HANNA VISKARI

Enterovirus Immunity and Maternal Enterovirus Infections

Connection to Type 1 Diabetes

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Tampere, for public discussion in the auditorium of Finn-Medi 5, Biokatu 12, Tampere, on August 27th, 2005, at 12 o’clock.
ACADEMIC DISSERTATION
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Tampere University Hospital, Centre for Laboratory Medicine,
Department of Clinical Microbiology
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Finland

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Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 1095
ISBN 951-44-6358-7
ISSN 1455-1616

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2005

Electronic dissertation
Acta Electronica Universitatis Tamperensis 454
ISBN 951-44-6359-5
ISSN 1456-954X
http://acta.uta.fi
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<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>CAR</td>
<td>coxsackievirus-adenovirus receptor</td>
</tr>
<tr>
<td>CAV</td>
<td>coxsackie A virus</td>
</tr>
<tr>
<td>CBV</td>
<td>coxsackie B virus</td>
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<tr>
<td>CFS</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CRS</td>
<td>congenital rubella syndrome</td>
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<tr>
<td>DAF</td>
<td>decay-accelerating factor</td>
</tr>
<tr>
<td>DIPP</td>
<td>the Finnish Diabetes Prediction and Prevention Study</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
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<td>EIU</td>
<td>enzyme immunoassay unit</td>
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<tr>
<td>EV</td>
<td>echovirus</td>
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<tr>
<td>GADA</td>
<td>glutamic acid decarboxylase antibody</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IAA</td>
<td>insulin autoantibody</td>
</tr>
<tr>
<td>IA-2A</td>
<td>tyrosine phosphatase-like protein antibody</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell antibody</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
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<tr>
<td>IPV</td>
<td>inactivated polio vaccine</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>OPV</td>
<td>oral polio vaccine, live attenuated</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>PV</td>
<td>poliovirus</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RV</td>
<td>rubella virus</td>
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Studying enterovirus immunity and maternal enterovirus infections – connection to type 1 diabetes

Enterovirus infections have been connected to type 1 diabetes. In the present study the possible role of intrauterine enterovirus infection as a risk factor for type 1 diabetes was evaluated. In addition, the epidemiology of enterovirus infections and herd immunity to enteroviruses was analysed in different populations to study their correlations with the risk of type 1 diabetes in these populations.

The possible role of first trimester enterovirus infection was studied in 1162 mothers, whose child later presented with type 1 diabetes and an equal number of control mothers by analysing enterovirus IgG and IgM class antibodies using EIA. Serum samples for virus analyses were taken at the end of the first trimester of pregnancy. In another series enterovirus infections were analysed during the whole period of pregnancy in 70 mothers whose children developed type 1 diabetes and in 133 HLA matched control mothers. In this series, virus analyses were carried out from cord blood samples and samples taken at the end of the first trimester. In spite of the observed trend for slightly higher frequency of IgM class antibodies in case mothers than in control mothers, the results suggest that enterovirus infection during pregnancy is not a major risk factor for type 1 diabetes. The results suggest that enterovirus infection during pregnancy is not a major risk factor for type 1 diabetes in the child, but may play a role in some susceptible subjects.

The epidemiology of enterovirus infections and type 1 diabetes was analysed in different countries in a randomly selected background population of infants (N=554), schoolchildren (N=887) and pregnant women (N=1176) by analysing enterovirus antibodies using EIA and plaque neutralisation assays. In order to assess the temporal trend in enterovirus infections, enterovirus antibodies were also measured from stored samples taken from pregnant women between 1983 and 2001 in Finland and Sweden (N=1999) and schoolchildren between the years 1975 and 2002 (N=438) in Finland. Enterovirus antibodies were less frequent in countries with a high incidence of type 1 diabetes (Finland and Sweden) compared to low incidence countries (Estonia, Germany, Hungary, Israel, Lithuania and the Karelian Republic of Russia). This difference was observed in all age groups. In addition, a linear decrease was observed in maternal enterovirus antibody levels over the past 20 years in Finland and Sweden. The results suggest that enterovirus infections are not particularly common in countries with high diabetes incidence. In contrast, there seems to be an inverse correlation between the incidence of type 1 diabetes and enterovirus infections in the...
background population. Accordingly, a new hypothesis (the polio hypothesis) was generated proposing that a low frequency of enterovirus infections in the background population increases the susceptibility of young children to the diabetogenic effect of enteroviruses.

Collectively, the results suggest that enterovirus infections during pregnancy are not a major cause of type 1 diabetes in children, but may play a role in a subgroup of patients. In addition, there seems to be an inverse relationship between the frequency of enterovirus infections and type 1 diabetes in the background population, leading to a hypothesis that weak herd immunity to enteroviruses and low protection of infants by maternal antibodies may increase the risk of type 1 diabetes and contribute to the increasing incidence of type 1 diabetes.
Hanna Viskari: Enterovirusimmuniteetin ja raskauden aikaisen enterovirusinfektion yhteys tyypin 1 diabetekseen

Enterovirusinfektion on yhdistetty tyypin 1 diabetekseen. Tässä työssä tutkittiin raskauden aikaisen enterovirusinfektion yhteyttä myöhemmin lapsella kehittyvään tyypin 1 diabetekseen tapaus-verrokki aineista. Lisäksi tutkittiin enterovirusten epidemiologiaa ja sen yhteyttä diabeteksen ilmaantuvuuteen viimeisen 20 vuoden aikana eri populaatioissa, joissa on suuret erot diabeteksen ilmaantuvuudessa.

Raskaudenaikaisen enterovirusinfektion osuutta myöhemmin lapsella kehittyvään diabetekseen tutkittiin kahdella näytesarjalla analysoimalla seerumin IgG ja IgM luokan vasta-aineita EIA menetelmällä. Alkuraskauden enterovirusinfektioita tutkittiin kolmannen raskauskuukauden lopulla otetusta näyteestä 1162 äidiltä, joiden lapsi myöhemmin sairastui tyypin 1 diabetekseen sekä yhtä monelta kontrolliäidiltä. Toisessa näytesarjassa tutkittiin koko raskauden aikaisia enterovirusinfektioita 70:n myöhemmin tyypin 1 diabetekseen sairastuneen lapsen sekä 133 HLA-kaltaistetun verrokkin napaseerumista ja äitien raskauden kolmannen raskauskuukauden lopulla otetusta näyteestä. Huolimatta alkuraskauden enterovirusinfektioiden pienestä ylimäärästä diabetekseen sairastuvien lasten äidelillä, enterovirusinfektioiden esiintyvyydessä ei havaittu suuria eroja raskauden aikana tapaus- ja verrokkiäitien välillä. Tulosten mukaan raskaudenaikainen enterovirusinfektio ei merkittävästi lisää syntyvän lapsen diabetesriskiä, mutta sillä voi olla osuutta pienellä osalla tapauksista.


Tiivistelmä
kehittämiseen (nk. poliohypoteesi), jonka mukaan vähäinen enterovirusten esiintyminen väestössä voi altistaa lapset enterovirusinfektioiden mahdollisille komplikaatioille, kuten tyyppi 1 diabetekselle.

Väitöskirjatutkimuksen löydösten mukaan raskaudenaikainen enterovirusinfektio ei ole suuri riskitekijä lapsen diabetekselle. Sen sijaan laumaimmuniteetin heikkeneminen ja vähentynyt äitien tarjoama eväsimmuniteetin määrä voi altistaa lapset enterovirusinfektion indusoimalle tyyppi 1 diabetekselle ja selittää osaltaan tyyppi 1 diabeteksen yleistymisen.
INTRODUCTION

Type 1 diabetes mellitus is a chronic disease where insulin-producing beta cells are selectively destroyed. Loss of beta cells results in elevated blood glucose levels in the individual and finally death if not treated with insulin injections. Patients with type 1 diabetes require lifelong insulin therapy and they are also predisposed to considerable morbidity and reduced life expectancy. The incidence of type 1 diabetes in Finland is the highest in the world, and has continued to rise steadily for decades. The pathogenesis as well as the reasons for the epidemiological manifestation of the disease are largely unknown. Both genetic and environmental factors are thought to play a role in the process.

Cumulative knowledge of the role of virus infections, especially enterovirus infections, in the pathogenesis of type 1 diabetes has led to a hypothesis that in certain individuals virus infections may initiate the process leading to type 1 diabetes even years before the manifestation of the disease. Markers of enterovirus infections have been found to be more common in children with type 1 diabetes than in controls, sometimes already in utero. In prospective studies, enterovirus infections have been shown to coincide with the occurrence of type 1 diabetes-associated autoantibodies, which are considered as the first sign of the ongoing beta-cell destruction process.

The purpose of this study was to evaluate the geographical and temporal epidemiology of enterovirus infections in association with incidence of type 1 diabetes. In addition, the role of enterovirus infections during pregnancy as a risk factor for type 1 diabetes was analysed.
REVIEW OF THE LITERATURE

1. TYPE 1 DIABETES

1.1 Epidemiology

Epidemiological data have revealed considerable variation in the incidence of type 1 diabetes between different countries and continents (Onkamo et al. 1999, Karvonen et al. 2000). The highest incidence has been recorded in Finland, where nowadays nearly 500 children present with the disease every year (Reunanen A, personal communication). The other high incidence areas include Sweden, Sardinia, Norway, the UK as well as parts of North America (Karvonen et al. 2000). Low incidences of type 1 diabetes have been recorded in Asia and South America, e.g. in China and Peru, where the incidence of type 1 diabetes is over 40 times lower than in Finland (Karvonen et al. 2000). The incidence also varies remarkably in geographically proximate countries. For example, the incidence of type 1 diabetes in Finland is six times higher than in the neighboring Russian Karelia, and three times higher than in Estonia despite the genetically very similar susceptibility to the disease (Podar et al. 2001, Kondrashova et al. 2005).

The incidence of type 1 diabetes has increased worldwide in recent decades. Incidence figures from the early 1900s are rare but some reliable data exist, suggesting that the major increase in the overall incidence figures started around mid-century (Gale 2002). The overall average annual increase in incidence rates in Europe and worldwide has been estimated to be around 3% per year, with a greater relative increase in some lower incidence countries e.g. in Eastern Europe (Onkamo et al. 1999, Green and Patterson 2001). The increase has been most rapid in children under five years (Karvonen et al. 1999, Dahlquist and Mustonen 2000).

Data on type 1 diabetes incidence in Finland were published for the first time for the year 1953, when the incidence was reported to be 13/100,000 children (Somersalo 1954). Since then the incidence has increased linearly (Gale 2002). The incidence has increased four to five fold and is now reported to have been as high as 48.5/100,000 in 1998 (Podar et al. 2001) and as much as 54/100,000 in 2003 (Reunanen A, personal communication).
1.2 Genetic susceptibility

Type 1 diabetes is the only organ-specific autoimmune disease that does not show female excess. The populations with high type 1 diabetes incidence show a small male excess, while in low diabetes incidence population there is a slight excess of females (Gale and Gillespie 2001). The average risk for type 1 diabetes among first-degree relatives is about 6% compared to the population risk of 0.4-0.6%. The offspring of a diabetic father has a twice higher risk than the offspring of a diabetic mother (Tuomilehto et al 1992, Redondo et al. 2001, Field 2002).

There are several genes associated with increased risk for type 1 diabetes. The human leukocyte (HLA) genes, designated as IDDM1, in the short arm of chromosome 6 are the major determinants of the genetic predisposition comprising approximately 40% of the familial clustering of the disease (reviewed in Schranz and Lernmark 1998, Kelly et al. 2001). The HLA-DQ molecules are of primary importance but HLA-DR gene products modify the risk conferred by HLA-DQ. In the same HLA region reside the major protective alleles. Thus the risk associated with type 1 diabetes is defined by the particular combination of predisposing and protective alleles. The highest risk among Caucasians is associated with DQA1*0301-DQB1*0302 and DQA1*0501-DQB1*0201. The major protective alleles for type 1 diabetes are DQA1*0101-DQB1*0602 and DQA1*0501-DQB1*0301 (Schranz and Lernmark 1998, Kelly et al. 2001, Ilonen et al. 2002). The mechanisms of HLA-conferred disease susceptibility and protection have remained open but several mechanisms have been proposed to be implicated in the role of HLA molecules in antigen presentation and activation of lymphocytes (Kelly et al. 2001).

The HLA-gene region has been the best characterized but other gene regions have also been observed to modify genetic susceptibility to type 1 diabetes. One of these regions is the polymorphism of the insulin gene region in the short arm of chromosome 11 (IDDM2). A series of genome-wide scans have been performed and numbers of genome linkages have been found but only few of these have been shown to be of clear importance. These include e.g. the loci in 16q22-q24 and cytotoxic T-lymphocyte antigen-4 (IDDM12, CTLA-4) (Buzzetti et al. 1998, Schranz and Lernmark 1998, Cox et al. 2001, Kelly et al. 2001).

1.3 Pathogenesis

Type 1 diabetes appears when approximately 80-90% of the insulin producing beta cells are destroyed in the islets of Langerhans (Foulis et al. 1986). The preclinical phase usually lasts for many years. Progression of the disease process towards clinical manifestation is accompanied by a reduction in the normal (first-phase) insulin response to orally or intravenously administered glucose (Bingley 1996). Studies on animal models and on humans have suggested that type 1 diabetes is a T-cell mediated autoimmune disease but the actual mechanisms for the beta cell destruction and breakdown of self tolerance in humans are not known (Roep 2002).
Autoantibodies to islet cell antigen (ICA), insulin (IAA), glutamic acid decarboxylase (GADA) and tyrosine phosphatase related IA-2 protein (IA-2A) appear in the peripheral blood as a marker of beta cell destruction in the preclinical phase (Kulmala et al. 1998, Schranz and Lernmark 1998). The presence of multiple autoantibodies has been observed to be a reliable surrogate marker for type 1 diabetes both in general population and first degree relatives, while most of the single autoantibody positive individuals never progress to clinical diabetes (Bingley et al. 1997, Knip 2002). Although autoantibodies can contribute to immune responses in many ways, there is no evidence that these antibodies have a direct pathogenetic role. For example, type 1 diabetes may develop in individuals with severe B-lymphocyte deficiency (Martin et al. 2001).

2. ENVIRONMENTAL FACTORS IN THE PATHOGENESIS OF TYPE 1 DIABETES

The genetic background contributes to the risk of type 1 diabetes but it is neither necessary nor sufficient to cause the disease. The concordance rates among mono- and dizygotic twins are approximately 50% and 11% respectively, supporting both the roles of environment and genetic factors in the etiology of the disease (Hawkes 1997, Field 2002, Hyttinen et al. 2003). In Finland, nearly 20% of the population have an increased HLA conferred predisposition to type 1 diabetes, but <1% have overt diabetes by the age of 20 (Ilonen et al. 1996).

