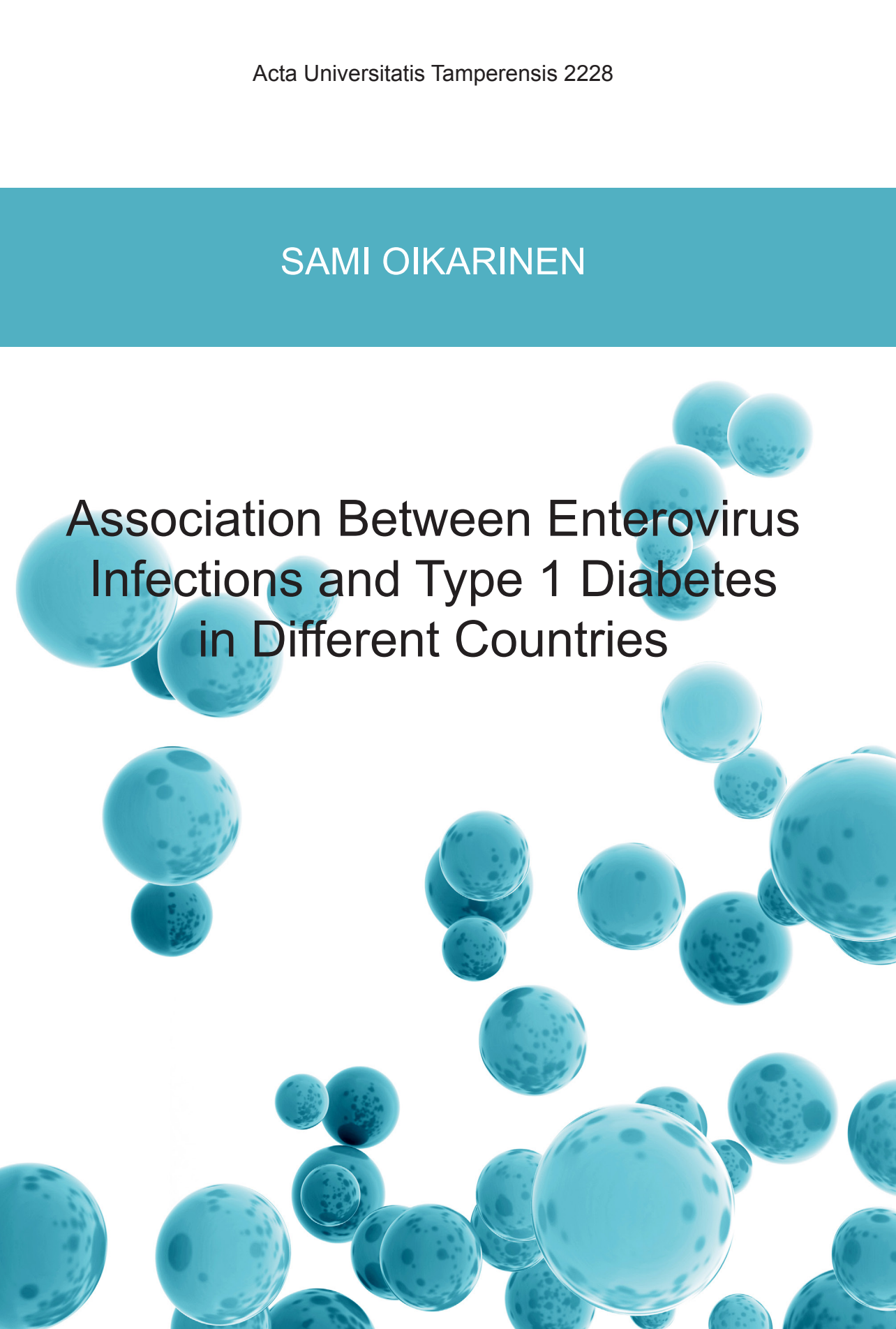SAMI OIKARINEN

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ABSTRACT

The present study evaluates a possible connection between enterovirus (EV) infections and type 1 diabetes (T1D) focusing on the following research questions: 1) The association between enterovirus infections and T1D was evaluated in different stages of the T1D process, 2) the specific role of the six coxsackie B viruses (CBVs) was studied in the development of T1D and 3) these associations were evaluated in six different countries.

The research population consisted of three independent case-control cohorts collected from various countries. One cohort, from Finland, included children who were followed from birth until the diagnosis of T1D. Another cohort, from the USA, included children followed from the appearance of T1D associated autoantibodies until the development of T1D. A third series included newly diagnosed T1D patients from Finland, Sweden, the UK, France and Greece. In total, these cohorts included 337 children who developed T1D, 90 autoantibody positive children and 389 autoantibody negative non-diabetic control children. Enterovirus infections were diagnosed using RT-PCR to detect enterovirus RNA in serum and stool samples. In addition, enterovirus specific antibodies were measured from serum samples using ELISA and plaque neutralization assays.

Enterovirus infections were more common in children who developed T1D than in control children. This difference was most marked during the time period of six months prior to seroconversion to T1D associated autoantibodies. In addition, autoantibody positive children who developed T1D had more enterovirus infections than children who did not develop the disease. However, the enterovirus RNA was not detected at the onset of clinical T1D in serum or stool samples.

When the risk association of six different CBV serotypes with T1D was analyzed in Finland, Sweden, the UK, France and Greece, CBV1 infections were more common in case children than in the control group. This finding was similar in all five study populations. The prevalence of antibodies against CBV2-6 did not differ between case and control groups.

The results of this study support the hypothesis that enterovirus infections may contribute to the development of T1D. CBV1 may especially have a specific role in triggering and accelerating the disease process. However, confirmation of causality

between the enterovirus infections and the disease needs additional studies, such as intervention trials with vaccinations or antiviral drugs.

TIIVISTELMÄ

Väitöskirjassani tutkin enterovirusten osuutta tyypin 1 diabeteksen synnyssä. Tutkimus jakautui kolmeen pääasialliseen kysymykseen: 1) Selvitin enterovirusten roolia tyypin 1 diabeteksen syntyprosessin eri vaiheissa, 2) tutkin kuuden eri coxsackie B viruksen (CBV) mahdollista riskivaikutusta ja 3) tutkimustulosten yleistettävyyttä tutkin kuudessa eri maassa.

Tutkimus perustuu kolmeen laajaan eri maista kerättyihin tapaus-verrokki aineistoihin: Suomessa syntymästä asti seurattujen tyypin 1 diabetekseen sairastuneiden ja heille valittujen verrokkilasten kohorttiin. Yhdysvalloista kerättyyn aineistoon, jossa lapsia seurattiin tyypin 1 diabeteksesta ennustavien autovasta-aineiden ilmaantumishetkestä sairauden puhkeamishetkeen asti. Lisäksi tutkimuksessa oli mukana poikkileikkausaineisto tyypin 1 diabeteksen syntyhetkeltä Suomesta, Ruotsista, Englannista, Ranskasta ja Kreikasta. Yhteensä tutkimukseen osallistui 816 lasta, joista 337 oli tyypin 1 diabetekseen sairastunutta lasta, 90 autovasta-aine positiivista ja 389 tervettä autovasta-aine negatiivista verrokkilasta. Tutkimuksessa käytettiin enterovirusspesifisiä RT-PCR menetelmiä virus RNA:n osoitukseen seerumi- ja ulostenäytesarjoista, sekä ELISA ja plakki neutralisaatiomenetelmää enterovirusspesifisten vasta-aineiden osoittamiseen seerumista.

Tutkimuksessa havaittiin, että enterovirusinfektiot ovat yleisempiä tyypin 1 diabetekseen sairastuvilla lapsilla kuin terveillä verrokkilapsilla. Suurin ero ryhmien välillä nähtiin erityisesti kuusi kuukautta ennen tyypin 1 diabetesta ennustavien autovasta-aineiden ilmaantumista. Lisäksi osoitettiin, että autovasta-ainepositiivisilla lapsilla, jotka myöhemmin sairastuivat tyypin 1 diabetekseen, oli enemmän enterovirusinfektioita kuin lapsilla, joille sairaus ei puhjennut. Sairastuvuusriski nousi tutkimuspopulaatiossa selvästi heti enterovirusinfektioiden jälkeen, mutta enterovirus RNA:ta ei löydetty enää tyypin 1 diabeteksen puhkeamishetkellä seerumi- tai ulostenäytteistä.

Selvitin myös mitkä aikaisemmissa tutkimuksissa tyypin 1 diabeteksen syntyyn liitetyt CBV serotyypit voivat olla yhteydessä tyypin 1 diabeteksen kehittymiseen. Tutkimme näiden virusten roolia viidessä Euroopan maassa: Suomessa, Ruotsissa, Englannissa, Ranskassa ja Kreikassa. Tutkimuksessa osoitettiin, että vasta-aineet

CBV1 serotyyppiä vastaan olivat yleisempiä tyypin 1 diabetekseen sairastuneilla lapsilla kuin kontrolliryhmässä. Tämä löydös oli samansuuntainen kaikissa viidessä tutkimukseen osallistuneessa maassa. Virusvasta-aineet muita CBV (2-6) viruksia vastaan eivät eronneet tapaus- ja verrokkiryhmien välillä missään maassa.

Tutkimustulokset vahvistavat aikaisempia havaintoja siitä, että enterovirukset, erityisesti CBV1 virus, voi mahdollisesti aiheuttaa tyypin 1 diabetesta. Viruksen ja taudin syy-seuraussuhteen varmistamiseen tarvitaan kuitenkin vielä jatkotutkimuksia. Yksi mahdollisuus on testata CBV rokotteen tai viruslääkkeiden vaikutusta hyvin kontrolloiduissa kliinisissä tutkimuksissa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The study is based on the following original publications, which are referred to in this thesis book by Roman numerals I-IV:

I   Sami Oikarinen, Sisko Tauriainen, Hanna Viskari, Olli Simell, Mikael Knip, Suvi Virtanen, Heikki Hyoty. PCR inhibition in stool samples in relation to age of infants. 2009. Journal of Clinical Virology 44:211-214.

II   Lars C. Stene, Sami Oikarinen, Heikki Hyöty, Katherine J. Barriga, Jill M. Norris, Georgeanna Klingensmith, John C. Hutton, Henry A. Erlich, George S. Eisenbarth, Marian Rewers. Enterovirus Infection and Progression from Islet Autoimmunity to Type 1 Diabetes: The Diabetes and Autoimmunity Study in the Young (DAISY). 2010. Diabetes 59: 3174-3180.

III   Sami Oikarinen, Mika Martiskainen, Sisko Tauriainen, Heini Huhtala, Jorma Ilonen, Riitta Veijola, Olli Simell, Mikael Knip, and Heikki Hyöty. Enterovirus RNA in Blood Is Linked to the Development of Type 1 Diabetes. 2011. Diabetes 60: 276-279.

IV   Sami Oikarinen, Sisko Tauriainen, Didier Hober, Bernadette Lucas, Andriani Vazeou, Amirbabak Sioofy Khojine, Evangelos Bozas, Peter Muir, Hanna Honkanen, Jorma Ilonen, Mikael Knip, Päivi Keskinen, Marja-Terttu Saha, Heini Huhtala, Glyn Stanway, Christos S. Bartsocas, Johnny Ludvigsson, Keith Taylor, Heikki Hyöty, and and the VirDiab study group. Virus Antibody Survey in Different European Populations Indicates Risk Association Between Coxsackievirus B1 and Type 1 Diabetes. 2014. Diabetes 63: 655-662

In addition to the communications mentioned above, this thesis contains unpublished data.

ABBREVIATIONS

ATCC	American Type Culture Collection
BA	baboon enterovirus
BSA	bovine serum albumin
Bp	base pair
CAR	coxsackie-adenovirus receptor
CAV	coxsackie A virus
CBV	coxsackie B virus
cDNA	complementary DNA
CI	confidence interval
CMV	cytomegalovirus
CPE	cytopathic effect
cps	counts per second
CTLA4	cytotoxic T lymphocyte antigen-4
DAF	decay-accelerating factor
DAISY	the Diabetes and Autoimmunity Study in the Young
DIPP	the Finnish Diabetes Prediction and Prevention Study
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EV	echovirus
GADA	glutamic acid decarboxylase antibody
GMK	green monkey kidney cells
HEPES	carboxymethyl cellulose
HEV A	human enterovirus A
HEV B	human enterovirus B
HEV C	human enterovirus C
HEV D	human enterovirus D
HLA	human leukocyte antigen
HR	hazard ratio
HRV	human rhinovirus
IAA	insulin autoantibody
IA-A2	tyrosine phosphatase-like protein antibody
ICA	islet cell antibody
ICAM-1	intracellular adhesion molecule 1
IFIH1	interferon induced with helicase C domain 1
IFN-γ	interferon-gamma

IgA	immunoglobulin A antibody
IgG	immunoglobulin G antibody
IgM	immunoglobulin M antibody
IL2RA	interleukin 2 receptor alpha
INS	insulin
JDFU	Juvenile Diabetes Foundation unit
kb	kilobase
kD	kilodalton
MDA5	melanoma differentiation-associated protein 5
MEM	minimum essential medium
NGS	next generation sequencing
NOD mice	non-obese diabetic mice
OR	odds ratio
P1	precursor protein 1
P2	precursor protein 2
P3	precursor protein 3
PCR	polymerase chain reaction
PTPN22	tyrosine phosphatase 22
PV	poliovirus
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RU	relative unit
(ss)RNA	single stranded RNA
SA	simian enterovirus
SD	standard deviation
SFV	semliki forest virus
SOCS mice	suppressor of cytokine signaling transgenic mice
SOCS-1	suppressor of cytokine signaling-1
SV	simian enterovirus
SVDV	swine vesicular disease virus
T1D	type 1 diabetes
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
UTR	untranslated region
VirDiab	Viruses in Diabetes EU study
VP	viral protein
VPg	genome-linked viral protein
ZnT8	zinc-transporter 8 autoantibody

1   INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease which is caused by the destruction of the insulin producing beta cells in the pancreas [1]. Without regular lifelong treatment with insulin injections, the disease is life-threatening. Despite treatment improvements, definitive prevention and cure of the disease is not available.

Incidence of T1D has increased in the past few decades in developed countries, especially in Finland where the incidence of the disease is the highest in the world. This steep increase exceeds population inheritance and suggests that environmental factors are important in the pathogenesis. It has been agreed that susceptibility for the disease is determined in several loci of the individual's genome, but environmental triggers are needed for development of the disease in genetically susceptible individuals. The nature of these environmental factors is still unknown and contested, but one of the most evident is enterovirus infection.

The aim of this thesis was to evaluate the role of enterovirus infections in different stages of the T1D process. In addition, the aim was to evaluate the association of serotype specific Coxsackie B virus (CBV) antibodies and T1D in a multicenter case-control study. Further, the association analyses were done across different European populations.

2 REVIEW OF THE LITERATURE

2.1 Human enteroviruses

2.1.1 Classification, structure and replication

Human enteroviruses belong to the *Picornaviridae* family. Traditionally, enteroviruses have been classified according to their antigenic properties and pathogenicity in animal models. However, the classification of the enteroviruses has changed during recent decades due to advances in modern molecular methods. Currently, enteroviruses are classified according to characteristics of the genome and properties of the replication cycle. Utilizing PCR based methods, intermediate strains of different species and also new species have been discovered, and these findings have emphasized the need for rearrangements in the Picornavirus family. Currently, the enterovirus genus includes human infecting enterovirus groups A-D, animal enteroviruses E-H, J and Rhinovirus groups A-C (Table 1).

Table 1. Classification of enterovirus genus

Species	Strains	Number of genotypes
Human enterovirus A	CAV2, CAV3, CAV4, CAV5, CAV6, CAV7, CAV8, CAV10, CAV12, CAV14, CAV16, EV-71, EV-76, EV-89, EV-90, EV-91, EV-114, EV-119, EV-92, EV-114, EV-119, EV-120, EV-121, SV19, SV43, SV46, BA13	25
Human enterovirus B	CBV1, CBV2, CBV3, CBV4 (incl. SVDV-2), CBV5 (incl. SVDV-1), CBV6, CAV9, E1 (incl. E8), E2, E3, E4, E5, E6, E7, E9 (incl. CAV23), E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E24, E25, E26, E27, E29, E30, E31, E32, E33, EV69, EV73, EV74, EV75, EV77, EV78, EV79, EV80, EV81, EV82, EV83, EV84, EV85, EV86, EV87, EV88, EV93, EV97, EV98, EV100, EV101, EV106, EV107, EV-110, EV-B111, EV-B112, EV-B113, SA5	63
Human enterovirus C	PV1, PV2, PV3, CAV1, CAV11, CAV13, CAV17, CAV19, CAV20, CAV21, CAV22, CAV24, EV95, EV96, EV99, EV102, EV104, EV105, EV-109, EV-116, EV-117, EV-118	18
Human enterovirus D	EV68 (incl. HRV87), EV70, EV94, EV-111, EV-120	5
Enterovirus E	Bovine enterovirus A	4
Enterovirus F	Bovine enterovirus B	7
Enterovirus G	Porcine enterovirus B	16
Enterovirus H	Simian enterovirus A, EV-H1	3
Enterovirus J	SV6, EV-J103, EV-J108, EV-J112, EV-J115, EV-J121	6
Rhinovirus A		80
Rhinovirus B		32
Rhinovirus C		55
Unassigned EV	EV-122, EV-123	2

Enteroviruses are small, positive-sense, single-stranded (ss)RNA viruses, which have a genome size of about 7 400 bases. The genome is monocistronic and flanked with 5'- and 3'-untranslated regions (UTR). A small virus-encoded protein VPg is attached to the 5' end of the molecule. The coding region encodes a single polyprotein, which is cleaved by virus-encoded proteases into three precursor proteins P1, P2 and P3 [2]. Region one (P1) consists of four structural proteins (VP1-4) and regions two and three (P2 and P3) code seven proteins and several forms of functional intermediates needed for viral RNA replication and processing of viral and host proteins. Structural proteins VP1, VP2 and VP3 form the surface of the capsid and VP4 is located inside the capsid. These proteins together assemble the icosahedral virus capsid, which is a structure of 60 identical subunits composed of four polypeptides (Fig. 1) [3, 4].
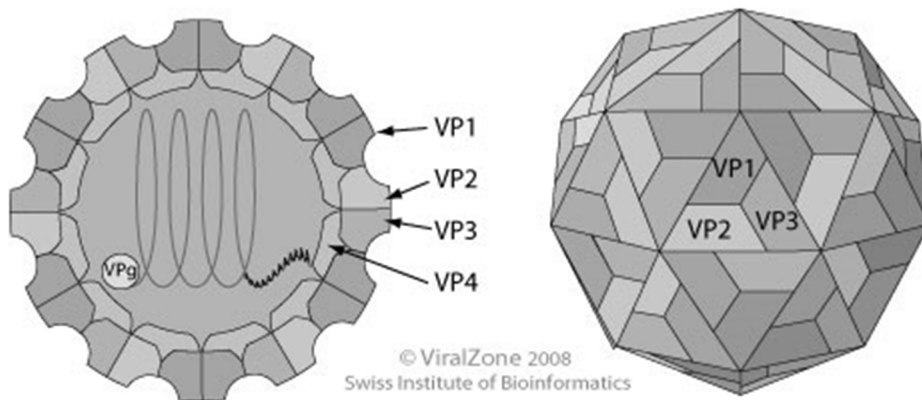
Figure 1. The stucture of enterovirus capsid. The virus capsid is formed by four structural proteins VP1-VP4. (Adapted from http://viralzone.expasy.org/)

Enteroviruses can replicate in several human cells and tissues, but the primary replication site is the mucosal tissue of the gut or the respiratory tract. Replication starts by the attachment of the virus to host receptors such as the intracellular adhesion molecule 1 (ICAM-1), the decay-accelerating factor (DAF), integrins (such as α2β1, αvβ3), the poliovirus receptor or coxsackie-adenovirus receptor (CAR), which mediate endocytosis of the virus. The capsid then undergoes conformational changes and the viral genomic RNA is released into the host cell cytoplasm via pores opened by the VP4 protein in the endosomal membrane of the host cell. The cleavage of the VPg from genomic RNA is needed before the RNA acts as a template for protein synthesis. The same RNA molecule is also used as a template for

synthesis of negative-sense RNA by the viral polymerase 3D. These RNA molecules are used in synthesizing large amounts of positive-sense RNA copies which are used as templates in the translation of viral proteins and become encapsidated into the assembling new virions [5]. The infection process is enhanced by shutting off the host cellular cap-dependent translation through the cleavage of translation initiation factors by viral protease 2A. A single infected cell can produce between 10^4 to 10^5 virus particles, which are released by the lysis of the cell. The lysis may occur due to increased permeability of the host cell membranes [6] caused by an accumulation of nonstructural proteins 2B [7] and 3A as well as intermediate proteins 2 BC [8] and 3AB [9, 10].

Enteroviruses cause acute infections, but also persistent infections have been demonstrated in cell cultures [11-13] and in animal models [14, 15], and viral persistence may play a role in dilated cardiomyopathy cases and in post-polio syndrome in humans [16-18]. The persisting virus has lost the effective replication and protein synthesis leading to slow-grade replication, which helps the virus hide from the host immune system. These changes of viral replication may be caused by deletions of nucleotides in the 5'UTR or VP1 region of the viral genome [19, 20]. Enteroviral persistence in the pancreatic islets has been suggested to be one possible mechanism for T1D [21, 22].

2.1.2 Epidemiology and clinical manifestation

Enteroviruses are one of the most common pathogens in the world, which infect all age groups, but are especially common in young children, elderly people and immunocompromised individuals. Enteroviruses are transmitted mainly by the fecal-oral route, but some serotypes are known to use the respiratory route. Serotypes utilizing the fecal-oral route are especially common in areas with poor sanitary conditions and a low standard of hygiene compared to developed countries, where prevalence of these serotypes has decreased [23]. In temperate climates seasonality of the enterovirus infections is clear, prevalence of the infections being highest in the late summer and autumn, whereas in tropical areas such seasonally dependent incidence is not seen and infections are more constant in all seasons [24].

Infections are most often subclinical or manifest as mild respiratory infections. In some cases a more severe outcome is seen, including cardiovascular diseases, neurological diseases, sepsis-like illness, meningitis, encephalitis, myocarditis, hand, foot and mouth disease, and pancreatitis. Enteroviruses may also cause chronic fatigue syndrome and dilated cardiomyopathy, which are chronic diseases [25-28].

Severity of the infection depends on the virus type, but also on host factors such as gender, immunological stage, genetic background and age of the subject; neonatal infections often have a more severe outcome than infections in older age groups [29]. It is also known that some diseases are linked to specific enterovirus serotypes. For example, CAV6, CAV16 and EV71 cause hand, foot and mouth disease, PVs poliomyelitis and CBV3 myocarditis In general, CBV infections tend to lead to more severe outcomes.

2.1.3   Diagnosis

Enterovirus infections are often difficult to diagnose based solely on clinical symptoms because they can cause a wide range of symptoms. Adding to the challenge is that many bacteria and other viruses may cause similar symptoms. An accurate diagnosis is critical to avoid unnecessary and inefficacious medications.

Traditional enterovirus diagnostics have been based on virus isolation and virus identification using neutralization with pools of serotype-specific antisera. This method is laborious and time-consuming, and often the patient has recovered prior to completion of these assays. Another problem is that the virus isolation is not done from primary tissue, but often from stool samples, where the virus may have been secreted for a prolonged period of time. Consequently, an isolated virus may show only temporal association but not be the real cause of the acute disease. In addition, cross-reaction between different serotypes and the alteration of the antigenic properties over time reduces the specificity of serotype identification using these hyperimmune sera [30]. Virus isolation is also biased, due to the difficulties of certain serotypes to grow in the cell cultures. The sensitivity of virus isolation is also usually lower than that of PCR based methods.

Diagnosis of human enteroviruses is demanding because in addition to the 111 subtypes known today, over 30 new genotypes have been found in the last few years, and most likely even more will be discovered in the future. Conventional cross-sectional pools of antisera used in neutralization do not recognize all of these new types and monotypic antisera are not available for all new types [31].

Neutralizing serotype and the genotype of the sequence of the VP1 region correlates well because the antigenic sites are located mostly in the VP1 region [31]. Several methods have been developed for sequencing part of the VP1 region to identify viral subtypes [32-36]. The chance to discover new serotypes increases

substantially using a couple of primer pairs or degenerated primers, because such primer sets allow amplification of a wider spectrum of the highly variable VP1 region. In addition, the next generation sequencing (NGS) method allows the unbiased detection of all enteroviruses including previously unknown types [37]. Even though PCR based methods have some disadvantages, such as false negative tests due to the low concentration of the virus in the sample, invalid sample material, loss of virus during the storage of the sample and presence of PCR inhibitors, they are still superior in enterovirus diagnosis due to their high sensitivity and speed, and they have replaced virus isolation in most diagnostic laboratories.

2.2 Type 1 diabetes

2.2.1 Pathogenesis of type 1 diabetes

Autoimmunity is a concept which refers to immune responses against an organism's cells and tissues. T1D is an autoimmune disease resulting from the selective destruction of the insulin-producing beta cells in the pancreas. The subclinical stage of this process typically starts from a few weeks up to several years prior to the actual onset of clinical disease. The disease may be subclinical for a long time and the symptoms, such as increased thirst, frequent urination, extreme hunger, weight loss, fatigue and blurred vision, occur when only about 10% of the beta cells are remaining [38].

2.2.1.1 Autoantibodies associated with type 1 diabetes

The first markers of the disease are autoantibodies which target pancreatic islet autoantigens. These autoantibodies include islet cell autoantibodies (ICA), insulin autoantibodies (IAA), autoantibodies against the 65-kDa isoform of glutamic acid decarboxylase 65 (GADA), protein tyrosine phosphatase-related IA-2 molecule (IA-2A) and zinc-transporter 8 autoantibody (ZnT8), which can be detected from a blood sample [39]. The lag time between seroconversion to autoantibody positivity and the onset of T1D varies from a few weeks to several years, and not all cases with autoantibodies will develop the disease. The risk increases according to the number of autoantibodies detected, with three to four autoantibodies raising the risk of T1D to over 60% [40]. According to current knowledge, these T1D associated

autoantibodies are not actively involved in the destruction of beta cells but can be used as markers of the disease process.

2.2.1.2   Genetic susceptibility for type 1 diabetes in humans

T1D has a strong inherited genetic component. The major risk genes map to the human leukocyte antigen (HLA region) Class II HLA haplotypes DR3-DQ2 (DRB1*03-DQA1*0501-DQB1*0201) and DR4-DQ8 (DRB1*0401-DQA1*0301-DQB1*0302) which are liable for an estimated 50% of the total genetic risk. In addition, more than 50 non-HLA genes have a smaller effect on the risk of the disease, such as tyrosine phosphatase (PTPN22) [41], insulin (INS) [42], interleukin 2 receptor alpha (IL2RA) [43] and cytotoxic T lymphocyte antigen-4 (CTLA4) [44, 45]. Interestingly, the majority of the T1D susceptibility genes mentioned above are involved in immune activation. One of the risk genes, called interferon, induced with helicase C domain 1 (IFIH1), encodes the intracellular pathogen receptor Melanoma Differentiation-Associated protein 5 (MDA5) that has been shown to be essential for the innate immune response to viral double-stranded RNA, such as double-stranded RNA replicative form of enterovirus genome, leading to a robust cytokine response and production of interferon-gamma (IFN-γ) and inducing apoptosis of infected cells [46-48].

2.2.1.3   Environmental factors in the pathogenesis of type 1 diabetes

Genetic factors determine the baseline risk of the disease, but there is also strong support for the involvement of environmental factors [40]. Firstly, less than 10% of children carrying HLA risk genes for T1D ever develop the disease [49]. Secondly, a pair-wise concordance of the development of T1D is only between 13-33% among monozygotic twins [50, 51]. Thirdly, the incidence of T1D has increased rapidly during the past few decades worldwide [52, 53]. An exceptionally rapid increase has been seen in developed countries, such as Finland where the incidence has doubled in 30 years, now being over 60 cases per 100 000 children under the age of 15 years per year [54]. Fourthly, about a 15-fold difference in the incidence of T1D has been described between genetically similar Caucasian populations living in Europe (Fig. 2). Fifthly, the incidence increases in population groups who have moved from a low- to a high-incidence region [55, 56]. Sixthly, seroconversion and the onset of

T1D comply with seasonal variation, being higher in cold months than in warm months at least in a temperate climate [57].

Different dietary factors have also been linked to the development of T1D. Cow milk proteins such as bovine serum albumin [58, 59], β-lactoglobulin [60], beta casein [61], and bovine insulin [62] have been suggested as potential risk factors. However, a recent clinical trial (TRIGR) showed that the use of hydrolyzed casein formula does not reduce the incidence of seroconversion to T1D-associated autoantibodies compared to conventional cow's milk-based formula in children at genetic risk of T1D [63]. Vitamin D supplementation has been associated with a reduced risk of T1D while low zinc in drinking water has been associated with the increased risk of T1D [64, 65]. Altogether, several dietary factors have been linked to the development of T1D, but findings have been inconsistent and none have been shown to be causally linked to the disease. However, it is possible that, in some subgroups of T1D patients, dietary factors may play a role or that dietary factors can have complex interactions with other risk factors, such as viruses, in the development of T1D.

2.2.2   The role of virus infections in type 1 diabetes

Seasonal incidence of T1D and observed case reports have contributed to the generation of the virus hypothesis in the etiology of T1D. Various viruses have been connected to T1D including cytomegalovirus (CMV) [66], parvovirus [67, 68], encephalomyocarditis virus [69], mumps, rubella and retroviruses [70], but the role of these viruses has been challenged or is still awaiting confirming reports from other studies. More evidence has been obtained for the possible role of rotavirus, congenital rubella, mumps and lately influenza A. The main reason for suspecting rotavirus as a diabetogenic virus was the sequence homologies observed between T cell epitopes within rotavirus protein and IA-2 and GAD autoantigens [71]. A population study in Australia showed the risk association between rotavirus infection and islet autoantibody positivity in at-risk children, but two studies in Finland did not confirm these Australian findings [72-74]. According to these studies the role of rotavirus in the etiology of T1D is tentative. In the coming years more data will accumulate as live attenuated rotavirus vaccines have been taken into the national vaccination programs in several countries. In Finland, rotavirus vaccination was included in the vaccination program in 2009. Interestingly, it seems that the increase in the incidence of T1D has leveled off in Finland, giving room for speculation of a possible protective effect of the rotavirus vaccine against T1D [75].

Rubella infection during the first trimester of pregnancy can cause serious organ damage in the fetus [76]. One of the clinical consequences of congenital rubella is diabetes, which has been reported in up to 40% of congenital rubella cases after a follow-up for 7-50 years [77]. However, these cases seem not to be typical autoimmune T1D cases, but the virus may cause diabetes by disturbing the normal development of beta cells in the pancreas [78]. An efficient vaccine was introduced in 1969 and the rubella virus has been largely eliminated in western countries. However, this has not changed the epidemiology of T1D. This may be due to the fact that congenital rubella infection was a rather uncommon event and its etiological fraction in T1D has probably been small.

The mumps virus has also been reported as a possible risk factor for T1D. However, a vaccination program started in the 1960s and it has not cut the rising T1D incidence in western countries [79].

Recently, influenza A H1N1 has been connected to the development of T1D in Italy [80]. In Sweden, the number of newly diagnosed T1D patients with a genotype of DQ2/8 and younger than 3 years decreased after influenza vaccination, but the frequencies of seroconversion to GADA and ZnT8QA autoantibodies increased. Therefore, it cannot be excluded that the vaccine affected the clinical onset of T1D in this population [81]. In Finland, influenza A infections were not associated with the islet autoimmunity in young children with an increased genetic susceptibility to T1D [82].

One of the most studied potential environmental risk factors for T1D is enterovirus. These studies are based on case reports, epidemiological associations, and isolation of the virus from the pancreas and stool samples, as well as various experimental studies in cell and animal models. The outcome of these studies is that enterovirus is currently considered as one of the most likely triggers of T1D, but this association still needs further confirmation. The role of enterovirus infection in T1D is summarized in more detail in the following paragraphs.