In many countries the increase in the incidence has been so rapid that it cannot be explained by increased genetic risk (Pitkäniemi et al. 2004). Two recent studies from the UK and Finland have shown that the predominance of high genetic risk genotype has decreased among type 1 diabetes patients while other genotypes have become more common. This suggests a greater environmental pressure leading to diabetes in subjects with only moderate or no genetic risk (Hermann et al. 2003, Gillespie et al. 2004). In addition, the incidence figures vary notably in geographically closely located areas in populations whose genetic risk for type 1 diabetes is about the same (Kondrashova et al. 2005). Studies in populations migrating from a low-incidence area to a high-incidence area have shown that the incidence of type 1 diabetes is higher among the offspring of migrants, supporting environmental pressure in the pathogenesis of diabetes (Siemiatycki et al. 1988, Bodansky et al. 1992, Feltbower et al. 2002).

The observed seasonality in the manifestation of clinical type 1 diabetes, seasonality in the occurrence of the disease-predictive autoantibodies and seasonality in month of birth reported in many studies support the role of infectious agents (see page 28-29). In addition, the role of environmental factors is also supported by studies showing geographical clustering of diabetes cases within countries and so-called “miniepidemics” (Law et al. 1997, Zhao et al. 2002, Samuelsson and Löfman 2004).
Thus, the current understanding of the pathogenesis of type 1 diabetes suggests that in genetically susceptible individuals environmental factors trigger an autoimmune process that leads to the destruction of the insulin secreting beta cells (Åkerblom et al. 2002). Among these environmental factors virus infections and dietary factors have most frequently been associated with type 1 diabetes.

2.1 Dietary factors

A number of dietary factors have been linked to type 1 diabetes. Possible protective effects have been linked to breastfeeding, nicotinamide, zinc and vitamins whereas N-nitroso compounds and cow’s milk may increase the risk. Despite many studies there is little firm evidence of the significance of nutritional factors in the etiology of type 1 diabetes (reviewed in Virtanen and Knip 2003).

Among vitamins, nicotinamide (vitamin B) has recently been studied in large prevention trials with negative results (Lampeter et al. 1998, Gale et al. 2004). Vitamin D supplementation during infancy has been shown to be inversely associated with the risk of type 1 diabetes in large case control and cohort studies (The EURODIAB Substudy 2 Study Group 1999, Hyppönen et al. 2001, Stene and Joner 2003). Vitamin D supplementation for infants has, however, been recommended in many countries for decades, but dose recommendations vary.

The likely candidates for dietary risk factors include early introduction of cow’s milk and short duration of exclusive breastfeeding. Increased risk for type 1 diabetes has been associated with early exposure to cow’s milk proteins in several human and animal studies, but contradictory results have been reported (reviewed in Åkerblom et al. 2002 and Virtanen and Knip 2003). Increased immunity to insulin and other proteins in CM has been reported when children were exposed to cow’s milk (Savilähti et al. 1993, Vaarala et al. 1999, Paronen et al. 2000). An independent role of cow’s milk as a risk factor has been observed, but the effect may also be related to short exclusive breast feeding (Kimpimäki et al. 2001a, Virtanen and Knip 2003). Furthermore, long breastfeeding has been shown to be an independent protective factor for type 1 diabetes (Sadauskaite-Kuehne et al. 2004). The role of milk in the pathogenesis of type 1 diabetes is currently being studied in a multicentre worldwide intervention trial (TRIGR). In the TRIGR pilot study the cumulative incidence of autoantibodies was somewhat smaller in the intervention formula (casein hydrolysate) than in the control formula group (Åkerblom et al. 2005).

The pathogenic mechanisms of dietary factors have also suggested to be related to the regulation of oral tolerance. A link between gut immune system and the islets infiltrating lymphocytes has been proposed (Vaarala 1999).
2.2 Virus infections

Virus infections have been connected to several chronic diseases including autoimmune diseases (e.g. MS disease, rheumatoid arthritis) and cancer (e.g. liver and cervical cancer), and a few of them are already or about to be preventable by a vaccine (Lai et al. 2003, Harper et al. 2004). The common feature for these diseases is the long prodromal latent period from the primary infection to the appearance of symptoms. Infection(s) has also been considered as major candidate for the environmental factors that could either initiate or accelerate the pathogenesis of type 1 diabetes. Several viruses have been associated with type 1 diabetes with the first speculations raised already a century ago. In animal models many viruses from the families of Picorna-, Retro-, Reo-, Parvo-, and Herpesviridae have been associated with type 1 diabetes as reviewed by Jun and Yoon (2003). From human viruses rotavirus-, mumps-, cytomegalovirus-, rubella- and enteroviruses have been connected to human type 1 diabetes (reviewed in Hyöty and Taylor 2002 and Jun and Yoon 2003).

Rubella virus infection during pregnancy has long been related to increased risk of diabetes in the offsprings suffering from the congenital rubella syndrome (CRS) (Menser et al. 1978, Ginsberg-Fellner et al. 1985). The pathogenetic process is not clear and many mechanisms have been proposed. In our recent study, we did not observe any increased frequency of type 1 diabetes related autoantibodies in CRS patients, suggesting that the diabetes may not be caused by an autoimmune mechanism (Viskari et al. 2003). Type 1 diabetes has also been connected to mumps epidemics and mumps virus has been shown to infect beta cells (Prince et al. 1978, Parkkonen et al. 1992). Rubella and mumps infections have, however, disappeared from many countries with a high incidence of diabetes due to vaccinations and cannot therefore contribute to the increasing incidence of type 1 diabetes. Rotavirus infections have been associated with type 1 diabetes-associated autoantibodies in an Australian study (Honeyman et al. 2000) but this finding could not be confirmed in a Finnish prospective birth cohort study (Blomqvist et al. 2002). Cytomegalovirus (CMV) has been connected to type 1 diabetes in rare case studies (Ward et al. 1979). In addition, increased markers of CMV infection in children with diabetes have been observed (Pak et al. 1988, Nicoletti et al. 1990). However, no evidence of excess CMV infections was found in children who developed ICA in a prospective study (Hiltunen et al. 1995). Recently, Ljungan virus, which belongs to the parechovirus genus in the picornavirus family (Niklasson et al. 1999), has been associated with diabetes in bank voles (Niklasson et al. 2003). In addition, increased levels of Ljungan virus antibodies were also observed at the onset of newly diagnosed type 1 diabetes in children (Niklasson et al. 2003).

Human enterovirus infections have commanded the greatest of scientific interest as they have been repeatedly associated with the risk of type 1 diabetes. The role of enteroviruses in the pathogenesis of type 1 diabetes is discussed in detail in the next section.
2.2.1. Enteroviruses

Human enteroviruses belong to the family of *Picornaviridae* together with aphtho-, cardio-, hepato-, parecho-, rhino-, erbo-, kobu- and teschoviruses. The genus of human enteroviruses consists of more than 60 serotypes which are further classified into five species according to the sequence analysis. These species include polioviruses (PV), human enterovirus A (HEV-A), HEV-B, HEV-C and HEV-D (Table 1) (Hyypiä et al. 1997, picornavirus homepage at [www.iah.bbsrc.ac.uk/virus/picornaviridae](http://www.iah.bbsrc.ac.uk/virus/picornaviridae)).

Picornaviruses are small icosahedral nonenveloped RNA viruses. They are among the simplest RNA viruses consisting of a protein capsid which surrounds the positive stranded RNA genome. The capsid consists of four structural proteins VP1, VP2, VP3 and VP4. VP1-3 forms the outer surface of the capsid and serve as the major antigenic sites during infection whereas VP4 is inside the capsid close to the viral RNA genome. The capsid surface has irregular clefts, which contain receptor binding sites, and interact with neutralizing antibodies (reviewed in Minor 1986 et al. and Racaniello 2001).

The RNA genome of enteroviruses consists of approximately 7,500 nucleotides. It is directly translated after entry into the cell and produces proteins required for viral replication and assembly. The RNA genome is covalently linked at the 5’end to a protein called VPg and at the 3’end poly(A)-tail. A highly structured 5´ non-coding region contains regions controlling genome replication and translation. The 3´ non-

<table>
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<th>Table 1. Human Enteroviruses</th>
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<tr>
<td><strong>Species</strong></td>
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<tr>
<td>Poliovirus (PV)</td>
</tr>
<tr>
<td>Human enterovirus A (HEV-A)</td>
</tr>
<tr>
<td>Human enterovirus B (HEV-B)</td>
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<tr>
<td>Human enterovirus C (HEV-C)</td>
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<tr>
<td>Human enterovirus D (HEV-D)</td>
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Table modified from Hyypiä et al. 1997 and [www.iah.bbsrc.ac.uk/virus/picornaviridae](http://www.iah.bbsrc.ac.uk/virus/picornaviridae) (14.6.2005)
coding region is short, containing a secondary structure pseudoknot, which takes part in RNA synthesis. A single long open reading frame following the 5'UTR encodes a long polypeptic, which is further cleaved to capsid proteins (VP1-4) and nonstructural proteins (2A-C, 3A-D). The nonstructural proteins take part e.g. in RNA synthesis and protein processing (reviewed in Racaniello 2001). The RNA-dependent RNA polymerase lacks proofreading activity and is thus prone to errors leading to quasispecies characteristic of the viruses (Holland et al. 1982).

RNA recombination occurs frequently in positive stranded RNA viruses and also in enteroviruses. Recombination plays an important role in the natural evolution of enteroviruses both within and between serotypes (Santti et al. 1999a, Oberste et al. 2004). For example, interserotypic exchange between Sabin vaccine strains is common in primary vaccinees and among vaccine-related paralysis cases (Furione et al. 1993, Cuervo et al. 2001). In studies of CBV and echovirus strains, recombination between serotypes was shown to occur almost entirely outside of the capsid region (Santti et al. 1999a). Moreover, there are genetic restrictions that may influence recombination making it possible only among members of a given species (e.g. HEV-B) (Lukashev et al. 2003, Chevaliez et al. 2004, Oberste et al. 2004). In addition, mutations in the antigenic sites of the capsid proteins have been implicated to affect the infectivity and infectivity of the virus (Chevaliez et al. 2004).

Polioviruses, which are the most thoroughly studied picornaviruses, are thought to multiply initially in the mucosal tissue of the upper respiratory tract and intestine. From there the virus spreads to primary replication sites in the lymphoid tissues of oropharynx and intestine (deep cervical and mesenteric lymph nodes), eventually leading to infection in systemic reticuloendothelial tissues such as lymph nodes, bone marrow, liver and spleen. After this stage a possible major viremia is thought to occur with e.g. central nervous system invasion (Pallansch and Roos 2001).

Collectively, enteroviruses can infect virtually all human tissues, including the pancreas (Roivainen et al. 2002). They use a variety of different specific receptors to initiate the infections in different cell types. These include the immunoglobulin superfamily proteins such as intracellular adhesion molecule –1 (ICAM 1) and decay-accelerating factor (DAF), as well as integrins (α2β1, αvβ3) and coxsackievirus-adenovirus receptor (CAR) (Racaniello 2001). Virus receptors alone cannot explain the tissue tropism of different enteroviruses, such as the tropism of coxsackie viruses to myocardium and polioviruses to motor neurons in anterior spinal cord but other mechanisms also play a role (Pallansch and Roos 2001, Racaniello 2001, Harvala et al. 2002). The presence of enterovirus infection induced destruction of beta cells and inflammation has been demonstrated in humans with fatal enterovirus infections supporting the in vivo islet cell tropism of enteroviruses in human pancreas (Yoon et al. 1979, Jenson et al. 1980, Ujevich and Jaffe 1980, Ylipaasto et al. 2004).

One of the prominent characteristics of enteroviruses is the cytolitic nature of their growth in cell culture (Pallansch and Roos 2001). However, some enteroviruses can also establish persistent infections in susceptible cells. Coxsackievirus and poliovirus
RNA have been found to persist for many years in human tissues. For example, the poliovirus genome has been present in the cerebrospinal fluid of patients with a poliomyelitis history and a persistent poliovirus infection has been connected to post polio syndrome (Julien et al. 1999). Persistent enterovirus infections in myocardium have also been connected to the clinical manifestation of cardiomyopathy and myocarditis in several studies (reviewed in Klingel et al. 2004). However, infectious viruses have not been isolated. CBV has also been found to persist in human pancreatic islet cells in vitro (Yin et al. 2002). In studies with coxsackievirus, activated or dividing cells supported productive infection while quiescent cells were protected from the cytopathic effects of the virus and were also more prone to persistent infection (Feuer et al. 2004). Also, receptor usage affects the establishment of persistent infection (Frisk 2001).

2.2.1.1. Epidemiology and clinical manifestations

Enteroviruses are transmitted mainly via fecal-oral or respiratory route, and the rate of transmission depends on socio-economic factors such as crowding and standard of hygiene (Pallansch and Roos 2001). Enteroviruses are often found in sewage as well as in surface and seawater and even tap water (Pallansch and Roos 2001, Lee and Kim 2002, Griffin et al. 2003). Swimming in contaminated water has been connected to epidemics of enterovirus infections as a route of transmission (Pallansch and Roos 2001). Human enteroviruses have also occasionally been detected in animals such as dogs, pigs and cattle (Grew et al. 1970, Waldman et al. 1996, Kadoi et al. 2001). However, humans are thought to be the only important natural reservoir of human enteroviruses and transmitters of viruses.

Enterovirus infections are common in all age groups. The infections are usually asymptomatic or perceived as mild respiratory illnesses but they may also lead to severe diseases such as meningitis, paralysis, myocarditis and systemic infections in newborn infants. Most primary infections with the highest amount of virus shedding already occur in infancy. Thus infants are also the most important transmitters of viruses especially in households (reviewed in Pallansch and Roos 2001).