2.3   Human enterovirus and type 1 diabetes

The connection between T1D and human enteroviruses has been studied for over 40 years. One of the first studies that showed a seasonal incidence for T1D, with autumn and winter peaks in England, suggested a possible association with enteroviral seasonality [83]. The same study group published an article, which showed higher titers of antibodies against CBV4 and CBV5 in newly diagnosed T1D

patients than in healthy controls [84]. Since then, various methods have been applied to study the role of enteroviruses in T1D pathogenesis in various sets of epidemiological studies in humans, and both animal and cell experiments. The main findings from these studies will be summarized in the next paragraphs, focusing on epidemiological studies.

2.3.1   Epidemiological evidence

2.3.1.1   Seasonality of onset of type 1 diabetes and related autoantibodies

The first detailed evidence pointing to a connection between virus infection and T1D was documented in the USA where seasonal incidence of T1D was observed with peaks through fall, winter and early summer months [85] . Similar results were recorded in the UK where new T1D cases were observed in the summer but peaking in winter months [83]. A review article which summarized results from 52 countries has later concluded that the seasonality of T1D occurs worldwide, although this phenomenon is stronger in the northern hemisphere, especially in areas with colder winter [86].

The seasonal pattern of autoantibody seroconversion time has also been reported. In Finland, children turn autoantibody positive usually during late summer and fall [57]. The seasonal pattern of autoantibody seroconversion was actually sharper than reported in the development of T1D. Altogether, the seasonal pattern in the incidence of T1D and in autoantibody seroconversion resembles that of enterovirus infections. Thus, if autoimmunity is caused by enterovirus infections, the process is initiated soon after a triggering infection, with a relatively constant lag phase. This hypothesis is supported by other prospective studies with a peak of enterovirus infections prior to development of autoantibodies, and also mouse models [87-89].

2.3.1.2   Geographical and temporal clustering of type 1 diabetes

It has been well documented that the incidence of T1D differs highly between different countries (Fig. 2) [52]. It can be concluded that in the developed high hygiene countries the incidence of T1D is higher compared to developing countries. Enterovirus infections follow an opposite trend. However, in theory this inverse

association may support a hygiene hypothesis in the etiology of T1D. It is also interesting that the trend in the incidence of T1D follows latitude, being the highest in the northern areas [90].
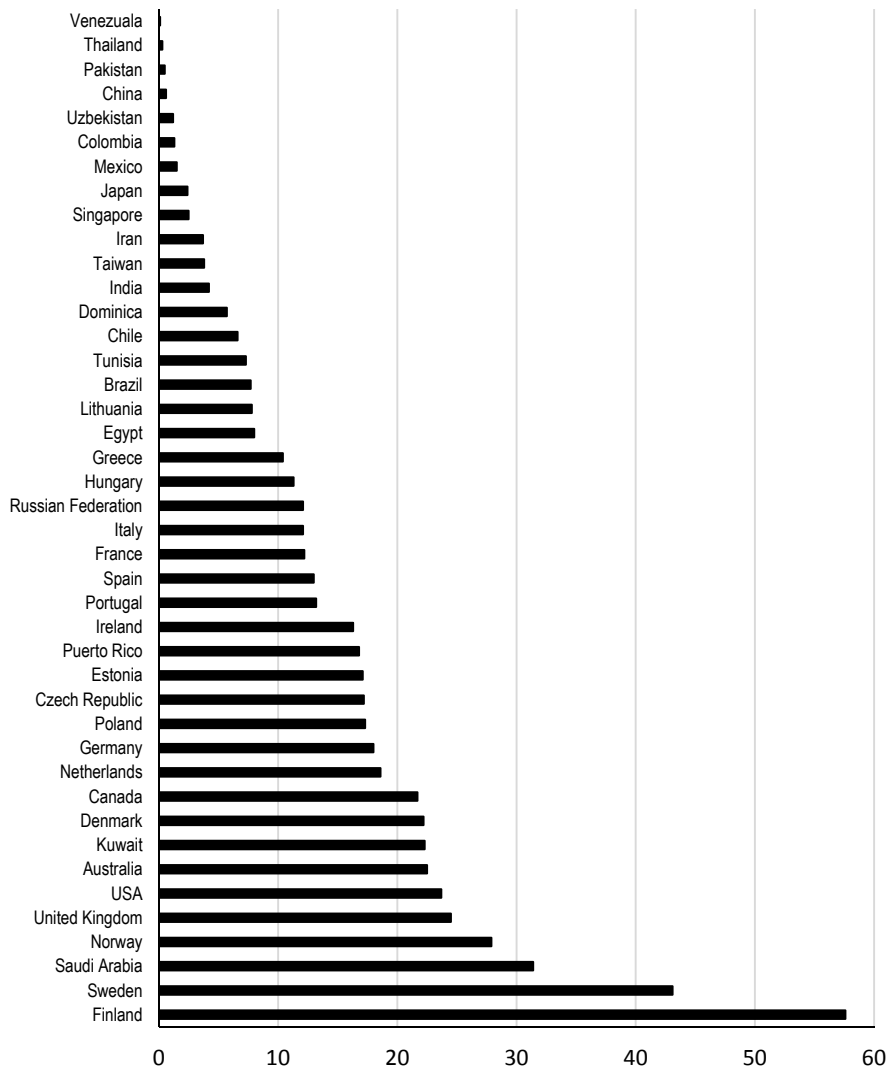
Figure 2. Incidence of T1D in various countries. (Adapted from International Diabetes Federation's Diabetes Atlas 2011)

Temporal clustering of T1D has also been observed in several areas. It is possible that this phenomenon may reflect the influence of some local environmental factors, such as an enterovirus outbreak, in triggering the disease [91-93]. Temporal clustering of the onset of T1D has also been documented in families, where the diagnosis of two or more family members has been done with short time intervals or even simultaneously. In some of these cases enterovirus has been detected, suggesting that enterovirus could have been the triggering factor of their T1D [94-97].

2.3.1.3   Case control studies

The role of enterovirus infections in the development of T1D has been studied mostly in retrospective case-control series, but also four longitudinal studies have been published (Table 2 and 3). These studies vary in many aspects such as type of samples collected and methods used for virus detection, which make comparison of the results complicated. Some of these studies have shown a risk association between enterovirus infections and islet autoimmunity or T1D, but some others have not found such an association. The main findings can be summarized with the following points: First, enterovirus RNA has been detected more often in cases than in controls at the onset of T1D from serum or plasma samples with the odds ratio ranging from approximately 10 to 12 [98]. Second, four longitudinal studies have been carried out detecting enteroviral RNA from serum or plasma samples and two of these studies reported enterovirus as a risk virus for seroconversion or T1D[89, 99]. Norwegian (MIDIA) study showed tendency towards an association at the sample interval prior seroconversion time [100]. One of these studies (DAISY) did not show such a connection, but this study combined data from serum, saliva and rectal swab samples and therefore it is possible that frequent enterovirus positivity of stool samples masked analysis of plasma samples. In addition, the number of cases was limited (N=26) [101]. Third, enteroviruses have been detected frequently from stool samples in three longitudinal studies, but none of these studies showed a risk association between enterovirus infections and islet autoantibodies or T1D [101-103]. However, a much larger study from Finland found more enterovirus infections several months before islet autoantibody appearance, based on virus detection from stools (Honkanen et al. unpublished data). It has been shown in several populations that enterovirus RNA is detected frequently from stool samples even in a healthy background population. Thus, a possible diabetogenic effect of specific enterovirus

serotypes may have been masked by these background infections in studies based on virus detection from stools [104-106]. Negative findings may be alternatively explained with the possibility that the primary replication site of diabetogenic enteroviruses is in the upper respiratory track and therefore viruses are not excreted in stools. Fourth, it seems clear that longitudinal studies have not provided evidence for viral persistence in T1D with blood sample collection intervals from three to six months. In stool samples, enterovirus infection had been detected from a few weeks up to four months showing no difference in virus excretion between cases and controls. Fifth, serological analysis in two studies showed an association with odds ratios of 1,8 and 3,8. The BABYDIAB study did not find an association, but this study had limited power because it included only 28 case children and a low number of appropriate samples [107]. Sixth, enterovirus incidence peaks a few months before the time of seroconversion, suggesting the triggering effect of these infections [89].

Table 2. Prevalence of enterovirus infections in case and control children at the onset of T1D based on the detection of viral RNA in blood or stool samples using RT-PCR assays.

Country	Case		Control		Reference
	N	Pos (%)	N	Pos (%)	
UK	14	64	45	4	[108]
UK	17	41	43	31	[109]
UK	110	27	182	5	[110]
France	12	42	27	0	[111]
France	56	38	37	0	[112]
Sweden	24	50	24	0	[113]
Australia	206	30	160	4	[114]
Japan	61	38	58	3	[115]
Germany	47	36	50	2	[116]
China	22	56	30	7	[117]

Table 3. Enterovirus infections in case and control children in longitudinal studies of T1D based on the detection of viral RNA using RT-PCR assays.

Study	Period	Case		Control			
		N	Pos (%)	N	Pos (%)	OR (95% CI)	P<
DAISY[1] [101]	Birth-Aab	26*	11,5	39	17,9	0,3 (0,02-2,89)	0,273
	Birth-T1D	26*	19,5	39	25,6	0,2 (0,01-4,15)	0,276
DiMe[2] [99]	Birth-T1D	11	22	34	2	14,9	0,001
DIPP[2] [89]	Birth-AAb	41	22	196	14	na	0,02
	6mo prior AAb	41	17	196	4	5,2 (1,6-16,7)	0,002
	AAb -T1D	16	24	na	16	na	0,1
MIDIA[3] [100]	Birth-AAb	45	7,6	92	10	0,6 (0,27-1,32)	0,2
	AAb	32	15,8	60	3,2	9,1 (0,95-86,0)	0,055
	AAb - T1D	45	10,5	92	5,8	2,8 (0,87-8,77)	0,086

Sample types in the studies: [1] Serum, saliva and rectal swab, [2] serum, [3] red and white blood cells with small amount of plasma
*Serum samples from 13 children were analyzed for EV

2.3.1.4 Population studies

A nationwide population based cohort study was performed in Taiwan utilizing data from Taiwan's National Health Insurance Research Database. They compared two groups of children, with and without enterovirus infection, and observed that enterovirus infections increased the risk of developing T1D (HR 1,48; 95% CI 1,19-1,83) [118]

Viskari et al. studied the frequency of enterovirus infections in seven Caucasian populations in Europe. Two of these countries had an exceptionally high incidence of T1D (Finland and Sweden) and five countries presented a low or intermediate incidence of diabetes (Estonia, Germany, Hungary, Lithuania, Russia). Enterovirus antibodies were significantly less frequent in countries with a high T1D incidence compared to countries with a low diabetes incidence (P<0.001) [23]. This inverse correlation between the incidence of type 1 diabetes and enterovirus infections is in line with the previously proposed polio hypothesis [119]. This hypothesis suggest that the complications of enterovirus infections become more severe, such as T1D, in a hygienic environment with a low rate of infections [23].

2.3.1.5 Enterovirus in the tissue samples of type 1 diabetes cases

Interesting results have been obtained during the last few years from studies analyzing different tissue samples using mainly immunohistochemistry and *in situ* hybridization methods to detect enterovirus protein or RNA. Pancreatic samples of T1D patients have been positive for enterovirus protein using immunohistochemistry, and in some studies these findings have been confirmed by *in-situ* hybridization or virus isolation. Virus positive cells locate in the islets and the majority of them seem to be beta cells. However, the results have not been consistent and criticism against specificity of immunohistochemistry has been presented [120-123]. In addition, intestinal biopsies from diabetic children have tested positive for the enterovirus protein or RNA more often than those from control children [124]. These tissue samples have been collected mainly at the time of clinical diagnosis of T1D, or even several years after the diagnosis. It is possible that enteroviruses may persist in organs long after the acute phase of the infection and are therefore detectable in samples collected after the diagnosis of T1D. On the other hand, negative findings of such samples do not exclude the possibility that enteroviruses have initiated the beta cell damaging process but have cleared from the host organs at the time of sample collection. Altogether, these studies suggest that a significant proportion of the T1D patients may have a prolonged or persistent enterovirus infection in the pancreas or gut mucosa.

The DiViD study in Norway collected unique tissue material from six newly diagnosed T1D patients [125]. In this study, a small piece of pancreatic tail was collected during laparoscopic surgery and these samples were tested with various methods such as immunohistochemistry, *in-situ* hybridization and RT-PCR to detect enteroviruses. Interestingly, enteroviral VP1 protein was detected in the pancreatic islets in all six patients and enteroviral RNA was detected in four patients by RT-PCR. None of the controls were positive for enterovirus VP1 protein or RNA. Only 1,7% of the islets were virus protein positive, suggesting the possibility that a low grade enteroviral infection may have been connected to the loss of beta cells and the development of T1D in these cases. Also in this study, stool samples were collected from five of the patients and one sample was positive for CAV22 and another for Echovirus 30. Based on the 5'UTR sequence, the Echovirus 30 virus strain found in the stool was different from the enterovirus strain in the pancreatic islets, suggesting that in addition to an acute systematic infection this patient may have had a persistent infection in the pancreas [21].

2.3.1.6 Various enterovirus genotypes are associated with type 1 diabetes

It would be critical to know which, if any, of over 100 different enterovirus genotypes may cause autoimmunity and T1D in humans. This data would be needed for vaccine development against possible diabetogenic enterovirus strains. It is not possible to develop a vaccine that is effective against all enteroviruses, but the presence of a limited number of candidate genotypes would make the development possible. This data would also facilitate studies evaluating the mechanisms of virus-induced diabetes.

In a few cases of recent onset T1D, enteroviruses have been isolated and typed, and in these case reports of three strains of CBV4, and one strain of each of CBV2, CBV5, Echovirus 9 and 11 have been detected [120, 121, 126-130]. In the familiar T1D where a sibling or a parent has developed T1D simultaneously, one echovirus 6, two CBV2 and two CBV5 strains have been associated with the onset of T1D [94-97]. Epidemics of echoviruses 4, 16 and 30 in Cuba have been linked to seroconversion with T1D associated autoantibodies or the onset of T1D.

In some studies, the genotype of detected enteroviruses has been evaluated by sequencing part of the viral genome. Altogether, the detected sequences have matched with CBV3, CBV4, CBV5, Echovirus 5 and several types of genotypes from HEV A and B groups [93, 108, 111, 113, 114, 131]. However, in all of these studies genotyping was done according to part of the 5'UTR sequence, and due to frequent recombination between 5'UTR and capsid region, enteroviruses can be classified only in two types, in group II consisting of species HEV A and B and in group I, species HEV C and D. Therefore, even if the authors reported specific genotype, genotyping in serotype level is not reliable.

All of the studies previously referred to were case reports or case series without appropriate control children, and there have not been studies systematically identifying enterovirus subtypes possibly associated with the induction of beta cell autoantibodies. The Finnish DIPP study is the first one to carry out systematic screening of neutralizing antibodies against 41 different enterovirus serotypes in children who seroconverted for T1D associated autoantibodies. In this study, only CBV1 serotype showed a significant risk association with an odds ratio (OR) of 1.5 [95% CI 1.0–2.2] for diabetes. Surprisingly, CBV3 (OR 0.4 [95% CI 0.2–0.8]) and CBV6 (OR 0.6 [95% CI 0.4–1.0) showed a protective association against development of T1D linked autoantibodies [132].

2.3.2   Murine models

The first evidence of the possible role of the diabetogenic effect of enterovirus in mice models was gained from experiments where mice developed T1D after infection with CBV4 [133]. Since then, mice have been widely used as an animal model for T1D. The challenge in these studies is that it may be difficult to estimate how relevant the murine results are for the human disease. For example, in most mice strains enteroviruses replicate mostly in exocrine tissue [134, 135], while in humans enteroviruses have tropism to the pancreatic islets [136-138]. However, the family of suppressors of cytokine signaling (SOCS) transgenic mice, which lack the interferon response in the beta cells, due to the expression of the suppressor of cytokine signaling-1(SOCS-1), develop robust infection in the islets, followed by hyperglycemia and loss of beta cells [139]. In NOD mice, which spontaneously develop diabetes, CBV3 and CBV4 enterovirus infection accelerates the disease process when the virus is given at an older age, while infection at a younger age can delay the disease process [134, 135, 140].

In conclusion, murine models have suggested that enteroviruses may have an important role in the development of T1D, but the effect is dependent on the virus strain, host age, genetic background and immunological stage of the host.

2.3.3   Pancreatic islet cell models

Pancreatic islets are the target of the autoimmune attack or enterovirus induced cell damage in the pathogenesis of T1D. In this process the beta cells are specifically destroyed, in contract to exocrine cells, alpha, delta, and PP cells which remain intact [1]. Pancreatic islet isolated from organ donors afford an opportunity to test factors involved in beta cell damage such as the enterovirus tropism to pancreatic islets and islet cell responses to the virus. The studies using islets cultivated as free-floating cell preparations has shown that both the genetic properties of the infecting enterovirus and the host cell response to the infection are important for the outcome of the infection. It has been shown that several enterovirus serotypes belonging in HEV-B, HEV-C and HEV-D species contain strains which have tropism to islet cells [141-144], but the replication pattern differs between the strains: some strains are highly cytolytic whereas others are slowly replicating without apparent cell cytolysis. Enteroviruses can also persistent for long periods in the cultured islet cells [145-147]. Replication of enteroviruses also induces also clear innate immune response in islet cells [148].

2.3.4   Possible mechanisms

The mechanism of enterovirus induced beta cell specific destruction in the pancreas is not fully understood, but three main hypotheses have been suggested: First, enteroviruses may infect the beta cell directly leading to beta-cell death and direct virus-induced diabetes. Second theory is based on molecular mimicry, which states that the autoimmune attack results from immunological cross-reactivity induced by similar structures between (entero)viral and host proteins. The third option is a "bystander activation model" which postulates that the interactions between the immune response caused by infection and virus itself set the stage for a "fertile field" where the host and target organ are "primed" for subsequent immunopathology [149].

Viruses may infect and damage beta cells directly leading to virus-induced or immune-mediated cell apoptosis, necrosis or impaired function of beta cells. Support for this mechanism is garnered from animal models and enteroviruses have been detected in the human pancreas particularly in beta cells [21, 120, 123].

An alternative model is so called "molecular mimicry", which postulates that a virus may have protein structures similar to components of the host. Therefore, an immune response of the host, which is directed against the viral proteins may, in addition, also recognize similar structures in host proteins and attack against own cells. Two such sequence similarities have been described in enteroviruses and islet autoantigens: the enteroviral non-structural protein 2C and islet autoantigen GAD65 carry homology sequence PEVKEK [150], and enterovirus VP0/VP1 proteins and IAR/IA-2 tyrosine phosphatase and heat shock protein 60/65 [151, 152].

Viral infection leads to a strong inflammatory response, which further attracts and activates aggressive immune cells and causes accumulation of these cells in the site of inflammation. In the case of islets this is called insulitis. In the "bystander activation" model, cellular immune reactivity is considered to be the direct reason for beta cell destruction, mainly mediated by T lymphocytes [153].

2.3.5   Relationship between the epidemiology of enterovirus infections and type 1 diabetes – The polio hypothesis

Different hypotheses have been developed to explain the rapidly increasing incidence of T1D. Environmental factors are the most likely explanation for this increase, and changes in the diet, vitamin D and other dietary factors have been proposed. Virus infections have also been linked to the increasing incidence of T1D.

The hygiene hypothesis suggests that a decreased exposure to infections may lead to deficient immune regulation and an increased incidence of immune mediated diseases [154]. In addition, enteroviruses have specifically been linked to an increasing incidence of T1D by the so-called "polio hypothesis". The polio hypothesis is based on the analogy with the epidemiological observations of polio paralysis caused by poliovirus, which is also an enterovirus [119].

The polio hypothesis explains the paradigm between the high incidence of T1D in countries having a low incidence of enterovirus infections in the background population. Polio paralysis was a rare event at the time when hygiene was poor and polioviruses circulated in the population, frequently infecting the majority of children by the age of 5 years and almost every individual by adulthood. As sanitation and hygiene improved the prevalence of poliovirus infections decreased, which paradoxically led to the first epidemics of polio paralysis at the end of the nineteenth century. These epidemics started in areas with a high standard of living especially in the USA and Scandinavia, continuing until a poliovirus vaccination was developed in the 1950s [119].

The epidemics and rapid increase of paralytic polio are explained by a decrease in inherited immunity in young children combined with delayed exposure to the first poliovirus infections. These unprotected children had a more severe outcome of the infection than children with maternal antibodies at the time of the first poliovirus infection. Similarly, a low circulation of enteroviruses in the background population may increase the incidence of T1D by making children more susceptible to enterovirus-induced diabetes. At the time of first enterovirus infections, children lacking maternal antibodies against these viruses are at an increased risk of a systemic infection, which may allow certain viruses to spread to secondary infection sites, such as the pancreas, and cause T1D. Several observations support this hypothesis: in the Finnish population the risk association of CBV1 infection for T1D was strongest in children who experienced CBV1 without maternal CBV1 antibodies [132]. Secondly, a clear decrease has occurred in enterovirus antibody levels during recent decades in pregnant women in Finland and Sweden, and at the same time T1D has increased in these countries [119, 155]. Thirdly, the prevalence of enterovirus infections is relatively low in Finland, compared to other European countries, while the incidence of T1D is the highest in the world [23].

3   OBJECTIVES OF THE PRESENT STUDY

The main aim of this study was to evaluate the association between enterovirus infections and T1D in human cohorts using a combination of different study designs and methods. The detailed aims were the following:

1. To develop a specific and sensitive RT-PCR method for the detection of enterovirus RNA in clinical samples.

2. To evaluate the possible role of enterovirus infections in the different stages of beta-cell damaging process leading to T1D.

3. To evaluate the possible role of CBV 1-6 serotypes in the development of T1D

4. To evaluate the possible role of CBV 1-6 infections in development of T1D in different populations

4   SUBJECTS AND METHODS

4.1   Subjects and sample material

The clinical cohorts used in Reports II, III and IV of this thesis are based on three independent studies: The Diabetes Prediction and Prevention Study (DIPP), The Diabetes and Autoimmunity Study in the Young Study (DAISY) and Viruses in Diabetes Study (VirDiab). Samples collected in these cohorts cover various phases of the development of T1D. In the DIPP study, samples were collected from birth to the onset of T1D, while in the DAISY study children were followed from the time of autoantibody seroconversion to the development of T1D. In the VirDiab study samples were collected at the onset of T1D (Fig. 3). These study populations were recruited from a wide geographical area including children from Finland, Sweden, England, France, Greece and the USA (Fig. 4). Altogether these three studies included 2905 serum and 1242 rectal swab samples collected from 337 children who developed T1D, 90 children who were positive for T1D associated autoantibodies and 389 control children. Samples were collected over 15 years from 1993 to 2007. The methodological publication (Report I) was based on 108 stool samples selected randomly from 27 children participating in the DIPP study.
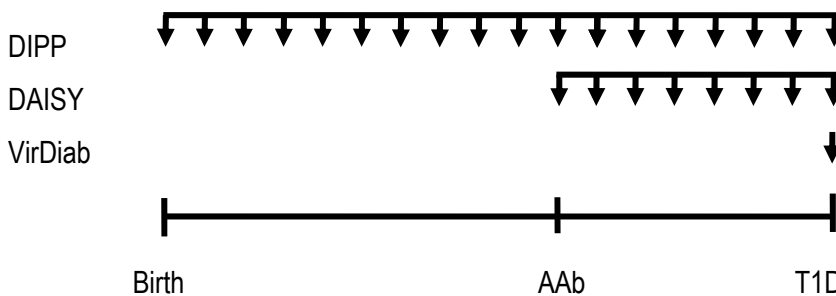
Figure 3.   Schematic presentation of sample collection in relation to T1D process in the DIPP, DAISY and VirDiab studies. Arrows indicate the time periods of sample collections related to T1D disease process.

Figure 4. Study centers on the map. The black rectangle indicates the centers of the DIPP study in Oulu, Tampere and Turku. The white triangle refers to the VirDiab study centers in Tampere, Linköping, London, Lille and Athens. The DAISY study center is indicated with a white rectangle in Denver.

4.1.1 DIPP study

The Diabetes Prediction and Prevention Study (DIPP) is a prospective birth-cohort study in which children at high or moderate genetic risk of developing T1D are followed from birth till the onset of T1D or 15 years of age. All parents with newborn infants at the University Hospitals in Oulu, Tampere and Turku are offered the possibility for screening T1D associated HLA-DQB1 alleles from a cord blood sample. Children with increased risk (HLA-DQB1*02/*0302), the *0302/x genotype [x ≠ *02, *0301, or *0602], and also boys with the genotype DQB1*02/y-DQA1*05/z [y ≠ *0301, *0302, *0602, *0603; z ≠ *0201] are recruited to the study on the consent of the parents. The protocol used in the present study has been described previously [156]. Serum samples were collected from birth at intervals of 3 to 6 months till the onset of T1D or the age of 15 years. The diabetes-associated ICA autoantibody was tested continuously from every sample and if the child became ICA positive all follow-up samples were screened also for IAA, GADA and IA-2A. After autoantibody seroconversion, the children were invited to a clinical visit every 3 months. Stool samples were collected from the high risk group every

month from the age of 3 months till two years of age. In addition, the clinical symptoms were recorded at every clinical visit using a questionnaire.

In Report I, 108 stool samples were selected randomly from 27 DIPP children aged from 3 to 24 months in Tampere DIPP center. For this period of time parents recorded the age when all new foods were added to the child's diet and this data was recorded by nurses at clinical visits at the age of 3, 6, 12, 18 and 24 months. Children were categorized in a group which was exclusively breastfed, and another group which received supplementary food.

In Report II, the frequencies of enterovirus infections were compared between case and control children at various time points during the development of T1D: 1) whole follow-up time period from birth to the onset of T1D, 2) time from birth to the time point of six months before seroconversion to autoantibody, 3) time window of 6 months before autoantibody seroconversion, 4) time period from autoantibody seroconversion to the diagnosis of T1D and 5) at the onset of T1D (Fig 5.). In this study, enterovirus analyses were carried out from all serum samples collected from case children who developed clinical T1D and from samples collected from one to six, non-diabetic and autoantibody negative control children matched for age, gender, HLA T1D associated DQ alleles, time of birth (± 1 month) and University Hospital district. A total of 333 serum samples from 38 case (boys 18) and 993 samples from 140 control (69 boys) children were analyzed.
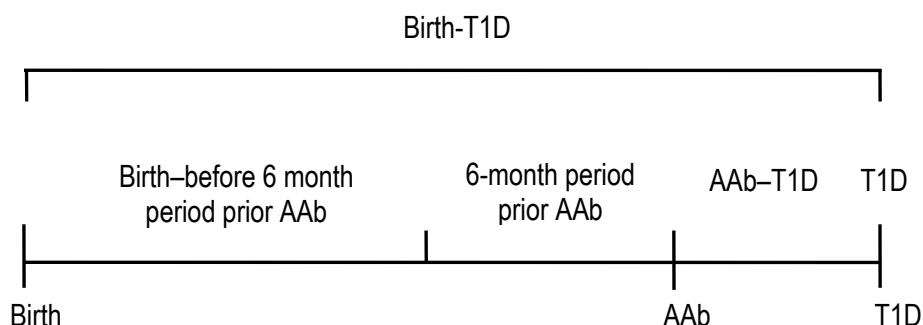
Figure 5. Outline of the analysis on role of enterovirus infections in different stages of T1D process in Report II. Birth-T1D = time from birth to onset of T1D. Birth - before 6 month period prior AAb = time from birth to 6 months before the seroconversion to positivity for the first autoantibody. 6 month period prior AAb = time window 6 months before autoantibody seroconversion, AAb-T1D = period from autoantibody seroconversion to diagnosis of T1D and T1D = at the onset of T1D.

4.1.2   DAISY study

The Diabetes and Autoimmunity Study in the Young (DAISY) study has followed two groups of children. One group was at an increased genetic risk of T1D without a diabetic relative, and in the other group of children, had a diabetic sibling or parent. Altogether, 2365 children in both groups were screened longitudinally for three islet autoantibodies IAA, GADA and IA-A2 at ages 9, 15 and 24 months and annually thereafter. Children who tested positive for at least one autoantibody were followed with 3 to 6 months intervals (median 4 months) [157].

Report III is based on a cohort of 140 children who tested positive for at least one islet autoantibody in two or more consecutive samples and who provided at least one serum or rectal swab sample for enterovirus testing. Altogether, 1081 serum and 1242 rectal swab sample were collected in 1295 clinical visits for enterovirus analyses. These samples were collected between 1993 and 2007 in Denver, United States of America.

4.1.3   VirDiab study

The Viruses in Diabetes Study (VirDiab) is a multicenter case-control study, which was carried out in five European countries during the years 2001-2005. Newly diagnosed T1D children were recruited in Tampere (Finland), Linköping (Sweden), London (England), Lille (France) and Athens (Greece). T1D children were matched pair-wise according to the time of sampling, gender, age and country. In Finland, the control children were recruited from DIPP study and they were matched with case children for the HLA-conferred risk for T1D. In other populations, control subjects were healthy school children or children from the local hospital coming for minor surgical operations. The diagnosis of T1D was based on the WHO criteria.

In Report IV, altogether 249 case-control pairs fulfilled these matching criteria and their samples were used for virus antibodies analyses. Antibodies against CBV1-6 serotypes were measured by a plaque reduction neutralization assay.

4.1.4   Study design for the analysis of PCR inhibition (I)

PCR inhibitors were tested in stool samples by spiking a standard amount of Semliki Forest Virus (SFV) RNA in nucleic acid fraction extracted from the stool sample before the RT-PCR reaction was performed. The amplification of SFV RNA in

spiked stool samples was compared to a positive control, which was a water sample spiked with the same amount of SFV RNA as the stool samples. The spiking of samples was done by mixing the SFV RNA in the master mix of RT-PCR reactions to minimize variation due to pipetting. All samples were tested with and without the addition of BSA to PCR mix to test if BSA inactivates possible inhibitors in the samples. In addition, the performances of two nucleic acid extraction kits were compared; one was based on silica columns and the other on magnetic glass particles. The definition of inhibition was categorized in three classes: the sample was considered to be completely inhibited when no amplification was observed, partially inhibited when the sample gave a maximum 25% of the signal of the positive control and not inhibited when the signal was more than 25% of positive control (Fig. 5).
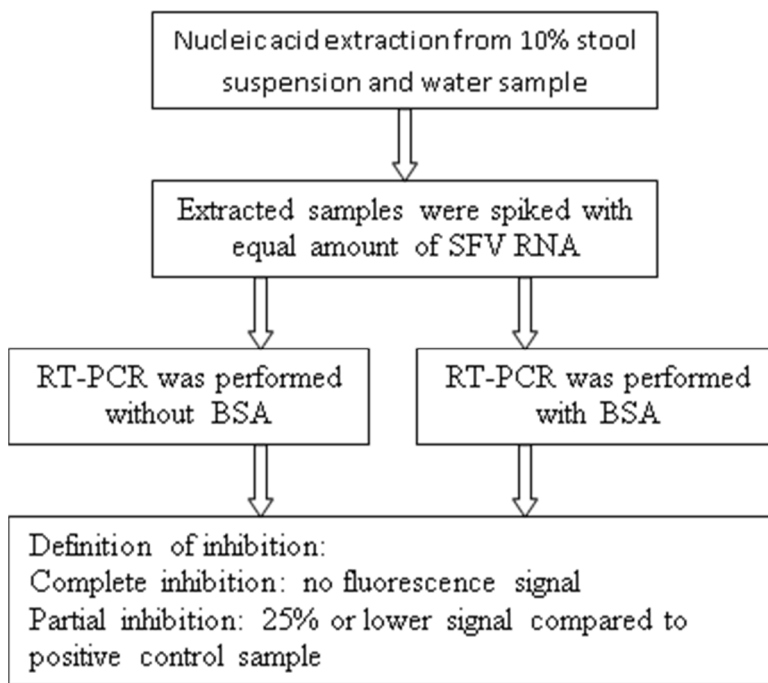
Figure 6. Schematic presentation of study design of RT-PCR inhibition test. Semliki forest virus RNA (SFV RNA) was used as a standard to estimate the amount of RT-PCR inhibition in the samples. SFV RNA was quantified by specific RT-PCR method to estimate the amount of RT-PCR inhibition in the samples. (Adapted from Fig. 1 in Report I)

4.1.5   Virus strains

In Report I, Semliki forest virus, which was used as the control virus for monitoring PCR inhibitors was obtained from the University of Turku (a gift from the department of virology and Dr. Ari Hinkkanen).