While enterovirus infections are usually mild or subclinical, they are on the other hand the major cause of aseptic meningitis. Enteroviruses have been observed to be the infectious agent in up to 50-90% of aseptic meningitis cases, depending on the epidemic situation (Berlin and Rorabaugh 1993, McIntyre and Keen 1993, Sawyer et al. 1994, Ramers et al. 2000, Chambon et al. 2001, Böttner et al. 2002, Cuney et al. 2003). In addition, most types of enteroviruses have been isolated from cases of meningitis (reviewed in Grist et al. 1978, Pallansch and Roos 2001). Koskininen and coworkers (2001) presented virological data from Finnish patients with clinical CNS symptoms from the period 1995-1996. They observed enteroviruses to be the largest group of etiological agents (30%) in meningitis cases of suspected viral etiology. However, in this study only serological methods were used.
The age of the host modulates the outcome of most enterovirus infections. Different age groups have different susceptibilities to infection. For example, in the case of poliovirus the incidence of the rare severe form of the disease, poliomyelitis, is low for the first 4-6 months of life, when the child has maternal antibodies which can neutralise the virus (reviewed in Horstmann 1955). Thereafter, the incidence of poliomyelitis rises with older children and adults about two to three times more prone to paralytic complications than children, as observed especially in a highly susceptible population without previous exposure to poliomyelitis (reviewed in Horstmann 1955, Nathanson and Martin 1979). However, infants not protected with neutralising antibodies were exposed to high case-infection ratio with high mortality. The same phenomenon is also seen in other enteroviral diseases (Moore et al. 1984). In general, enterovirus encephalitis and meningitis are more common in 5-14 year-olds than other age groups (Pallansch and Roos 2001). On the other hand, enteroviruses can cause more severe diseases in newborns than in older children, involving multiple organs especially when maternal antibodies are not present. For example, pre- or perinatal transmission of echovirus or coxsackievirus infection from the mother have been shown to cause severe disease with increased mortality (Modlin 1986, Dagan 1996, Bryant et al. 2004). Recent papers also suggest enteroviruses as the major etiological cause for severe febrile illness in neonates (Byington et al. 1999, Rosenlew et al. 1999, Verboon-Macielel et al. 2002). There is a male-female difference of 2:1 in the occurrence of enteroviral infections. The male excess is more pronounced in the severe forms of the disease, e.g. meningitis and carditis (Pallansch and Roos 2001).

There are several reports of possible transplacental infection of enteroviruses but there is no known congenital enterovirus syndrome. Some rare associations have been suggested for coxsackievirus infections and anomalies in the newborn (Moore and Morens 1984, Keyserling 1997). Vertical transmission of enteroviruses has been shown to lead to fetal death in utero or prematurity (Greenberg and Siegel 1956, Pallansch and Roos 2001). However, maternal enterovirus infection during pregnancy does not lead to infection of the fetus in the majority of cases (Greenberg and Siegel 1956, Amstey et al. 1988).

Enteroviruses are known to be endemic but also epidemics such as meningitis also occur frequently. The endemic and epidemic viruses vary annually and geographically. Enterovirus infections show seasonality in temperate countries with most cases reported in autumn. In tropical areas enteroviruses circulate throughout the year (Pallansch and Roos 2001). Information on the epidemiology of enteroviruses usually comes from clinical laboratories or hospital records based on severe enterovirus diseases such as aseptic meningitis. A number of varying serotypes are found yearly. Different serotypes predominate in different years and one serotype can cause epidemics at interval of few years (Nelson et al. 1979, Strikas et al. 1986, Hovi et al. 1996, Maguire et al. 1999, The National Enterovirus Surveillance System 2000).

There are some reports about temporal trend in nonpolio enterovirus infections. Nairn and Clements (1999) reported such a trend since the 1970’s in isolates in the Glasgow area with echovirus infections becoming more common but coxsackieviruses
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becoming more rare. A recent paper from North America reported a decreased frequency of all cases of enteroviral disease since 1990 (Sedmak et al. 2003). They also detected echoviruses most frequently, an observation which has also been seen in other countries (Trallero et al. 2000, The National Enterovirus Surveillance System 2002, Christensen and Nordbo 2003). In contrast, an earlier report by Shattuck et al. (1992) noted an increasing trend of enteroviral meningitis in neonates over the period 1974-1988 while the overall number of reported enterovirus cases remained in the range of normal variation (The National Enterovirus Surveillance System 1985, 1986, 1987, 1988 and 1989, Strikas et al. 1986).

Some seroepidemiological surveys have been performed in the healthy background population showing the frequent and subclinical nature of enterovirus infections. Seropositivity as a sign for previous enterovirus infection ranged from 0% to 90% according to age and serotype assessed (Kogon et al. 1969, O'Neill et al. 1983, Hammond et al. 1985, Weber et al. 1994, Galama et al. 1997, Lönnrot et al. 1999a). Weber and coworkers (1994) performed a large (N=2759) seroepidemiological survey in 1990-92 in Germany where neutralising antibodies were analysed against a panel of enteroviruses: CBV1-6, CAV9, EV 6, 9, 11, and 30, and PV 1, 2 and 3. Age-related increase in the antibodies was most marked in the age groups of 1-14 years but continued to over 60 years of age in some serotypes (CBV2 and CAV2). Of all samples, 97% showed seropositivity for at least one serotype. 7% of subjects were seronegative for polioviruses. No gender difference was observed in this study.

One study has compared enterovirus epidemiology between two countries with different incidences of type 1 diabetes (Lönnrot et al. 1999a). Neutralising antibodies against CBV4 and CBV5 in 11 year-old schoolchildren in Lithuania and Finland were analysed. Seropositivity for these two coxsackieviruses was significantly higher in the children in Lithuania where the incidence of type 1 diabetes is lower than in Finland (seropositivity against CBV4 and CBV5 75% and 65 % in Lithuania vs. 63% and 38% in Finland) (Lönnrot et al. 1999a).

In Finland, in 1972-74, a survey of enteroviruses in the stools of preschool children detected a prevalence of coxsackie and echoviruses of 11% in virus isolation (Lapinleimu and Stenvik 1981). During the late 90’s, the prevalence of non-polio enteroviruses in healthy <5 year-old Italian children was 5% according to virus isolation from stool samples (Patti et al. 2000). Jenista and coworkers (1984) followed new-borns during an enterovirus season in New York in 1981 and found that the incidence of non-polio enterovirus infections was 13% during the first month of life according to virus culture. Of these infections 79% were asymptomatic. The incidence of enterovirus infections in Finland during the 1990’s was also fairly high: Juhela and co-workers (1998) reported that 30% and 60% of healthy children had had enterovirus infection according to serology by the age of 6 months and 12 months respectively. The high rate of infections is also reflected by the frequent presence of enteroviruses in sewage, which is used for means of poliovirus surveillance in the population (Hovi et al. 1996, Sedmak et al. 2003). In the Scottish blood donor material of adults,
enterovirus RNA was found in the blood 1/1800-1/9000 of the donors depending on month of sampling (Welch et al. 2003).

2.2.1.2. Immune response

Enteroviruses are usually eradicated relatively rapidly by the immune system even though the virus may replicate in the gut for several weeks. Humoral and cellular responses to both structural and non-structural viral proteins occur. Enterovirus infections induce a rapid production of heterotypic IgM class antibodies turning to class IgG and IgA response. Specific neutralising antibodies are formed a few days after onset of disease (reviewed in Tracy et al. 1995). IgG and IgA class antibodies may persist for years while IgM response usually only lasts for a few months. Re-infection with enteroviruses provokes a secondary response with rises in specific IgG titers accompanied with titer rises of other related enterovirus antibodies (Moore and Morens 1984). Protection against enterovirus infections depends mainly on neutralising antibodies. Major antigenic sites for neutralising antibodies reside on VP1 and to a lesser extent on VP2 and VP3 proteins (Minor et al. 1986). The neutralising antibodies are stable and can provide a life-long serotype specific protection. However, they do not protect against infection but against the severe form of the disease as seen in the case of polio vaccination (Beale 1990). The importance of humoral immune responses in protection against enterovirus diseases has been implicated in patients with agammaglobulinemia, who suffer from persistent enterovirus infections (McKinney et al. 1987).

Enterovirus specific T-cell responses are widely activated during natural enterovirus infection. T-cell responses help to control and boost B-cell induced humoral responses but the role of cytotoxic T cell responses in enterovirus infections is not clear. They may directly clear the virus by causing lysis of virus-infected cells and possibly relates to virus-induced tissue pathology (Henke et al. 1995, Huber 2004). Enterovirus induced T-cell responses are known to show wide cross-reactivity between serotypes, which may play a role in the group specific immunity against enteroviruses (Cello et al. 1996, Juhela et al. 1999). Enteroviral T-cell epitopes have so far been identified in VP1-4 proteins and 2C (Cello et al. 1996, Marttila et al. 2001, Marttila et al. 2002, Varela-Calvino et al. 2004).

2.2.1.3. Diagnosis

The diagnosis of enteroviral infection cannot be based on symptoms alone due to the wide variety of clinical manifestations as well as frequent subclinical infections. An antiviral drug (pleconaril) is available for enteroviral diseases, but it has side effects which limit its clinical use to a very few selected cases of severe disease (Hayden et al. 2004, Abzug 2004). A specific diagnosis, however, is important, for example, in the surveillance of community outbreaks of enteroviral diseases. The surveillance of polioviruses demands differentiation between polio and nonpolio enteroviruses. In
addition, the differentiation between vaccine-derived and natural poliovirus is very important as the vaccine-derived poliomyelitis has remained a problem after wild poliovirus infections have disappeared in most countries.

Laboratory diagnosis of enteroviral disease is challenging. The traditional gold standard of laboratory diagnosis relies on virus isolation in cell culture and typing of isolated viruses with serotype-specific neutralising antisera panels. Enteroviruses can be isolated from stools, CNS fluid, throat swabs, urine or blood (Pallansch and Roos 2001). Most often enteroviruses are found in stools or rectal swabs where they are present in high titres and the virus may be shedding for weeks or even some months (Kogon et al. 1969, Chung et al. 2001). Cell culture, however, is a demanding and labour-intensive method, and has relatively low sensitivity. In addition, properly taken and stored samples with relatively high amount of virus are needed. The reference antisera are not available for all serotypes. In addition, the antisera may not be able to recognize new virus variants as the reference antisera have been raised against reference strains 40-50 years ago (Chevaliez et al. 2004). Moreover, there are coxsackie A virus serotypes which do not grow in cell lines but only in suckling mice.

Serological diagnosis is also complicated due to the vast number of serotypes and the cross-reacting heterotypic antibody responses induced during infection. A serotype-specific diagnosis can be achieved by measuring neutralising antibodies in paired samples when a fourfold increase in the antibody titre is observed. The neutralisation method is reliable but laborious (Pallansch and Roos 2001). Group-specific diagnosis can be accomplished with antibodies measured with immunoassay methods. The rise in the IgG antibody titer in the EIA test can be measured from samples taken during the acute and convalescent phase of disease (2-4 weeks time interval) (Torfason et al. 1988). In 50-80% of enterovirus infections group-specific IgM antibodies are measurable, leading to diagnosis from a single acute-phase sample. (Bell et al. 1986, Day et al. 1989, Frisk et al. 1989, Muir et al. 1990, Samuelson et al. 1993, Swanink et al. 1993, Bendig and Molyneaux 1996).

Enterovirus genome can be detected from clinical samples using a reverse transcriptase polymerase chain reaction (RT-PCR) method. It is a fast and relatively cheap and above all, a highly sensitive method which is currently widely used in clinical laboratories. A selection of PCR protocols has been established for enteroviruses, some of them with real-time quantitative PCR (reviewed in Romero 1999, DeBiasi and Tyler 2004). The primers are targeted to amplify the 5′-untranslated region of the virus genome, which is similar in all enterovirus serotypes making a diagnosis of all enteroviruses possible even from a very meager clinical sample. Further sequencing of an amplicon, e.g. in the VP1 region may enable partial serotyping of the virus (Santti et al. 1999b, Oberste and Pallansch 2003, Thoelen et al. 2004). The high sensitivity is, however, sometimes a problem, as small amounts of the viral RNA may be present in stools for prolonged periods diminishing the clinical significance of positive findings. The high sensitivity is also a challenge for the laboratory due to the risk of contamination of negative samples.
2.2.1.4. Connection to type 1 diabetes

Enteroviruses have been connected to type 1 diabetes in many studies. The epidemiology of diabetes resembles that of enteroviruses: seasonality in the incidence of type 1 diabetes has been shown to peak after enterovirus epidemics (Gamble and Taylor 1969, Rewers et al. 1987, Wagenknecht et al. 1991). The first serological reports were made as early as 1960’s, showing more neutralising antibodies in patients with type 1 diabetes against coxsackieviruses than in control subjects (Gamble et al. 1969). Ten years later, additional evidence supporting the role of enteroviruses in the pathogenesis of type 1 diabetes came from the isolation of enterovirus (coxsackie B virus) from the pancreas of a patient who died of acute onset type 1 diabetes (Yoon et al. 1979). This virus strain further caused beta-cell necrosis and diabetes in mice. Since then the role of enteroviruses has been suggested in several but not in all seroepidemiological studies (reviewed in Hyöty and Taylor 2002, Haverkos et al. 2003, Green et al. 2004). Subsequent reports have also demonstrated enteroviruses in the pancreatic islets in diabetic and prediabetic individuals (Dotta et al. 2002, Gianani et al. 2004).

Studies showing enteroviral genome in the blood of patients with type 1 diabetes more often than controls have further supported the role enteroviruses. Enterovirus RNA has been found in 27-64% of newly diagnosed type 1 patients compared to 0-5% of the control subjects (reviewed in Hyöty 2004). Recently, ongoing prospective study settings have provided information about the long prodromal stage before clinical type 1 diabetes. These studies have provided further evidence for the role of enteroviruses, especially when temporal connections have been found between enterovirus infections and the subsequent appearance of diabetes related autoantibodies (reviewed in Hyöty and Taylor 2002). However, not all prospective studies have observed an excess of enterovirus infections (Füchtenbusch et al. 2001, Graves et al. 2003). Methodological differences may explain part of this inconsistency as those studies with positive correlation usually used more sensitive methods (reviewed in Hyöty and Taylor 2002).

The initiation of the process leading to type 1 diabetes has been suggested to begin in some cases already in utero. In line with this exposure to maternal enterovirus infections has been suggested to increase the risk of type 1 diabetes in the offspring (Table 2). A few studies have observed more enterovirus infections during the first trimester of the pregnancy in mothers whose child subsequently developed type 1 diabetes than in control mothers (Hyöty et al. 1995, Dahlquist et al. 1999a). The risk effect of maternal enterovirus infections was observed in the Finnish Childhood diabetes in Finland (DiMe) study with increased frequency of enterovirus IgM during the first trimester in pregnant women whose children developed diabetes (Hyöty et al. 1995). This risk effect was restricted to very young children who manifested with the disease before the age of 3 years. Dahlquist and coworkers (1995a, 1995b) have
<table>
<thead>
<tr>
<th>Country</th>
<th>Endpoint</th>
<th>N (Case/Control)</th>
<th>Risk effect</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweden</td>
<td>Clinical T1D &lt;15yrs</td>
<td>85/172</td>
<td>+</td>
<td>EV-RNA (PCR), Serology (IgM)</td>
</tr>
<tr>
<td>Finland</td>
<td>Clinical T1D &lt;7yrs</td>
<td>96/96</td>
<td>+(^a)</td>
<td>Serology (IgM, IgA, IgG)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>21/104(^b)</td>
<td>-</td>
<td>Serology (IgM, IgA, IgG)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>19/84(^b)</td>
<td>+/-</td>
<td>Serology (IgM, IgA, IgG and neutralising antibodies)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>41/196(^b)</td>
<td>-</td>
<td>EV-RNA (PCR), Serology (IgM, IgA, IgG)</td>
</tr>
<tr>
<td>Germany</td>
<td>Appearance of AABs</td>
<td>16/110(^b)</td>
<td>-</td>
<td>Serology (IgM, IgG)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>21/104(^b)</td>
<td>-</td>
<td>EV-RNA (PCR), Serology (IgM, IgA, IgG)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>21/104(^b)</td>
<td>-</td>
<td>Serology (IgM, IgA, IgG)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>19/84(^b)</td>
<td>+/-</td>
<td>EV-RNA (PCR), Serology (IgM, IgA, IgG and neutralising antibodies)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>41/196(^b)</td>
<td>-</td>
<td>EV-RNA (PCR), Serology (IgA, IgG)</td>
</tr>
<tr>
<td>Finland</td>
<td>Appearance of AABs</td>
<td>18/190(^b)</td>
<td>+</td>
<td>EV-RNA (PCR), Serology (IgM, IgA, IgG)</td>
</tr>
</tbody>
</table>

AAB, autoantibody; T1D, type 1 diabetes; EV-RNA, presence of enterovirus RNA using PCR; Serology, IgA, IgG or IgM class antibodies to enterovirus antigens; \(^a\) in subgroup of children diagnosed <3 years of age; \(^b\) HLA-matched case/control
observed a risk effect of maternal enterovirus infections during the later stages of pregnancy in Swedish mothers according to serology. In addition, they found a small risk effect in a recent large case control study where they analysed entroviral RNA in the offspring in blood spot taken a few days after delivery (Dahlquist et al. 2004). The risk effect of maternal entrovirus infection has also been studied in ongoing prospective studies in HLA matched case-control study settings with contradictory results (Table 2). Two of these prospective studies showed more enterovirus infections during the second and third trimesters of pregnancy in mothers whose children subsequently developed diabetes-related autoantibodies (Sadeharju et al. 2003a, Sadeharju et al. 2005). No effect was observed in other studies (Füchtenbusch et al. 2001, Lönnrot et al. 2000, Sadeharju et al. 2001, Salminen et al. 2003). Altogether, the number of study subjects has been small in studies evaluating the possible risk effect of maternal enterovirus infections and larger studies are needed to find the answer to this question.

Different animal models have proposed the role of different virus infections in the pathogenesis of type 1 diabetes. In the group of picornaviruses the diabetogenic variant of encephalomyelocarditis virus (EMCV-D) causes diabetes in over 90% of the infected genetically susceptible mice (Jun and Yoon 2003). The genetic properties of the virus modulate the diabetogenicity of the virus strain and on the other hand the genetic background of the host also has an effect, as only some mouse strains are susceptible. The same phenomenon has been shown in different murine and nonhuman primates models studying CBV:s (reviewed in Jun and Yoon 2003). In addition, in the EMCV model, the dose of infective virus given to the animals resulted in different pathogenesis. In animals infected with a high dose of EMCV the replication of the virus within the beta cells played a major role, whereas in animals infected with a low dose of EMCV, the activated macrophages had a greater role in pathogenesis (Jun and Yoon 2003). The mechanism of beta cell destruction also varied in vitro when human islet cells were inoculated with different doses of CBV5 (Rasilainen et al. 2004). However, virus infections can also have a protective role in the animal models spontaneously developing diabetes e.g. bio-breeding diabetes-prone (BB-DP) rat and the non-obese diabetes (NOD) mouse (Tracy et al. 2002, Jun and Yoon 2003). For example, in NOD mice, inoculation of 4- or 8-week-old mice with different CVB strains reduced the incidence of diabetes 2 to 10-fold (Tracy et al. 2002). The timing of the infection may have an effect, as early CBV infection blocked the development of type 1 diabetes in the NOD mouse model while the later inoculated infection accelerated development of the disease (Serreze et al. 2000).

Several mechanisms have been proposed to explain the relationship between enteroviruses and type 1 diabetes. Immune response cross-reacting between pathogen epitope and host molecules (molecular mimicry) has been proposed, especially when cross-reactivity between non-structural enterovirus protein P2C and autoantigen GAD-65 molecules was discovered. However, the supporting evidence from human studies is meager (Atkinson et al. 1994, Marttila et al 2001, Varela-Calvino et al. 2004). Enteroviruses may also initiate the autoimmune process leading to type 1 diabetes through bystander activation of autoreactive T-cells due to inflammation in
the pancreas, resulting in tissue damage and release of islet antigens (Horwitz and Sarvetnick 1999, Roep 2003). Recently, the role of T-regulatory cells has been studied intensively as the weak point in the immune system to fail when an autoimmune process develops (Homann and von Herrath 2004). A direct lysis of beta cells by enterovirus infection as well as persistent infection has also been suggested (Frisk 2001, Roivainen et al. 2002). However, the mechanisms are largely unresolved and several options persist.

3. SOCIO-ECONOMIC AND POPULATION EFFECTS ON THE RISK OF TYPE 1 DIABETES

Several studies have found links between improved living standards with decreased exposure to micro-organisms and increased risk of immune-mediated diseases in childhood (Bach 2002). Several domicile and perinatal risk factors for type 1 diabetes have been described, but their risk effect has varied between different case-control studies. Many studies have used questionnaires as a method (Verge et al. 1994, Wadsworth et al. 1997, Pundziute-Lyckâ et al. 2000, Marshall et al. 2004), but some were also based on prospectively collected data (Dahlquist and Kallen 1992, Patterson et al. 1994, Jones et al. 1998, Bache et al. 1999, Dahlquist et al. 1999b, Bingley et al. 2000, Stene et al. 2004). The EURODIAB Substudy 2 Study Group (2000) utilized both questionnaires and clinical records.

The finding that older maternal age is associated with increased risk of type 1 diabetes has been very consistent (Patterson et al. 1994, Bache et al. 1999, Bingley et al. 2000, McKinney et al. 2000, reviewed in Larsson et al. 2004). The risk effect of being the firstborn child has also been found in many studies (Wadsworth et al. 1997, Bingley et al. 2000, Stene et al. 2001), but has not been confirmed in all reports (Jones et al. 1998, EURODIAB Substudy 2 Study Group 2000). Small family size or low household crowding have been more common in children with diabetes (Patterson et al. 1994, Verge et al. 1994, Pundziute-Lyckâ et al. 2000, Marshall et al. 2004). Furthermore, better education or higher socioeconomic status in the family has also been related to increased risk of type 1 diabetes (Siemiatycki et al. 1988, Patterson et al. 1994, Patterson et al. 1996, Marshall et al. 2004). However, opposite findings have also been reported (Siemiatycki et al. 1988, McKinney et al. 2000). An ecological analysis observed a correlation between the incidence of type 1 diabetes and indicators of national prosperity in different European populations (Patterson et al. 2001).

Several case-control studies have shown indications that pre-eclampsia, caesarian section, higher gestational age or complicated delivery increase the risk of type 1 diabetes (Dahlquist and Kallen 1992, Patterson et al. 1994, Jones et al. 1998, McKinney et al. 1999). However, the findings have not been supported in many other studies (Patterson et al. 1994, Dahlquist et al. 1999b, Stene et al. 2003b). Blood group incompatibility between the fetus and mother have been shown to be associated with
increased risk of type 1 diabetes or the appearance of diabetes associated autoantibodies (reviewed in Larsson et al. 2004). After birth, accelerated height gain and increased weight gain have been associated with increased risk of type 1 diabetes (reviewed in Virtanen and Knip 2003). Stressful events as potentiators of the process leading to type 1 diabetes have also been proposed (Blom et al. 1991, Thernlund et al. 1995).

Several studies have reported an inverse relationship between incidence of type 1 diabetes and population density (Patterson et al. 1996, Staines et al. 1997, Parslow et al. 2001), whereas other studies have reported a higher risk in urban areas or no relationship at all (Cherubini et al. 1999, Pundziute-Lyckå et al. 2003, Schober et al. 2003). In Finland, a strong inverse correlation has been reported between population density and incidence of type 1 diabetes (Karvonen et al. 1997). In another Finnish report, the incidence was also found to be higher in rural than urban areas, whereas the increase in the incidence was higher in urban areas (Rytkönen et al. 2003). In an Austrian study an inverse correlation was observed between the proportion of children under 15 years of age in the population and the incidence of type 1 diabetes (Schober et al. 2003). Another study showed a higher incidence of diabetes in areas with low levels of population mixing based on the number and diversity of incoming migrants (Parslow et al. 2001).

A number of reports have suggested that attendance at day care protects against diabetes, but larger studies are needed to confirm this association (reviewed in Kaila and Taback 2001). The role of day care is supported by the finding that diabetic children have had fewer social contacts between 6 and 11 months (Marshall et al. 2004). The same study found fewer animal contacts among diabetes cases (Marshall et al. 2004). Data on infections suggest that one or more infections during first half year or first year of life may reduce the risk of type 1 diabetes (Blom et al. 1991, Gibbon et al. 1997, Pundziute-Lyckå et al. 2000). A severe neonatal infection, on the other hand, was instead a risk factor for diabetes in a large multicentre study among neonatal and newborns (Dahlquist et al. 1999b, EURODIAB Substudy 2 Study Group 2000). In a prospective study, maternal symptoms of a respiratory or gastrointestinal infection showed negative correlation with autoantibodies among girls (Stene et al. 2003a). The same phenomenon of fewer childhood infections has also been connected to increased frequency of atopy and asthma and on the basis of this the hygiene hypothesis has been proposed (Strachan 2000, Matricardi and Ronchetti 2002).

There has been seasonality both in the incidence of type 1 diabetes and in the birth month of children later developing diabetes. A larger number of new type 1 diabetes cases is diagnosed during winter and a smaller during the warmer summer months (Gamble and Taylor 1969, Joner and Sovik 1989, Levy-Marchal et al. 1995, Karvonen et al. 1998, Padaiga et al. 1999, Green 2001, Ursic-Bratina et al. 2001, Gyurus et al. 2002, Kordonouri et al. 2002, Weets et al. 2004). The seasonality is more pronounced in areas of moderate incidence than lower incidence of type 1 diabetes (Padaiga et al. 1999). There is also seasonality in the appearance of type 1
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diabetes associated autoantibodies showing an excess in winter (Kimpinäki et al. 2001b). On the other hand, the children who later developed type 1 diabetes have been reported to be born more frequently than expected during early spring and summer months (Jongbloet et al. 1998, Laron et al. 1999, Rothwell et al. 1999, Songini et al. 2001, Ursic-Bratina et al. 2001, Kordonouri et al. 2002). In Shanghai, China, a similar of excess of birth was seen from November to January (Ye et al. 1998). Seasonality of birth has not been observed in all studies including the centres of the large EURODIAB study (Rothwell et al. 1999, Kida et al. 2000, Muntoni et al. 2002).

Ecological studies have demonstrated a gradient in the risk of IDDM, with the incidence increasing with the distance from the Equator and with decreasing yearly average temperature and mean sunshine hours (Diabetes Epidemiology Research International Group 1988, Dahlquist and Mustonen 1994).

Taken together, higher maternal age, lower population density, higher socio-economic status of the family or lack of early infections seem to be related to higher risk of type 1 diabetes. However, findings are not consistent but vary between studies carried out in different countries with different incidences of diabetes.

4. POPULATION AND HOST DYNAMICS IN ENTEROVIRUS INFECTIONS

The immune status of the population at risk (herd immunity) affects the spread of the virus, especially when humans are the only hosts for the virus. The occurrence of epidemics is due to the actions of three parameters determining the disease incidence: the proportion of the population susceptible, the proportion of susceptible subjects infected and case/infection ratio. The proportion of susceptibles depends on the past history of virus circulation, the proportion infected further depends on the dynamics of transmission of the virus, influenced for example by the density of susceptible persons and the season of the year. The case/infection ratio varies greatly due to number of subclinical infections: for example the case/infection ratio for poliovirus (member of the enterovirus genus) is less than 1/100, meaning that fewer than 1% of infected individuals develop paralytic poliomyelitis, while most of those infected remain symptom-free (Nathanson 2001).

Different age distributions of viral disease are seen in different areas, even in the same population at different times, infected with endemic viruses that at some time point reach 100% of the population. This reflects the different rates of transmission of the virus. For example, in the case of poliomyelitis areas where poliovirus was transmitted readily, all the cases were seen among young children. Conversely, in areas of better hygiene and lower rate of transmission, poliomyelitis cases were seen frequently in older age, 30 years and above (reviewed in Horstmann 1955, Nathanson 2001).
Population size affects the perpetuation of a virus in the population. Further, the proportion of susceptible population, turnover rates of susceptible persons as well as density of the population play a role. The viral determinants include the transmissibility, incubation time, and duration of infectiousness. For poliovirus, which showed clear seasonality, it was estimated that a population of 100,000 was just sufficient to perpetuate poliovirus in the population. During the introduction of vaccine and fade-out of the disease 1967-1972, the poliomyelitis cases declined in every enterovirus season and viruses were reintroduced to fewer areas every year before they disappeared (reviewed in Nathanson 2001).

Personal and environmental hygiene reduces the spread of infections even in our era of “cleanliness” (Aiello and Larson 2002). The fecal-oral route of transmission makes enteroviruses highly communicable, resulting in infection in non-immune members of the community e.g. day care and further to families. One serotype usually causes epidemics at intervals of a few years after the number of susceptible individuals has increased sufficiently. Enterovirus infections are more prevalent among individuals in lower socio-economic conditions or those living in urban areas. The intrafamily transmission of enteroviruses is known to be rapid, depending on duration of virus-shedding, household size, number of siblings, and socio-economic status. Immune status also plays a role, as those with previous neutralising antibodies to the serotype are not usually infected (reviewed in Pallansch and Roos 2001).

The fetus receives protective neutralising antibodies through the placenta (IgG class), and these persist in the child’s circulation after birth for 6-9 months. In addition, the child receives additional protection by IgA class maternal antibodies in breast milk. In fact, breastfeeding itself also protects against a number of infections (Pisacane et al. 1994, Beaudry et al. 1995, Pundziute-Lyckå et al. 2000) including enterovirus infections (Jenista et al. 1984, Sadeharju et al. 2005). These passively acquired antibodies protect newborn infants from serious illnesses for the first 6-9 months, during which time the child can develop his/her own specific immunity by acquiring infections and thus receiving “a natural vaccination”. Without these protective antibodies the newborn would be vulnerable to severe forms of diseases. This is particularly true for enteroviruses, as protection against enteroviral disease depends on mainly on neutralising antibodies, and because enteroviruses are already common in early childhood. The titre and the serotype repertoire of these passively acquired antibodies depend on the enterovirus infection history of the mother and are important in determining the risk of infection and for modulating the severity of the disease (Pallansch and Roos 2001). However, in some cases the neutralising antibodies may not be detected in the newborn even though they are present in the mother (Hammond et al. 1985).