In Report IV, CBV1-6 prototype strains were obtained from American Type Culture Collection (ATCC) and in addition, wild-type viruses CBV1 and CBV3 were isolated from Finland.

4.2   Virus detection and typing methods

4.2.1   RT-PCR based methods

4.2.1.1   Viral RNA extraction method

Viral RNA was extracted from a 10% stool suspension (Report I), rectal swab solution (Report II), serum (Reports II and III), plasma (Report III) and cell culture supernatant (Reports I, II and III). Extractions were done from 140µl of the samples using a silica column based RNA extraction kit according to the manufacturer's protocol (QIAamp viral RNA kit, Qiagen, Hilden, Germany) (Reports I, II and III). A subgroup of samples in Report I was also selected for nucleic acid extraction by MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) using automated nucleic acid extraction robot according to the manufacturer's protocol (MagNA Pure LC Instrument, Roche, Mannheim, Germany) (Table 4).

Table 4. Summary of sample materials, virus detection methods and statistical methods used in the publications.

	Study			
	Report I (PCR inhibitors)	Report II (DAISY)	Report III (DIPP)	Report IV (VirDiab)
Sample type	Stool suspension	Serum, rectal swab	Serum, plasma	Serum
HLA typing	not analyzed	analyzed	analyzed	analyzed
Detection method	RT-PCR	RT-PCR ELISA (IgM, IgG, IgA)	RT-PCR	Neutralization antibody assay
RNA extraction method	Viral RNA Kit Total nucleic acid extraction kit	Viral RNA Kit	Viral RNA Kit	n.a.
Statistical method	Wilcoxon test	Cox regression Monte Carlo permutation test	Conditional logistic regression Mann Whitney test	Conditional logistic regression

n.a. = not applicable

4.2.1.2   Virus detection using RT-PCR methods

Enterovirus detection from stool suspension (Report I), rectal swab solution (Report II), serum (Reports II and III), plasma (Report III) and cell culture supernatant (Report I, II and III) was done using enterovirus specific two step RT-PCR. This enterovirus-specific method amplifies a 115bp long fragment of enteroviral 5'UTR, which was lineary quantified using a liquid hybridization assay [158]. RT-PCR amplification was done using a mixture of RT-buffer (Promega, Madison, WI, USA), 0,5mM dNPT (Pharmacia Biotech, Uppsal, Sweden), 4U of RNase inhibitor (Promega), 50pmol (4-) reverse transcriptase enzyme (Promega) and 10µl of the RNA template. The total volume of reaction was 40µl and reactions were carried out at 37°C for 60min. A parallel reaction was also performed with the addition of 0,5% BSA (Report I). The PCR reactions were performed using 0,2mM dNTP, 1U of DyNazyme DNA polymerase (Finnzymes, Espoo, Finland), 0,2µM of enterovirus-specific primers (4- and 636+bio). The total reaction volume was 100µl. PCR amplification started with denaturation of cDNA and primers at 94°C for 3min, followed by 40 cycles at 94°C for 30s, 53°C for 45s and at 72°C for 1 min. The final extension was done at 72°C for 7min [158]. A parallel reaction was also performed in Report I with the addition of 0,5% BSA in the PCR mixture.

The PCR amplicons were quantified by a liquid-phase hybridization assay using europium labelled probes (PerkinElmer, Turku, Finland). A biotinylated PCR product was first allowed to bind to a microtiter well, coated with streptavidin. After denaturation of the PCR amplicon, the well was washed to remove the free-floating DNA strand. Detection of single strand DNA was performed with a europium labelled probe specific for enterovirus sequence (PerkinElmer Wallack, Turku, Finland). Finally, the europium fluorescence was quantified in a time-resolved manner by a Victor multilabel counter (PerkinElmer Vallack, Turku, Finland) [158].

The cut-off limit for a positive sample was set to the level corresponding five times the signal of the negative control (water sample). All positive samples were re-tested and finally a sample was interpreted as a positive sample if a minimum two out of three tests were positive. All test runs included one virus positive control and two virus negative control samples. The samples were blinded without knowing the case-control status of the child.

The SFV was used as a standard RNA template for monitoring PCR inhibitors in stool samples (Report I). A specific primer pair was designed for amplification 201bp long region of the 5' noncoding region of SFV. RT-PCR and PCR reactions were performed parallel with and without BSA. PCR amplicons were quantified using a

samarium labeled SFV specific probe. This method with primer and probe sequences is described more detailed in Report I.

4.2.1.3 Sequencing analysis

PCR amplicons were purified using a MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Further extracted amplicons were sequenced with primers used in PCR (BigDye v. 3.1. Applied Biosystems) by an automated sequencer (Applied biosystems) (Report II).

4.2.2 Plaque reduction neutralization assay

The plaque reduction neutralization test was used in Report IV for quantification of neutralization antibody titers against CBV 1-6 ATCC prototypes in the VirDiab samples. The CBV1 and CBV3 antibodies were also measured against wild-type virus strains isolated from Finland. The test serum was spiked with 100 pfu of the virus and incubated for 1 h at 37°C followed by overnight incubation at RT. This mixture was pipetted on a monolayer of green monkey kidney cells (GMK) on six-well plates in a plaque assay medium containing the Minimal Essential Medium (MEM) supplemented with 1% fetal bovine serum, 40U/ml Penicillin-Streptomycin, 0,0023% Glucose, 1*L-Glutamine, 1,5mM MgCl2 and 1,5mM Carboxymethyl Cellulose (HEPES). Both virus positive and negative control wells were included in all test runs. The number of plaques was counted after incubation at 37°C for 48h. Sera were tested using 1/4 and 1/16 dilutions and the cut-off limit for a seropositive sample was more than 80% reductions of the plaques. The antibody positive series were titered for higher dilutions against ATCC CBV strains (titers 1/64, 1/256 and 1/1024). These analyses were conducted using samples with blinded identity.

4.2.3 ELISA assay

Enterovirus antibodies were measured from a subset of samples of Report II using an enzyme-linked immunosorbent assay (ELISA). These samples were collected between the years 1993-2004. Capture ELISA (EIA) was used to measure IgM antibodies against a cocktail of purified and heat treated CBV3, CAV16 and E11 viruses. In addition, IgG and IgA class antibodies were tested using a purified and

heat treated CBV4 virus and a synthetic enterovirus peptide antigen KEVPALTVETGAT-C as antigens in indirect ELISA. This synthetic peptide is common for various enterovirus serotypes. In all mentioned ELISA assays the definition of infection was based on a two-fold or higher increase in the antibody level between two consecutive samples, when reaching a signal-to-background ratio of three. The analyses were done blinded without knowing the case-control status of the child [89].

4.3   Autoantibody analyses

T1D associated autoantibodies GADA, IA-2A and IAA were analyzed with specific radiobinding assays [159] and ICA was analyzed by immunoluorescence [160]. These analyses were done in Professor Mikael Knip's laboratory at the University of Oulu, Finland.

4.4   HLA typing

The HLA-DQB1 and -DQA1 alleles were determined as earlier described [161]. These analyses were carried out at Professor Jorma Ilonen's laboratory at the University of Turku, Finland.

4.5   Statistical methods

In Report I the proportion of inhibited samples per child was calculated separately for children less than 6 months and over 6 months old and the means of both groups were compared using Wilcoxon test. A p value <0,05 was considered statistically significant.

In Report II Cox regression analysis was used for analyzing the association of enterovirus infections related to the progression of T1D during the follow-up in the DAISY study. A rapid effect model was also used to compare enterovirus positive sample intervals to the negative sample interval prior to progression to T1D progression. A cumulative effect model was used to estimate the rate of progression to T1D with the number of cumulative enterovirus infections during the follow-up time. Because only three children developed T1D in the sample intervals following enterovirus detection in serum, a Monte Carlo permutation test (10000 repeated

permutations) was applied to assess the validity of the standard inference based on the Cox regression model. All analyses were done using STATA 11 (StataCorp; College Station, TX, USA).

In Reports III and IV conditional logistic regression analysis was applied for the estimation of odds ratios and 95% confidence intervals for factors associated with T1D. The possible effect of HLA type for enterovirus infections was evaluated using Mann Whitney test (Report III). These analyses were ran by STATA 12.1 (StataCorp LP; College Station, TX, USA).

5   RESULTS

## 5.1   Presence of PCR inhibitors in stool samples (Report I).

The presence of PCR inhibitors was tested in 108 stool samples randomly collected from 3- to 24-month old children participating in the DIPP study. Complete inhibition was detected in 12% (13/108) of the samples and partial inhibition (75 – 92% reduction in the PCR amplification) in 7% (8/108) of the samples. However, 56% of the samples did not contain PCR inhibitors at all and those samples showed a similar level of the PCR signal as a positive standard (Fig. 7).
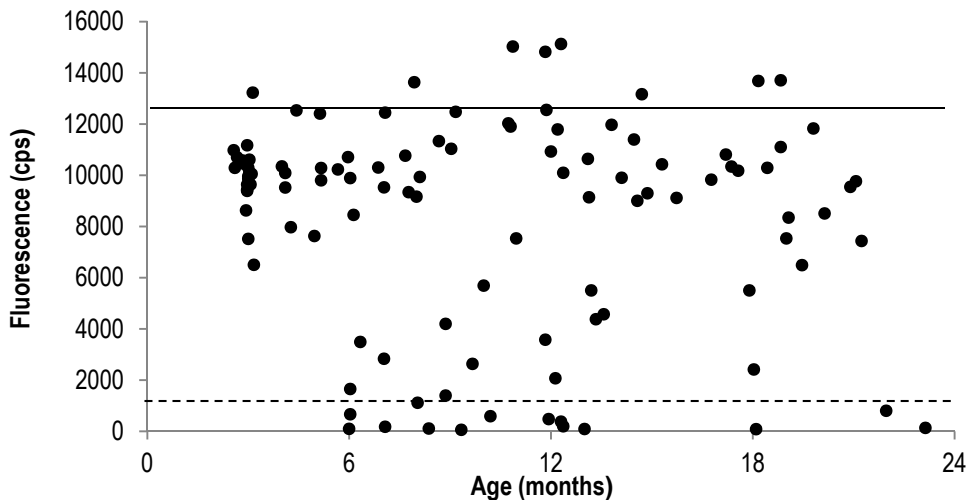
Figure 7.   Effect of the age on the occurrence of PCR inhibitors in stool samples. The black horizontal line indicates the median count of the positive control sample (amplification of SFV in water sample using RT-PCR). Samples under the horizontal dashed line are showing complete inhibition of PCR amplification. (Adapted from Figure 3 in Report I)

PCR inhibition was associated with the age of the children; none of the samples (0/31) collected from children younger than six months old were inhibited. Inhibition was detected in 23% (10/43) of the samples in the age group from six to 13 months and in 9% (3/34) in the age group from 13 to 23 months (Fig. 7). None of the 20 samples collected during exclusive breastfeeding contained inhibitors.

BSA was tested for its ability to inactivate PCR inhibitors in these stool samples. All samples were clearly positive when BSA was added to RT and PCR reactions, including even those samples which were completely inhibited when BSA was not used in the RT-PCR reactions (Fig. 8; median 182 cps without BSA vs. 8506 cps with BSA). The RT-PCR signal of the samples without inhibition was slightly weaker with BSA than without BSA, indicating that BSA slightly reduced the sensitivity of the PCR (median 11223 cps without BSA vs. 9789 cps with BSA), but the overall effect was favorable (Table 5).
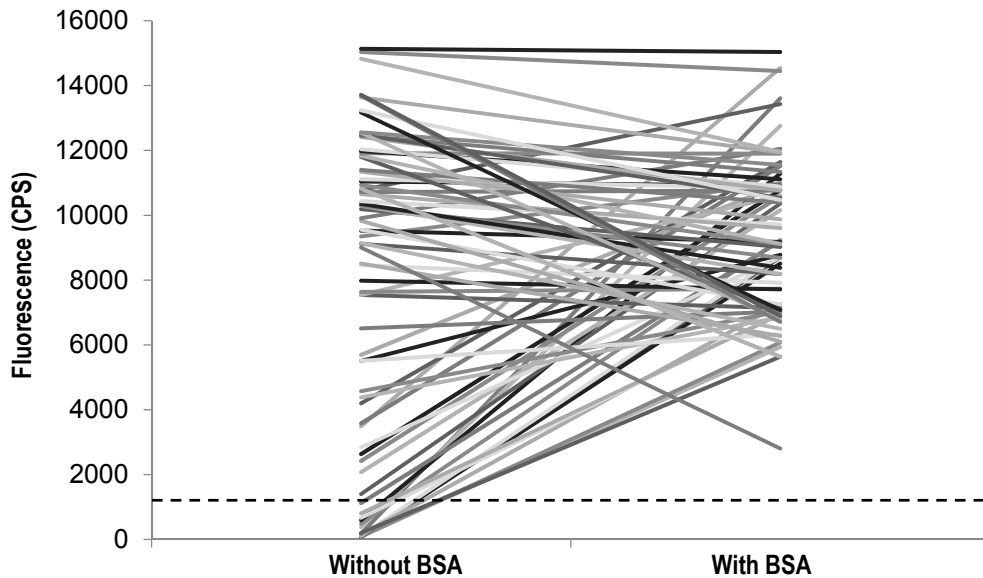
Figure 8. The effect of the addition of BSA on RT-PCR amplification. SFV RNA was amplified from stool samples by two step RT-PCR with and without BSA. The horizontal dash line indicates the detection limit of the method.

Table 5. RT-PCR signal (cps) of positive control (water) and stool samples spiked with standard amount of SFV RNA. The test was performed with and without the addition of BSA.

	no BSA mean cps (SD)	BSA added mean cps (SD)
SFV spiked water	12 268 (1 339)	10 630 (2 455)
SFV spiked stool suspension	9 790 (5 086)	9 146 (2 404)

Two commercial RNA extraction methods, based on silica columns and magnetic coated glass particles, were tested for their ability to remove PCR inhibitors using the 13 samples with the highest amounts of PCR inhibitors and 15 samples that showed no inhibition in the original screening. Nine of these samples showed complete inhibition using both methods. Four samples showed complete inhibition when silica columns were used, while the amplification was much stronger when magnetic particles were used. In contrast, another four samples showed complete inhibition when magnetic particles were used, but gave a much stronger signal when silica columns were used. Both methods removed inhibitors equally well, but some variation between individual samples was seen. The addition of BSA removed the inhibition effect of stools on RT-PCR amplification similarly, in both RNA extraction methods.

5.2 Enterovirus Infection and Progression from Islet Autoimmunity to Type 1 Diabetes (Report II).

The study population included 140 children who seroconverted at least for one islet autoantibody in the DAISY cohort. Altogether, 50 of these children developed T1D during the follow-up at a median age of 8,7 years. The presence of enterovirus RNA was analyzed from 1081 serum and 1242 rectal swab samples that were collected at clinic visits prior to the diagnosis of T1D using RT-PCR. Both sample types were available from 1028 children (collected at the same clinical visit). The median sample interval was 4 months.

Enterovirus RNA was detected in 17 serum (1,6%) and 45 rectal swab samples (3,6%) and an enterovirus infection was diagnosed at altogether 54 clinical visits (4,2%). In eight of these visits enterovirus RNA was detected simultaneously in both serum and rectal swab samples. Thirty-one out of 140 children (22,1%) were enterovirus positive at least in one serum or rectal swab sample. Most often, children were positive only in one sample, but 12 children were positive in more than one visit. The prevalence of enterovirus infections in serum or rectal swab samples was close to 10% in the age group less than 2,5 years, but it declined in the older age groups, being about 1% in children older than 7,5 years. Boys and children positive for multiple autoantibodies tended to be more often enterovirus positive.

Children who were enterovirus positive during the follow-up progressed to T1D more frequently than enterovirus negative children: 61,3% of children who were at least once enterovirus positive in the serum or rectal swab eventually developed

clinical T1D, compared to 28,4% of those who were enterovirus negative in all samples (P=0,001).

Of the 50 children who progressed to T1D during the follow-up, three children were enterovirus positive in the serum sample immediately preceding the progression to T1D (of which one was positive in both the serum and rectal swab sample), while 36 were diagnosed enterovirus negative. The sample from this time point was missing from 14 children. These three enterovirus positive children were all siblings of patients with T1D, were young at seroconversion for multiple islet autoantibodies, and two children carried the HLA DR3/4 genotype. Statistical analyses showed that the risk of developing T1D increased in the sample interval after the serum was tested positive for enterovirus RNA compared with the interval after an enterovirus RNA negative serum sample, the hazard ratio (HR) being 7,02 (95% CI 1,95-25,3). Interestingly, the presence of enterovirus RNA in a stool sample was not a risk factor for progression to T1D in the following sample interval (HR 0,79; 95% CI 0,10-5,92). Enterovirus in the serum showed a trend to be a risk for T1D, but the result was not statistically significant. The study population did not show a cumulative effect of the number of enterovirus infections as a risk for development of T1D. None of the tested serum (17 samples) and rectal swabs (14 samples) were enterovirus positive at the onset of T1D.

5.3   Enterovirus RNA in Blood Is Associated with the Initiation of Islet Autoimmunity and Development of Type 1 Diabetes (Report III).

A possible risk association between the detection of enterovirus RNA in serum and later development of T1D was evaluated in a nested case-control study carried out in the prospective birth cohort study in Finland (DIPP study). The study cohort consisted of 38 children who developed T1D during the follow-up and 140 autoantibody negative and non-diabetic control children who were pair-wise matched with case children. The proportion of children who were positive for enterovirus RNA in serum at least once during the follow-up was significantly higher in the case group, compared to the control group (32% vs. 14% of children were at least once positive for enterovirus, respectively; p<0,002). In the group of case children a total of 5,1% of the samples (17/333) were enterovirus positive compared to 1,9% of the samples (19/993) in control children (p<0,01).

The study design made it possible to analyze the frequency of enterovirus RNA positivity in different stages of the autoimmune process. In the case children, enterovirus RNA was detected more frequently in the time period ranging from birth to onset of T1D, compared to the corresponding period in the control group (OR=4,7; 95% CI 1,9-12,0). Overall, the frequency of enterovirus positivity was the lowest in the time period ranging from birth to the 6 months long period preceding the first detection of autoantibodies when 2,4% of the samples were positive in the case and 0,7% in the control group (OR=3,1; 95% CI 0,4-22,4). On the other hand, enterovirus positivity peaked in the case group during a period of six months preceding the first autoantibody positive sample, and at the time point 15,2% of the samples in the case group and 3,3% of samples in the control group were enterovirus positive (OR 7,7; 95% CI 1,9-31,5). During the time period ranging from autoantibody seroconversion to diagnosis of T1D, altogether 3,9% of the samples were enterovirus positive in case children and 2,2% in the control children (OR=3,2 [95% IC 1,1-8,9]) (Fig. 9).
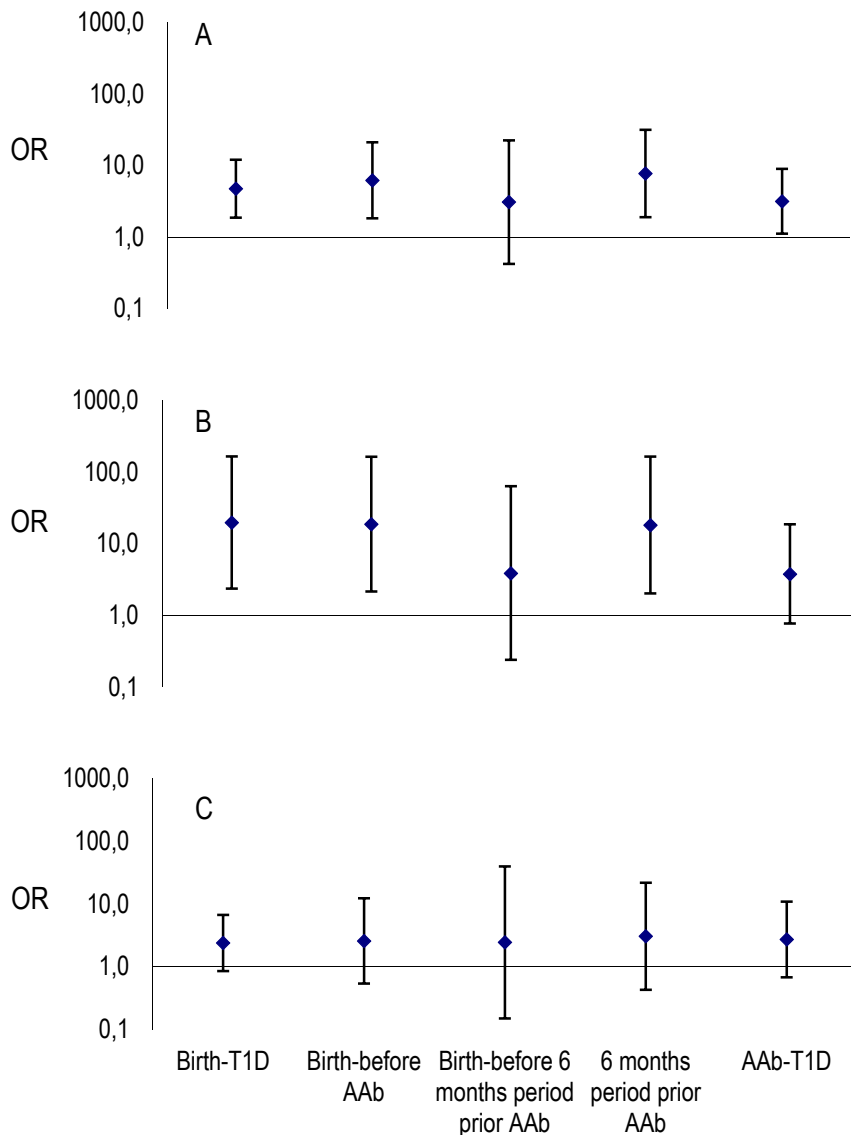
Figure 9. The association between the detection of enterovirus RNA in serum and the development of clinical T1D in different stages of the disease process. The figure shows odds ratios (black squares) and 95% confidence intervals (error bars). "Birth-T1D" = whole time from birth to onset of T1D; "Birth-before AAb" = time from birth to autoantibody seroconversion; "Birth-before 6 months period prior AAb" = time from birth to six months before autoantibody seroconversion; "6 month period prior AAb" = time window six months before autoantibody seroconversion; "AAb-T1D" = period from autoantibody seroconversion to onset of T1D. Figure A represents the whole cohort, figure B shows the data for boys and figure C for girls. (Adapted from Table 1 in Report III)

The sliding window analysis was applied to carry out time-dependent analyses of the association between enterovirus RNA positivity and appearance of islet autoantibodies. The analysis with the zero point anchored to the first autoantibody positive sample indicated the same peak in enterovirus RNA, around 6 months before autoantibody seroconversion among the case children (OR=5,6; 95% IC 1,8-18,0). At other time points such clustering of infections was not seen (Fig. 10). Analogously, when the zero-point was anchored to the onset of clinical T1D, a clear peak in enterovirus positivity was seen in case children about two years before the onset of diabetes. No such peaks occurred in the control group.
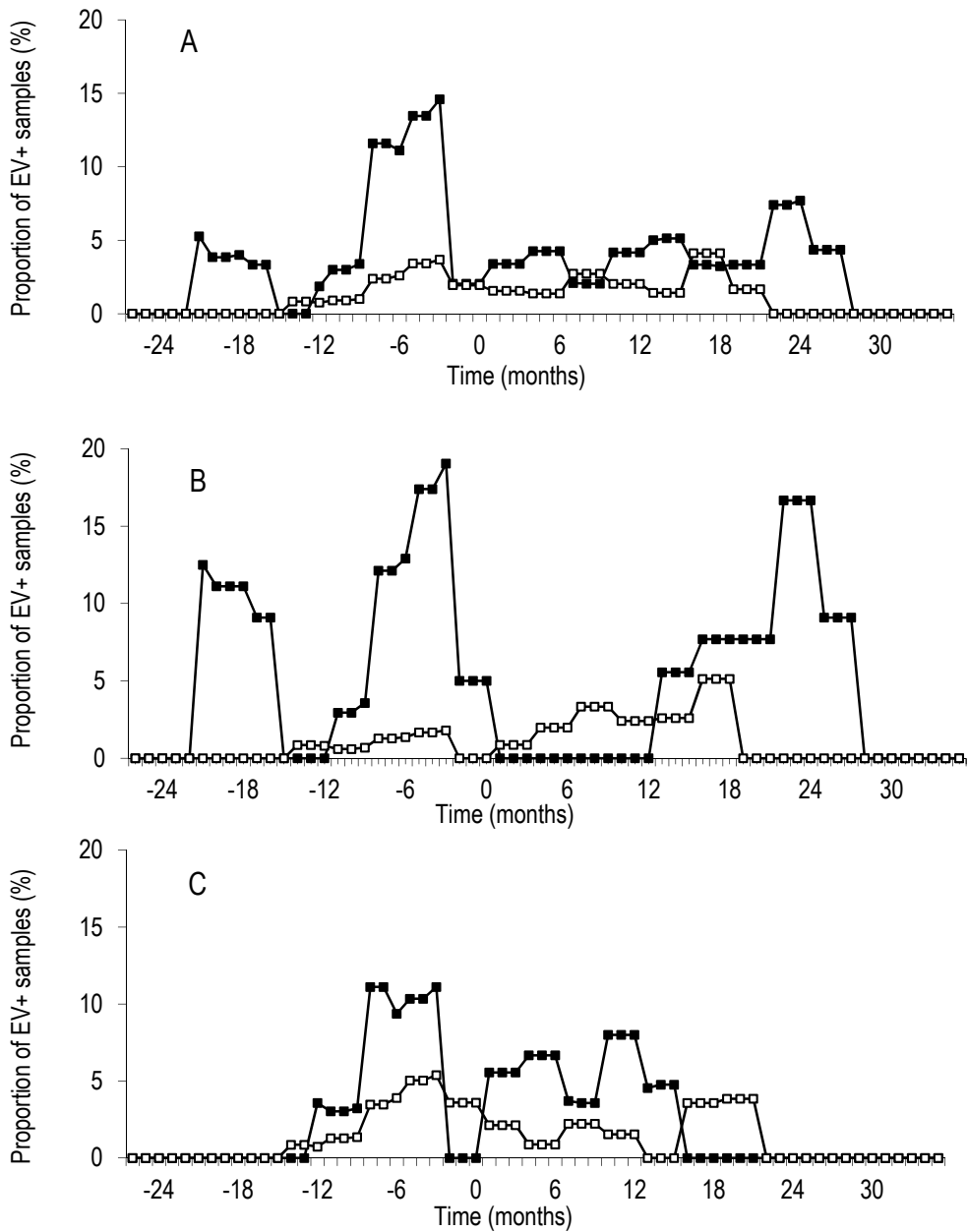
Figure 10. The occurrence of enterovirus RNA in serum across different stages of the autoimmune process in the "sliding window" analysis. Enterovirus positivity peaked six months prior to the detection of first autoantibodies. X-axis indicates time from the first autoantibody positive sample (time point 0) and y-axis the proportion of enterovirus positive samples by calculating moving average for each six-month window. Figure (A) presents the whole cohort, figure (B) boys and figure (C) girls. Black squares = case children. White squares = control children.

Enterovirus positive samples were equally frequent in boys and girls (2,5% vs. 3,1%) but the risk association between enteroviruses positivity and T1D was stronger among boys than among girls (OR=10,5; 95% CI 2,2-51,2 vs. OR=2,1; 95% IC 0,8-5,3, respectively). The stronger risk association among boys was similarly seen in infections occurring before and after autoantibody seroconversion. The highest risk was related to infections occurring in boys during the 6-month period prior to the first autoantibody positive sample (OR=18,2; 95% CI 2,0-164,5 p=0,01, in boys vs. OR=3,1; 95% IC 0,4-21,8 P<0,26, in girls).

The age of the child influenced the frequency of enterovirus RNA in serum samples. The lowest frequency of positive samples (1,0%) was found among children younger than six months, while the rate of positivity increased to 3,5% at the age of 6-18 months and to 5,0% in children older than 18 months old. At the age of two years the frequency of virus positive samples dropped to 4,3% and further to 2,0% in children older than two years (Fig. 11). The first samples which were enterovirus positive were taken at a younger age in the case children compared to the control children (median age of first enterovirus positive sample was 10 vs. 16 months, respectively).
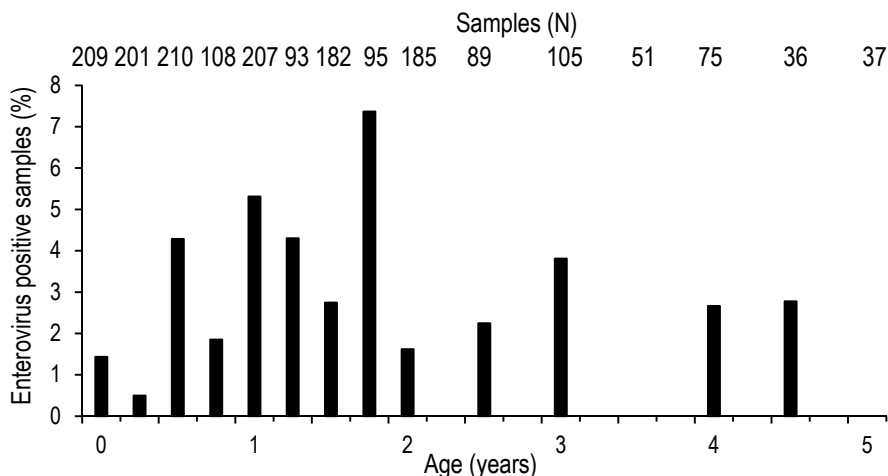
Figure 11. The proportion (%) of enterovirus positive serum samples according to the age of the child. The number of analyzed samples is shown above the columns. (Adapted from Figure 2A in Report III)

Four children (three cases and one control) had more than one enterovirus positive sample (two of these children had two positive samples and another two children had three positive samples). No signs of persistent infection were observed, because none of these samples were subsequent and also virus negative samples occurred between the positive ones. In addition, different virus genotypes were present in serially positive samples in each of these children.

Enterovirus RNA was detected during different seasons of the year, but the frequency showed some variation. The samples taken during the spring months (February-May) were characterized by low frequency of the virus (1,4 - 2,2%), while the rate of positivity started to increase in August peaking to 5,6% in September (Fig. 12).
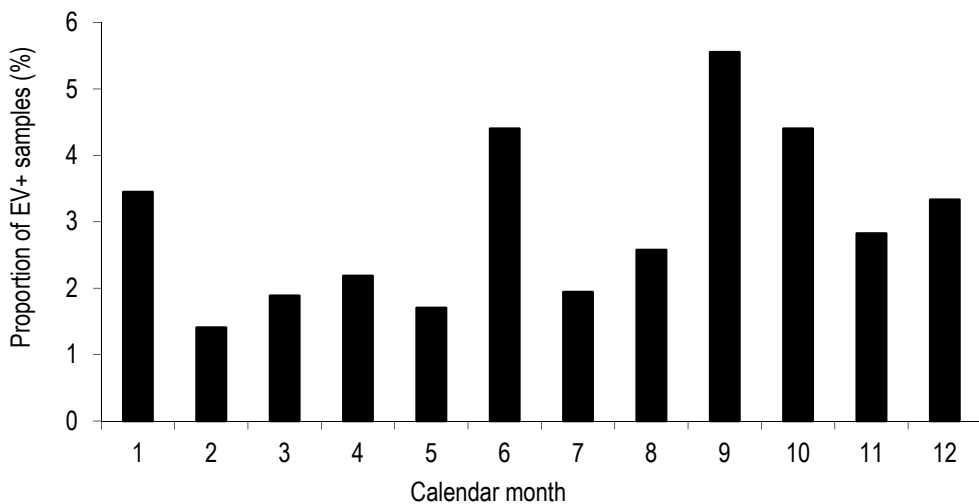

Figure 12. Proportion (%) of enterovirus positive serum samples according to the calendar month of sample collection.

5.4 Virus Antibody Survey in Different European Populations Indicates Risk Association Between Coxsackievirus B1 and Type 1 Diabetes (Report IV).

Antibodies against the CBV1-6 serotypes were analyzed in newly-diagnosed T1D patients and controls using a plaque neutralization assay. Patients and controls were recruited in Finland, Sweden, England, France and Greece. CBV reference strains were used in the antibody assay for all CBVs. In addition, wild-type strains of CBV1

and CBV3 were also used to compare the antibody response to reference and wild-type virus strains.

The prevalence of neutralizing antibodies against CBV1 and CBV6 was lower than that of antibodies against CBV2, CBV3, CBV4 and CBV5. CBV antibodies varied also considerably between the countries. Finland was an exception since the prevalence of serotypes CBV 1-5 were considerably lower in Finland than other countries. CBV6 was relatively rare in all countries (Fig. 13).
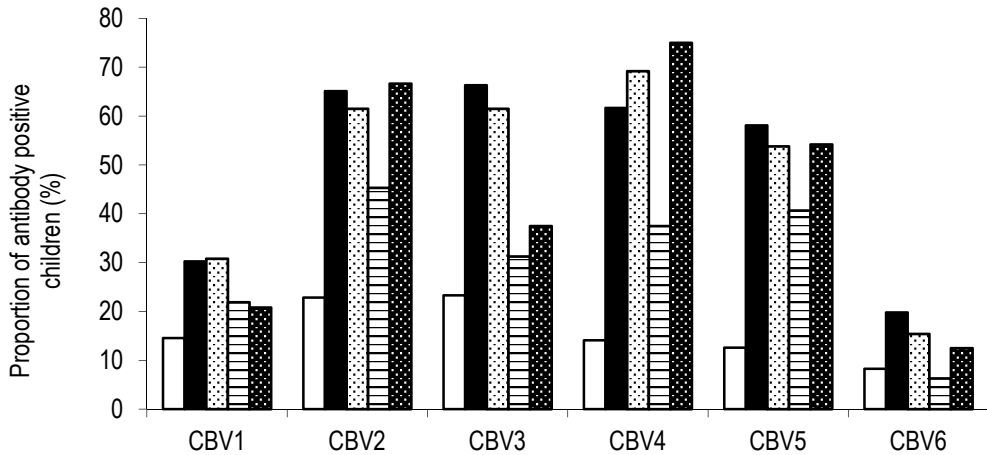

Figure 13. Proportion (%) of children who were seropostive for different CBV ATCC prototype strains in different countries. White bars represent Finland, black bars France, white dotted bars England, horizontally striped bars Greece and black dotted bars Sweden. (Adapted from Figure 1 in Report IV)

The proportion of CBV seropositive children increased according to age. At the age of less than two years 75% of the children had no antibodies against CBV viruses, while at the age of four years 46% children were negative for all CBV viruses. The prevalence of antibodies increased further at older ages and a plateau was reached at the age of eight years, when only about 10% of the children were still negative for all CBV viruses and close to 50% of the children had antibodies against at least three different CBV serotypes (Fig. 14).
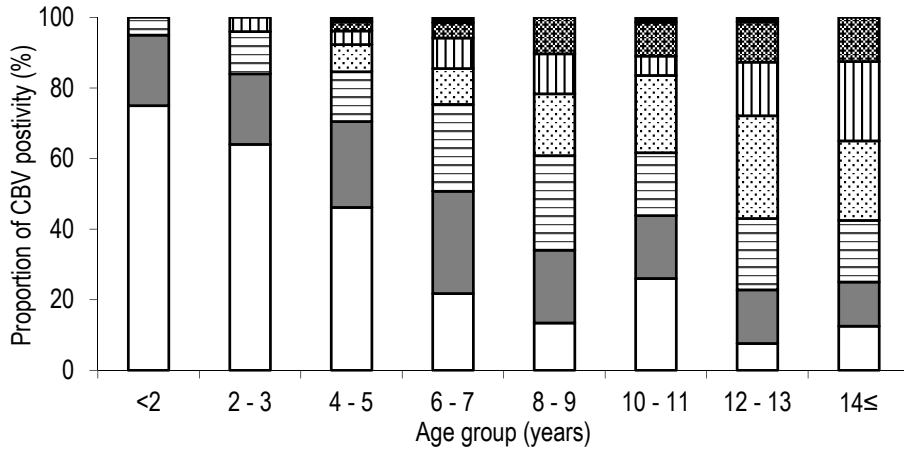
Figure 14. Positivity for CBV (1-6) serotypes in various age groups. The 100% stacked columns present relative proportions of children according to the number of seropositivity against the six different CBV serotypes. White bars represent children without any CBV infections; gray bars, positivity for one CBV serotype; horizontally striped bars, positivity for two CBV serotypes; white dotted bars, positivity for three CBV serotypes; vertically striped bars, positivity for four serotypes; black dotted bars, positivity for five serotypes and black bars proportion of children positive for all six different CBV serotypes. (Adapted from Figure 2A and B in Report IV)

The prevalence of CBV 2, 3, 4 and 5 antibodies increased by the age of children, and at the age of 8 years the prevalence of each of these viruses ranged from 50% to 70%. Contrary to these serotypes, the prevalence of CBV1 and CBV6 antibodies did not increase so clearly by the age but varied between 15-30% (CBV1) and 0-15% (CBV6) in all age groups. Interestingly, the first antibodies against CBV5 and CBV6 were detected in as late as four year old children (Fig. 15).
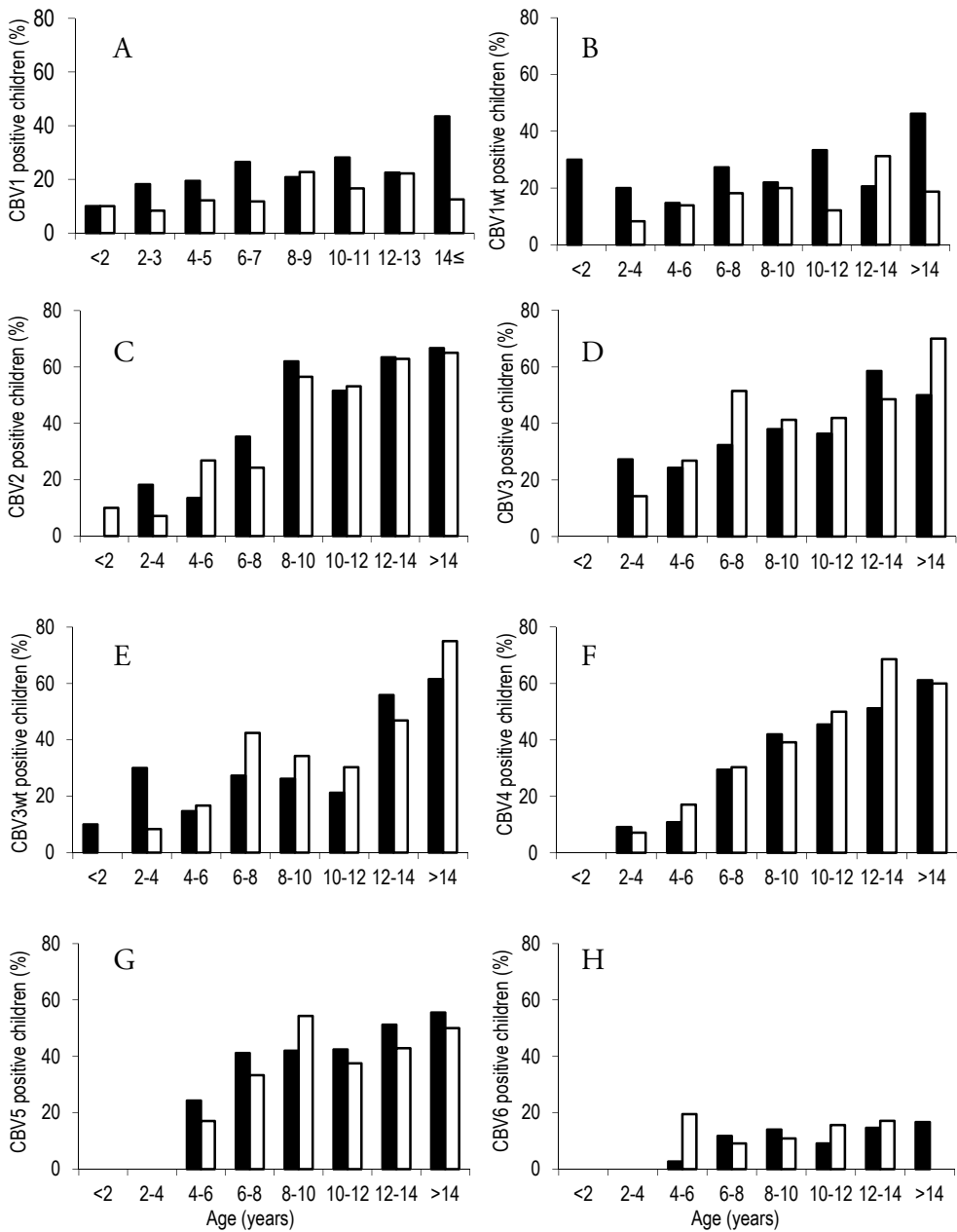
Figure 15. Proportion (%) of children positive for CBV1-6 antibodies in different age groups. Black bars indicate the case and white bars control children: (A) children positive for prototype CBV1 and (B) wild type CBV1 strain, (C) CBV2, (D) CBV3, (E) wild type CBV3 strain, (F) CBV4, (G) CBV5 and (H) CBV6 strain. (Adapted from Figure 2B in Report IV)

The antibodies against the CBV1 reference strain were more frequent among diabetic children than among controls (24% vs. 16%; OR=1,7; 95% CI 1,0-2,9; p=0,04; Table 6.). This result was confirmed by testing antibodies against wild-type CBV1 strain (OR=1,8; 95% CI 1,1-3,0; p=0,03). The risk character of CBV1 was not associated with HLA genotype, age or gender of the child. Other CBV serotypes did not differ between the case and control groups. Children with T1D-associated HLA-DQ genotypes were less frequently positive for CBV2, CBV4 and CBV5 than children with other HLA-DQ. The CBV antibodies were compared between case and control children separately in Finland and in other countries. None of the CBV2-6 antibodies differed between case and control groups in either of the tested populations, but CBV1 antibodies showed a trend for increased frequency in case subjects both in Finland and in other countries (OR=2,2; 95% CI 0,8-5,7 and OR 1,5; 95% CI 0,8-3,0, respectively).

Table 6. Neutralizing antibodies against CBV 1-6 serotypes in children with T1D and in matched control subjects. (Adapted from Table 2 in Report IV)

| | Antibody prevalence (%) | | | | |
	T1D (N=249)	Controls (N=249)	OR	95% CI	*P*
CBV1-all	28,5	18,5	1,7	1,06-2,74	0,03
CBV1-ATCC	24,4	15,9	1,7	1,02-2,92	0,04
CBV1-wt	24,9	17,3	1,8	1,05-3,00	0,03
CBV2-ATCC	44,9	42,7	1,1	0,73-1,72	0,59
CBV3-all	37,3	40,6	0,8	0,55-1,20	0,29
CBV3-ATCC	37,2	40,3	0,8	0,56-1,24	0,38
CBV3-wt	30,0	33,7	0,8	0,54-1,32	0,45
CBV4-ATCC	35,5	38,4	0,9	0,52-1,46	0,60
CBV5-ATCC	38,0	34,5	1,1	0,69-1,90	0,61
CBV6-ATCC	10,3	11,6	0,8	0,42-1,45	0,44

ATCC = prototype virus strain from American Type Culture Collection; wt=wild type virus strain
CBV- all = antibodies against either ATCC or wt virus strain

6 DISCUSSION

6.1 RT-PCR inhibitors in stool samples

PCR inhibitors can be a problem when microbes are detected or quantified in clinical samples using PCR-based technologies [162, 163]. In the methodological study (Report I) PCR inhibition was tested using RNA extracts from stool samples which were spiked with the standard amount of viral RNA. Semliki Forest virus (SFV) RNA was used in this experiment since this virus should not be present in human samples. The amplification of viral RNA in these samples was compared to water samples which were spiked with the same amount of viral RNA. Significant inhibition was seen in 19% of the analyzed samples suggesting that PCR inhibitors were frequent in stool samples. Previous studies have shown variable estimates for the proportion of inhibited samples, the highest numbers being up to 44%, but some studies did not detect any inhibition at all. The comparison of different studies is challenging, because standardization of the criteria for inhibition is not possible between studies.

Report I showed for the first time that PCR inhibition depends on the age of the study subject as it was not seen in stool samples collected before the age of six months. One of the most likely explanations for this phenomenon is related to diet and maturation of the gastrointestinal system: PCR inhibition did not exist during a simple diet consisting of breast milk, while a more complex diet consisting of additional food items was associated with inhibition. Thus, dietary factors themselves or diet-associated microbial changes in the gut may lead to an accumulation of PCR inhibitors in stools.

In Report I, two different nucleic acid extraction methods were tested: one was based on magnetic beads (MagnaPure LC Total Nucleic Acid Isolation Kit) and the other on silica columns (Qiagen Viral RNA Kit). Both methods removed PCR inhibitors equally well but some variation was seen in individual samples suggesting they may eliminate different kinds of PCR inhibitors during RNA extraction. This finding may also partly explain the variation in the frequency of samples containing PCR inhibitors (range 0-44%) observed in different studies [164-166] and shows that inhibition is difficult to remove from nucleic acid extracts by silica membrane or

magnetic beads, which are both commonly used in microbial detection. Altogether, this finding indicates that careful optimization of the PCR reaction is a critical step in the development of reliable molecular methods for the detection of microbes in clinical samples [163].

We optimized the PCR method by adding BSA to both RT and PCR reactions to eliminate the effect of inhibitors as suggested in a previous study [167]. This addition had two opposite effects: all tested stool samples became positive when BSA was added, including also those which were totally inhibited without BSA. On the other hand, the addition of BSA decreased the efficacy of PCR amplification in some of those samples, which did not contain any or only minor amounts of PCR inhibitors (Fig 8.). In summary, even though the addition of BSA reduced the sensitivity of the RT-PCR method, it also effectively inactivated RT-PCR inhibitors and therefore decreased the amount of false negative samples. Based on this, we confirmed an earlier finding that the addition of BSA would be a useful way to eliminate false negative PCR results in the analysis of clinical samples [168].

The possible effect of inhibition also needs to be considered in the interpretation of the real time PCR results, which may not actually be quantitative, because amplification of the template is a sum of concentration of the template and presence of PCR inhibitors (Fig. 16). In addition, other factors such as primer mismatching, variation in the efficacy of nucleic acid extraction and pipetting errors can all affect the quantification of the template [163, 169]. This may have crucial consequences in several PCR applications; it may cause false negative results in detection of microbes or lead to the underestimation of virus load in the sample, which, in turn, may lead to inaccurate treatment of the patient. This emphasized not only the importance of optimization of the PCR method for each type of sample material, but also the importance of the use of internal controls in all tested samples to detect the possible presence of inhibition [162].
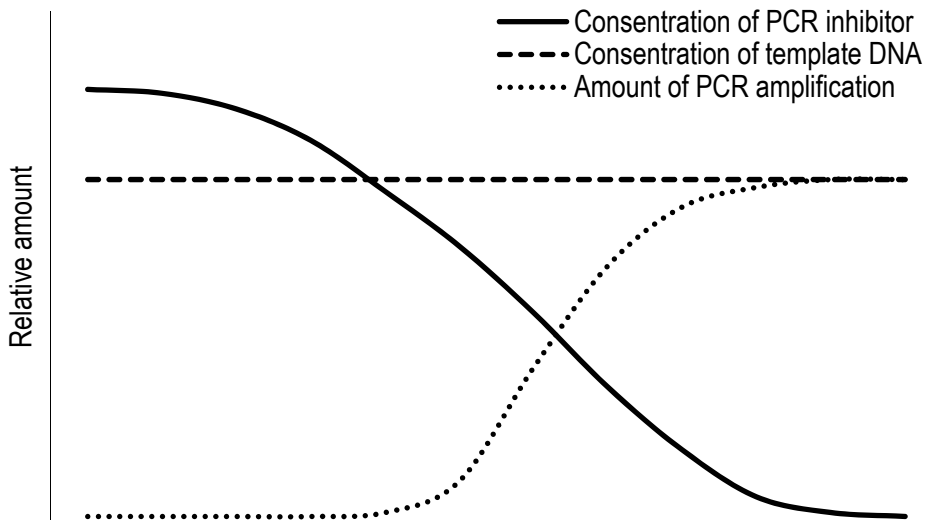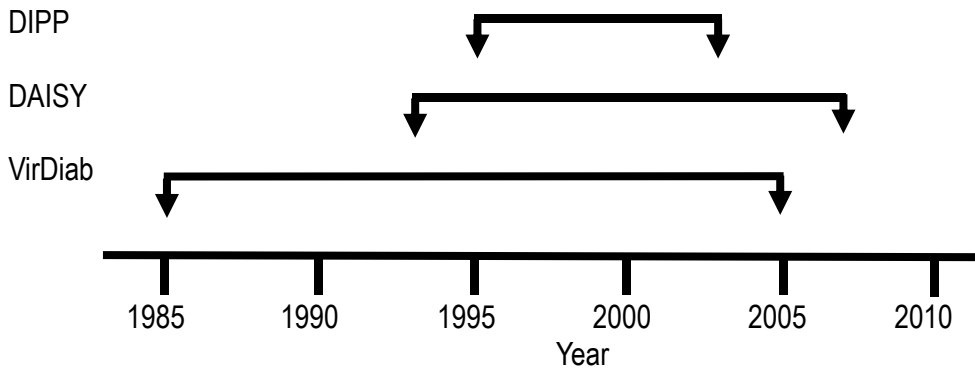
Figure 16. A schematic presentation of the effect of PCR inhibitors on the amplification of DNA template in PCR reaction.

6.2 Strengths of the combination of the study populations and various methods applied

The three populations used in this thesis create a unique possibility to study the possible role of enteroviruses in different stages of the beta-cell damaging process leading to the development of T1D. One important point is that these study populations are independent, which gives more power for generalization of the results. The strength in combining these study series is that the samples were collected in different phases of the T1D process. Altogether, these series consisted of 337 children who developed T1D, 90 children who were positive for T1D-associated autoantibodies and 389 control children, resulting in a final sample size of 2905 serum and 1242 rectal swab samples. This is large cohort compared to earlier studies, which included from 14 to 206 case and from 24 to 196 control children (Table 2 and 3). The combination of these series also covers a large geographical area both in Europe and in North America, which makes it possible to generalize the results, at least to a certain extent to western countries, where the prevalence of T1D is generally quite high. In addition, the series included study subjects from Finland which has the highest incidence of T1D in the world. Time-wise the sample

collection covers a time period of more than 15 years. Neutralizing antibodies (Report IV) stay elevated long after the infection (at least a decade), which makes it possible to screen the whole enterovirus infections history of a child from one sample (Fig. 17). Due to these reasons the study population actually covers more than 20 years from 1985 to 2007. Besides a neutralization assay and ELISA assay, another indirect detection method was used for the analysis of enterovirus antibodies (Report II). In addition to serological methods, a direct virus detection method was applied to detect enterovirus RNA using RT-PCR from serum (Reports II and III) and rectal swab samples (Report II). In summary, the combination of these three independent populations and use of several kinds of methods in the diagnosis of enterovirus infections created a powerful setup to address the question concerning the possible role of enteroviruses in the pathogenesis of T1D.

Figure 17. A schematic presentation of the years covered by the assays used to diagnose enterovirus infections in different study series of the present study.

6.3   Human enterovirus infections in T1D

6.3.1   Enterovirus infections in boys and girls

In the DIPP study enterovirus positive serum samples were equally frequent in boys (2,5%) and girls (3,1%). In the DAISY study enterovirus RNA was detected more often in boys than in girls in stool samples (5,5% vs. 1,5%), but in serum samples such a difference was not seen (1,7% vs. 1,4%). However, the risk association

between enterovirus infections and T1D was stronger in boys than in girls: in the DIPP study this risk association was particularly seen in boys who were enterovirus positive 6 months before the detection of the first autoantibody. In addition, in the DAISY study all three children, who developed T1D soon after the detection of enterovirus RNA in serum, were boys. This is in line with an earlier finding that maternal enterovirus infection was a risk factor for the development of T1D in boys, but not in girls [170]. Similarly, an enterovirus surveillance report in USA showed that boys predominated among patients aged under 20 years [171]. Thus, it is possible that enterovirus infection spreads also to the pancreas more readily in boys than in girls.

6.3.2   Seasonality of enterovirus infections and autoimmunity

In the DIPP cohort enterovirus RNA was detected in serum most frequently during the autumn and winter months. The highest prevalence was seen in samples which were taken in September (5,5%) and the "high season" continued until January. However, enterovirus RNA was detected in serum all year around. During the spring and summer months 1,5-2,0% of serum samples were enterovirus positive. The only exception to this pattern was June, when the frequency was slightly higher (4,5%). Similar seasonality has also been observed earlier in stool samples collected in the DIPP study[172].

Interestingly, the appearance of islet autoantibodies in DIPP children followed a similar seasonal pattern [172]. Assuming that enterovirus infections play a role in the initiation of the autoimmune process, these findings suggest that autoantibodies are induced quite rapidly after the infection and with a relatively constant lag-period after the infection. In fact, such a short lag time has been observed in diabetes-susceptible mice, which overexpress the autoantigen three days after enterovirus infection and further develop autoantidobies and hyperglycemia [173, 174].

6.3.3   Enterovirus infections in various age groups of children

In Finland, breastfeeding lasts for an average of eight months. This period is important since it provides protection against enterovirus infections by transferring maternal enterovirus antibodies to the child [175]. In addition to the breast milk, maternal antibodies are also transferred to the child via the placenta. In fact, the presence of maternal antibodies may be one reason for the low frequency of

enterovirus RNA in serum in infants younger than 6 months in the DIPP study, and the clear increase in virus positivity at the age of 12-18 months when maternal antibodies are not any longer present (1% vs. 5% of the samples were enterovirus positive in these two age groups). A similar increase has previously been observed at this age in the prevalence of enterovirus positive stool samples [23, 171]. However, the frequency of virus positive sera decreased again in children older than 2 years (2% of samples were positive). As infections are still common at this age, the decrease may be due to a more effective clearance of infections as the child's immune system has matured and become more effective. This hypothesis got support from the DAISY study, in which a similar trend was seen: the highest proportion of enterovirus RNA positive serum and stool samples (10% were positive altogether) was seen before the age of 2,5 years while the prevalence subsequently decreased to about 1% in children older than 7,5 years. In the DIPP study, the age when enterovirus RNA peaked in serum samples overlapped with the age when the incidence of autoantibody seroconversions peaked. Thus, there seems to be a susceptibility period when enterovirus infections frequently lead to a systemic spread of the virus in young children. At this age, children may be particularly susceptible for a viral spread to the pancreas and possible diabetogenic effect of the virus. The fact that the incidence of autoantibody seroconversions occurs also at this early age supports this hypothesis.

6.3.4   Role of enterovirus infections in different phases of T1D disease process

In the DIPP and DAISY cohorts enterovirus RNA in serum was more common in children who developed T1D than in their matched control subjects. In the DIPP study the strongest risk association was seen in the time frame spanning 6 months prior to the detection of the first autoantibodies. MIDIA study showed similar tendency in sample taken in the autoantibody seroconversion interval [100]. In addition, case children had more enterovirus infections during the time period spanning from autoantibody seroconversion to T1D. MIDIA study did not show similar association [100]. In the DAISY cohort children were followed after the detection of the first autoantibodies until they developed T1D. The same phenomenon was also seen in these analyses as the detection of enterovirus RNA in serum after autoantibody seroconversion was associated with a more rapid progression to T1D. An especially high progression risk was seen soon after virus detection (in the next sample interval following enterovirus positive sample). These studies emphasize the temporal association of enteroviruses both in initiation of the

autoimmunity and close to the onset of the disease. This kind of time-relationship between enterovirus infections and the appearance of islet autoantibodies has also been observed in our previous studies suggesting that enterovirus infections may play a role in the initiation of the beta cell damaging process [172]. Interestingly, earlier results based on RT-PCR method from DAISY study did not show connection between T1D process and enteroviruses[101]. The difference between these studies can most likely to be explained by the fact that earlier DAISY study analysed only serum samples from 13 children and these results were not analysed separately from saliva and stool samples, therefore the frequent enterovirus positivity of stool and saliva samples masked analysis of plasma samples. Our findings from DIPP and DAISY studies emphasize the possible role of enterovirus infections also in the acceleration of the beta cell damaging process. As far as I know, this is the first time that such a temporal association has been found. The result fits well with the hypothesis that serial hits by consecutive enterovirus infections may lead to cumulative beta cell damage that eventually progresses to clinical T1D (Figure 18).
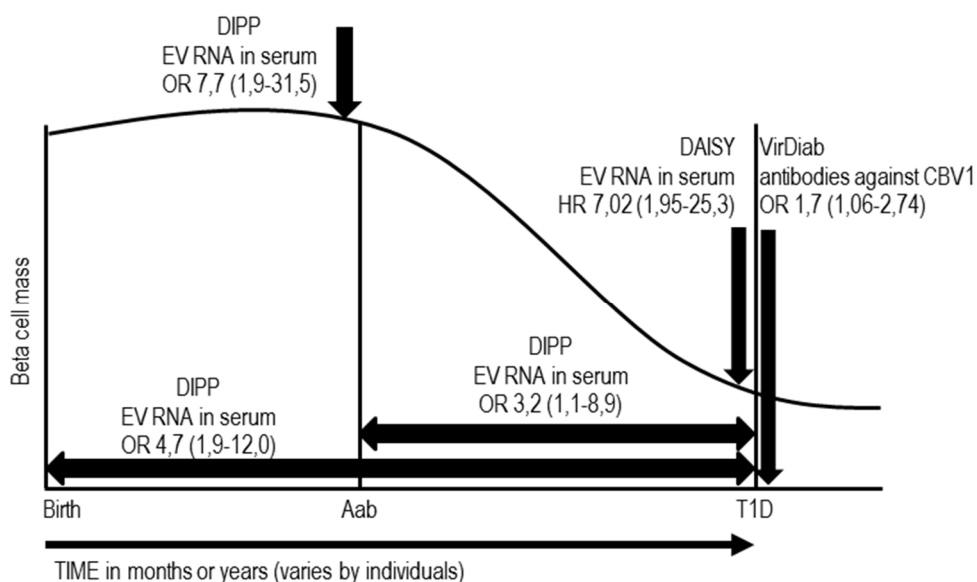
Figure 18. The summary of the main findings of enterovirus associations to T1D process in DIPP, DAISY and VirDiab cohorts of the present study.

Interestingly, all samples that were collected at the onset of T1D were enterovirus negative in both the DIPP and DAISY studies. Similarly, all the serum and blood samples tested for enterovirus by RT-PCR in VirDiab populations at the onset of

T1D were negative (unpublished data). This result is in conflict with the majority of other published studies. Collectively, nine such studies have been published, most of them based on samples collected soon after the onset of the clinical disease, and altogether an average of 31 % of the patients and 6 % of the control subjects had been positive for enterovirus RNA in serum or whole blood samples [108, 110-114, 116, 131, 176, 177]. It is difficult to find an explanation for this conflict, but it is unlikely that this contrast between our studies in Finland, the USA, Sweden, the UK, France, and Greece reflects population differences, because some of the earlier studies have been done partly in the same countries. It is more likely that the reason is related to methodological differences in sample collection, RNA extraction, primer design or RT-PCR optimization.

It is obvious that due to the short viremic phase, which usually ranges from a few days to about 2 weeks, the number of enterovirus infections is largely underestimated when a viral genome is detected in serum or blood in the follow-up of studies [24, 178]. Assuming that the analysis of each serum sample by enterovirus RT-PCR can detect infections during the previous 14 days, the serial serum sample collection covered only 5,1% of the whole follow-up time of case children in the DIPP study (2,1% of control children). Similarly, in the DAISY study sample collection covered about 5,8% of the whole follow-up time. In addition, the duration of viremia may be even shorter than 2 weeks, especially in older children, which further decreases the sensitivity to detect viral RNA in the follow-up sera. When the predicted length of viremia and the observed frequencies of enterovirus RNA in serum in different stages of the beta-cell damaging process are taken into account, one can estimate that the total number of infections in the DIPP study could have been about 1,4 viremic infections per year among case children and 0,6 infections per year in control children (N of positive samples / detection period covered by the collected samples / follow-up years). As the mean age of onset of T1D was 2 years and 10 months, this prediction leads to a total average of 3,7 viremic enterovirus infections experienced before the onset on T1D and 1,6 infections in control children. This estimation demonstrates that the true number of viremia episodes has been higher than observed in the randomly collected follow-up samples in DIPP and DAISY cohorts. Therefore, it is possible that enterovirus infections may also be involved in a higher proportion of T1D cases than detected in these cohorts.

It is possible that the difference in the prevalence of enterovirus infections between case and control children reflects prolonged enterovirus viremia rather than increased frequency of infections in the case group. This could be due to a weaker immune response in pre-diabetic children than in control children. However,

previous studies have suggested that the immune response to enteroviruses is actually higher in children who carry T1D associated HLA genes [179], which does not support this hypothesis. In addition, it is also possible that prediabetic children could have a persistent or chronic infection in the pancreas or other organs, which could lead to increased detection of viral RNA in peripheral blood. The possible occurrence of a persistentor prolonged enterovirus infection in gut mucosa was suggested in recent studies where enterovirus protein and RNA were detected in duodenal biopsies collected from T1D patients. Viral RNA was detected more often in cases than in control subjects (20-75% vs. 10-22% of subjects were virus positive in immunohistochemistry and 50-74% vs. 0-29% in in situ hybridization) [124, 180]. In addition, the pancreatic islets of T1D patients have been found to be more often enterovirus positive than those of control individuals by immunohistochemistry (61% vs. 6%) [122]. However, detection of enterovirus RNA in serum in the present study did not provide evidence of chronic or persistent infection since the virus strains in serially positive children was different at different time-points and virus negative samples were found between virus positive samples.

In the DAISY study enterovirus RNA was also tested from rectal swab samples, but virus positivity in rectal swabs did not show a risk association with the development of T1D. Enterovirus RNA is detected commonly in stool samples and the frequency of "background" infections is high (nearly 10% of rectal swabs were enterovirus positive in children younger than 2,5 years in the DAISY study). Thus, it is possible that the high detection rate of different enteroviruses in stools, in general, may mask a possible excess of a specific group of enteroviruses in case children (our RT-PCR amplified all different enterovirus types). In addition, many enterovirus infections are restricted to mucosal surfaces and are not invasive, and such infections may not be able to spread to the pancreas and cause beta-cell damage. Thus, detection of enteroviruses in stools may be a weaker marker of the development of T1D compared to viremia, which allows the spread of the virus to susceptible secondary replications sites such as beta cells in the pancreas. Another possible explanation is that the primary replication of diabetogenic viruses may occur in the respiratory track, and it may be challenging to detect these viruses from rectal swabs.

In the DAISY study enterovirus specific antibodies were tested from serum by ELISA. Infections which were diagnosed by these antibody analyses were not associated with the progression of islet autoimmunity to T1D. This finding parallels the results from enterovirus detection in rectal swabs, and it is again possible that background infections, which are often not viremic, may mask the possible effect of

T1D-associated infections since broadly reactive pan-enterovirus ELISA assays were used.

These results suggest that the presence of enterovirus in serum, which indicates invasive infection, may be associated with initiation and progression of islet autoimmunity. However, detection of enterovirus RNA in rectal swabs or diagnosis of enterovirus infections by serological assays was not associated with progression of islet autoimmunity to T1D. In the present study these assays were not used to evaluate a possible association between enterovirus infections and initiation of islet autoimmunity. Our previous studies in the prospective DiMe, DIPP and TRIGR cohorts suggest that serologically verified enterovirus infections are also associated with the initiation of islet autoimmunity [87, 99, 132, 181], and recent still unpublished findings from the DIPP study suggest that detection of enterovirus RNA in stools also precedes the appearance of islet autoantibodies (Honkanen et al. unpublished data).