The association between day-care and increased frequency of respiratory and gastrointestinal infections has been reported earlier (Collet et al. 1994, Rylander and Megevand 2000, The National Institute of Child Health and Human Development Study of Early Child Care 2001, Bradley 2003). An increased rate of infections in
Review of the Literature

children attending day care has been documented for microbes transmitted via either respiratory or the fecal-oral route (Rylander and Megevand 2000, Venczel et al. 2001). Outbreaks of enteroviruses have been observed in day-care centers but the number of subclinical enterovirus infections has not been assessed (Mohle-Boetani et al. 1999, Reintjes et al 1999). Interestingly, one study reported that early exposure to infections in day care reduces the number of infections later in life (Bradley 2003).

In summary, population dynamics play a role in the spreading of enterovirus infections and host factors such as immunity and age in addition to the characteristics of infecting virus determine the clinical manifestation of the disease.
OBJECTIVES OF THE PRESENT STUDY

This study aimed to address the following questions:

1. Does maternal enterovirus infection during pregnancy increase the risk of future type 1 diabetes in the exposed fetus?
2. Is there a correlation between the epidemiology of enterovirus infections and type 1 diabetes in different European populations?
3. Has the epidemiology of enterovirus infections changed in recent decades and does it correlate with the rising incidence of type 1 diabetes in Finland and Sweden?
SUBJECTS AND METHODS

1. SUBJECTS

1.1 Intrauterine series (I,II)

The intrauterine series included the sub-series described below (series 1-3). Series 1 and 2 covered infections occurring during the first trimester of pregnancy while Series 3 covered the whole period of pregnancy. These series were based on serum samples taken since 1982 from all pregnant women in Finland at the end of the third month of pregnancy for the national screening of infectious diseases. These sera have been stored at -20 °C at the National Public Health Institute. The stored sera of pregnant women were analysed for the presence of enterovirus antibodies in order to test whether enterovirus infection during pregnancy was a risk factor for subsequent type 1 diabetes in the offspring. National registries were used for the identification of children with type 1 diabetes.

Series 1 included 948 samples from mothers whose children manifested with diabetes before the age of 15 years during the period 1987-1995 and corresponding samples from 948 control mothers. These children were first identified from a national diabetes register (Tuomilehto et al. 1999). The control mothers were randomly selected (one for each case mother) by taking the maternal sample located next to the case mother in the freezer and thus matched according to calendar time. The mean age of the index mothers was 28.0 ± 5.1 SD years and the mean age of control mothers was 27.9 ± 5.1 SD years. Samples were taken from the mothers during the years 1982-1995. (Report I)

Series 2 included 680 children developing diabetes before 7 years of age in the period 1983-1995 who were identified from a national registry based on the drug reimbursement allowances of the Social Insurance Institution of Finland and 680 control mothers. The registry includes all cases developing diabetes before 30 years of age since 1965 (Tuomilehto et al. 1995). One control child without diabetes of the same age and sex and living in the same municipality as the case was chosen from the Finnish Central Population Registry. The unique identification codes of the biological mothers of case and control children were identified from the Population Registry and
cross-linked with the serum bank registry. The mean age of the case mothers was 28.3 ± 5.0 SD years and that of the control mothers 28.1 ± 5.3 SD years. The samples were taken during the years 1983 to 1994 in different parts of Finland. The monthly distribution of the samples was approximately the same in case and control women. (Report I)

There was an overlap among the case mothers of the Series 1 and 2: 68.5 % of the case mothers of Series 2 were included also in Series 1. Control mothers did not overlap between the two series and they were selected using different criteria. (Report I)

Series 3 consisted of samples covering the early and later stages of pregnancy in HLA matched case control study setting. Study series included children who took part in the Type 1 Diabetes Prevention and Prediction (DIPP) Study running in three university hospitals in Finland (Turku, Oulu and Tampere) (Salminen et al. 2003). These children were screened at birth for HLA-DQ alleles which are associated with increased or decreased risk for type 1 diabetes. Children who developed clinical type 1 diabetes by the age of seven years were identified from hospital records. The case children (N=70) were diagnosed during the years 1994-2004 and the median age at diagnosis of type 1 diabetes was 2.4 years (range 0.8-6.7 years). Two non-diabetic control children were selected from the same DIPP cohort for each case child and matched for the time (month) and place (city) of birth, gender and the HLA-DQB1 alleles. Two samples for each index or control subject were traced: serum sample taken from the mother at the end of first trimester of pregnancy (stored at -20 °C at the National Public Health Institute) and at birth from the child (cord blood) as part of the protocol of the DIPP Study. The mean age of the index mothers was 29.4 ± 4.9 SD years and the mean age of control mothers was 30.6 ± 5.0 SD years. Samples were taken from the mothers during the years 1994-2000. Cord blood samples were available for 70 case and 133 control children and both cord blood and first-trimester sample of the mother were available from 61 case and 110 control children. 30% of the children carried the high risk alleles (DQB1*02-DQA1*05/DQB1*0302), 47% carried the moderate risk alleles (DQB1*0302/x, x≠ DQB1*0301, DQB1*0602 or DQB1*0603) and the rest (23%) had low risk alleles. (Report II)

1.2. Subjects with aseptic meningitis (III)

The incidence of aseptic meningitis was analysed in 0 to 14-year-old children living in the Pirkanmaa Hospital District. Incidence cases were identified from the hospital case records traced by diagnostic codes according to the following criteria: typical symptoms of aseptic meningitis (headache, fever, nausea, meningeal signs or anorexia as well as irritability in infants, Hammer and Connolly 1992) together with 10 or more leukocytes per mm$^3$ of cerebrospinal fluid (CSF). Cases with confirmed bacterial or other non-enteroviral infections (like parotitis) were excluded. The laboratory techniques used included viral culture and serolocal assays, which have not substantially changed during the study period. The average incidence of enterovirus
Subject and Methods

meningitis was calculated in five-year periods per 0 to 15 year-old background population living in the Pirkanmaa Hospital district. The size of the 0 to 15 year-old population remained approximately the same during the study period, the mean number of children being 83,267 in the period 1980-84, 81,822 in the period 1985-89 and 83,467 in the period 1990-94.

1.3. EPIVIR series (III-V)

The EPIVIR project is an international research project supported by the EU as a part of the INCO-Copernicus Programme (http://www.cordis.lu/inco2). It includes the following types of series.

1.3.1. Cross-sectional series (IV, V)

Eight countries with different incidences of type 1 diabetes were included in the analyses of geographical differences in enterovirus epidemiology. The high incidence countries were Finland and Sweden, while low/intermediate incidence countries included Estonia, Germany, Hungary, Lithuania, Russia and Israel (see page 41). Three series of serum samples were collected including infants (N=553, mean age 1.0±0.2 SD years), schoolchildren (N=887, mean age 12.3±1.4 SD years) and pregnant women (N=1176, mean age 28.6±5.5 SD years) representing the background population (Table 3).

The infant series were recruited throughout the year during the period 1998-2001 either randomly from children participating in population screening projects or from children attending outpatient clinics for the evaluation of minor ailments (minor surgical procedures, allergies, etc.). In Finland and Sweden these infants were healthy children taking part in the ongoing birth-cohort studies ABIS (Ludvigsson et al. 2001) and DIPP (Salminen et al. 2003) and in Lithuania and Estonia they were comparable healthy children followed-up from birth. In Germany, Hungary, and Russian Karelia infants were recruited from the outpatient clinics of the local hospitals (children with severe infections or malignant or immunological diseases were excluded). The samples were collected throughout the year during the period 1998-2001.

The schoolchildren were recruited from schools as campaigns in all other countries but Finland, where they were randomly selected healthy children from general population (recruited in context of the DIPP Study). The samples were collected during the period 1995-2001, and 79% of the samples were taken from October to December.

Samples from pregnant women were collected at the end of the first trimester of pregnancy from randomly selected women during their regular visits to a prenatal clinic in all countries. The samples were collected throughout the year during the period 1999-2001.
The time period of sampling was within the same range in both high and low incidence countries. The participation rate in the infant series was 82% (range 68-90%), in the schoolchildren series 88% (range 69-95%), and in the pregnant women series 90% (range 86%-98%) showing no difference between the high and low incidence countries. The socio-economic status of the study population was not matched between the countries in order to avoid overmatching. The study population was Caucasian.

As polioviruses are enteroviruses, histories of polio vaccinations were also recorded. The polio vaccination programme covers almost all children in each country but the type of vaccines varies. In Finland and Sweden an inactivated polio vaccine (IPV) has been used exclusively with the only exception that one dose of live attenuated vaccine (OPV) was given in Finland to the whole population in 1985. In Lithuania and Germany the schoolchildren and pregnant women had received OPV, while the infants received IPV. In Estonia, Russian Karelia and Israel OPV vaccine was used exclusively. In Hungary, a combination of IPV and OPV vaccines was given to the infants, while schoolchildren and pregnant women received OPV.

Information about the day-care history was obtained from 65% of infants by a questionnaire including information on whether the child had been taken care of at home or outside the home, and at what age the child started day-care outside the home. This information was obtained from 73% of the infants from the low incidence countries and from 44% of the infants from the high incidence countries.

1.3.2. Time-trend series (III, IV)

Stored serum samples from pregnant women were analysed for possible changes in enterovirus epidemiology over the past 20 in from the Finnish and Swedish mothers. Series of samples, which were taken during July in each of the years 1983, 1989, 1995 and 2001, were randomly selected from the Finnish serum bank. They represented different age groups of women living in various parts of Finland. In Sweden, stored serum samples had been collected from mothers at delivery in the town of Linköping. Samples were randomly selected from the period of June-July in 1985, 1990, 1995 and 2000 (Table 4).

In addition to these EPIVIR series a separate time series was available from children aged 10-14 years representing the background population in Finland (Table 4). Serum samples were collected from schools in the course of campaigns in the years 1975 and 1983. The samples from the years 1998-2002 were healthy siblings of the index cases taking part in the ongoing Finnish DIPP Study. The age of the children did not differ between the 1983 and 1998-2002 cohorts. However, the mean age of the girls was significantly higher in 1975 compared to 1983 and 1998-2002 (p<0.0001, Table 4).
Table 3. Subjects in the EPIVIR cross-sectional series

<table>
<thead>
<tr>
<th>High incidence countries</th>
<th>Pregnant women</th>
<th>Schoolchildren</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland</td>
<td>N 104</td>
<td>mean age±SD 28.8±4.7</td>
<td>Sample collection period (mo/yr) 9/99-3/00</td>
</tr>
<tr>
<td>Sweden</td>
<td>N 128</td>
<td>mean age±SD 29.7±4.0</td>
<td>Sample collection period (mo/yr) 6/99-12/99</td>
</tr>
<tr>
<td>Low incidence countries</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estonia</td>
<td>N 100</td>
<td>mean age±SD 28.1±8.1</td>
<td>Sample collection period (mo/yr) 2/00-4/01</td>
</tr>
<tr>
<td>Germany</td>
<td>N 110</td>
<td>mean age±SD 32.9±4.6</td>
<td>Sample collection period (mo/yr) 11/99-2/01</td>
</tr>
<tr>
<td>Hungary</td>
<td>N 100</td>
<td>mean age±SD 27.5±4.7</td>
<td>Sample collection period (mo/yr) 3/00-5/00</td>
</tr>
<tr>
<td>Lithuania</td>
<td>N 154</td>
<td>mean age±SD 26.5±5.1</td>
<td>Sample collection period (mo/yr) 5/99-9/99</td>
</tr>
<tr>
<td>Russia</td>
<td>N 103</td>
<td>mean age±SD 25.9±5.5</td>
<td>Sample collection period (mo/yr) 5/00-12/00</td>
</tr>
<tr>
<td>Israel(^a)</td>
<td>N 377</td>
<td>mean age±SD 29.1±5.0</td>
<td>Sample collection period (mo/yr) 2/00-3/00</td>
</tr>
</tbody>
</table>

\(^a\) In Israel samples were not collected from schoolchildren or infants
2. METHODS

2.1 Infection induced immunity

The frequency of enterovirus infections was studied by analysing enterovirus antibodies in the study series using EIA (group specific antibodies) and a neutralisation assay (serotype-specific antibodies). All samples except IgM class coxsackie B 5 (CBV5) antibodies were analysed in the Virus Laboratory at the Medical School, University of Tampere (IgM class CBV5 antibodies were analysed by RIA at the National Public Health Institute of Finland, Report I). Equal numbers of samples from cases and controls or different countries and years were included in the same run. The frequency and levels of these antibodies were taken as an indicator of past exposure to enteroviruses, reflecting the frequency of enterovirus infections in a given population (Roivainen et al. 1998a). A summary of the methods used for the analysis of different sample series is provided in Table 5.

2.1.1 Enterovirus and tetanus antibodies (I-V)

IgG class antibodies were measured separately against a panel of antigens including highly purified coxsackievirus B4 (CBV4), poliovirus type 1 (PV1, strain Sabin), echovirus 11 (EV11) and a synthetic enterovirus peptide (sequence KEVPALTAVETGAT-C), which is a common epitope for enteroviruses (Cello et al. 1993, Hovi et al. 1993, Samuelsson et al. 1995, Oberste et al. 1999). The assays were
<table>
<thead>
<tr>
<th>Study series</th>
<th>Method</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunoassay(^a)</td>
<td>Neutralization assay</td>
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<tr>
<td><strong>Intrauterine series</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series 1</td>
<td>IgM (CBV5)</td>
<td></td>
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<tr>
<td>Series 2</td>
<td>IgG (CBV4, peptide antigen)</td>
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<tr>
<td></td>
<td>IgM (CBV3, CAV16, EV11)</td>
<td></td>
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<tr>
<td>Series 3</td>
<td>IgG (CBV4, peptide antigen)</td>
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</tr>
<tr>
<td></td>
<td>IgM (CBV3, CAV16, EV11)</td>
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<tr>
<td><strong>EPIVIR time-trend series</strong></td>
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<tr>
<td><em>Pregnant women</em></td>
<td></td>
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<tr>
<td>Finland</td>
<td>IgG (CBV4, peptide antigen)</td>
<td>CBV4, CBV5, EV9, CAV9(^b)</td>
</tr>
<tr>
<td></td>
<td>tetanus- IgG(^b)</td>
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<tr>
<td>Sweden</td>
<td>IgG (CBV4, peptide antigen)</td>
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<tr>
<td><strong>Schoolchildren</strong></td>
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<tr>
<td>Finland</td>
<td>IgG (CBV4, peptide antigen)</td>
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<tr>
<td><strong>EPIVIR cross-sectional series</strong></td>
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<tr>
<td><em>Infants</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Schoolchildren</em></td>
<td>IgG (CBV4, PV1, peptide antigen)</td>
<td>CBV4, CBV5, EV9, EV11</td>
</tr>
<tr>
<td><em>Pregnant women</em></td>
<td>IgG (CBV4, PV1, peptide antigen)</td>
<td>CBV4, CBV5, EV9, CAV9(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Antigen and immunoglobulin isotype measured by EIA (RIA was used for intrauterine Series 1)

CBV, coxsackie B virus; CAV coxsackie virus A; EV, echovirus, PV, poliovirus; peptide antigen, synthetic enterovirus peptide antigen
\(^b\) years 1983 and 2001, \(^c\) Finnish, Estonian and Karelian samples
performed as previously described (Lönnrot et al. 2000). CBV4 and EV11 were first heat-treated (30 min at +56 °C) to expose antigenic epitopes, which are cross-reactive between various enterovirus serotypes. The antibody results are given as EIU (enzyme immunoassay unit) with reference to the same negative and positive control samples used in all analyses. The value of 15 EIU was used as a cut-off point for seropositivity. Antibodies against tetanus toxoid (National Public Health Institute, Helsinki, Finland) were measured as previously described (Kurikka et al. 1996) to analyse the possible effect of long storage on antibody levels in Report V (regular tetanus vaccinations for children started in 1957 in Finland and did not change substantially during the study period).