6.3.5   Role of enterovirus persistence in type 1 diabetes

Enterovirus persistence has been suspected to play a role in the pathogenesis of T1D. This hypothesis has been supported by the high prevalence (61%) of enterovirus protein in the pancreatic islets of recent-onset T1D patients, but only in a minority (6%) of control subjects [122]. In the present studies no evidence of persistent infection was found, as enterovirus was not detected in consecutive serum samples and none of the enterovirus strains which were detected from repeatedly positive children were of the same genotype (DIPP or DAISY studies). However, these results cannot exclude the possibility of persisting infection, because virus replication in the pancreas seems to occur at a very low level and in such cases the infection may generate only small amounts of viruses into the blood. Thus, the virus load can be under the detection limit of the RT-PCR methods when serum samples are tested [21]. In addition, it is not known if viral persistence in the pancreas could lead to viremia at all, and therefore the virus may not be detectable in peripheral blood even though it can be detected in the pancreas and intestine of T1D patients as suggested earlier [122-124, 180].

6.3.6   Enterovirus serotypes associated with the development of type 1 diabetes

Enterovirus in serum was found to be a risk factor for the development of T1D both in DIPP and DAISY cohorts. Unfortunately, genotyping of these enteroviruses by sequence analysis from serum samples is challenging due to the very low amount of virus in the serum. Therefore, it was not possible to genotype the viruses by sequencing the VP1 region of the viral genome either in the DIPP or DAISY cohorts. In addition, even if genotyping would have been possible, the number of enterovirus positive samples was too low to compare the risk effect of enterovirus genotypes between case and control children. In fact, it may be difficult to identify possible T1D-associated enterovirus genotypes by sequencing, due to the limited statistical power of such studies. However, other methods can be used to address this question. Analysis of neutralizing antibodies is one good option since it provides information about the exact type of enteroviruses and also covers those infections which are not diagnosed by direct virus detection due to non-optimal timing of the sample. Laitinen et al. has carried out a wide screening of neutralizing antibodies in the DIPP cohort which partially overlapped the cohort analysed in Report III. They used a plaque neutralizing assay to measure antibodies against 41 different enterovirus serotypes and observed a temporal association between CBV1 infections and the appearance of islet autoimmunity at the same time point when enterovirus RNA peaked in serum in the present study [132]. These results suggest that CBV1 may be one of those enterovirus types which may induce islet autoimmunity. It may have caused the peak in the detection of enterovirus RNA a few months before autoantibody seroconversion as observed in the present study (Report III).

The serotype specific risk association between various CBV serotypes and T1D was evaluated in the VirDiab study using the same plaque neutralization assay as in the study by Laitinen et al. (Report IV). The risk effect of CBV1 was again detected, but other CBVs were not linked to development of T1D. Laitinen et al. found a similar risk association for CBV1 in the Finnish child population, but in addition, they observed a protective association for CBV3 and CBV6. Thus, the results of Report IV are in line with the observations by Laitinen et al., suggesting that CBV1 may induce islet autoimmunity. The significance of this finding is emphasized by the fact that it was made in two independent studies which were based on different populations and study designs, and also the antibody analyses were done in two independent laboratories [132].

The prevalence of CBV1 antibodies was quite low in the VirDiab cohort, which suggests that only part of the T1D could be related to these infections. However, two observations suggest that the prevalence of CBV1 infections may be underestimated: Firstly, it has been shown that the prevalence of enterovirus antibodies increases by age, but in the VirDiab study the prevalence of CBV1 antibodies remained at the same level (10-25%) in all age groups. Secondly, Laitinen et al. [132] presented that the prevalence of CBV1 antibodies in the DIPP population was about 55% compared to 15% in the VirDiab study, even though children in the DIPP cohort were younger than in the VirDiab cohort. In summary, these points suggest that part of antibody responses may be transient and the actual number of infections higher than seen in the VirDiab study.

However, in this thesis only the association of CBV group viruses and T1D was tested, therefore we cannot exclude the possibility that other enteroviruses may also be associated with T1D.

6.3.7   Geographical and temporal significance of the results

The risk association between enterovirus infections and T1D was analyzed in six independent populations, which represent a period of more than 20 years. These cohorts were collected in Finland, Sweden, the UK, France, Greece and the USA, and all these countries have shown a steep increase of T1D incidence during the past few decades. In all of these populations enterovirus infections were associated with increased risk of T1D using different enterovirus detection methods. Enterovirus RNA in serum showed a risk association both in the Finnish DIPP study (OR 7,7; 95%CI 1,9-31,5) and the American DAISY study (OR 6,2; 95%CI 1,8-21,2). Similar findings, from two independent populations from different continents, support the significance of this phenomenon. In addition, this risk association was seen in CBV1 antibodies (OR 1,7; 95% CI 1,06-2,74) in European countries. Results of all studies were coherent, pointing in the same direction, suggesting that enterovirus infections may play a role in the development of T1D in children, at least in developed countries.

These studies emphasize the risk character of CBV1 infections in the development of T1D. However, it is obvious that other enteroviruses may also be associated with T1D in other time periods or geographic regions, as suggested previously [21, 94-97, 120, 121, 126-130].

6.4   Limitations of the study

Prospective follow-up studies are enormously labor intensive and expensive to carry out. Despite the huge investment made in the DIPP and DAISY studies for several years, the number of end points was still one restricting factor in the virus analyses in these cohorts. In addition, the majority of enterovirus infections are missed by direct virus detection in serum even when the most sensitive RT-PCR assays are used, since the duration of viremia is short and collection of samples rarely overlap these viremic periods. This limitation also partly applies to the analyses of the stool collection series. It is practically impossible to collect such a frequent sample series, which would be representative for all infections. Further, it is possible that those enteroviruses which are replicating in the respiratory track have an association with the development of T1D. The optimal sample types for the detection of those infections include nasal and throat swabs which were not collected in the present study series.

    In the present study, neutralizing antibodies were analyzed only against CBV group enteroviruses. This decision was based on the results from earlier studies, which have suggested that particularly CBV group enteroviruses are associated with T1D. However, it is possible that other enteroviruses may also play a role in the pathogenesis and antibodies against a wider panel of enteroviruses should be screened to address this question.

6.5   Future prospects

The studies presented in this thesis showed that large epidemiological studies carried out in different populations are powerful tools in the evaluation of viral etiology of T1D. They can provide important new information about the nature of the virus-T1D association. For example, the present study sheds new light on the role of enteroviruses in T1D and the role of specific enterovirus serotypes which may contribute to the disease process. However, epidemiological studies alone cannot prove the causality. Therefore, it would be important to also focus research on intervention studies which can evaluate possible causality of enterovirus-T1D association and test antiviral strategies for their ability to prevent T1D. For example, antiviral drugs (such as pleconaril, ribavirin or fluoxetine) could be used in autoantibody positive children and patients with recently diagnosed T1D to test the hypothesis that they could eradicate chronic enterovirus infection from the pancreas

and thereby improve the function of beta-cells and even prevent the disease. Another important research area is vaccines, which could be tested in clinical trials. The development of vaccines against those viruses which have associated with T1D, such as enteroviruses, would make it possible to test their efficacy in the primary prevention of T1D.

One can argue that it would be important to know the mechanism of the disease process before such vaccines can be tested. However, the mechanism of poliovirus paralysis caused by polioviruses is still largely unknown, even though the effective multivalent live-attenuated and also inactivated poliovirus vaccines were developed in the early 1950s. Eradication of the polioviruses has been successful using these vaccines. Before vaccinations started, these common viruses caused paralysis all over the world but are now rare, causing only few endemic polio infections and paralytic cases in Afghanistan and Pakistan. Another example of enterovirus vaccine is the vaccine against enterovirus 71, which has caused human hand, foot, and mouth disease epidemics and severe encephalitis in Asia. Altogether, three enterovirus 71 vaccines have been tested in human phase III trials and have showed excellent efficacy [182]. These vaccines are good models for the development of multivalent formalin-inactivated vaccine against other enteroviruses.

Vaccine against CBV group enteroviruses would probably be optimal for clinical trials to test its efficacy against T1D. The first steps toward such a CBV vaccine have been taken: formalin-inactivated experimental CBV1 vaccine has been produced and tested in mouse models. The study showed that the vaccine was safe and it conferred protection against CBV1 infection [183]. Two honored pioneers in the field of T1D research, D. R. Gamble and K. W. Taylor showed the first time possible connection between CBV infections and T1D. They wrote later in the British Medical Journal (1973): "It is to be hoped that these investigators will not abandon their project when they may be close to obtaining a significant answer to this important problem". Even though finding the answer has taken probably longer than these two respected professors thought, it is important to follow their stance and finalize the project.

7 CONCLUSIONS

The main aim of this thesis work was to evaluate the possible role of enterovirus infections in the pathogenesis of T1D in three independent case-control cohorts. Two of these cohorts were prospective follow-up series including children living in Finland and the USA and one cohort was a cross-sectional, multi-center study including children from Finland, Sweden, England, France and Greece. Enterovirus infections were tested either by detecting viral RNA (RT-PCR) or virus antibodies (ELISA, plaque neutralization assay) in study subjects.

In these study populations enterovirus infections were detected more often from pre-diabetic and diabetic children, suggesting that enterovirus may be a risk factor for the disease. The infections showed an association with the initiation of the disease process but also with its progression to clinical diabetes. Further, one specific enterovirus type, CBV1 serotype, showed a risk association with T1D in European populations.

In conclusion, the present study supports the hypothesis that enterovirus infections may play a role in the pathogenesis of T1D. However, intervention trials with vaccines or antiviral drugs would be needed to prove the causality of the observed association between enterovirus infections and T1D.

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Sami Oikarinen

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10   ORIGINAL COMMUNICATIONS

PCR inhibition in stool samples in relation to age of infants

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ABSTRACT

Background: PCR is rapidly replacing traditional methods in diagnostic virus laboratories. PCR inhibitors, which are often present in clinical samples, may lead to false negative test results.
Objectives: The aim was to study the presence of PCR inhibitors in stool samples collected from 3- to 24-month old children.
Study design: Total RNA fraction extracted from stool samples was spiked with a standardized amount of Semliki Forest Virus RNA and amplified using specific PCR primers. The presence of PCR inhibitors was detected by a decrease in amplification rate compared to spiked water samples. Inhibition in different age groups and dietary origin of PCR inhibitors were analyzed by comparing the samples taken during exclusive and non-exclusive breastfeeding periods. The inactivation of PCR inhibitors was also assessed.
Results: Complete inhibition was seen in 12% (13/108) and partial inhibition in 19% (21/108) of the samples. Inhibition was seen in none of the stool samples (0/31) taken from infants younger than 6 months compared to 17% of samples (13/77) taken from 6 to 24 months old infants ($p < 0.036$). Breastfeeding was more common in younger age group. Addition of bovine serum albumin (BSA) into the reaction mixtures eliminated the effect of inhibitors leading to all samples being positive.
Conclusions: PCR inhibitors are frequent in stool samples. They may originate from dietary components and can lead to false negative PCR results. The addition of BSA to the cDNA and PCR reactions proved to be an easy and effective method for eliminating the inhibitory effect of these compounds.

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1. Background

Methods based on polymerase chain reaction (PCR) have replaced many traditional virus detection assays in clinical virus laboratories. In spite of their superior sensitivity, they have also weaknesses, which limit their use in virus diagnostics. One of these problems is caused by PCR inhibitors, which are often present in clinical samples and may lead to false negative results.[1] Such inhibition can be detected using an internal control in the PCR reaction.

Although PCR inhibitors seem to be common in stool samples, disturbing detection of viruses and other microbes, little attention has been paid on the potential biases caused by these poorly defined PCR inhibitors. The quality and quantity of inhibitors varies between samples, and several kinds of PCR inhibitors have been characterized including phenolic compounds, glycogen, fats, cellulose, constituents of bacterial cells, non-target nucleic acids and heavy metals.[2,3] However, it is not known if the concentration of these inhibitors in stool samples correlates with differences in dietary factors, gut microbiota or other factors in our environment or lifestyle. Furthermore, previous studies aiming at characterization of these inhibitors have been based on small sample series. Indeed, the overall analytical impact of PCR inhibitors is still a matter of controversy.

Various methods have been developed to remove or inactivate these inhibitors. Many nucleic acid extraction methods can eliminate part of the PCR inhibitors but their efficiency varies and is far from complete. Consequently, the selection of the nucleic acid extraction method is a trade-off between sensitivity of nucleic acid extraction and the efficacy of removal of inhibitors.[4] Previous studies have shown that inhibitors can partly be inactivated or bound by several compounds such as betaine, bovine serum albumin (BSA), formamide, glycerol, gp32, nonidet P-40 and tween.[5] The amount of PCR inhibitors can also be reduced after nucleic acid extraction, but these methods are time consuming and reduce the yield of nucleic acids, limiting their value in clinical diagnostics.

2. Objectives

This study evaluates the frequency and effect of PCR inhibitors in a large number of stool samples to assess how frequently they could lead to false negative findings in PCR analyses. In addition, we analyzed inhibition in different age groups and how these inhibitors correlate with the diet (simple diet based on breastfeeding vs. complex diet based on supplementary food). Finally we studied the efficiency of BSA in the removal of these inhibitors.

3. Study design

3.1. Subjects

Stool samples (N = 108) were collected from 27 infants aged from 3 to 24 months (mean age 10 ± 6 months) during their prospective observation starting from birth. These children participated in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study.[6] For the first 2 years of the child's life the family was asked to record the age at introduction of all new foods and end of breastfeeding on a continuous form, which was checked by trained study nurses at 3, 6, 12, 18 and 24 months visits.[7] The children were categorized into two groups: children who were exclusively breastfed, and children who received supplementary food.

3.2. Nucleic acid extraction

Stool specimens were first suspended (1:10) into HBSS (Hank's balanced salt solution) supplemented with gentamycin sulphate (Biological Industries, Israel), Fungizone (Amphotericin B), penicillin G, and 4% BSA (Calbiochem, USA) and RNA was extracted from 140 μl of this suspension using silica columns (QIAamp Viral RNA Kit, Qiagen, Hilden, Germany). From selected samples RNA was extracted also by MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) using automated nucleic acid extraction robot (MagNA Pure LC Instrument, Roche, Mannheim, Germany), which is based on magnetic coated glass particles. Extractions were performed according to the manufacturer's protocols.

3.3. Detection of PCR inhibition

The nucleic acid fraction which was extracted from the stool sample suspension was first spiked with a standardized amount of Semliki Forest Virus (SFV) RNA prior to RT-PCR reaction. This SFV RNA was originally extracted from SFV-infected Syrian hamster kidney (BHK-21) cells using silica columns (QIAamp Viral RNA Kit) and was added to the cDNA mastermix to ensure that it was present in equal titres in all experiments (100,000 copies of SFV RNA/reaction). SFV RNA diluted in water was used as a positive control, to which the magnitude of possible inhibition was compared (Fig. 1). The RT reactions were performed using RT-buffer
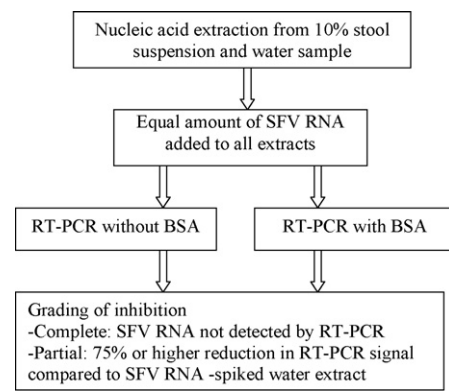

Fig. 1. Summary of study design.

(Promega, Madison, WI, USA), 0.5 mM dNTP (Pharmacia Biotech, Uppsala, Sweden), 4 U of RNase inhibitor (Promega), 50 pmol SFV-specific reverse primer (5′-TGCATGGTCATTTGGTGTGACC-3′), 20 U of Moloney murine leukemia virus reverse transcriptase enzyme (Promega) and 10 μl of the RNA template. Parallel reactions were performed with 0.5% of BSA added to the reaction mixture. The total volume of RT-PCR reaction was 40 μl. Reactions were incubated at 37 °C for 60 min. PCR reactions were done using 0.2 mM dNTP, 0.2 μM of SFV-specific primers (biotinylated forward primer 5′-ATGGCGGATGTGTGACATAC-3′; reversed primer 5′-TGCATGGTCATTTGGTGTGACC-3′), 1 U of DyNazyme DNA Polymerase, (Finnzymes, Espoo, Finland) and 10 μl of template cDNA. Parallel reactions were performed using 0.35% of BSA in the PCR mixture. The total reaction volume was 100 μl. cDNA template and primers were denatured at 94 °C for 3 min, followed by 40 cycles each consisting at 94 °C for 30 s, at 53 °C for 45 s and at 72 °C for 1 min. Final extension was at 72 °C for 7 min. PCR amplicons were linearly quantified by a liquid-phase hybridization assay[8] using a samarium labelled probe (5′-GACGGAAATGCCTTCTG-3′) (PerkinElmer, Turku, Finland) specific for SFV.
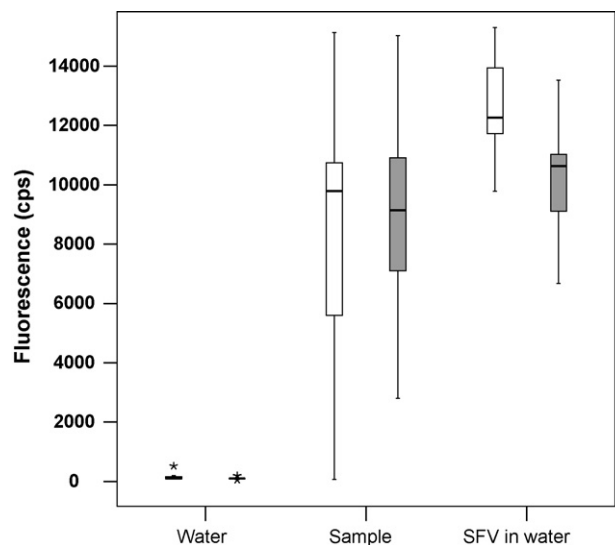

Fig. 2. Amplification of Semliki Forest Virus RNA from spiked stool and water samples using RT-PCR. The Y axis represents detection of amplification products using a Semliki Forest Virus specific probe in a liquid-phase hybridization assay (fluorescence counts per second). White bars indicate results obtained without BSA in RT-PCR reaction and gray bars results with BSA. Negative control represents background signal of non-spiked water control. The median for each dataset is indicated by the black center line, and the box presents the middle 50% of the dataset. The ends of the vertical lines indicate the minimum and maximum data values, unless potential outliers are present as asterisk.
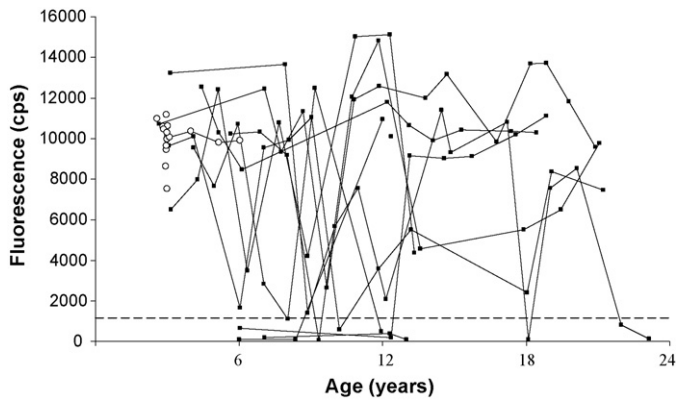
Fig. 3. Effect of age and diet on the level of PCR inhibitors in stool samples. White circles indicate exclusive and black circles non-exclusive breastfeeding samples. Sample series collected from each individual are connected with line. Dash line indicates the detection limits.

3.4. Statistical analyses

Frequency of inhibited samples was analyzed under 6 months and from 6- to 24-month old groups. Firstly, means of both groups were calculated for every child and further the means were tested using Wilcoxon test. A p value <0.05 was regarded significant.

4. Results

The presence of RT-PCR inhibitors was first tested in 108 stool samples. The amplification of SFV RNA was clearly lower in stool sample extracts compared to water sample extracts. Median fluorescence intensity in the hybridization assay was 12.268 counts per second (cps) in water samples and 9.790 cps in stool sample extracts (Fig. 2). When the cut-off limit for SFV RNA positive samples was set to a level representing five times the cps of non-spiked water sample extract (this is usually used as the cut-off limit in our diagnostic PCR tests), the amplification of SFV RNA (100,000 copies of SFV RNA per reaction) turned completely negative in 12% (13/108) of the stool samples. In addition, almost complete inhibition (75% or higher reduction in cps values) was detected in 7% (8/108) of the samples. Accordingly, altogether 19% (21/108) of the stool samples included such a high amount of PCR inhibitors that a false negative result in diagnostic RT-PCR assays was possible. The presence of PCR inhibitors in the stool samples was tightly associated with age of the child, as none of the 31 stool samples taken from younger than 6 months old infants were negative, whereas 13 (17%) out of the 77 samples taken from children aged 6–24 months were inhibited ($p < 0.036$). In the age group of less than 6 months, 61% of the samples were collected in the exclusive breastfeeding period, in the age group from 6 to 24 months only one sample was collected in the period of breastfeeding, the sample was not inhibited (Fig. 3).

All samples were also tested in parallel using BSA in the RT and PCR reactions. Addition of BSA eliminated the RT-PCR inhibition effectively leading to comparable results in SFV RNA-spiked stool and water samples (median 9.146 cps vs. 10.630 cps; Fig. 2). Even the 13 samples with complete inhibition turned clearly positive after the addition of BSA to the RT-PCR reactions (median 182 cps without BSA vs. 8.506 cps with BSA).

We further analyzed the ability of two different nucleic acids extraction methods, silica columns and magnetic coated glass particles, to remove PCR inhibitors using the 13 samples, which contained the highest amounts of PCR inhibitors (complete inhibition in original SFV RNA amplification) as well as the 15 samples which showed no inhibition. Both methods performed equally well, although some variation between individual samples was seen
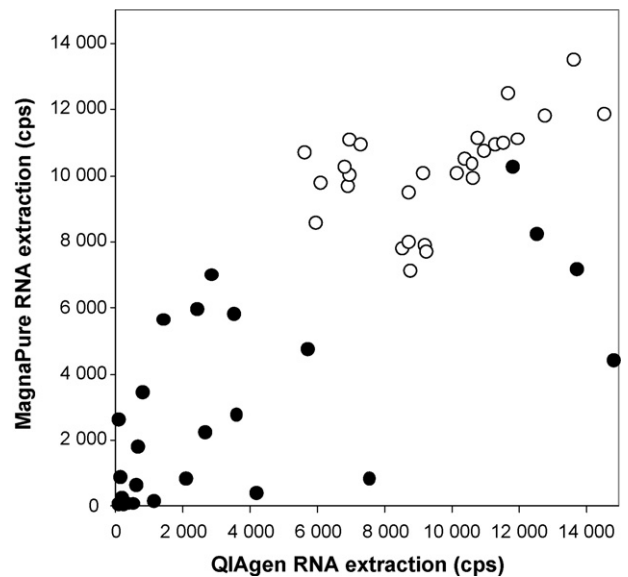
Fig. 4. Correlation between the two nucleic acid extraction methods in the detection of Semliki Forest Virus RNA in spiked stool sample without (black circle) and with (white circle) addition of BSA to RT-PCR reaction. The results of magnetic beads based method (MagnaPure RNA extraction) are shown on the Y axis and the results of the silica based method (QIAgen RNA extraction method) are presented on the X axis.

(Fig. 4). The addition of BSA abolished inhibition equally well with both extraction methods.

5. Discussion

The present study shows that PCR inhibitors are relatively common in stool samples and that their effect can strongly influence the results of PCR assays. Altogether 19% of stool samples contained so high amounts of PCR inhibitors that they may lead to false negative results when viruses are searched for using a PCR-based assay. This creates a real problem in clinical virus laboratories as stool samples are quite commonly used for the detection of viruses. The amount of SFV RNA used in our study represents average viral RNA concentrations observed in stool samples during acute virus infections. SFV RNA was spiked into extracted RNA but not to the original stool sample to avoid the effect of possible variation in the RNA extraction step on the content of SFV RNA in the PCR reaction.[9]

In previous reports, the proportion of stool samples containing PCR inhibitors has varied substantially, ranging from 0% to 44%.[10,11,12] In the present study significant inhibition was observed in 19% of the samples. This variation may be due to different sample materials, methodologies (sensitivity to different inhibitors varies between polymerase enzymes) and the definition of the inhibition itself. The inhibition has usually been categorized as a binary variable, even though it is clearly a continuous variable. Thus, the presence of inhibition has also varied depending on the cut-off limit used in various studies.

The frequency of PCR inhibitors may be even higher in the adult population compared to that observed in the young infants, as we observed no inhibition in younger that 6 months old infants. This kind of effect can cause bias to the results in research projects where viruses are analyzed from stool samples, especially if an age of the study participants varies. This kind of age dependent effect on PCR inhibition has not been described earlier and further studies are needed to identify possibly connection to dietary and maturation of digestion system of infants which may be responsible for this effect. The present study emphases the need of internal controls to detect PCR inhibitors when clinical samples are analyzed using RT-PCR. In addition, one can try to eliminate the effect of these
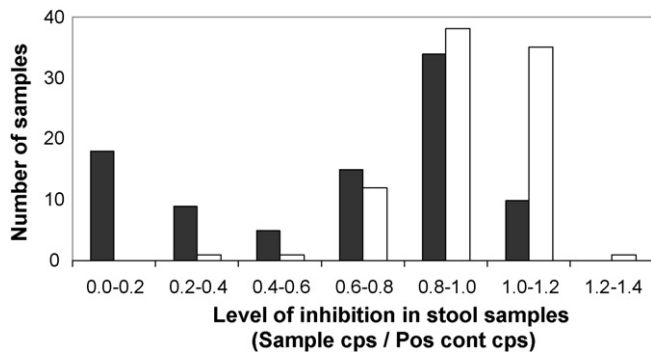
Fig. 5. Shift in PCR amplification after addition of BSA. Black bars indicate results obtained without BSA in RT-PCR reaction and white bars results with BSA.

inhibitors. We tested the effect of BSA added to both RT and PCR reactions as a factor eliminating the effect of inhibitors, as a previous study has suggested that BSA may have such a favourable effect.[13] BSA treatment decreased the efficacy of PCR amplification in samples, which did not contain any PCR inhibitors (water samples spiked with SFV RNA). However, all stool samples were positive when BSA was added to the RT and PCR reactions (Fig. 5). This indicates that the addition of BSA reduced the sensitivity of the RT-PCR method, but at the same time BSA effectively inactivated the RT-PCR inhibitors. Accordingly, the net effect of BSA was clearly favourable in stool samples eliminating the false negative samples. Based on these studies, we conclude that addition of BSA is an efficient method for decreasing false negative PCR results in clinical virus laboratories.

Conflict of interest

None declared.

Acknowledgements

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Enterovirus Infection and Progression From Islet Autoimmunity to Type 1 Diabetes

The Diabetes and Autoimmunity Study in the Young (DAISY)

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OBJECTIVE—To investigate whether enterovirus infections predict progression to type 1 diabetes in genetically predisposed children repeatedly positive for islet autoantibodies.

RESEARCH DESIGN AND METHODS—Since 1993, the Diabetes and Autoimmunity Study in the Young (DAISY) has followed 2,365 genetically predisposed children for islet autoimmunity and type 1 diabetes. Venous blood and rectal swabs were collected every 3–6 months after seroconversion for islet autoantibodies (against GAD, insulin, or insulinoma-associated antigen-2 [IA-2]) until diagnosis of diabetes. Enteroviral RNA in serum or rectal swabs was detected using reverse transcriptase PCR with primers specific for the conserved 5′ noncoding region, detecting essentially all enterovirus serotypes.

RESULTS—Of 140 children who seroconverted to repeated positivity for islet autoantibodies at a median age of 4.0 years, 50 progressed to type 1 diabetes during a median follow-up of 4.2 years. The risk of progression to clinical type 1 diabetes in the sample interval following detection of enteroviral RNA in serum (three diabetes cases diagnosed among 17 intervals) was significantly increased compared with that in intervals following a negative serum enteroviral RNA test (33 cases diagnosed among 1,064 intervals; hazard ratio 7.02 [95% CI 1.95–25.3] after adjusting for number of autoantibodies). Results remained significant after adjustment for ZnT8-autoantibodies and after restriction to various subgroups. Enteroviral RNA in rectal swabs was not predictive of progression to type 1 diabetes. No evidence for viral persistence was found.

CONCLUSIONS—This novel observation suggests that progression from islet autoimmunity to type 1 diabetes may increase after an enterovirus infection characterized by the presence of viral RNA in blood. *Diabetes* 59:3174–3180, 2010

Type 1 diabetes results from destruction of the insulin-producing β-cells in the pancreatic islets (1). The majority of patients carry the HLA *DRB1*03-DQB1*0201* or *DRB1*04-DQB1*0302* susceptibility haplotype or both, but these are not sufficient for development of disease. For many years, viral infections have been suspected to play a role, but the specific etiologic agent(s) in human type 1 diabetes remains elusive. While several viruses have been linked to type 1 diabetes, seroepidemiology, histopathology, animal studies, and in vitro experiments have provided the strongest overall evidence for enteroviruses, although results have been somewhat conflicting and not conclusive (2–4).

Autoantibodies to islet autoantigens are present for years prior to diagnosis of type 1 diabetes (1), and prospective studies testing whether enterovirus could predict islet autoantibodies have yielded conflicting results, with positive results in Finnish studies (5–7) and no association found elsewhere (8,9).
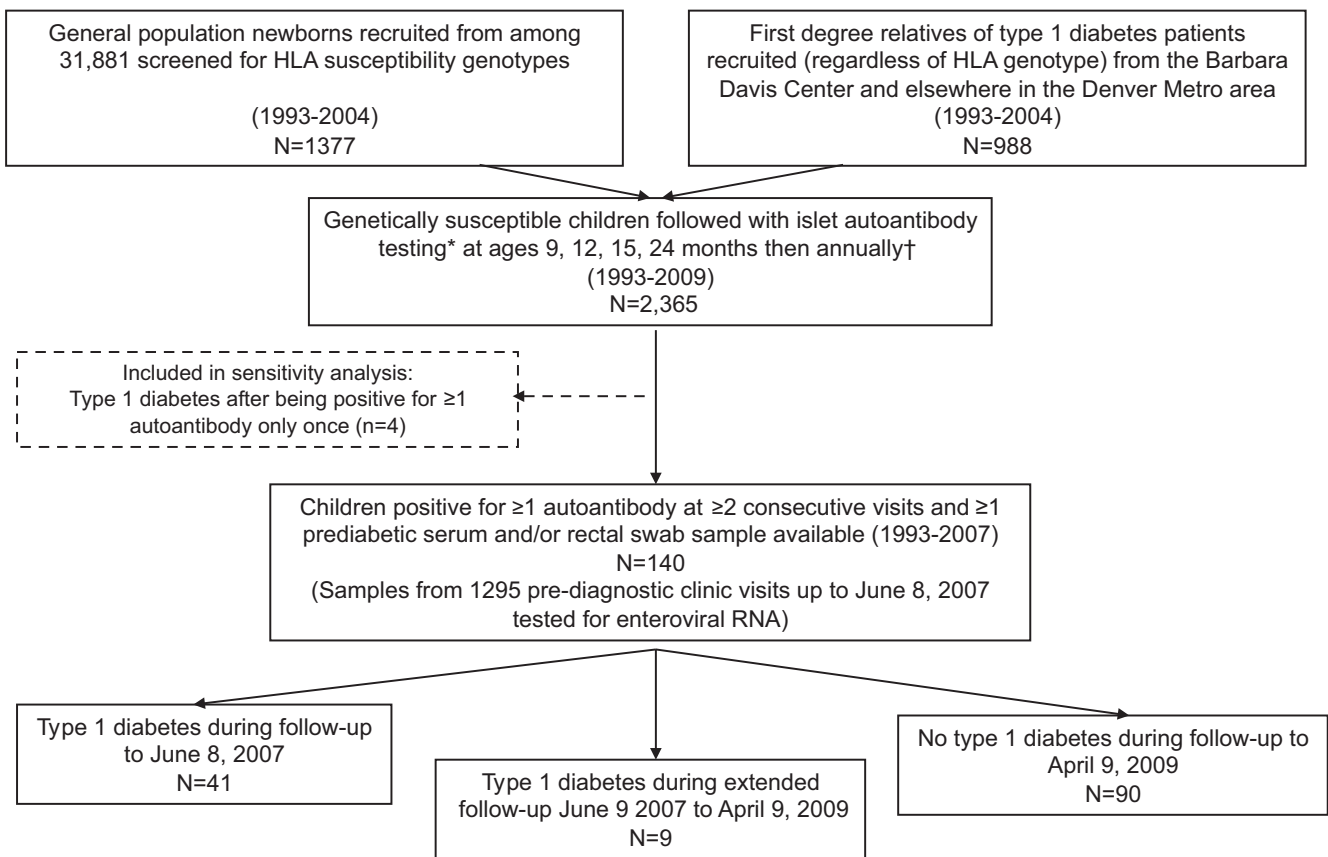
Results from animal models suggested that viral infections usually cannot initiate the autoimmune disease process leading to diabetes but may accelerate an already initiated disease process. Studies in various strains of NOD mice have shown that enteroviral infections may accelerate the progression to diabetes only if they occur after autoreactive T-cells have already accumulated in the islets (10–13). In an attempt to evaluate for the first time whether such a general model of disease progression rather than initiation by enteroviruses applies to human type 1 diabetes, we tested whether enteroviral infections predict progression to type 1 diabetes in children repeatedly positive for islet autoantibodies.

RESEARCH DESIGN AND METHODS

From 1993 to 2004, children born at St. Joseph's Hospital in Denver carrying *HLA* genotypes that confer increased risk for type 1 diabetes and siblings or offspring of people with type 1 diabetes (regardless of their genotype), identified from the Barbara Davis Center for Childhood Diabetes, were enrolled in the Diabetes Autoimmunity Study in the Young (DAISY). Informed consent was obtained from parents of all children, and the study was approved by the Colorado Multiple Institutional Review Board. Children were followed longitudinally from soon after birth and screened for islet autoantibodies at ages 9, 15, and 24 months and annually thereafter. Siblings or offspring of individuals with type 1 diabetes were enrolled after 9 months of age (median age 1.33 years [range 0.02–7.9]). Children who tested positive for islet autoantibodies were scheduled for more frequent follow-up, with visits at 3–6 month intervals.

The current study is a cohort analysis of all children who tested positive for one or more islet autoantibody on two or more consecutive clinic visits and provided at least one sample for enterovirus testing during follow-up for type

General population newborns recruited from among 31,881 screened for HLA susceptibility genotypes

(1993-2004)
N=1377

First degree relatives of type 1 diabetes patients recruited (regardless of HLA genotype) from the Barbara Davis Center and elsewhere in the Denver Metro area
(1993-2004)
N=988

Genetically susceptible children followed with islet autoantibody testing* at ages 9, 12, 15, 24 months then annually†
(1993-2009)
N=2,365

Included in sensitivity analysis:
Type 1 diabetes after being positive for ≥1 autoantibody only once (n=4)

Children positive for ≥1 autoantibody at ≥2 consecutive visits and ≥1 prediabetic serum and/or rectal swab sample available (1993-2007)
N=140
(Samples from 1295 pre-diagnostic clinic visits up to June 8, 2007 tested for enteroviral RNA)

Type 1 diabetes during follow-up to June 8, 2007
N=41

Type 1 diabetes during extended follow-up June 9 2007 to April 9, 2009
N=9

No type 1 diabetes during follow-up to April 9, 2009
N=90

FIG. 1. Flow chart illustrating formation of the study cohort. *Samples were tested for the three islet autoantibodies: anti-GAD65, anti-insulin, and anti–IA-2. †If positive for ≥1 islet autoantibody at or after 12 months of age, frequency of blood sampling was increased to every 3–6 months.

1 diabetes. Figure 1 shows a flow chart illustrating how the study cohort was formed. A batch of samples collected 1993–2004 was sent for enterovirus testing in 2005, and another batch of samples collected during 2005–June 2007 was sent in 2008. The children were further followed for diagnosis of type 1 diabetes, and the current analysis includes information on autoantibody status and diabetes up to April 2009. Type 1 diabetes was clinically diagnosed based on American Diabetes Association criteria (14), and details of procedures and clinical characteristics have been described elsewhere (15,16).

Laboratory methods. *HLA* genotyping was done at Roche Molecular Systems, Alameda, CA, as previously described (17). Children with genotypes *DRB1*04-DQB1*0302/DRB1*03-DQB1*0201* were defined as high risk, and *DRB1*04-DQB1*0302/DRB1*04-DQB1*0302* or *DRB1*03/*03* or *DRB1*04-DQB1*0302/X* (where *X* is not *DRB1*04, DQB1*0302, DRB1*03,* or *DR2,DQB1*0602*) was categorized as conferring moderate risk for type 1 diabetes.

At each clinic visit, venous blood and rectal swabs were collected. Blood samples were immediately processed, aliquoted, and stored at −70°C until testing. Rectal swabs were immediately placed in 1 ml transport medium (veal infusion broth or M4−3 medium) and stored at −70°C as previously described (8). Radioimmunoassays were used to measure serum autoantibodies to insulin, GAD_{65}, and IA-2 (BDC512) in George Eisenbarth's laboratory as previously described (18–21), with rigorous duplicate testing and confirmation of all positive and a subset of negative results (22). ZnT8 autoantibodies were measured in John Hutton's laboratory, as previously described, using a dimeric construct incorporating monomeric forms of the COOH-terminus with the polymorphic 325 Arg and Trp variants joined by a flexible linker (23,24). This autoantibody was measured in stored, available samples (81% of samples with valid serum enterovirus RNA measurements).

All enterovirus assays were carried out in Heikki Hyöty's laboratory at the University of Tampere. All virus analyses were done blindly, without knowledge of the disease status of the child. RNA was extracted from 140 μl serum and from 140 μl rectal swab solution according to the manufacturer's protocol (QIAamp viral RNA kit; Qiagen, Hilden, Germany). The presence of enterovirus RNA was detected with RT-PCR using primers specific for the 5′ noncoding region conserved among Picornaviridae and subsequent enterovirus-specific hybridization with lanthanide chelated probes, providing sensitive and specific detection of practically all known enterovirus serotypes (25). All samples with a RT-PCR signal five-fold or higher than a negative control were tested two more times, and a sample was interpreted as positive if at least two out of the three tests were five-fold or higher than the negative control. The 5′ noncoding region of detected enteroviruses was partially sequenced, and sequences were analyzed as described in detail in the online appendix (http://diabetes.diabetesjournals.org/cgi/content/full/db10-0866/DC1).

Enterovirus antibodies were measured in the batch of sera collected 1993–2004, using enzyme immunoassay as described previously (26–28). Antibodies tested were immunoglobulin M antibodies against an antigen cocktail containing coxsackievirus B3, A16, and echovirus 11, as well as IgA and IgG antibodies against purified coxsackievirus B4 and a synthetic enterovirus peptide antigen, KEVPALTVETGAT-C, derived from an immunodominant region of capsid protein VP1 (29), which is a common epitope for many enteroviruses (30). The purified viruses were heat treated to expose antigenic determinants common for various enterovirus serotypes (26).

Definition of infection. Our primary, a priori definition of infection at a given visit was positivity (as defined above) for RT-PCR detection of enterovirus RNA in serum or rectal swab. Additional analyses were done separately for serum PCR and rectal PCR and for the subset of samples tested for enterovirus antibodies. A sample was defined as positive for serology if there was a twofold or higher increase in level (optical density value) of any of the measured antibodies in the subsequent visit (samples usually 3–6 months apart), with an additional requirement that the signal-to-background ratio should exceed three. Additional two-fold or higher increases in enterovirus antibodies in a third (or later) consecutive sample drawn within 9 months of a previous one were not counted as an additional infection. These criteria were the same as those used in our previous prospective studies (6).

Statistical analysis. Using Cox regression, we compared the rate of progression to type 1 diabetes under two different models, which we have called the rapid effect model and the cumulative effect model. Both treat enterovirus infection as a time-dependent variable. In the rapid effect model, we estimated the rate of progression to diabetes in the sample interval following detection of enterovirus (median 4 months) compared with sample intervals where enterovirus was not detected. The exposure status returned to zero at the next clinic visit unless enterovirus was also found here. In the cumulative effect model, we estimated the rate of progression to diabetes according to the cumulative number of infections acquired during follow-up, which also allows

TABLE 1
Characteristics of the cohort and results of Cox regression survival analysis of progression from islet autoimmunity to type 1 diabetes

	Progressed to type 1 diabetes ($n = 50$)	No type 1 diabetes ($n = 90$)	Unadjusted HR (95% CI)	Adjusted HR (95% CI)†
Age (years) at diagnosis of type 1 diabetes	8.7 (1.9–15)			
Follow-up (years) from onset of islet autoimmunity	4.1 (0.2–11)	4.6 (1.6–14)		
Positive for >2 islet autoantibodies at the first and/or second positive visit	36 (72%)	21 (23.3%)	4.57 (2.46–8.51)	4.24 (2.26–7.95)
Female sex	26 (52%)	48 (53.3%)	1.18 (0.67–2.06)	1.41 (0.79–2.50)
First-degree relative with type 1 diabetes‡	35 (70%)	53 (58.9%)	1.23 (0.67–2.26)	1.13 (0.61–2.10)
HLA DRB1*04-DQB1*0302/DRB1*03-DQB1*0201	26 (52.0%)	27 (30.0%)	1.84 (1.06–3.21)	1.51 (0.86–2.67)
Non–Hispanic white ethnicity§	46 (92.0%)	72 (80.0%)	1.94 (0.70–5.39)	1.45 (0.51–4.13)
Age (years) when first islet autoantibody positive¶	3.1 (0.7–12)	5.2 (0.7–13)	0.93 (0.85–1.02)	1.01 (0.091–1.11)

Data are median (range) or n (%) unless otherwise indicated. †Estimates from Cox regression model simultaneously adjusting for multiple autoantibodies in first two visits, HLA high risk genotype, presence of first-degree relative with type 1 diabetes, and age when first positive for islet autoantibodies. ‡Of these, 35 had an affected father only, 16 had an affected mother only, 34 had an affected sibling, and 3 had a sibling and a parent with type 1 diabetes. §Ethnic group was self-reported. There were 118 non–Hispanic whites, 19 Hispanics, one African American, and two children of mixed ethnicity in the cohort. ¶HRs per year increase in age when first positive for islet autoantibodies.

for detection of delayed effects. Each individual first contributed follow-up time with zero infections, and the exposure variable increased by one at each visit when a new infection was detected. Because few individuals had repeated infections, the cumulative exposure variable had to be grouped (0 vs. ≥1 for serum RNA; 0, 1, and ≥2 for rectal swab RNA). The main time variable was time from the first clinic visit at which a child tested positive for islet autoantibodies to type 1 diabetes diagnosis or to the most recent visit (up to 9 April 2009) at which the child was known not to have diabetes. Because enteroviral RNA was relatively rarely detected in serum and, consequently, the number of events during the exposed periods were limited, we also carried out a Monte Carlo permutation test with 10,000 repeated permutations of the enterovirus variable to assess the validity of the standard inference based on the Cox regression model. All analyses were done using Stata, version 11 (StataCorp, College Station, TX). A 95% CI for the hazard ratio excluding the value 1.00 or a P value <0.05 was regarded as statistically significant.

RESULTS

A total of 140 children seroconverted for islet autoantibodies at a median age of 4.0 years. Of those, 50 developed type 1 diabetes at a median age of 8.7 years after a median follow-up of 4.1 years from the initial appearance of islet autoantibodies (Table 1). The samples tested for enterovirus were collected prior to June 2007, and 41 of the 50 children had developed type 1 diabetes by that time while another nine progressed to type 1 diabetes between June 2007 and April 2009 (Fig. 1).

Positivity for two or more islet autoantibodies at the first or second positive visit strongly predicted progression to type 1 diabetes, independent of other factors (Table 1). Those who progressed to type 1 diabetes tended to more often carry the high-risk HLA genotype, to have a first-degree relative with type 1 diabetes, and to seroconvert for islet autoantibodies at an earlier age, but these factors did not significantly predict progression to type 1 diabetes independently of positivity for multiple islet autoantibodies in at least one of the two first positive visits (Table 1). The number of positive islet autoantibodies treated as a time-dependent variable was also highly predictive of progression to type 1 diabetes, and positivity for ZnT8-autoantibodies significantly predicted progression both before and after adjusting for the other three islet autoantibodies (supplemental Table 1).
Enterovirus infections. Enteroviral RNA results were available from serum and rectal swabs collected at 1,081 and 1,242 prediagnostic clinic visits, respectively. Results were available for either serum or rectal swab at 1,295 visits and from both types of specimens at 1,028 visits. The

median interval between the visits was 4 months. Enteroviral RNA was detected at a total of 54 of 1,295 visits (4.2%). At eight of these 54 visits, enteroviral RNA was detected in both serum and rectal swab. Of the 140 children in the cohort, 31 (22.1%) had at least one serum or rectal swab sample positive for enteroviral RNA. While 19 of these 31 were positive only once, some had up to six positive visits. Only two children were ever positive twice for serum enteroviral RNA.

The prevalence of enterovirus RNA in serum or rectal swabs declined with age from nearly 10% for the age-group <2.5 years to ~1% for the age-group ≥7.5 years (supplemental Fig. 1). Enteroviral RNA tended to be more frequent in boys and at visits positive for multiple islet autoantibodies, but these differences were mostly of borderline statistical significance and not consistent among serum and rectal swab samples (supplemental Table 2). Of the 17 serum samples and 14 rectal swab samples collected on the day of the diabetes diagnosis, none were positive for enteroviral RNA.

Viral sequence was obtained from 8 of 17 positive serum samples and from 33 of 45 positive rectal samples. The sequences were deposited in the GenBank sequence database under accession no. HM746666–HM746706 (supplemental Table 3). Sequences are shown in supplemental Fig. 2 together with reference strain sequences listed in supplemental Table 3. Samples on which sequencing was not successful contained low concentrations of viral RNA. All samples but one clustered into enterovirus genogroup II, which contains, among others, the coxsackie B viruses (31) (supplemental Fig. 3). The sequence data indicated that viruses detected simultaneously in serum and rectal samples represented the same virus strain and only a single nucleotide substitution was once observed between such strains. Viruses detected in successive samples taken from the same individual represented different enterovirus strains. Thus, no evidence of viral persistence was found.
Progression to type 1 diabetes following enterovirus infections. The progression to type 1 diabetes in the 17 intervals following detection of enteroviral RNA in serum was significantly more rapid (three type 1 diabetes cases diagnosed) than that in the 1,064 intervals following negative enteroviral RNA serum test (33 type 1 diabetes cases diagnosed; hazard ratio [HR]6.36) (Table 2). Further adjustment for number of positive conventional islet autoan-

TABLE 2

Progression from islet autoimmunity to clinical type 1 diabetes in sample interval (median ~4 months) following infection detected by enterovirus RNA in serum or rectal swab sample

Type of sample	Person-years of follow-up	Cases progressing to type 1 diabetes in interval*	Unadjusted HR (95% CI)	HR (95% CI) adjusted for islet autoantibodies
Serum				
No enterovirus RNA in previous sample	494	33	1.00 (ref.)	1.00 (ref.)
Enterovirus RNA in previous sample	6.5	3	6.36 (1.89–21.4)†	7.02 (1.95–25.3)
Rectal swab				
No enterovirus RNA in previous sample	537.1	32	1.00 (ref.)	1.00 (ref.)
Enterovirus RNA in previous sample	21.2	1	0.93 (0.12–6.90)	0.79 (0.10–5.92)

Data are n unless otherwise indicated. *Forty-one of 140 children in the study cohort progressed to type 1 diabetes during the period wherein collected samples were tested for enterovirus, of which serum enterovirus RNA results were available from the clinic visit preceding diagnosis in 36 (of which 3 were positive) and rectal swab enterovirus RNA was available in 33 (of which 1 was positive, and serum from the same visit was also positive for enterovirus RNA). Enterovirus exposure variables coded according to the rapid effect model described in research design and methods. †Cox regression model: $P = 0.003$. Permutation test based on Cox regression model with 10,000 permutations of the enterovirus variable: $P = 0.0075$.

tibodies did not alter the result (Table 2). Because only three children were diagnosed in the interval after being positive for enteroviral RNA in serum, we employed a permutation test to make sure that the results of the standard inference based on Cox regression were valid. With 10,000 permutations, the (Monte Carlo) P value was 0.0075, thus confirming the highly significant result. After restricting the analysis to the 81% of samples with available data on ZnT8 autoantibodies, there was still a significant relation between serum enteroviral RNA and progression to type 1 diabetes (HR 6.21 [95% CI 1.82–21.2]), and this essentially was not affected by adjustment for ZnT8-autoantibody positivity in models without (8.50 [2.21–32.6]) or with (9.08 [2.30–35.8]) additional adjustment for the number of other islet autoantibodies.

The three children who progressed to type 1 diabetes in the interval following a positive serum enteroviral RNA test all had typical characteristics of high risk for progression to type 1 diabetes. They had an early age at seroconversion for multiple islet autoantibodies and an affected sibling, and two of three carried the HLA DR3/4 genotype (supplemental Table 4). They also had a near-average interval length between clinic visits, and all were male and of non–Hispanic white ethnicity. Results were similar and remained statistically significant after restriction of the analysis to these respective subgroups (supplemental Table 5). Furthermore, the results were essentially unchanged after including four children who progressed to type 1 diabetes after being positive only once for islet autoantibodies (all four were negative for enteroviral RNA, adjusted HR 6.56 [95% CI 1.84–23.5]).

Presence of enteroviral RNA in rectal swabs did not predict progression to type 1 diabetes in the following sample interval (adjusted HR 0.79 [95% CI 0.10–5.92]) (Table 2).

Analysis of progression to type 1 diabetes according to the cumulative number of enterovirus infections during follow-up, which allows for delayed effect, showed no significant relation with progression to type 1 diabetes for either serum or rectal swab enteroviral RNA or for serologically defined infections (supplemental Table 6). We also ran a Cox regression model simultaneously including variables modeling enterovirus according to the rapid effect model and the cumulative effect model. The results confirmed that the nonsignificant tendency toward an association for the cumulative effect variable was entirely due to the rapid effect, while the rapid effect of serum enteroviral RNA was unaltered and still significant (5.79 [1.23–27.3] for rapid effects model and 1.07 [0.37–3.11] for cumulative effect model).

There was also no relation between infections defined as increases in enterovirus antibodies and progression to type 1 diabetes according to the rapid effect model (supplemental Table 7). (Note that antibodies were only tested in the subset of samples collected during 1993–2004).

Finally, there were 19 children (61.3%) who progressed to type 1 diabetes among the 31 with one or more enteroviral RNA–positive serum or rectal swab samples compared with 31 (28.4%) among the 109 children in whom enteroviral RNA were not detected during follow-up ($P = 0.001$). The proportion of visits where both serum and rectal swabs were positive for enteroviral RNA was higher among those who progressed to type 1 diabetes (6 of 425 prediagnostic visits [1.4%]) than among nonprogressors (2 of 603 visits [0.3%]), but this difference was not statistically significant.

DISCUSSION

To our knowledge, this is the first study to specifically assess the role of viral infections in the progression from islet autoimmunity to clinical type 1 diabetes in humans. We found that the rate of progression from islet autoimmunity to diabetes was significantly increased in sample intervals (of an average of 4 months) after the detection of enteroviral RNA in serum but not after detection of enteroviral RNA in rectal swab samples.

Strengths and limitations. Given the amount of data available and many possible ways of analyzing data, we took great care to make all decisions a priori regarding algorithms for defining infections and methods of analysis. We used a formal cohort design and employed two main models (rapid effect and cumulative effect) to analyze two main indicators of enterovirus infections: enterovirus RNA in serum or in rectal swabs. Admittedly, our a priori–defined main exposure, presence of enterovirus RNA in either serum or rectal swabs, did not significantly predict progression to type 1 diabetes (supplemental Table 7). However, in preplanned subanalyses of serum and rectal swab enteroviral RNA examined separately, we found the presence of enterovirus RNA in serum to be a highly significant predictor of progression. Also, in the Finnish

studies of enterovirus as a risk factor for islet autoimmunity, enteroviral RNA in serum samples have been more predictive than enteroviral RNA in stool samples (4). The number of children who progressed in sample intervals after the detection of viral RNA in serum was limited. However, rather than relying on standard inference alone, we confirmed the highly significant result using a permutation test, which is not susceptible to bias with small sample sizes. Furthermore, the result was consistent and remained significant in subgroups defined by characteristics of those who progressed to type 1 diabetes after enteroviral RNA was found in serum.

As a marker of islet autoimmunity, we used repeated presence of at least one islet autoantibody. This probably does not always reflect insulitis or activation of autoreactive T-cells, but autoantibodies are currently the best way of predicting type 1 diabetes in humans (1).

Interpretation. Approximately 8% of the children progressing to type 1 diabetes had enteroviral RNA in their serum a few months prior to diagnosis. While our finding supports the hypothesis that infections resulting in enteroviral RNA in serum lead to a more rapid progression to clinical disease in some high-risk individuals, it may also suggest that enterovirus infection is a relatively uncommon cause of progression to type 1 diabetes. These observations may be explained by at least three potential scenarios.

First, we may be seeing only the tip of an iceberg because enterovirus is normally present in blood for only a few days during infection in immunocompetent hosts (5,32). Thus, the sampling intervals (median 4 months) are probably too wide to catch most of the causal infections, and enterovirus infections could turn out to be a major cause of progression from islet autoimmunity to diabetes. On the other hand, while viral shedding in feces rarely lasts more than 1 or 2 months (33), the prevalence of enteroviral RNA in rectal swab samples collected at ages <2.5 years in the current study was of a magnitude (8.7%) similar to that seen in other longitudinal studies with stool samples collected monthly from healthy children aged 3–28 months in Norway (11.5%) (33) and 3–22 months in Finland (6.0%) (34). To explain the lack of association between enteroviral RNA in rectal swabs, we may speculate that not all instances of gut infection are associated with a period with enteroviral RNA in the blood.

Second, enterovirus may establish low-grade persistent infection in children with islet autoimmunity, but the quantity of viral RNA in serum and feces may be below the detection limit in most such cases. Some studies have indicated presence of enterovirus in pancreatic tissue in a sizeable proportion of patients dying soon after onset of type 1 diabetes (35–38). Although the results varied depending on methodology and quality of specimens, detection of enterovirus in β-cells clearly strengthens the case for its role in the pathogenesis. In addition, a recent study suggests that the virus is present in the intestinal mucosa of diabetic patients (39). Enteroviral RNA was only detected at one time point in children diagnosed with type 1 diabetes during the sample interval following a positive serum enteroviral RNA test, which does not support the hypothesis of viral persistence. Sequence analysis did not give support for persistent infection because all sequences obtained from children with multiple infections were from different genotypes. Furthermore, it is notable that none of the samples collected at the day of diabetes diagnosis were positive for enteroviral RNA. This is consistent with

a previous study of serum samples from Finland (5) but apparently inconsistent with the majority of studies of enterovirus RNA in plasma or serum taken from patients soon after diagnosis, which have found ~30% of patients to be positive (40–42). We have no explanation for this except a suggestion that an international laboratory standardization workshop could shed more light on these differences.

Third, enterovirus infection may be just one of many factors that can accelerate progression to diabetes, e.g., through nonspecific activation of autoreactive T-cells. Additional host and environmental factors are also likely to play a role. It is currently unclear whether certain enterovirus serotypes are more diabetogenic in humans than others. The most frequently implicated serotype, coxsackievirus B4 (43), was responsible for 2.4% of the enterovirus infectious episodes in the Norwegian study of healthy children (33). There may also be differences within serotypes because enteroviruses are known to mutate rapidly (4,32).

Another possibility that cannot be discarded is that progression to type 1 diabetes is enhanced because the viral infection induced insulin resistance sufficient to precipitate clinical disease. Nonspecific febrile illness or other infectious symptoms in a period prior to diagnosis seems to be quite commonly reported, but few studies have been able to obtain comparable data in age-matched controls (44) and the large majority of enterovirus infections are asymptomatic (32). Furthermore, while biopsy studies and previous cross-sectional or retrospective studies of enterovirus infections in patients with type 1 diabetes cannot exclude the possibility that the disease influenced the risk of infection, our longitudinal design allowed us to draw stronger inference in this regard. The fact that none of the children who were tested on the day of diagnosis were positive for enteroviral RNA, including those who were enterovirus positive in the interval before diagnosis, shows that reverse causation was unlikely.

A number of potential mechanisms for how viral infections may induce or accelerate autoimmune diabetes have been proposed, mostly based on animal models or in vitro studies (43,45). Mechanisms in humans are likely to be complex, but may initially involve, for example, activation of the innate immune system, secretion of interferon-α, and perhaps upregulation of major histocompatibility complex molecules on β-cells (46). Results from animal models cannot automatically be generalized to humans, but studies in strains of NOD mice have indicated a requirement for preceding β-cell damage and release of β-cell antigens taken up by antigen-presenting cells (13,47), as previously reviewed (43). The "fertile field hypothesis" proposes that different viruses may increase the risk of diabetes in susceptible time windows after an infection, while outside this window a similar viral infection would be resolved with no further consequences for the host (3).

Future studies and final conclusion. Despite the huge undertaking of screening and prospectively following a large number of children for several years, the number of end points was still limited and independent replication in future studies would strengthen the results. Children who progressed to type 1 diabetes immediately after detection of enterovirus RNA all had clinical characteristics consistent with high risk of progression such as early development of multiple islet autoantibodies. Future studies could investigate further the potential role of additional host and

viral factors in this process. Prospective studies are challenging, and up to now such studies have mainly focused on the initiation of autoimmunity as the end point (7–9,26), with mixed results. The Environmental Determinants of Diabetes in the Young (TEDDY) study (48) has the potential to provide answers concerning the role of enterovirus and progression to type 1 diabetes with greater power and avoiding some of the limitations of the study presented here.

In conclusion, the rate of progression from islet autoimmunity to type 1 diabetes was significantly increased in the approximately 4-month interval following detection of enteroviral RNA in serum.

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Online supplemental material
Content:
- text describing methods for enterovirus sequence analysis
- references for the sequence analysis methods
- 7 Online Supplemental Tables,
- 3 Online Supplemental Figures (Fig 3A and B on separate pages)

Methods for enterovirus sequencing and sequence analysis
The 5'NCR of detected enteroviruses was partially sequenced (75 bp). PCR amplicon of the screening PCR (1) was purified using Minelute Gel Extraction kit (Qiagen) and sequenced with fluorescent labeled terminators (BigDye v. 3.1 Applied Biosystems) using an automated sequencer (Applied Biosystems) according to manufacturer's protocol. Chromatogram sequence files were inspected with Sequencher (4.10.1. Gene Codes Corp.). Multiple sequence alignments were done using Clustal X (2) and alignments were edited with Genedoc (Nicholas KB, H. B. Niclohas and D.W. Deerfield.: GeneDoc: analysis and visualization of genetic variation, available at http://www.nrbsc.org/gfx/genedoc/ebinet.htm). Genetic distances were calculated using the Kimura two-parameter model with transition/transversion ratio 2.0 using PHYLIP (J. Felsenstein: PHYLIP Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle 1993, available from http://evolution.genetics.washington.edu/phylip.html). Phylogenetic trees were constructed using the neighbor-joining method (PHYLIP) and visualized by TreeView software (3). The accession numbers for and description of previously determined sequences for reference strains used in the phylogenetic analyses are shown in Online Supplemental Table 3. Multiple alignment of the currently obtained sequences together with the selected reference sequences are shown in Online Supplemental Figure 2, and phylogenetic trees are shown in Online Supplemental Figure 3A and B.

References for online supplemental material
1. Lönnrot M, Sjöroos M, Salminen K, Maaronen M, Hyypiä T, Hyöty H: Diagnosis of enterovirus and rhinovirus infections by RT-PCR and time-resolved fluorometry with lanthanide chelate labeled probes. *J Med Virol* 59:378-384, 1999
2. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876-4882, 1997
3. Page RDM: TreeView: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12:357-358, 1996

Online Supplemental Table 1. Prediction of progression from islet autoimmunity to clinical type 1 diabetes according to ZnT8-autoantibody positivity and according to number of other positive autoantibodies (against insulin, GAD and IA-2) according to Cox regression models treating autoantibodies as time-dependent variables.

		Person-years of follow-up	Cases progressing to type 1 diabetes in such intervals	Hazard ratio (95% CI) Unadjusted	Adjusted for number of other 3 islet autoantibodies†
ZnT8-autoantibodies*	Negative	261.2	5	1.00 (reference)	1.00 (reference)
	Positive	179.6	29	7.21 (2.75-18.9)	4.62 (1.46-14.6)
Number of other 3 autoantibodies†	0	194.1	4	1.00 (reference)	
	1	246.3	14	4.57 (1.48-14.1)	Not applicable
	2	207.5	22	5.90 (2.03-17.2)	Not applicable
	3	57.4	10	10.2 (3.16-33.1)	Not applicable

* Data available in 81% of samples with valid serum enterovirus data.

† Number of positive islet autoantibodies, codes as 0-3 (anti-insulin, -GAD, or –IA2).

Online supplemental Table 2. Relevant factors potentially associated with enteroviral RNA positivity in serum and rectal swab samples.

	Percent of tested serum samples positive for enteroviral RNA	p-value*	Percent of tested serum samples positive for enteroviral RNA	p-value*
Boys	1.7		5.5	
Girls	1.4	0.70	1.5	0.02
HLA DR3/4†	2.3		3.9	
Other HLA types	1.0	0.10	3.4	0.51
FDR‡	1.5		3.3	
Not FDR	1.6	0.96	4.1	0.89
Non-Hispanic white	1.6		3.8	
Other ethnicity	1.2	0.69	2.1	0.25
≥2 AAb at same visit	2.2		4.7	
≤1 AAb at same visit	0.9	0.09	2.6	0.07
ZnT8-AAb positive	2.1		5.6	
ZnT8-AAb negative	1.5	0.50	2.9	0.07

* P-value for difference in frequency of enteroviral RNA positivity based on logistic regression model with random intercept for each individual to account for possible intra-individual correlation in infection, run in Stata version 11.
† *HLA DRB1*04-DQB1*0302/DRB1*03-DQB1*0201*
‡ FDR: Have 1st degree relative with type 1 diabetes.
AAb: Autoantibody.

Online Supplemental Table 3. Human enterovirus type and strain with associated GenBank accession numbers for sequences of reference strains used in phylogenetic analysis, and GenBank accession numbers for the enterovirus sequences obtained in the current study.

Human enterovirus strain	Accession number
CAV2: Coxsackievirus A2, strain Fleetwood	AY421760
CAV6: Coxsackievirus A6, strain Gdula	AY421764
CAV9: Coxsackievirus A9, strain Griggs.	D00627
CAV10: Coxsackievirus A10, strain Kowalik	AY421767
CAV15: Coxsackievirus A15 strain G-9	AF465512
CAV16: Coxsackievirus A16 strain, Taiwan/5079/98	AF177911
CAV19: Coxsackievirus A19 strain Dohi	AF329689
CBV1: Coxsackievirus B1, isolate CVB1Nm	EU147493
CBV3: Coxsackievirus B3 isolate CVB3-m(NANCY)	U30926
CBV4: Coxsackievirus B4, strain VD2921	AF328683
CBV5: Coxsackievirus B5, strain Faulkner	AF114383
CBV6: Coxsackievirus B6, strain Schmitt	AF114384
E3: Echovirus 3, strain PicoBank/DM1/E3	AJ849942
E4: Echovirus 4, strain Pesacek	AY302557
E5: Echovirus 5, strain Noyce	AF083069
E7: Echovirus 7, strain UMMC	AY036578
E11: Echovirus 11, isolate HUN-1108	AJ577589
E18: Echovirus 18, strain Metcalf	AF317694
E24: Echovirus 24, strain DeCamp	AY302548
E30: Echovirus 30, strain Echo30/Zhejiang/17/03/CSF	DQ246620
E31: Echovirus 31, strain Caldwell	AY302554
Ent70: Enterovirus 70, strain J670/71 (isolate ATCC VR-836)	DQ201177
PV1: Poliovirus 1 strain Sabin 1 isolate S302	GQ984141
Code used for samples sequenced in the current study*	Accession number
K1097R	HM746666

A0195R	HM746667
A0195S	HM746668
B0395R	HM746669
B0896R	HM746670
L0896S	HM746671
C0801R	HM746672
C1101R	HM746673
D0601R	HM746674
D0799R	HM746675
M1198R	HM746676
N1198R	HM746677
O0702R	HM746678
P0899R	HM746679
Q0503S	HM746680
E0301R	HM746681
E0602R	HM746681
E0703R	HM746683
E0602S	HM746684
R1003R	HM746685
F0501R	HM746686
F0702R	HM746687
F0996R	HM746688
F0702S	HM746689
G0201R	HM746690
G0404R	HM746691
G0800R	HM746692
G0801R	HM746693
G1002R	HM746694
G0404S	HM746695
G0800S	HM746696
H0401R	HM746697
H1098R	HM746698
S0596R	HM746699
I0299R	HM746700
I0600R	HM746701
I0900R	HM746702
J0900R	HM746703
J1101R	HM746704
J1103R	HM746705
J1101S	HM746706

* Coding of study samples: First letter (A-S) is a code for child, the next four digits is the date of the sample collection (mmyy), and the final S or R indicates serum sample (S) or rectal swab sample (R).