IgM class antibodies to heat-treated CBV5 were analysed by using RIA in the enterovirus laboratory of National Public Health Institute as previously described (Hyöty et al. 1995, Hiltunen et al. 1997) (Series 1, Report I). In this RIA, sera were scored antibody positive if the mean cpm of two wells exceeded the mean cpm of negative control plus 2 SD. IgM class enterovirus antibodies against a mixture of three enterovirus antigens [coxsackievirus B3 (CBV3), coxsackievirus A16 (CAV16) and EV11] were measured by using a capture EIA as previously described (Lönnrot et al. 2000) (Series 2 and 3, Report I and II). The cut-off point for seropositivity was absorbance of three times the negative control. All IgM positive sera were reanalysed using the mock-infected control antigen and each of the three virus antigens separately to study the specificity of IgM responses. The sample was taken to be IgM positive if the reactivity (absorbance) against the mock-infected control antigen did not exceed 50% of that observed against the virus antigen. IgM positive samples in Series 3 were additionally analysed for the presence of IgM class Epstein-Barr virus (EBV) antibodies (Enzygnost*Anti-EBV/IgM™, Dade Behring, Marburg, Germany) to assess the specificity of IgM responses.

2.1.2 Enterovirus neutralising antibodies (IV,V)

Neutralisation antibody assay was performed in order to assess the serotype-specific enterovirus immunity. The presence of neutralising antibodies against CBV4, CBV5, echovirus 9 (EV9) and EV11 were analysed using the plaque neutralisation assay (Roivainen et al. 1998b). Two serum dilutions were used for the detection of high (titre > 256, report V) and low (titre > 4, reports III, V) levels of neutralising antibodies. The viruses (ATCC reference strains) were first incubated with a four-fold or 1/256 dilution of serum for 1 hour at 36 °C followed by an overnight incubation at room temperature. The virus was then added on monolayers of Green Monkey kidney cells on 6-well plates (Nunclon™, NUNC, Denmark). The amount of infectious virus was measured by counting the plaques after 46 hours of incubation at 36 °C. The serum was taken as antibody positive if it blocked more than 80 % of the virus infectivity.
2.2. Detection of enterovirus RNA (I)

Randomly selected 152 index mothers and 152 control mothers were tested for the presence of enterovirus RNA in serum using an RT-PCR assay and a liquid-phased hybridization based detection system of enterovirus specific sequences as described (Lönnrot et al. 1999b). In addition, samples positive for EV11 IgM in EIA in Series 2 were tested in the same way. This RT-PCR assay amplifies all enteroviruses and is a highly sensitive method for the detection of enterovirus RNA (described in detail in Lönnrot et al. 1999b).

2.3. HLA genotyping (II)

The presence of HLA class II haplotypes associated with the risk for or protection against type 1 diabetes were analysed as described earlier in the DIPP study protocol (Nejentsev et al. 1999, Laaksonen et al. 2002, Salminen et al. 2003).

2.4. Incidence of type 1 diabetes (III-V)

The incidence data for type 1 diabetes in Report III were obtained from the central drug register of the Social Insurance Institution as previously described (Åkerblom and Reunanen 1985). The mean incidences of type 1 diabetes in different countries in Reports IV and V (/100,000) were from previous publications covering approximately the same time period in the 1990’s (Green and Patterson 2001, Podar et al. 2001, Kondrashova et al. 2005). The incidence figures were taken as 12.3 in Estonia, 40.8 in Finland, 12.0 in Germany, 9.4 in Hungary, 7.3 in Israel, 7.8 in Lithuania, 7.3 in Russia and 25.7 in Sweden. Finland and Sweden were considered to be high incidence countries and Estonia, Germany, Hungary, Lithuania, Russia and Israel to be low incidence countries.

3. STATISTICAL ANALYSES

In report I, antibody positivity between cases and controls was analysed using McNemar's test and multivariate analysis of the risk of coxsackie antibody positivity were done using conditional logistic regression for 1:1 matched case-control design. In Report II, Mantell-Haenszel odds ratio was used for the analysis for the presence of enterovirus infection in a matched case-control study setting with multiple or varying of control subject per case. Mann-Whitney and paired t-test (antibody levels) and ANOVA (age) tests were used in the analysis of continuous data in all reports. In Report III and V, Chi-Square and Fisher's exact test were used in the analysis of nonparametric data. In the EPIVIR cross-sectional series the comparisons in enterovirus immunity were made between high and low incidence countries and in each age group separately (Reports IV,V). A logistic regression model was applied to
explain seropositivity in the neutralisation assay by geographical area (high/low incidence) or time point as covariates (binomial logistic regression). Multinomial logistic regression was used to test the dose effect of multiple positivity for neutralising antibody in terms of absolute titres separately and the dose effect of titre for each serotype separately. Age was considered to be a confounding factor, and accordingly the logistic regression analyses were adjusted for maternal age (Report IV). In report IV, the differences in antibody levels between different years in the time-trend series were tested by the non-parametric test of Cuzicks for linear trend (Altman 1991). The software packages used were STATA, version 6.0 (Stata Corporation, College Station, Texas, USA), SPSS, version 10.1 (SPSS Inc., Chicago, Illinois, USA) and CIA (Altman et al. 2000). A P value of 0.05 or less was considered statistically significant.
Results

RESULTS

1. MATERNAL ENTEROVIRUS INFECTION AS A RISK FACTOR FOR TYPE 1 DIABETES (I,II)

There were no major differences in enterovirus infections during pregnancy between those mothers whose children later developed type 1 diabetes and the mothers of control children. In Series 1, altogether 3.1 % of the 948 mothers with children with diabetes had IgM class antibodies against heat-treated CBV5 at the end of the third month of pregnancy. The corresponding prevalence in the control mothers was 4.1 % (NS). In Series 2, altogether 7.1 % of case mothers had IgM class antibodies against the mixture of CBV3, CAV9, EV11 antigens compared to 5.3 % of control mothers (NS). When IgM positive cases of Series 2 were reanalysed for IgM against each of the three enterovirus antigens separately, EV11 binding IgM tended to be more frequent in case than in control mothers (6.2 % vs. 3.7 %, p<0.05) but this difference did not remain statistically significant when adjusted for the number of comparisons (p=0.15 after Bonferroni’s correction). CBV3 binding IgM was found in 2.6 % of case mothers and 2.6 % of control mothers, and CAV16 binding IgM in 0.7 % of case and 1.3 % of control mothers, indicating respectively no differences between the groups.

In Series 3, enterovirus infection throughout the whole pregnancy period was observed with either increase in IgG levels or presence of IgM in 21.3 % of the case children's mothers and in 11.8% of the control children's mothers (NS). IgM class antibodies were observed in 13.1% of case and in 4.5% of control children's’ mothers in the first trimester samples (mixture of CBV3, CAV9, EV11) (p=0.043). This difference between case and control mothers was observed both in mothers whose children carried high risk HLA- genotype as well as in mothers whose children had low risk HLA genotype. Enterovirus infection during the second and third trimester of pregnancy was observed in 7.6% of sample pairs (6 cases and 10 controls) according rise in IgG class antibody levels (NS). No IgM antibodies were detected in any of the cord blood samples. None of the enterovirus IgM positive samples was positive for EBV IgM antibodies.

IgG class enterovirus antibody levels did not differ between case and control subjects in the cord blood or at the end of the first trimester of pregnancy (CBV4, synthetic peptide antigen). In Series 3, the median IgG antibody levels against CBV4 antigen
were 42 EIU and 48 EIU in first trimester samples and 49 EIU and 50 EIU in cord blood, for cases and controls respectively. In Series 2 the median IgG levels were 44 EIU for cases and 45 EIU for controls. IgG was not analysed in Series 1.

The frequency of enterovirus infections or the levels of enterovirus antibodies were not associated with the gender of the child, the age of the mother or the age of the child at the manifestation of type 1 diabetes. In Series 3, the enterovirus infections during pregnancy were evenly distributed over the study period. In Series 2, the difference between case and control mothers was the greatest in the years 1985 and 1990.

In a previous study, maternal enterovirus infections during pregnancy were associated with manifestation of diabetes in children aged less than 3 years (Hyöty et al. 1995). However, in the present study no difference was found in this particular age group in any of the study series: 3.0% of case women and 3.6% of control women were positive for CBV5 IgM (Series 1) and 5.8% vs. 5.0% for IgM against the mixture of CBV3, CAV16 and EV11 antigens (Series 2). In Series 3 the excess of IgM positive was observed similarly in age groups of 0-2 years (14% vs. 5%) and 3-7 years (12% vs. 4%).

Enterovirus RNA was found in serum by RT-PCR in one case mother (0.7 %) in a subgroup of 152 case and 152 control mothers randomly selected from Series 2. In addition, all IgM positive mothers in series 2 were tested for the presence of enterovirus RNA in serum. Two of the 40 EV11 IgM positive case mothers (5%) and two of the 30 echovirus IgM positive control mothers (6.7%) were positive for enterovirus RNA in serum. In addition IgG levels were significantly higher in the 84 IgM positive mothers than in the 1276 IgM negative mothers, suggesting that high IgG level reflects recent/acute infection (median values 71 vs. 44 EIU respectively; p<0.001, Report I).

2. OCCURRENCE OF ASEPTIC MENINGITIS (III)

The criteria for aseptic meningitis were fulfilled in 145 cases. Enterovirus meningitis was confirmed in 20% of these cases. A declining trend was observed in the incidence of these meningitis cases decreasing linearly from 16.8 (/100 000 children) in 1980-84 to 12.0 in 1985-89 and 8.7 in 1990-94 (p<0.001, Figure 1). However, in the very young infants (<6 months old) no decrease was found. In fact, their proportion increased from 12% (8/65) in 1980-84 to 29% (10/34) in 1990-94 (p<0.05). The seasonal distribution of cases (peak between July and November) and the predominance of boys (male/female ratio 1.8) were both typical for enterovirus meningitis (Figure 2).
Results

Figure 1. Mean annual incidence of type 1 diabetes in children (<15 years old) from 1980 to 2003 in Finland (line with circles, Reunanen, personal communication). The mean annual incidence of aseptic meningitis (/100,000 children) living in Pirkanmaa Hospital district (line with squares). The distribution of enterovirus antibody levels against purified CBV4 antigen (white box plots) and synthetic enterovirus peptide antigen (hatched box plots) in Finland in years 1983, 1989, 1995 and 2001.

Figure modified from Report IV.

Figure 2. Seasonal distribution of children hospitalized for aseptic (enterovirus) meningitis in the University Hospital of Tampere during the years 1980-1994 (black bars represent boys and white bars girls).
3. TIME TRENDS IN ENTEROVIRUS EPIDEMIOLOGY (III, IV)

Incidence of type 1 diabetes increased in Finland linearly during the study period in all children from 33.0 per 100,000 in 1982-1984 to 48.6 in 2000-2002 (Figure 1). The incidence increased in all age groups although the greatest increase was observed in the youngest age groups, particularly in children aged 0-4 years. A significant decrease was observed in enterovirus antibody levels in pregnant women between 1983 and 2001 both in Finland and in Sweden (Figure 1, Reports III, IV). This decrease was observed in antibodies against both the highly purified CBV4 as well as the synthetic enterovirus peptide antigen (p<0.0001 for both in Cuzicks test for linear trend). A significant decrease was observed also in neutralising antibodies for CBV4 and CBV5 (p=0.023 and 0.024 respectively, Table 4, Report IV). However, the prevalence of EV9 or CAV9 antibodies did not differ between these years.

In Finland, enterovirus antibody levels in pregnant women correlated with population density. Antibody positivity was slightly more frequent in areas with higher population density: In rural or suburban areas (100 inhabitants /km 2 or less) the antibody positivity against the synthetic enterovirus peptide antigen was 44%, whereas in areas of more than 100 inhabitants/km2 it was 56% (p=0.052 in Chi-Square).

The antibody levels against tetanus toxoid that were induced by tetanus vaccinations did not decrease during this period but rather showed a slight increasing trend (median IgG levels were 36 EIU in 1983 and 52 EIU in 2001 in Finland) (Report IV).

In addition to pregnant women, a decrease in enterovirus antibody levels was also observed in 10 to 14-year-old children between the years 1983 and 2002. This decrease was again observed in antibodies against both the CBV4 and the synthetic enterovirus peptide antigens (p=0.007 and p<0.0001 respectively) (Figure 3). No gender difference was observed in the antibody levels in this series. Among girls, an additional sample was available from 1975, and a significant decrease was again observed for both enterovirus antigens when these additional samples were included in the comparison (Cuzicks test for linear trend p<0.0001) (Report IV).

4. GEOGRAPHICAL DIFFERENCES IN ENTEROVIRUS EPIDEMIOLOGY (IV,V)

The levels of enterovirus antibodies increased according to age from a median of 1 EIU in infants to 32 EIU in schoolchildren and 59 EIU in pregnant women (IgG levels against synthetic enterovirus peptide antigen). Among schoolchildren boys had significantly lower antibody levels than girls. Median PV1, CBV4 and synthetic enterovirus peptide antigen IgG levels were 28, 48, 24 EIU among boys and 40, 72, 39 EIU among girls respectively (p<0.001 for each antibody specificity, Report V).
Markers of enterovirus immunity were significantly more frequent in countries with low-incidence of type 1 diabetes compared to the high-incidence countries in all age groups in at least one serological assay. In infants, the IgG levels of antibodies against CBV4, EV11, PV1 and synthetic enterovirus peptide antigen were all significantly lower in the high-incidence countries when measured by EIA (p<0.001) (Figure 5). The proportion of seropositive infants was also significantly lower in the high-incidence countries. In the low-incidence countries 29%, 26% and 72% were positive for synthetic enterovirus peptide antigen, CBV4 and EV11 antigens respectively compared to 3%, 10% and 56% in high-incidence countries (p<0.0001) (Report V).