Online Supplemental Table 4. Details of laboratory results at clinic visits from first testing positive for islet autoantibodies in the three children who developed type 1 diabetes (T1D) in the sample interval following detection of serum enteroviral RNA.

ID	Date	Age (yrs)	Positive autoantibodies (IAA, GADA, IA2-A)	ZnT8-A	HbA1c‖	T1D diagnosis	Enterovirus RNA in serum	Enterovirus RNA in rectal swab sample	Enterovirus serology¶
132*	Sep-1994	0.8	IAA	−	−	−	−	−	+
	Jan-1995	1.1	IAA	−	5.4	−	−	−	−
	Apr-1995	1.3	IAA, GADA	−	5.2	−	−	−	−
	Nov-1995	1.9	IAA, GADA	−	4.7	−	−	−	−
	Feb-1996	2.2	IAA, GADA	−	5.2	−	−	−	−
	May-1996	2.4	IAA, GADA	•	−	−	•	−	•
	Aug-1996	2.7	IAA, GADA	+	6.3	−	+	•	•
	Sep-1996	2.8	•	•	6.6	T1D	•	•	
539†	Jan-1999	1.3	IAA, IA2-A	−	−	−	•	•	•
	May-1999	1.6	IAA, IA2-A	•	4.9	−	•	−	−
	Jun-1999	1.7	IAA, IA2-A	+	5.3	−	−	•	−
	Aug-1999	1.8	IAA, IA2-A	+	5.1	−	−	−	•
	Nov-1999	2.1	All three	+	5.7	−	+	+	•
	Jan-2000	2.3	•		5.9	T1D	•	•	
600‡	Apr-1998	1.5	IAA, IA2-A	•	−	−	•	−	•
	Jul-1998	1.8	IAA, IA2-A	+	5.3	−	−	−	−
	Oct-1998	2.1	IAA, IA2-A	+	5.1	−	+	−	−
	Aug-1999	2.8	IAA, IA2-A	+	7.0	T1D	−	−	•

+: Positive; −: negative; •: missing data. If a child was diagnosed outside the regular study visit, there was often no sample available for testing of autoantibodies or enterovirus RNA. Other instances of missing data were mostly because there was no sample left for enterovirus testing.

‖ HbA1c was measured using a DCA2000 meter (Bayer, Leverkusen, Germany), details described in ref. (14).

¶ + if significant increase in one or more of the five measured enterovirus antibodies in the following sample, − if no increase (see methods for more details). ID 132 experienced a significant rise in coxsackievirus B4 IgA.

* ID 132: Male, non-Hispanic white, sibling with type 1 diabetes, high risk (DR3-DQ2/DR4-DQ8) genotype.

† ID 539: Male, non-Hispanic white, sibling with type 1 diabetes, high risk (DR3-DQ2/DR4-DQ8) genotype.

‡ ID 600: Male, non-Hispanic white, sibling with type 1 diabetes, HLA DR4-DQ8/DR1 genotype.

Online Supplemental Table 5. Progression to type 1 diabetes (T1D) after serum enterovirus RNA in sub-groups: First degree relatives of persons with type 1 diabetes, sample intervals positive for multiple islet autoantibodies (AAbs), those who seroconverted for AAbs before 4 years of age, those with the HLA DR4-DQ8/DR3-DQ2 high risk genotype, to boys only, and to non-Hispanic white ethnicity only (rapid effect model).

Subgroup	Enterovirus RNA in previous sample interval	Person-years of follow-up	Cases progressing to T1D in interval	Hazard ratio (95% CI)
First degree relatives of T1D	No	328.0	25	1.00 (reference)
	Yes	5.0	3	6.88 (1.98-24.0)
Multiple islet autoantibodies	No	193.6	22	1.00 (reference)
	Yes	3.8	3	6.67 (1.88-31.3)
Islet AAbs at <4yrs of age	No	261.3	24	1.00 (reference)
	Yes	6.5	3	4.43 (1.27-15.4)
HLA DR4-DQ8/DR3-DQ2	No	173.1	18	1.00 (reference)
	Yes	4.1	2	6.05 (1.25, 29.3)
Boys	No	249.2	13	1.00 (reference)
	Yes	3.8	3	39.0 (6.29-242)
Non-Hispanic white ethnicity	No	422.4	30	1.00 (reference)
	Yes	6.2	3	6.62 (1.94-22-6)

Online Supplemental Table 6. Risk of progression from islet autoimmunity to clinical type 1 diabetes according to cumulative enterovirus (EV) infections defined by presence of enterovirus RNA in serum or rectal swab samples, or serologically defined enterovirus infections.

Cumulative number of enterovirus infections		Person-years*	Children progressing to T1D	Hazard ratio (95% CI)	
				Unadjusted	Adjusted†
EV RNA, serum or rectal swab	0	557.9	31	1.00 (reference)	1.00 (reference)
	1	91.9	14	2.34 (1.24-4.42)	1.82 (0.94-3.50)
	≥ 2	61.7	5	0.95 (0.35-2.56)	0.63 (0.23-1.73)
EV RNA, serum	0	646.8	40	1.00 (reference)	1.00 (reference)
	≥ 1	64.7	10	1.90 (0.93-3.87)	1.51 (0.73-3.09)
EV RNA, rectal swab	0	584.0	36	1.00 (reference)	1.00 (reference)
	1	68.3	9	1.91 (0.91-4.01)	1.46 (0.69-3.13)
	≥ 2	59.2	5	0.91 (0.34-2.42)	0.62 (0.23-1.70)
Serology‡	0	364.5	22	1.00 (reference)	1.00 (reference)
	1	227.3	15	0.78 (0.39-1.54)	0.87 (0.44-1.71)
	≥ 2	119.7	13	0.96 (0.44-2.07)	0.88 (0.40-1.90)

* Total person-time under observation using cumulative number of infections as a time-varying variable.

† Adjusted for number of positive islet autoantibodies (0-3 positive out of anti-insulin, -GAD, and -IA-2, as time-dependent variable).

‡ Number of infections defined by number of serial increases in one or more of five measured enterovirus antibodies, or enterovirus IgM > 3 times the background optical density in the first visit (see methods section for details). Note that enterovirus antibodies were only measured in the subset of samples collected 1993-2004.

Online Supplemental Table 7. Progression from islet autoimmunity to clinical type 1 diabetes in sample interval (median ~4 month) following infection detected by enterovirus RNA in serum or rectal swab sample, or following a serologically defined enterovirus infection (rapid effects model).

Type of sample/definition of infection	Infection in previous sample	Person-years of follow-up	Cases progressing to type 1 diabetes in interval*	Hazard ratio (95% CI) Unadjusted	Adjusted for islet autoantibodies†
Serum or rectal swab enteroviral RNA	No	552	37	1.00 (reference)	1.00 (reference)
	Yes	25.4	3	1.91 (0.58-6.29)	1.64 (0.49-5.52)
Serologically defined infection‡	No	285.1	11	1.00 (reference)	1.00 (reference)
	Yes	46.55	2	1.36 (0.28-6.53)	1.15 (0.24-5.62)

* Forty-one of 140 children in the study cohort progressed to type 1 diabetes during the period where collected samples were tested for enterovirus, of which a rectal swab enterovirus RNA result was available in 33 (of which 1 was positive, and serum from the same visit was also positive for enterovirus RNA). The enterovirus exposure variable was coded according to the rapid effect model described in the methods section.

† Number of positive islet autoantibodies, codes as 0-3 (anti-insulin, -GAD, or –IA2). Results remained essentially unchanged after also including anti ZnT8 autoantibodies, analyzed in a subset of samples, see main text of results section for details.

‡ Defined by serial increases in one or more of five measured enterovirus antibodies, or enterovirus IgM > 3x background in the first visit (see methods section for details). Note that enterovirus antibodies were only measured in the subset of samples collected 1993-2004.

Online Supplemental Figure 1. Prevalence of enterovirus (EV) RNA in serum (A), rectal swab samples (B), serum or rectal swab sample (C), or according to diagnostic increase in serum enterovirus antibodies (D), by age-group in progressors to type 1 diabetes and non-progressors.
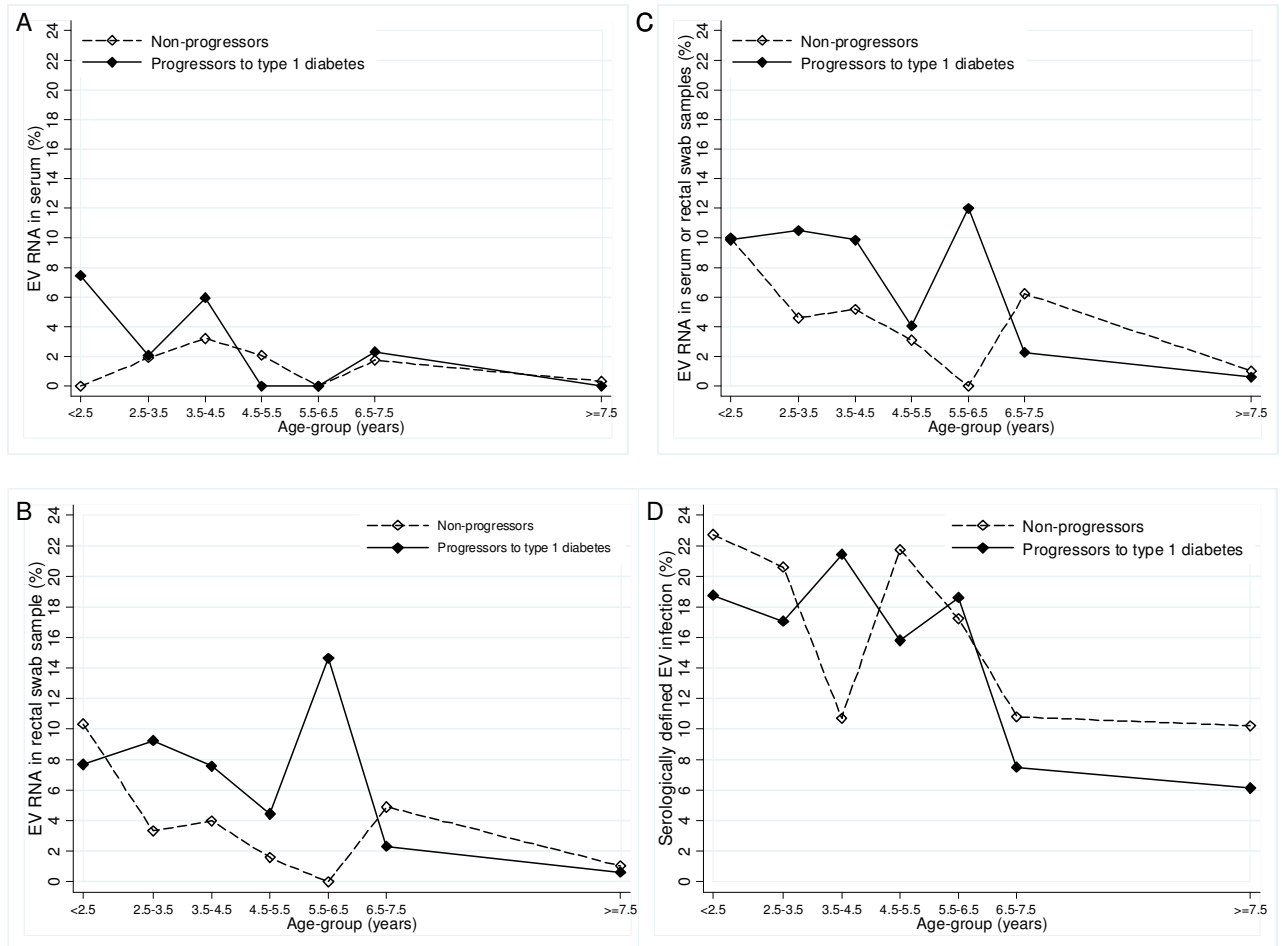
Online Supplemental Figure 2. Multiple sequence alignment of study samples and reference strain sequences produced using Clustal X. Eight of 17 positive serum samples and 33 of 45 positive rectal samples (altogether 41 samples) were successfully sequenced in the present study. Coding of study samples: First letter (A-S) is a code for child, the next four digits is the date of the sample collection (mmyy), and the final S or R indicates serum sample (S) or rectal swab sample (R). The GenBank accession numbers for these sequences are listed in Online Supplemental Table 3. Abbreviations and accession number of the reference strain samples: see Online Supplemental Table 3. Nucleotides 472-544 of the 5' non coding region are shown (nucleotide positions refer to the CBV4 sequence with the GenBank accession number AF328683). The following children later developed type 1 diabetes at the indicated month and year: A:0300; B:0103; D:0808; G:1005; H:1106; J:1204; K:0706; L:1296; M:0703; N:0101; O:0603;P:0804;R:0704.

Online Supplemental Figure 3. Phylogenetic tree of enteroviruses detected in DAISY children and selected reference strains. The tree was constructed on the basis of part of the 5' non coding region (74 bp) of 8 of 17 positive serum samples and from 33 of 45 positive rectal samples (altogether 41 samples) which were sequenced in the present study. Reference strains are described in Online Supplemental Table 3. Panel A illustrates all viruses and Panel B shows those viruses which were detected in same individual from multiple samples. Coding of study samples: First letter (A-S) is a code for child, the next four digits is the date of the sample collection (mmyy), and the final S or R indicates serum sample (S) or rectal swab sample (R). The GenBank accession numbers for these sequences are listed in Online Supplemental Table 3. The following children later developed type 1 diabetes at the indicated month and year: A:0300; B:0103; D:0808; G:1005; H:1106; J:1204; K:0706; L:1296; M:0703; N:0101; O:0603;P:0804;R:0704.

A
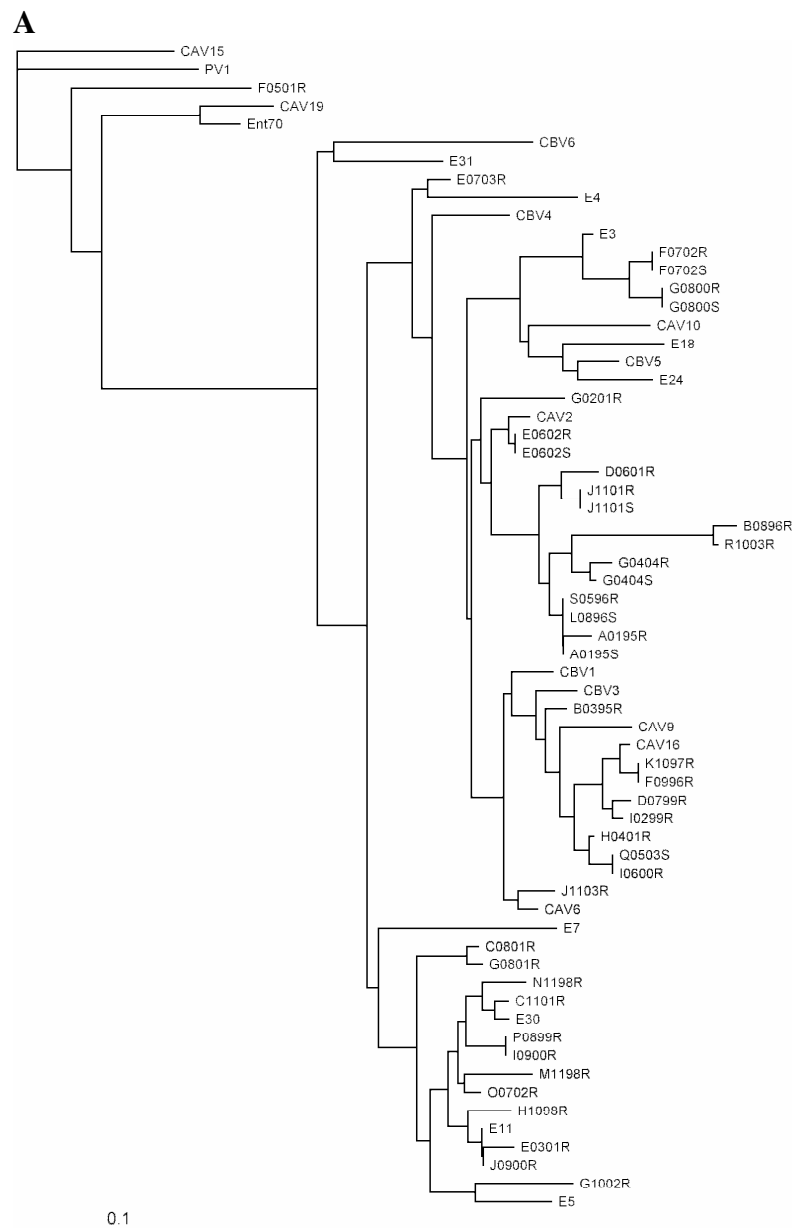
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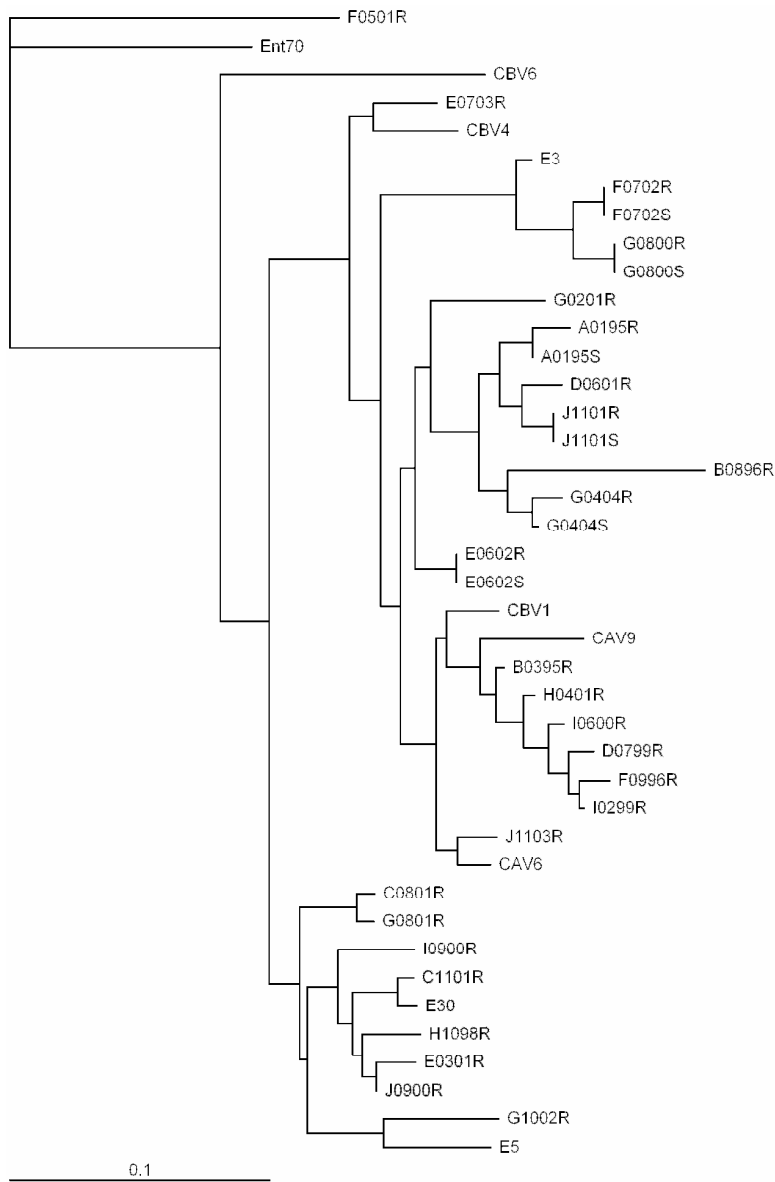
Enterovirus RNA in Blood Is Linked to the Development of Type 1 Diabetes

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OBJECTIVE—To assess whether the detection of enterovirus RNA in blood predicts the development of clinical type 1 diabetes in a prospective birth cohort study. Further, to study the role of enteroviruses in both the initiation of the process and the progression to type 1 diabetes.

RESEARCH DESIGN AND METHODS—This was a nested case-control study where all case children ($N = 38$) have progressed to clinical type 1 diabetes. Nondiabetic control children ($N = 140$) were pairwise matched for sex, date of birth, hospital district, and HLA-DQ–conferred genetic susceptibility to type 1 diabetes. Serum samples, drawn at 3- to 12-month intervals, were screened for enterovirus RNA using RT-PCR.

RESULTS—Enterovirus RNA–positive samples were more frequent among the case subjects than among the control subjects. A total of 5.1% of the samples (17 of 333) in the case group were enterovirus RNA–positive compared with 1.9% of the samples (19 of 993) in the control group ($P < 0.01$). The strongest risk for type 1 diabetes was related to enterovirus RNA positivity during the 6-month period preceding the first autoantibody-positive sample (odds ratio 7.7 [95% CI 1.9–31.5]). This risk effect was stronger in boys than in girls.

CONCLUSIONS—The present study supports the hypothesis that enteroviruses play a role in the pathogenesis of type 1 diabetes, especially in the initiation of the β-cell damaging process. The enterovirus-associated risk for type 1 diabetes may be stronger in boys than in girls. *Diabetes* **60:276–279, 2011**

E nterovirus infections are among the major candidates for environmental risk factors for type 1 diabetes. Previous studies have suggested that enterovirus epidemics associate with an increase in the incidence of type 1 diabetes, and an increased frequency of enterovirus antibodies has been reported in patients with type 1 diabetes (1,2). Several studies have detected enterovirus genome in the blood of diabetic patients, but it is unknown whether the finding reflects persistent or acute infection (3). Virus has been detected both in pancreas and in intestinal mucosa and has also shown a tropism for islets (4,5). On some occasions, coxsackievirus B and echoviruses have even been isolated from diabetic children (6). The recent discovery that genetic polymorphism in the *IFIH1* gene (innate immune system sensor for enteroviruses) affects diabetes susceptibility has further supported the possible role of enteroviruses (7). Experimental data support these findings because enteroviruses can cause diabetes in mice and damage β-cells in human islet cell cultures in vitro (3).

Type 1 diabetes–associated autoantibodies in peripheral blood reflect initiation of the β-cell–damaging processes. However, the progression toward clinical diabetes is usually slow, and possible triggering infections can occur long before the presentation of clinical type 1 diabetes. Consequently, prospective follow-up series are essential for the identification of such triggers. A few prospective studies have been carried out on the possible role of enterovirus infections, but the results have been conflicting (8–11).

The aim of this study is to test risk effect of enterovirus RNA in blood for the development of type 1 diabetes in a prospective birth cohort study. Blood samples were collected with short intervals, which made it possible to detect enterovirus RNA directly from the serum in different stages of the disease process. We have previously documented the risk effect of enteroviruses in children who developed β-cell autoimmunity. Now, the aim is to confirm these findings in children who have developed type 1 diabetes and to study the role of these viruses in both the initiation of the process and its progression to diabetes.

RESEARCH DESIGN AND METHODS

Study series. The study series included children who took part in the Finnish type 1 Diabetes Prediction and Prevention (DIPP) study (12). In DIPP, the families of all newborn infants at the University Hospitals of Oulu, Tampere, and Turku are offered a possibility for screening of newborn infants for HLA risk genes for type 1 diabetes. Families with a child who carries increased genetic susceptibility to diabetes are invited to participate in prospective follow-up starting from birth. Blood samples are taken in 3- to 12-month intervals and regularly analyzed for type 1 diabetes–associated autoantibodies. Islet cell antibodies (ICAs) have been used for the primary screening, and all samples of ICA-positive children were tested for autoantibodies against insulin (IAA), glutamate decarboxylase (GADA), and the protein tyrosine phosphatase–related islet antigen 2 (IA-2A). Children who seroconverted to autoantibody positivity were observed subsequently at an interval of 3 months.
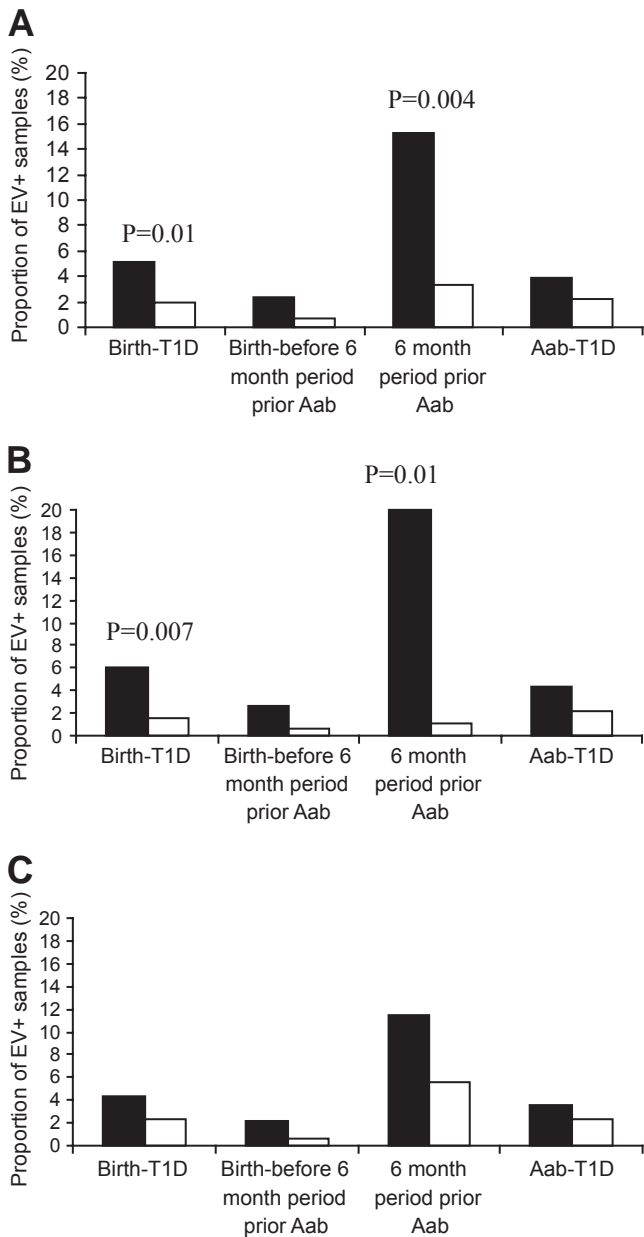
The current study is based on a nested case-control design, where the definition of the case status was based on the diagnosis of clinical type 1 diabetes. For every case child, one to six healthy autoantibody-negative control children were matched pairwise for sex, date of birth (±1 month), hospital district, and HLA-DQ–conferred genetic susceptibility to type 1 diabetes. The study population comprised a total of 38 case children (18 boys) and 140 control children (69 boys). Serum samples were collected for virus

A

B

C

FIG. 1. Enterovirus (EV) RNA positivity in serum samples during different stages of the diabetic disease process. 6 month period prior AAb, 6-month time window before autoantibody seroconversion; Aab-T1D, period from autoantibody seroconversion to diagnosis of type 1 diabetes; birth-before 6 month period prior Aab, time from birth to 6 months before the seroconversion to positivity for the first autoantibody; birth-T1D, time from birth to diagnosis of type 1 diabetes. *A*: the whole cohort. *B*: boys. *C*: girls. ■, case children; □, control children. The differences between case and control children were tested using conditional logistic regression analysis (*P* values shown).

analyses from November 1994 to April 2003, and the total number of samples analyzed was 1,326. Our earlier study population overlaps with the current study, with 129 samples from 21 case children and 474 samples from 85 control children (11).

The study was approved by the ethics committees of the participating university hospitals. Parents gave written informed consent.

Genetic and autoantibody analyses. The presence of HLA DR-DQ haplotype associated with type 1 diabetes was determined using typing for DQB1 alleles in the first phase and informative DQA1 and DRB1 alleles in the second phase as previously described (13). ICA, IAA, GADA, and IA-2A were analyzed as previously described (14).

Enterovirus RT-PCR. RNA was extracted from 140 μl of serum or plasma using a QIAamp viral RNA kit (Qiagen, Hilden, Germany). Screening for enterovirus RNA was done by RT-PCR followed by hybridization of PCR

TABLE 1
Risk effect of the detection of enterovirus RNA in serum for later development of clinical type 1 diabetes

Time period	OR	95% CI	*P*
Boys and girls			
Birth-before Aab	6.2	1.8–21	<0.004
Birth-before 6 month			
period prior Aab	3.1	0.4–22.4	<0.27
6 month period prior Aab	7.7	1.9–31.5	<0.004
Boys			
Birth-before Aab	18.8	2.2–163.7	<0.008
Birth-before 6 month			
period prior Aab	3.9	0.2–63.3	<0.34
6 month period prior Aab	18.2	2.0–164.5	<0.01
Girls			
Birth-before Aab	2.6	0.5–12.3	<0.24
Birth-before 6 month			
period prior Aab	2.4	0.2–39.7	<0.53
6 month period prior Aab	3.1	0.4–21.8	<0.26

6 month period prior AAb, 6-month time window before autoantibody seroconversion; birth-before 6 month period prior Aab, time from birth to 6 months before the seroconversion to positivity for the first autoantibody; birth-before Aab, time from birth to the seroconversion to positivity for the first autoantibody.

amplicons using an enterovirus-specific probe. The detection limit for the method is <0.015 fg RNA, which is equivalent to fewer than four copies of enteroviral RNA genome (15). All RNA-positive samples were retested twice, and a result of at least two positive tests out of three tests was interpreted as a positive sample.

Statistical analyses. The significance of difference in the number of enterovirus infections between case and control children was tested using conditional logistic regression analysis (STATA statistical software, College Station, TX). To adjust for more frequent sample collection from autoantibody-positive children than control children, conditional logistic regression analysis was calculated as proportions (%) of positive samples. Effect of HLA type for EV infections was evaluated using Mann Whitney test.

RESULTS

Enterovirus RNA was detected in 2.7% of serum samples (36 of 1,326). In case children, a total of 5.1% of the samples (17 of 333) were enterovirus RNA positive compared with 1.9% (19 of 993) in control children ($P < 0.01$) (Fig. 1).

The frequency of enterovirus RNA positivity was further analyzed during different stages of the preclinical disease process. The frequency of enterovirus RNA in the case children peaked during the 6-month period before the appearance of the first autoantibody when 15.2% of the samples from the case children and 3.3% of the samples from the control children were positive (odds ratio [OR] 7.7 [95% CI 1.9–31.5], $P < 0.004$). The lowest frequency of enterovirus RNA was observed during the time period from birth to 6 months before the autoantibody positivity when 2.4% of the samples were positive among the case children and 0.7% in the control group (OR 3.1 [95% CI 0.4–22.4], $P < 0.27$). After autoantibody seroconversion, 3.9 vs. 2.2% of the samples were enterovirus RNA positive, respectively (Table 1 and Fig. 1).

The risk effect of enterovirus RNA was stronger among boys than in girls (Table 1). This was similarly seen in infections occurring before and after autoantibody seroconversion. The highest risk was related to infections that occurred in boys during the 6-month period prior to the first autoantibody-positive sample (OR 18.2 [95% CI 2.0–164.5], $P < 0.01$) (Table 1).
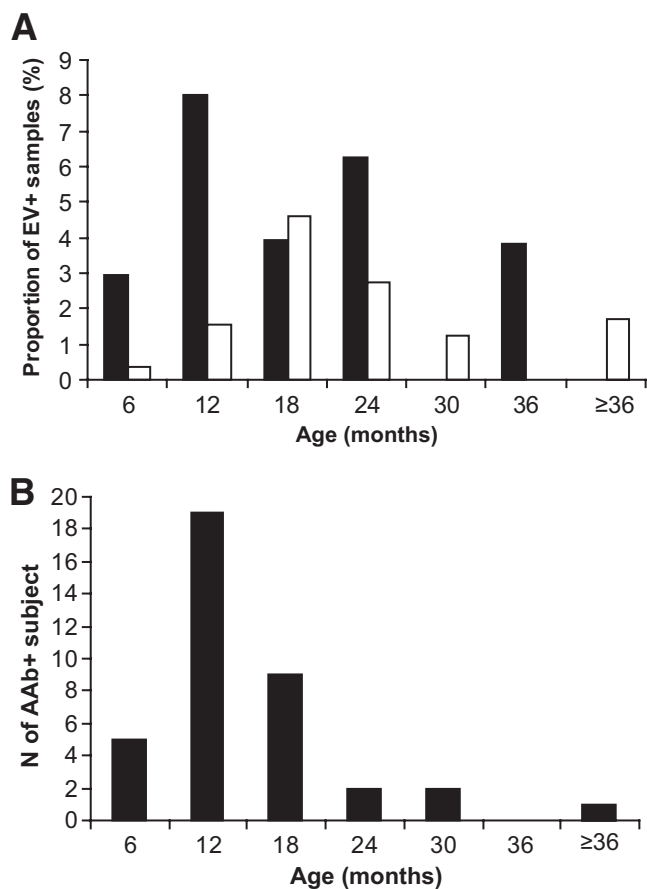
FIG. 2. Age of the first detection of enterovirus (EV) RNA (*A*) and autoantibodies (*B*) in serum. ■, case children; □, control children.

The age of the child had an influence on the frequency of enterovirus RNA in serum. In children who were younger than 6 months of age, only 1.0% of the samples were enterovirus RNA positive compared with 3.5% of samples in 6- to 18-month-old children and 5.0% of the samples in children aged 18–24 months. At the age of 2 years, the frequency of virus-positive samples decreased to 4.3%; the frequency decreased further to 2.0% in children older than 2 years. Case children had the first enterovirus RNA positivity earlier than control children (median 10 vs. 16 months, respectively) (Fig. 2*A*). The age when autoantibodies were first detected varied from 4 months to 3 years and 5 months (median 12 months) (Fig. 2*B*.).

In three case children, more than one follow-up sample was enterovirus RNA positive (two positive samples in one child and three positive samples in two children). None of these samples were consecutive, and there were virus-negative samples in between the positive ones. Different virus genotypes were present in repeatedly positive samples in each of these children.

More samples were enterovirus RNA positive in case children with high-risk HLA DR3-DQ2/DR4-DQ8 genotype than in children who carried moderate-risk genotypes with the DR4-DQ8 haplotype (6.8 vs. 2.2%) ($P < 0.002$).

DISCUSSION

In the present study, enterovirus RNA was detected in serum long before the diagnosis of clinical type 1 diabetes. The frequency of virus peaked during the 6-month window that preceded the first appearance of diabetes-associated autoantibodies. This temporal relationship has also been observed in our previous studies, suggesting that enterovirus infections may play a role in the initiation of the β-cell–damaging process (8,11). Enterovirus RNA was also more common in the case than in the control children after the initial autoantibody seroconversion but was not detected in samples taken close to the presentation of diabetes. The absence of enterovirus RNA at the diagnosis of diabetes is in contrast to the majority of the retrospective case-control studies where altogether an average of 31% of the patients and 6% of the control subjects have been positive for enterovirus RNA (3). The controversy might be due to methodological differences, e.g., the sensitivity of the PCR applied or the type of samples collected (16). On the other hand, it may also reflect a true difference in enterovirus epidemiology. In fact, we have previously observed that enterovirus infections are less frequent in Finland compared with many other countries (17,18). In any case, the present study emphasizes the importance of those infections that occur during the early stages of the diabetogenic pathway, possibly playing a role in the initiation of the β-cell– damaging process rather than the later stages of the process.

As enterovirus viremia usually lasts for a maximum of 2 weeks, the number of enterovirus episodes is largely underestimated if samples are collected at longer intervals. Accordingly, we can estimate the true number of enterovirus RNA–positive episodes (*N* of positive samples/ detection period covered by the collected samples × total follow-up time), which would be 154 episodes in case and 254 in control children (mean 3.7 vs. 1.6 episodes per child). However, it is also possible that the difference between the case and control children reflects prolonged enterovirus episodes in the case group.

Our previous studies among DIPP children suggest that the detection of the first diabetes-associated autoantibody and enterovirus RNA in stools shows similar seasonal variation (19). In the present study, the same seasonal pattern was seen in the detection of enterovirus RNA in serum (frequent in the autumn and winter). In addition, viral RNA was most common in serum at the age when autoantibodies were most frequently induced. Autoantibodies became detectable soon after detection of enterovirus RNA in the serum. In mouse models, this time interval has also been short (20).

It has previously been shown that children are protected against enterovirus infections by maternal antibodies and that this protection is at least partly mediated by antibodies in breast milk. In Finland, children are breastfed for an average of 8 months (range 0.15–23months) (21). This may explain partly the low frequency of viral RNA observed in infants aged <6 months. Viral RNA was most frequent in 12- to 18-month-old children, suggesting that there is a susceptibility period at that age.

Enterovirus persistence has been shown to play a role in chronic cardiomyopathies, and it may also be involved in type 1 diabetes (5,22,23). In the present study, no signs of persistent systemic infection were seen; enterovirus genomes that were detected from repeatedly positive children represented different viral genotypes. However, in persisting infections, the virus replication can occur at a very low level and the virus may not be detectable in peripheral blood even though it may be present in the pancreas or other organs (4,23–25).

The risk effect of enterovirus RNA on autoantibody development was stronger among boys than among girls.

Boys are also known to be more susceptible to general complications of enterovirus infections. The higher number of enterovirus RNA–positive samples in children with the DR3-DQ2/DR4-DQ8 genotype merits further investigation to find out whether this genotype is particularly susceptible to diabetogenic effect enteroviruses.

In conclusion, the present study supports the hypothesis that enteroviruses play a role in the pathogenesis of type 1 diabetes. The presence of virus in serum was shown to be a risk factor for the development of β-cell–specific autoimmunity, which progress to clinical diabetes, especially among boys.

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Virus Antibody Survey in Different European Populations Indicates Risk Association Between Coxsackievirus B1 and Type 1 Diabetes

Enteroviruses (EVs) have been connected to type 1 diabetes in various studies. The current study evaluates the association between specific EV subtypes and type 1 diabetes by measuring type-specific antibodies against the group B coxsackieviruses (CVBs), which have been linked to diabetes in previous surveys. Altogether, 249 children with newly diagnosed type 1 diabetes and 249 control children matched according to sampling time, sex, age, and country were recruited in Finland, Sweden, England, France, and Greece between 2001 and 2005 (mean age 9 years; 55% male). Antibodies against CVB1 were more frequent among diabetic children than among control children (odds ratio 1.7 [95% CI 1.0–2.9]), whereas other CVB types did not differ between the groups. CVB1-associated risk was not related to HLA genotype, age, or sex. Finnish children had a lower frequency of CVB antibodies than children in other countries. The results support previous studies that suggested an association between CVBs and type 1 diabetes, highlighting the possible role of CVB1 as a diabetogenic virus type.

A connection between enterovirus (EV) infections and human type 1 diabetes has been documented in a variety of studies (1–3). Meta-analyses of studies on direct detection of EVs in blood or tissues have indicated a clear risk effect (odds ratios [ORs] 5.5–17.4) (4), whereas serological studies have shown inconsistent results (5). Accordingly, invasive infection, as reflected by the presence of EV in blood or tissues, rather than superficial

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See accompanying commentary and original article, pp. 384 and 446.

(i.e., mucosal) infection is possibly needed for the development of β-cell damage.

On the other hand, many serological studies have been based on methods that cannot differentiate EV types from one another. In a scenario where only some of the >100 different EV types would induce type 1 diabetes, their effect could easily be missed if only pan-EV assays are used (antibodies against diabetogenic EV types can be masked by antibodies against all other EV types). For example, one can argue that if the etiology of poliomyelitis would have been evaluated in retrospective case-control studies, it would have been impossible to identify the three EV types that cause polio paralysis (poliovirus types 1–3) with such broadly reactive pan-EV antibody assays because the background frequency of EV infections was high when poliovirus antibody studies were conducted. This aspect is particularly relevant if antibodies are measured a long time after the causative infection, such as at the time when type 1 diabetes becomes clinically apparent.

The current study was designed to detect antibodies specifically against each of the six group B coxsackievirus (CVB) serotypes in patients with type 1 diabetes and matched control subjects. We focused on the CVB group of EVs because previous studies have shown that human pancreatic islets strongly express the coxsackievirus and adenovirus receptor (CAR) (6). CAR is the major receptor for CVBs and is not used by other EV types (7). Because the receptor binding partly explains the tropism of different EV serotypes to various organs (e.g., polioviruses to the central nervous system), the expression of CAR in the pancreatic islets fits with possible tropism of CVB group EVs to these cells. In fact, previous studies have documented the presence of EV RNA and EV proteins in the pancreatic islets of patients with type 1 diabetes (8–10), but the serotype of the involved EVs has not been identified. CVBs have also been linked to diabetes in mouse models, case reports, and case-control studies. In the early studies of the 1960s, Gamble et al. (11) reported neutralizing antibodies against CVB group EVs more frequently in patients affected by type 1 diabetes than in control subjects. Later studies identified species B EV sequences in the blood of type 1 diabetic patients, and CVB4 has been isolated from the pancreata of two patients with newly diagnosed type 1 diabetes (12,13). In addition, we have recently observed that of 41 different EV serotypes screened in prospectively observed children carrying HLA genes conferring susceptibility to type 1 diabetes, only CVB serotypes modulated the risk of β-cell autoimmunity and clinical type 1 diabetes (14).

In the current study, the past exposure to different CVB serotypes was analyzed in children with type 1 diabetes and control subjects recruited in five European countries. The study was based on the measurement of neutralizing antibodies, which are specific and sensitive indicators of past infection by a given EV serotype, thus reflecting the infection history of the child (serological scar).

RESEARCH DESIGN AND METHODS

The study population included 249 patients with newly diagnosed type 1 diabetes and 249 control subjects who were matched pairwise according to the time of sampling, sex, age, and country (Table 1). They were recruited in Finland (103 case-control pairs), Sweden (58 pairs), England (13 pairs), France (43 pairs), and Greece (32 pairs) between 2001 and 2005 in the European Union–funded Viruses in Diabetes (VirDiab) study. In Finland, the patients and control subjects were also matched for the HLA-conferred risk for type 1 diabetes since control subjects were recruited from the Diabetes Prediction and Prevention (DIPP) birth cohort study, which observes children who carry HLA risk genes (15). In other countries, control subjects were healthy schoolchildren or children coming to the local hospital for minor elective surgery. Children with malignant or autoimmune diseases, with chronic infections (e.g., hepatitis), and from infectious disease wards were excluded as control subjects. A case child's family members or classmates were also not accepted as control subjects. However, children with sporadic and common acute infections were not excluded. The diagnosis of type 1 diabetes was based on World Health Organization criteria. Serum samples were collected for virus antibody analyses shortly after the diagnosis of type 1 diabetes (mean 3 days; range 0–31 days). In the control group, corresponding serum samples were collected an average of 68 days after the diagnosis of type 1 diabetes in the matching case child. Because neutralizing antibodies reflect past infections and their prevalence was not associated with the season of the year, we accepted a long time difference in older children (range 512 days before to 514 days after the corresponding case sample). Blood was collected in EDTA tubes at the same time for genetic studies. Samples were stored at −80°C until analyzed.

Virus Antibodies

Neutralizing antibodies were measured against all six CVB serotypes (American Type Culture Collection [ATCC] prototype strains) with a plaque neutralization assay at the Department of Virology, University of Tampere, Finland. In addition, antibodies against CVB1 and CVB3 serotypes were measured with the use of wild-type virus strains (PicoBank strains isolated in Finland). The serotype of all viruses was confirmed by sequencing the VP1 coding region of the viral genome (16). The serum was first mixed with 100 plaque-forming units of the virus and incubated for 1 h at 37°C followed by overnight incubation at room temperature. This mixture was then transferred to a monolayer of green monkey kidney cells on six-well plates in plaque assay medium containing minimal essential medium supplemented with 1% FBS,

Table 1—Characterization of patients with type 1 diabetes and control subjects

	Type 1 diabetic patients	Control subjects
n	249	249
Recruitment period	May 2001–January 2005	May 2001–April 2005
Age (years), mean (range)	9.0 (1.1–22.7)	9.0 (1.0–23.5)
Male sex (%)	55	55
Time of sampling since type 1 diabetes diagnosis in case child (days), mean (range)	3 (0–31)	68 (−512 to 514)
HLA-DQB genotype (%)		
DR3	20.6	12.3
DR3/DR4	27.0	8.8
DR4	37.0	46.8[1]
x	15.3	32.2
Autoantibody positive (%)		
ICA	95.0	5.8
IAA	36.3	0.6
GADA	59.4	0.0
IA-2A	74.5	0.0

[1]The high frequency of HLA-DR4 in the control group is attributable to the Finnish control children who were matched with case children also for HLA-DR and were participating in the prospective DIPP study that recruited children with type 1 diabetes–associated HLA-DR alleles (15).

40 U/mL penicillin-streptomycin, 0.0023% glucose, 1 × L-glutamine, 1.5 mmol/L MgCl$_2$, and 1.5 mmol/L carboxymethyl cellulose (HEPES). The number of plaques was counted after 48 h of incubation at 37°C. All test runs included both virus-positive and virus-negative control wells (17). Sera were tested in two dilutions (1/4 and 1/16), and the sample was judged seropositive if either of these dilutions inhibited >80% of the plaques. Samples were also analyzed for higher titers (1/64, 1/256, and 1/1,024) of antibodies against ATCC CVB strains.

Diabetes-Associated Autoantibodies

Islet cell antibody (ICA) was detected by indirect immunofluorescence, whereas insulin autoantibody (IAA), GAD antibody (GADA), and insulinoma-associated protein 2 autoantibody (IA-2A) were quantified with radiolabel binding assays as previously described (18).We used cutoff limits for positivity of 2.5 JDRF units for ICA, 3.48 relative units (RU) for IAA, 5.36 RU for GADA, and 0.43 RU for IA-2A, representing the 99th percentile in >350 Finnish children. The disease sensitivity and specificity of the ICA assay were 100 and 98%, respectively, in the fourth round of the International Workshops on Standardization of the ICA assay. The disease sensitivity of the IAA assay was 58% and the specificity 100% in the 2005 Diabetes Autoantibody Standardization Program Workshop. The same characteristics of the GADA assay were 82 and 96%, respectively, and those of the IA-2A assay were 72 and 100%, respectively.

HLA Genotyping

Alleles in the HLA-DQB1, -DQA1, and -DRB1 genes were defined by panels of sequence-specific oligonucleotides and the presence or lack of disease-associated HLA-DRB1*03-DQA1*05-DQB1*02 and HLA-DRB1*04-DQA1*03-DQB1*03:02 haplotypes documented in each subject (19). Altogether, 189 patients and 171 control subjects were available for genetic analyses, providing 148 HLA-typed case-control pairs.

Statistical Analyses

Descriptive statistics are presented as proportions, frequencies, and means for demographic data and proportions and frequencies for genotypes and autoantibodies. ORs and 95% CIs were estimated for factors associated with type 1 diabetes, using conditional logistic regression to account for the matched-pair study design. Two-tailed $P < 0.05$ was considered statistically significant. Data were analyzed using Stata 12.1 (StataCorp LP, College Station, TX) statistical software.

RESULTS

Neutralizing Antibodies Against CVBs in Different Countries and Age-Groups

The prevalence of antibodies against different CVB serotypes varied. CVB2, CVB3, CVB4, and CVB5 were the most common serotypes, whereas CVB1 and CVB6 were less frequent (Fig. 1). CVB antibody prevalence also varied considerably among countries. The most striking difference was that Finland had fewer CVB infections than other countries: All six CVB serotypes were most uncommon in Finland except CVB6, which was relatively rare in all countries (Fig. 1). The antibody prevalence increased by age in children <8 years old; thereafter, a plateau was observed (Fig. 2A). Altogether, 28% of the children were seropositive for at least one CVB serotype
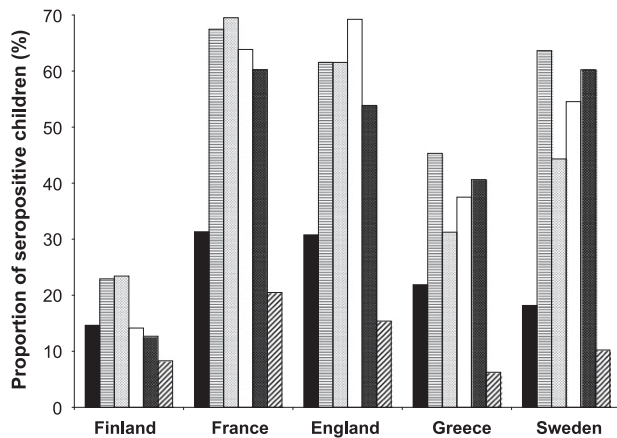
Figure 1—Prevalence of neutralizing antibodies against different CVB serotypes in various countries (neutralizing antibody titer ≥4 by plaque assay). Black bars represent CVB1; horizontally striped bars, CVB2; white dotted bars, CVB3; white bars, CVB4; black dotted bars, CVB5; and skew striped bars, CVB6.

before the age 4 years compared with >80% of children >8 years ($P < 0.001$). This age association varied for different CVB serotypes. CVB1 was frequent already at a very young age but did not show as sharp an increase at older ages as did CVB2 to CVB5 (Fig. 2B). The average antibody levels also showed clear variation among CVB types: Antibody responses against CVB3 and CVB4 were the strongest, whereas the antibody responses against CVB1 and CVB6 were the weakest (Fig. 3).

Neutralizing Antibodies Against CVBs in Patients With Type 1 Diabetes and Control Subjects

Neutralizing antibodies were first measured against all six CVB types by ATCC CVB prototype strains. Antibodies to CVB1 were the only ones whose prevalence differed between the case and control subjects, being more frequent in diabetic children (Table 2). This finding was confirmed with a wild-type CVB1 strain that gave almost identical results of a significantly higher prevalence of CVB1 antibodies among patients (Table 2). The risk character of CVB1 was seen in both sexes and in different age-groups (data not shown). This risk character was also observed when different antibody levels were used as a cutoff for seropositivity (titer 1/16, OR 1.7 [95% CI 0.9–3.4], $P = 0.133$; titer 1/64, 1.6 [0.8–3.5], $P = 0.198$; titer 1/256, 2.0 [0.7–5.9], $P = 0.206$) and when the effect of CVB1 was adjusted for the effect of the HLA-DQ genotype (adjusted OR for ATCC CVB1 1.6 [0.8–3.3], $P = 0.173$), although these analyses were not statistically significant. (Only a subgroup of children was analyzed for different antibody levels and HLA alleles.)

Because the prevalence of CVB antibodies was considerably lower in Finland than in other countries, we compared CVB antibodies between case and control subjects separately in Finland and the other countries. CVB1 showed a similar trend for increased frequency in
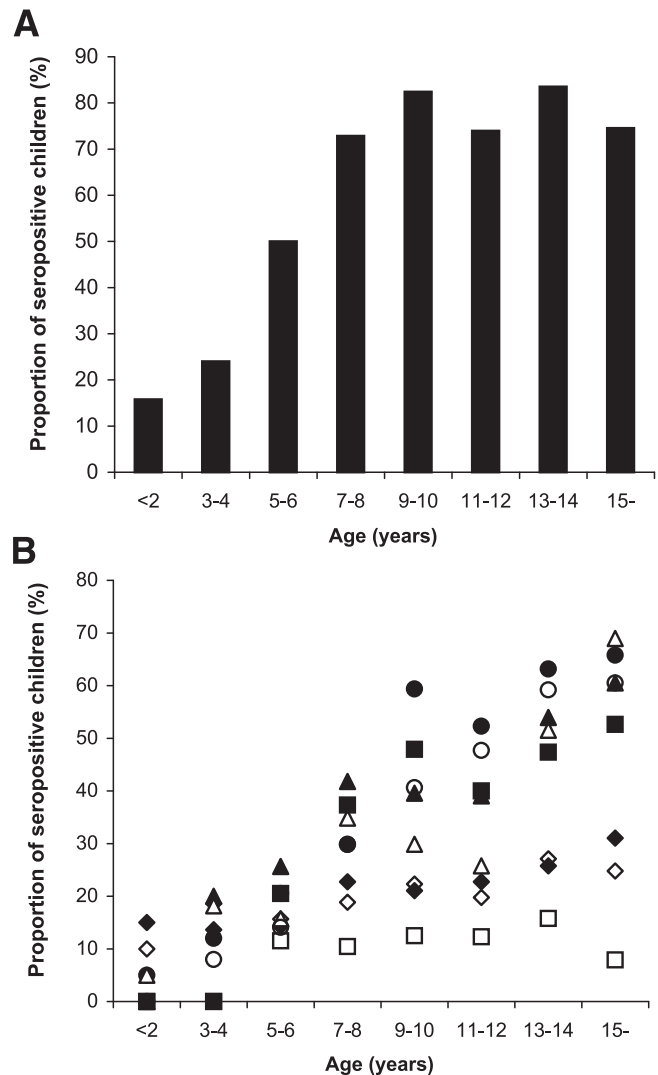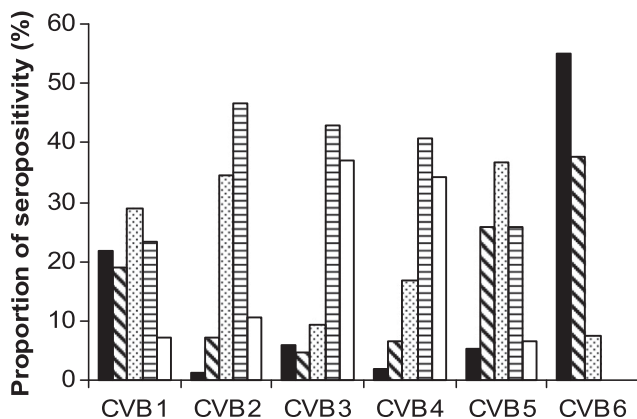

Figure 2—Proportion of children positive for CVB antibodies in different age-groups (neutralizing antibody titer ≥4 by plaque assay). A: Children positive for at least one CVB serotype. B: Children positive for individual CVB serotypes. ◇, children positive for CVB1; ◆, CVB1 wild type; ●, CVB2; ▲, CVB3; △, CVB3 wild type; ○, CVB4; ■, CVB5; □, CVB6.

case subjects both in Finland (OR 2.2 [0.8–5.7]) and in other countries (1.5 [0.8–3.0]), although the difference was not statistically significant in these subgroups.

None of the other CVB serotypes differed between case and control subjects. The overall cumulative number of antibodies to different CVB types also did not differ between these groups (mean 1.8 vs. 1.7, respectively).

DISCUSSION

The results of the current study are in line with those from previous studies, suggesting an association between CVBs and type 1 diabetes. In the current study, CVB1 was the only CVB serotype showing this association. The significance of this finding is emphasized by the very same virus type recently being observed to increase the

Figure 3—Relative proportion of low and high antibody titers among children seropositive for different CVB serotypes. Altogether, 249 patients and 249 control subjects were analyzed for the presence of different titers of antibodies against ATCC CVB strains. Black bars represent a titer of 1/4; skew striped bars, titer 1/16; dotted bars, titer 1/64; horizontally striped bars, titer 1/256; and white bars, titer 1/1,024. Statistical significance when the distribution of CVB1 antibody titers was compared with that of other serotypes are as follows: CVB1 vs. CVB2, $P = 0.0001$; CVB1 vs. CVB3, $P = 0.0001$; CVB1 vs. CVB4, $P = 0.0001$; CVB1 vs. CVB5, $P = 0.008$; and CVB1 vs. CVB6, $P = 0.0001$.

risk of type 1 diabetes in the large, prospective DIPP study (14). In DIPP, EV infections were identified by measuring neutralizing antibodies against 41 different EV serotypes in children who were followed from birth and who developed multiple type 1 diabetes-associated autoantibodies as well as in control children. The risk association of CVB1 was seen already before type 1 diabetes–associated autoantibodies appeared, suggesting a potential role of CVB1 infections in initiating β-cell damage (14).

The identification of CVB1 as a risk virus in these two independent studies supports the biological significance of this finding because the detection of the same signal in both studies just by chance is unlikely, especially with the study designs being different in many ways. The current study is based on a cross-sectional design that included children who had recently been diagnosed with clinical type 1 diabetes, whereas the DIPP study was based on a prospective birth cohort where samples had been taken during the prediabetes period. In principle, the detection of neutralizing antibodies in the current study covered all past CVB infections, including those that occurred after the β-cell damaging process began. In addition, antibodies were measured against ATCC reference strains, whereas only wild-type strains were used in DIPP; additionally, DIPP samples were analyzed in another laboratory. Of most importance, the current study covered different countries and populations, whereas the DIPP study was carried out only in Finland, and the children analyzed in these two studies were conceived during different time periods. Nonetheless, the risk character of different CVBs still may vary according to time and place, and we cannot exclude the possibility that other CVB types, such as CVB4, could also be associated with type 1 diabetes in a certain time period or geographic region, as suggested in previous studies (11–13).

One limitation of the current study is that the case and control subjects were not completely matched for type 1 diabetes–associated HLA genotypes. Previous studies suggested that these genotypes may modulate antibody responses against EVs (20) and detection of EVs in patients with type 1 diabetes (21). The HLA genotype is also known to modulate the immune response and the course of many other viral infections (22,23). However, such an HLA effect can hardly explain the elevated CVB1 antibodies in patients with type 1 diabetes because the

Table 2—Neutralizing antibodies against different CVB serotypes in patients with type 1 diabetes and control subjects

	Antibody prevalence (%)			
	Type 1 diabetic patients ($n = 249$)	Control subjects ($n = 249$)	OR (95% CI)	P value
CVB1-all	28.5	18.5	1.7 (1.06–2.74)	0.03
CVB1-ATCC	24.4	15.9	1.7 (1.02–2.92)	0.04
CVB1-wt	24.9	17.3	1.8 (1.05–3.00)	0.03
CVB2-ATCC	44.9	42.7	1.1 (0.73–1.72)	0.59
CVB3-all	37.3	40.6	0.8 (0.55–1.20)	0.29
CVB3-ATCC	37.2	40.3	0.8 (0.56–1.24)	0.38
CVB3-wt	30.0	33.7	0.8 (0.54–1.32)	0.45
CVB4-ATCC	35.5	38.4	0.9 (0.52–1.46)	0.60
CVB5-ATCC	38.0	34.5	1.1 (0.69–1.9)	0.61
CVB6-ATCC	10.3	11.6	0.8 (0.42–1.45)	0.44

Neutralizing antibody-positive serotypes were samples having a titer ≥4 by plaque assay. all, antibodies against either ATCC or wt strain; wt, wild type.

risk effect of CVB1 remained when adjusted for the effect of HLA, and it was seen in Finland where case and control subjects were matched for diabetes-associated HLA genotypes. In addition, the same risk effect of CVB1 was recently observed in the DIPP study, where case and control subjects were matched for type 1 diabetes–associated HLA genotypes (14). An additional limitation of the current study is that the time of sample draw was not exactly the same in patients and their matched control subjects (i.e., the sample from control subjects was taken an average of 68 days after the diagnosis of type 1 diabetes in the case patient). However, considering that neutralizing antibodies remain elevated for years after infection and that the samples were taken randomly all year round and at a later time point in control subjects, the timing of sample draw is an unlikely explanation for the increased frequency of CVB1 antibodies in the case patients.

The measurement of neutralizing antibodies allowed us to study the past exposure to individual CVB serotypes in an unbiased way. These antibodies persist for years or decades and are a sensitive indicator of past infection. They are also highly specific for the EV serotype used in the assay, suggesting that elevated CVB1 antibodies in patients with type 1 diabetes are not elicited by unspecific immune reactivity. The specific nature of this observation is supported by the fact that the difference in CVB1 antibodies between case and control subjects was seen at both low and high antibody levels and that the antibodies against all other CVB types did not differ between subject groups. Furthermore, neutralizing antibodies are biologically active because they neutralize the infectivity of the virus and correlate with immune protection against that EV serotype. On some occasions, however, the neutralizing antibody response can remain low and even be transient, especially if the infection is caused by a low dose of the virus (24). Therefore, the prevalence of neutralizing antibodies in this kind of cross-sectional retrospective survey may underestimate the true number of past infections, making it difficult to assess the proportion of diabetes cases that could be causally linked to CVBs.

On the basis of the current findings, the neutralizing antibody levels against CVB1 and CVB6 were lower than those against other CVB types, and they did not increase by age as clearly as antibodies against other serotypes did, suggesting that a proportion of CVB1 and CVB6 infections may have remained undiagnosed. This kind of variation in neutralizing antibody responses against different serotypes has also been described after vaccinations with live poliovirus (i.e., poliovirus type 1 and 3 induce lower antibody responses than poliovirus type 2). Such a low neutralizing antibody response may also have biological significance, making the virus able to evade the host's immune response. This is seen clearly in patients with antibody deficiencies who can develop severe and chronic EV infections. This scenario has been proposed

for human parechoviruses, which are close relatives of EVs (25).

In our previous study in the prospective DIPP birth cohort, CVB3 was observed to have a strong protective effect against type 1 diabetes (14). Further analyses indicated that this effect could be mediated by immunological cross-protection against CVB1 infections, attenuating its diabetogenic effect. However, this kind of protective effect of CVB3 was not detected in the current study. One possible explanation is the chronological order of CVB1 and CVB3 infections. In the DIPP series, we were able to identify the exact timing of infections by measuring antibodies in longitudinal follow-up samples, and the protective effect of CVB3 was related to early CVB3 infections that occurred before CVB1 (14). Accordingly, the possible protective effect of early CVB3 infections may have remained unrecognized in the current study because this serological scar may have been faded by antibodies induced by CVB3 infection occurring after CVB1 infection.

The prevalence of CVB antibodies was lower in Finland than in other counties, supporting our previous studies that found a relatively low frequency of EV infections in Finland (26). In contrast to EV infections, the incidence of type 1 diabetes is exceptionally high in Finland (the highest in the world at ~60 per 100,000 children per year) (27). The frequency of EV infections has also declined over the past decades in both Finland and Sweden, whereas the incidence of type 1 diabetes has increased in both countries (17). On the basis of these findings, we launched the polio hypothesis, which claims that a low frequency of EV infections in the background population increases the risk of EV-induced β-cell damage (28). This hypothesis is based on an analogous experience from another EV disease, paralytic poliomyelitis, which is the well-known complication of poliovirus infection and similarly associated with a low frequency of poliovirus infections in the population (28). The polio hypothesis has recently been supported by mouse studies, showing that the absence of maternal EV antibodies increases the risk for severe outcomes of an EV infection in offspring (29). However, in the present study this relationship was not absolute because, for example, Sweden had a relatively high frequency of infections but has a high incidence of type 1 diabetes.

In conclusion, the current study supports the idea that CVBs may include diabetogenic virus types and indicates that CVB1 may be one of them. However, according to the present data, predicting how large a fraction of type 1 diabetes could be causally related to CVB infections is difficult. Experience from other EV diseases, such as polio, suggests that a very small proportion of infected individuals will ever develop the disease, leading to a scenario where the antibody prevalence can be almost similar in case and control subjects in this kind of cross-sectional study (30). Therefore, prospective studies are needed to confirm the risk effect

of CVB infections and to evaluate their etiologic fraction. In any case, the present observations together with previous studies showing that CVB1 infection is frequent (31) and able to cause insulitis and islet cell damage in young babies (32) as well as pancreatitis, transient diabetes (33,34), and persisting infection in mice (2) make it an attractive target for further studies addressing the possible role of EVs in the pathogenesis of type 1 diabetes.

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