In the schoolchildren series neutralising antibodies were significantly less frequent in the high-incidence countries, even though EIA antibodies did not differ between the high-incidence and low-incidence countries in this age group (Table 6). In the low-incidence countries 56% of the schoolchildren had neutralising antibodies against three or all four serotypes tested (CBV4, CBV5, EV9 and EV11) compared to 39% of the children in the high-incidence countries (p<0.001) (Report V).

In the pregnant women series, the neutralising antibodies were again more frequent in the low-incidence countries (Estonia and Karelia) compared to the high-incidence country (Finland) for all four viruses tested showing a 20-45% difference in the antibody prevalence (Table 6). When the frequency of high titre antibodies was analysed separately (>256), only EV9 antibodies were more frequent in Estonia and Karelia, while antibodies against other serotypes did not differ. The presence of multiple antibodies (3 or 4 of the four viruses tested) was significantly more frequent in the low-incidence countries of Estonia and Karelia compared to Finland, and this was seen for both serum dilutions (Table 6). The prevalence of neutralising antibodies did not differ between Estonia and Karelia. As in the schoolchildren series, enterovirus antibodies measured using EIA (against the synthetic enterovirus peptide antigen, PV1 and CBV4 antigens) did not differ between Estonia and Karelia compared to Finland in pregnant women. When antibody results from all low-incidence countries (Estonia, Germany, Hungary, Israel Lithuania and Russian Karelia) were compared to the two high-incidence countries (Finland and Sweden), no difference was found either, except that the levels of CBV4 antibodies were slightly higher in high-incidence countries than in low-incidence countries (median level 70 EIU vs. 59 EIU, p=0.037) (Report IV).

Samples collected during autumn showed higher antibody levels than samples collected in springtime in infants and schoolchildren but not in pregnant women (p<0.0001 in infant and schoolchildren series for CBV4 and PV1 antigens, p<0.01 in infants against synthetic enterovirus peptide and EV11 antigens). Children in low-incidence countries had higher antibody levels than children in high-incidence countries throughout the year.

Poliovirus antibody levels were significantly higher in countries using OPV than in countries using IPV among infants and schoolchildren (in schoolchildren the median poliovirus IgG was 36 EIU vs. 27 EIU respectively, p<0.001). However, the infants
vaccinated exclusively with IPV in Finland and Sweden (high-incidence countries) had again lower antibody levels against CBV4, EV11 and synthetic enterovirus peptide antigens than IPV-vaccinated infants in the low-incidence countries Lithuania and Germany, suggesting that the difference in these antibodies was not due to different polio vaccine types (Figure 4).

The age at starting day-care outside the home affected enterovirus antibody levels (information on day-care history was available on 359 infants). Children who had started day-care before the age of 9 months had higher enterovirus antibody levels and were more often seropositive than children who had not started day-care by that age: 40% vs. 21% of the children were positive for synthetic enterovirus peptide antigen IgG (p<0.01), 42% vs. 20% for CBV4 IgG (p<0.01), and 72% vs. 65% for EV11 IgG (p=0.01) respectively.

**Figure 4.** Distribution of antibody levels in infants in countries with high (Finland and Sweden) and low (Estonia, Germany, Hungary, Lithuania, Russia) incidence of type 1 diabetes. The distribution of antibody levels in low incidence countries using only IPV as poliovirus vaccine in infants (Lithuania and Germany) is presented separately. Each box plot represents the median (black horizontal line) and the 25th and 75th percentiles. The error bars represent the lowest and highest values that are not outliers. Enterovirus, synthetic enterovirus peptide antigen; CBV4, coxsackievirus B 4; EV11, echovirus 11; PV1, poliovirus strain Sabin 1.
## Results

**Table 6.** Proportion (%) of seropositive subjects (neutralising antibodies) in EPIVIR series (Reports IV,V).

<table>
<thead>
<tr>
<th>Series</th>
<th>Antibody specificity</th>
<th>High-incidence countries</th>
<th>Low-incidence countries</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Finlan 1983*</td>
<td>Finland 2001*</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>titre&gt;4</td>
<td>66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58</td>
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<td>41</td>
</tr>
<tr>
<td></td>
<td>multiple neutralising antibodies (3 or 4)</td>
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<td>39</td>
</tr>
<tr>
<td></td>
<td>titre&gt;256</td>
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<td>41</td>
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<td></td>
<td></td>
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<td>multiple neutralising antibodies (3 or 4)</td>
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<td>11</td>
</tr>
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<td>60&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>34</td>
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<td></td>
<td>multiple neutralising antibodies (3 or 4)</td>
<td>39</td>
<td>56&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Year when samples were collected

CBV, Coxsackie B virus; CAV, Coxsackie A virus; EV, echovirus

<sup>a</sup>p<0.05; <sup>b</sup>p<0.0001; <sup>c</sup>p<0.01 when compared to year 2001 samples from Finland.
Results

The correlation between the two antibody detection methods was assessed by comparing CBV4 antibody results in the neutralisation and EIA assays in all samples where both assays were performed (schoolchildren and pregnant women): 56% of the samples were positive in both assays and 7% of the samples were negative in both assays. 30% of the samples which were negative in the neutralisation assay were positive in the EIA test, and 7% of the samples positive in the neutralisation test were negative in the EIA test. The synthetic peptide antigen was used as an enterovirus group antigen to detect general enterovirus immunity. This antigen also detects antibodies induced by poliovirus vaccination. The level of these antibodies was higher in subjects with multiple antibody positive subjects in the neutralisation assay (p<0.0001, in Mann-Whitney U test when 3-4 neutralising antibodies were compared to 0-1 neutralising antibodies) (Figure 5).

![Figure 5](image-url)

**Figure 5.** Distribution of enterovirus antibody levels against synthetic enterovirus peptide antigen according to number of enterovirus antibodies in neutralisation antibody assays against the four serotypes tested.
1. ENTEROVIRUS INFECTIONS DURING PREGNANCY

Intrauterine infections may cause malformations and functional defects in affected organs and they may also play a role in the induction of beta-cell damage leading to type 1 diabetes. Congenital rubella is a well-known example of intrauterine virus infection which is associated with diabetes later in life (Menser et al. 1978, Ginsberg-Fellner et al. 1985). Intrauterine exposure to maternal enterovirus infection either during the first trimester or later stages of the pregnancy has also been reported to be associated with increased risk of type 1 diabetes in the child (Dahlquist et al. 1995a, and 1995b, Hyöty et al. 1995). However, these studies have been based on relatively small series.

In the present study the markers of enterovirus infections were analysed in a larger number of pregnant mothers whose children developed type 1 diabetes. The role of infections during the first trimester of pregnancy was analysed in a large series of mothers from samples taken at the end of first trimester of pregnancy. In addition, infections during the whole duration of pregnancy were analysed using samples taken at the end of the first trimester of pregnancy and cord blood samples. In spite of the observed trend for slightly higher frequency of IgM class antibodies in case mothers than in control mothers, the results suggest that enterovirus infection during pregnancy is not a major risk factor for type 1 diabetes. Enterovirus infection during pregnancy may, however, play a role in some susceptible subjects. In both these series the strongest possible endpoint, clinical type 1 diabetes, was used to minimize the variation due to individual differences in the progressions of the beta-cell damage. Moreover, the children were also matched for possible confounding factors including gender and HLA conferred susceptibility alleles (HLA matching only in Series 3, Report II).

The assays used for the detection of enterovirus specific IgM in this study and in most previous publications are deliberately cross-reactive, i.e. they are intended to detect IgM responses induced by a wide range of different enterovirus serotypes also including others than those included in the antigen preparations. However, some of enterovirus infections were probably missed because the coverage of the antigen panel is much lower than the number of enterovirus serotypes. The infections detected by IgM antibodies were intended to include primary enterovirus infections, which are more likely to spread to the fetus. We found enterovirus RNA and higher levels of enterovirus IgG significantly more often in the IgM positive than in the IgM negative
mothers, suggesting that at least part of the IgM positive samples did indeed represent genuine acute enterovirus infections. On the other hand, none of the enterovirus IgM positive samples was positive for EBV IgM antibodies, confirming the specificity of the observed IgM responses (report II). Previously, we assessed the specificity of the enterovirus IgM capture method with sera showing IgM positivity for parvovirus \( (N=16) \), varicella zoster/ cytomegalovirus \( (N=12) \), rubella/measles \( (N=12) \), mycoplasma pneumoniae \( (N=4) \), hepatitis A \( (N=5) \) or rheumatoid factor \( (N=2) \) and by testing healthy background population \( (N=84) \). Only two hepatitis A and one EBV IgM positive samples showed reactivity in this enterovirus IgM assay. Hepatitis A (picornavirus) may thus cross-react in our test in some cases. However, the incidence of hepatitis A in Finland is very low and cannot be important confounding factor in the present study.

In Report I, analysis of a randomly selected subset of sera revealed that the apparent overall prevalence of RNA positivity among the mothers was very low. The viremic phase in acute enterovirus infections is considered to last for a much shorter time than the subsequent IgM response. The long storage of the sera at \(-20^\circ C\) may additionally have reduced the amount of intact virus RNA. Consequently, no systematic screening for enterovirus RNA was carried out.

IgG levels in maternal serum at delivery have been shown to correlate with those in cord blood (Sadeharju et al. 2003a). Thus the increase in IgG class antibodies between the first trimester and cord blood samples reflects maternal infection probably during the 2\(^{nd}\) or 3\(^{rd}\) trimester of pregnancy. The re-infections inducing only IgG class antibody response could not be detected with the one sample available in Report I. In Report II we were able to use both kinds of samples and measure not only IgM responses on a certain time point but also increases in IgG class antibodies between the first trimester and cord-blood samples. Enterovirus infection was observed according to rise in IgG levels in 8\% of the mothers, but no detectable IgM was observed in the cord blood samples even though the fetus is known to be capable of producing IgM class antibodies already in utero (Vesikari 1972, Revello and Gerna 2002). This may reflect the lack of fetal infection in these cases but may be due to lower sensitivity of IgM antibody assay in detecting the more monotypic responses in an immunologically naive fetus.

Altogether, the results suggest that first trimester maternal enterovirus infection is not a major risk factor for type 1 diabetes in the offspring, but may play a role in a small sub-group of diabetes cases. Hypothetically, an infection during pregnancy could even serve as a protective factor in a case where the infection does not spread to the fetus but only boosts the mother's enterovirus immunity, which is then transported to the fetus (maternal IgG). However, in cases when the infection spreads to the fetus, the infection could be harmful, increasing the risk of type 1 diabetes. For example, a case report of intrauterine type 1 diabetes has been reported with concurrent enterovirus infection (Otonkoski et al. 2000). Dahlquist and coworkers recently analysed 600 case-control pairs for the presence of enteroviral RNA in the blood sample taken on days 2-4 of life. They found significantly more enterovirus RNA in cases (4.5\%) who
later developed type 1 diabetes than in control children (2.3%) (Dahlquist et al. 2004). Indeed, an enterovirus infection in the neonatal period may be of more importance especially when the fetus has low levels of maternal immunoglobulins and/or HLA conferred susceptibility to type 1 diabetes.

2. EPIDEMIOLOGY OF ENTEROVIRUS INFECTIONS AND TYPE 1 DIABETES

This is the first study to systematically evaluate the relationship between the epidemiology of enterovirus infections and type 1 diabetes at the population level. The results suggest that populations with a high incidence of type 1 diabetes (Finland and Sweden) have lower frequency of enterovirus antibodies than populations with lower diabetes incidence. This is in line with an earlier study carried out on schoolchildren in Finland and Lithuania (Lönnrot et al. 1999a). In the present study, the differences were seen in all age groups including infants, schoolchildren and pregnant women. Furthermore, enterovirus antibodies have shown a clear decrease over the past 20 years in Finland and Sweden, while the incidence of diabetes has simultaneously increased. These findings suggest that an inverse relationship may exist between the frequency of type 1 diabetes and enterovirus infections at the population level.

Ecological studies such as the present one may be biased by several confounding factors, which should be kept in mind when drawing conclusions. In international comparisons, the distribution of HLA risk alleles for type 1 diabetes is one possible source of bias, as HLA can modulate the immune response to enterovirus antigens (Bruserud and Thorsby 1985, Sadeharju et al. 2003b). These HLA allele combinations vary between countries, and high-risk combinations may be more prevalent in the countries with high diabetes incidence, thus contributing to the international variation in diabetes incidence (Ronningen et al. 2001). However, the difference observed between high and low-incidence countries is opposite to that expected, as a strong antibody response to enteroviruses is associated with HLA risk alleles for type 1 diabetes (Sadeharju et al. 2003b).

Another possibility could be population selection bias. In this study the sample collection of pregnant women was carried out in a regular prenatal clinic in each country where the participation rate is known to be high. The schoolchildren series was mainly collected from schools as part of campaigns also with high participation rate. The infants were most difficult to recruit; however a good participation rate was achieved in various countries.

The differences were seen in all age groups, supporting the assumption that they reflect true differences in enterovirus epidemiology. However, there was some variation depending on the method used. The EIA method indicated no difference in enterovirus antibodies between high and low-incidence countries in the older age
groups (pregnant women and schoolchildren) while a clear difference was seen in the neutralisation assay in these age groups. These two methods are not directly comparable as they assess different aspects of enterovirus immunity. The plaque neutralisation assay is the most specific assay available for enterovirus antibody measurements. It is generally used as the golden standard as it measures the “biological” ability of antibodies to inhibit the infection. EIA measures antibodies binding to several different epitopes while neutralising antibodies target serotype-specific sites in the viral capsid proteins and are highly specific and sensitive markers of past infection. In contrast to the neutralising antibodies, the antibodies measured by EIA cross-react between serotypes, being thus more group-specific than serotype-specific. This was also observed in our study, where 30% of the CBV4 antibodies detected with EIA were negative in the neutralisation assay. In EIA, the analyses were done using different cut-off points for seropositivity (10 EIU, 15 EIU, 20 EIU) all yielding similar results, and data based on a cut-off point of 15 EIU which is generally used as a cut-off in our enterovirus EIA.

The enterovirus antibody status of pregnant women reflects their exposure to enterovirus infections (frequency of infections) as well as the protection of the offspring against enterovirus infections conferred by these maternal antibodies when transferred to the infant either transplacentally or in breast milk. The decreasing enterovirus antibody levels over the past 20 years suggest that the overall exposure rate to enteroviruses has decreased both in the Finnish and Swedish population. Tetanus antibody levels did not decrease during the same period, thus excluding possible artefact due to the long storage of the time-trend series at –20 °C. In addition, studies using the same sample source of first trimester samples of pregnant women found no linear declining trend in infection immunity, thereby supporting the view that we observed a true decrease in enterovirus immunity (Laukkanen et al. 2003, Stolt et al. 2003). Rising standards of living and hygiene and other factors inhibiting the spread of the virus are probably the reasons for this decrease. This is in line with the rapid decrease in hepatitis A virus infections which, like enteroviruses, are transmitted through the fecal-oral route (since the 1970’s there has been no endemic hepatitis A in Finland) (Pohjanpelto and Lahdensivu 1984). The finding implies that the proportion of new-borns who lack protective antibodies has increased, making the new-borns now more susceptible than before to enterovirus infections. For example, in year 2000 altogether 42% of Finnish pregnant women lacked neutralising antibodies to CBV4 and were thus unable to protect their children against this particular serotype, which has most often been associated with type 1 diabetes. The corresponding figure in Estonia and Karelia was 14%.

The incidence of enterovirus meningitis has decreased in children, while the proportion of those under 6 months old cases has increased, also supporting the role of maternal antibodies in protection against enteroviruses. The diagnosis of aseptic meningitis was based on hospital records traced by diagnostic codes. Thus, the number of aseptic meningitis cases may be underestimated. However, the same kind of increase in the frequency of neonatal enterovirus meningitis or severe neonatal enterovirus disease has also been reported in other countries (Shattuck and
Discussion

Chonmaitree 1992, Lin et al. 2003). In our study, cases with enterovirus diagnosis based on serology or virus culture from CSF were included in the study. In addition, cases with typical symptoms of enterovirus meningitis/sepsis were included, thus excluding cases with specific microbial diagnosis indicating other infections than enterovirus infections as well as patients with typical symptoms of parotitis or any other non-enterovirus infection. The diagnostic routine of the microbiological laboratory did not markedly change during the study period 1980-1994. Thereafter, the PCR method for the diagnosis of enterovirus infections was implemented the years after 1994 were thus not included in the present series. The distribution as well as male predominance in the patients supports the fact that the observed meningitis cases represent enteroviral disease. In addition, enteroviruses are known to be the major cause for aseptic meningitis in several populations (Pallansh and Roos 2001).

Transmission of enterovirus infections is influenced by various socio-economic factors as well as the climate, which probably contributed to the differences observed in the present study between countries. Starting day-care outside the home at an early age was associated with higher enterovirus antibody levels, probably reflecting the role of close contacts among children in the spreading of enterovirus infections. However, we did not find any difference in children’s age on starting day-care between the high and low-incidence countries, suggesting that this does not explain the difference in enterovirus infections between these countries. Day-care has been shown to be associated with decreased risk for type 1 diabetes (Kaila and Taback 2001). Accordingly, it is possible that day-care may modulate both the child’s exposure to enterovirus infections and the risk for type 1 diabetes.

Girls had higher levels of enterovirus antibodies than boys in the cross-sectional series of infants and schoolchildren. Such differences have previously been observed at least for mumps and measles (vaccine) antibodies (Hyöty et al. 1985, Miller et al. 1995). It is known that complications of enterovirus infections are more common among males than females with the male-female ratio being around 2:1 (Moore and Morens 1984). Accordingly, it is possible that the reduced antibody levels in males reflect lower immune responsiveness and related susceptibility to severe enterovirus infections. The difference between boys and girls could not be confirmed in the secular trend series of schoolchildren, probably due to smaller sample size.

The children vaccinated with OPV had significantly higher poliovirus antibody levels than children vaccinated with IPV. Among infants this difference may be explained by the lower number of vaccine doses by the age of 1 year in the IPV group compared to the OPV group. However, the same difference was also seen among schoolchildren in both groups who had received several vaccinations. This is in contrast to previous findings indicating that IPV induces a stronger humoral immune response than OPV measured after the first 2-3 doses (McBean and Modlin 1987, Beale 1990, Faden et al. 1990). However, some studies have shown that long-term antibody titres are quite similar (Faden et al. 1993). In addition, an earlier comparative study showed higher T-cell immunity in Estonian (OPV vaccinated) than in Finnish (IPV vaccinated) children (Juhela et al. 1999).
Taken together, the high incidence of type 1 diabetes seems to be associated with a low frequency of enterovirus infections in the background population.

3. THE POLIO HYPOTHESIS

The observed inverse relationship between type 1 diabetes and enterovirus infections can be interpreted in various ways. First, if enterovirus infections are a true risk factor for type 1 diabetes, as implied in several but not all reports (Füchtenbusch et al. 2001, Hyöty and Taylor 2002), one might expect that the incidence of these diseases should correlate at the population level. Accordingly, the inverse relationship observed in the present study could be taken as evidence against the role of enterovirus infections in type 1 diabetes. However, the observed inverse correlation may also reflect a specific role of enteroviruses in type 1 diabetes. This possibility is supported by previous experience from another enterovirus disease, poliomyelitis, where the risk of virus-induced motor-neuron damage increased when the frequency of poliovirus infections in the population decreased. On the other hand, it is also possible that certain diabetogenic virus variants circulate in countries with a high diabetes incidence but were not detected by the present methods possibly due to limited coverage of serotypes screened and possible antigenic drift in enterovirus strains (neutralisation assay).

Poliomyelitis is an old disease and cases have been demonstrated from early history. Poliovirus infections used to be endemic, but their frequency decreased rapidly towards the end of the 19th century. Until then poliovirus infections were usually mild and paralytic complications rare. However, when the rate of infections decreased as hygiene and general living standards improved, the incidence of paralytic disease paradoxically increased (reviewed in Nathanson and Martin 1979, Rogers 1990). This is in contrast to all other common infective diseases at the time the as their incidences were declining. The basis of this phenomenon was a delay in the age at initial poliovirus infection; some children were not exposed to polioviruses until later in childhood when they were no longer protected by maternal antibodies and were thus more severely affected by the infection (reviewed in Nathanson and Martin 1979). In addition, the vulnerability of the central nervous system to poliovirus infection increased with increasing age. As hygiene, sanitation and housing improved, the proportion of children escaping infection in infancy rose and the number of paralytic diseases rose in parallel.

The role of genetic susceptibility factors in poliomyelitis has been suggested by the higher incidence in monozygotic twins as well as higher frequency of cases among relatives (Wyatt 1975). The susceptibility has shown to be associated with HLA-linked genetic factors (van Eden et al. 1983). In addition, cases of poliomyelitis and deaths from the disease were more common in males than females. For example, in
the 1916 New York epidemic, poliomyelitis was at first clearly a children’s disease (reviewed in Nathanson and Martin 1979, Rogers 1990). The case-fatality rate was high in young infants and again in adults, increasing with age. Polio victims in rural counties of New York were older than in urban children and they were more likely to die of the infection. A higher proportion of cases was recorded in Queens and Staten Island than in densely populated Manhattan. These same phenomena were also observed in Sweden based on poliomyelitis case rates over a 20-year period (reviewed in Nathanson and Martin 1979). It was shown very early in the polio puzzle that polio epidemics could spread via individuals without any symptoms at all, showing that the same virus could cause a clinical disease depending mainly on host susceptibility factors (Rogers 1990).

Based on the findings of the present study a new hypothesis was proposed. According to this so-called polio hypothesis, the same kind of mechanism as operated in polio may also operate for enterovirus strains causing loss of pancreatic beta-cells. A low frequency of enterovirus infections in the background population may increase the risk of diabetes by making children more susceptible to enterovirus-induced beta-cell damage, thus contributing to the international variation in diabetes incidence rates and to the marked increase in incidence rates seen in most developed countries after World War II. Type 1 diabetes shares many features with poliomyelitis from the epidemiologic point of view: gender bias, seasonality, HLA susceptibility, increasing incidence, high incidence in “high hygiene” areas, inflammation and selective destruction of the target cells and finally, according to our observation, low frequency of infections in the background population. In fact, poliomyelitis was once suggested to be an autoallergic disease, where poliovirus infection induced immune mediated paralysis in genetically predisposed individuals (Wyatt 1976). One major difference between these diseases, however, is the long prodromal subclinical phase of type 1 diabetes.

The proposed polio hypothesis shares the same features with the hygiene hypothesis in the sense that a lower frequency of infections predisposes children to future disease. However, in the hygiene hypothesis, the basis of the pathomechanism rests on disrupted immune development, whereas the polio hypothesis proposed suggests a viral infection as the initiator of the disease process. The lack of education of the immune system with early infection may additionally play a role, as suggested in the hygiene hypothesis (Curotto de Lafaille and Lafaille 2002, Homan and von Herrath 2004).

The mother plays a crucial role in the immunity of the newborn baby by offering neutralising antibodies which protect the offspring for the first 3-9 months after birth. During this time the child can be “naturally vaccinated” with microbes and develop his/her own specific immunity. Accordingly, the proportion of mothers who lack enterovirus antibodies is increasing and children also experience their first infection later, when maternal antibodies have already disappeared. This could increase the susceptibility of young children to the diabetogenic effect of enteroviruses (Figure 6). Genetically predisposed children could be at higher risk and the nature of the immune
**Discussion**

**Figure 6.** Development of enterovirus immunity in two populations with low and high incidences of enterovirus infections in the background population. In a population with a high prevalence of enterovirus infections in background population, the infant acquires the first enterovirus infection during the first months of life before maternal enterovirus antibodies have disappeared. In a population with a low prevalence of enterovirus infections, the infant may have a low level of maternal IgG class antibodies and, in addition, the first infection may be delayed to the period when maternal antibodies have already disappeared. Thus the child is more vulnerable to severe forms of enterovirus diseases.

response (persistent/lytic) could affect the time of clinical manifestation. The idea of the polio hypothesis has later been supported by Zinkernagel (2001 and 2003) who has also proposed a connection between maternal antibodies and childhood infections and other autoimmune diseases. All in all, the timing of infection or acquired immunity as well as other susceptibility (e.g. HLA) may play an important role in the pathogenesis of type 1 diabetes.
4. FUTURE PROSPECTS

This is the first study to systematically compare epidemiology of enteroviruses and type 1 diabetes in different populations. The results suggest that enterovirus infections are not particularly common in countries with high type 1 diabetes. In contrast, an inverse correlation was observed with type 1 diabetes and enterovirus infections in the background population. These data give a new epidemiological perspective for the understanding of the role of enteroviruses in the pathogenesis of type 1 diabetes and opens up new possibilities for future research. The polio hypothesis proposed should be tested further in other study series and in different populations. One of the most important future studies would be a prospective study carried out on different populations, where the interplay between maternal antibodies, herd immunity and the course of infections on individual levels could be assessed.

One of the major difficulties in the international studies such as the present EPIVIR Project is the control of the study protocol and the collection of study material in similar ways in each center. The goal of 100 samples in each country in each sample series was mostly achieved. The sample size in each study group was big enough to assess the main question of the study i.e. whether there were major differences in the epidemiology of enterovirus infections in association with the incidence of type 1 diabetes. However, larger sample sizes from individual countries would have enabled better subgroup analyses. The major advantage of this study was that the laboratory analyses were done in the same laboratory throughout the EPIVIR Study. In the future, it would be interesting to look back on the possible changes in enterovirus infections in some other countries where the incidence of type 1 diabetes has been steadily increasing for the last decade (Podar et al. 2001).

The role of enterovirus infection during pregnancy as a risk factor for type 1 diabetes in the offspring was studied in pregnant women covering the whole duration of pregnancy. Even though enterovirus infections are very common and in spite of relatively wide arsenal of methods, the frequency of the infections may have been underestimated. However, the study series covering the first trimester of pregnancy was substantially larger than in any other study carried out so far. In addition, the effect of confounding factors was reduced by careful matching between cases and controls. The present study does not support any major role of enterovirus infections during pregnancy in the pathogenesis of type 1 diabetes. However, it leaves open the possibility that enterovirus infections may play a role in a small subgroup of patients. Particularly the second and third trimesters of pregnancy and the neonatal period needs to be evaluated in larger series.

The pathomechanisms of beta cell death are still unknown. The ongoing and planned prospective studies will provide excellent tools for understanding the process and the interplay between different host and environmental factors in pathogenesis. In the
future more attention could additionally be paid to studies searching for tools for intervention therapies such as enterovirus vaccine development. It would also be important to identify the mechanisms of enterovirus-induced beta cell damage, particularly if the vaccine is to be developed to exclude possible harmful effects. In the case of poliomyelitis, the exact pathological events were never discovered but the disease was beaten with the two working vaccines of Salk and Sabin. In the future, the same could be true for the prevention of type 1 diabetes if enteroviruses were indeed a true cause of type 1 diabetes. The development of a vaccine would require more detailed information about the serotypes which should be included in it; a question to which the prospective studies should give answers.
SUMMARY AND CONCLUSIONS

The purpose of this study was to explore the epidemiology of enterovirus infections in relation to type 1 diabetes and the role of infections during pregnancy as risk factors for type 1 diabetes.

The major findings were:

1. Enterovirus infections during pregnancy are not a major risk factor for type 1 diabetes in the offspring, but may play a role in some susceptible subjects.

2. Enterovirus infections are less common in countries with high incidence of type 1 diabetes. In addition, enterovirus infections have become less frequent in recent decades. This suggests an inverse correlation between enterovirus infections in the background population and the incidence of type 1 diabetes. The absence of enterovirus infections could predispose children to the diabetogenic effect of enteroviruses according to the proposed polio hypothesis.
ACKNOWLEDGEMENTS

This work was carried out at the Department of Virology, Tampere Medical School, University of Tampere, Tampere University Hospital and Tampere Graduate School in Biomedicine and Biotechnology during the years 1997-2005.

I wish to express my deepest gratitude to my supervisor, Professor Heikki Hyöty. His expertise, enthusiastic attitude and support are much appreciated. His open minded and innovative way of thinking of scientific problems has been the best possible example. His philosophies of other areas of life have also been great fun.

I wish to thank the Head of the Department of Virology, Professor Timo Vesikari, for providing excellent working facilities, for valuable collaboration and guidance.

I also want to express thanks to Professor Mikael Knip for valuable collaboration and for giving me the benefit of his expertise and guidance over the years.

The official reviewers Professor Timo Hyypiä and Docent Timo Otonkoski, are warmly thanked for their careful review and constructive comments. Virginia Mattila is warmly thanked for the revision of the language.

It has been my privilege to collaborate with many big study settings. I wish to thank all the co-authors of the original papers. The principal investigators of the DIPP Study, Professor Olli Simell, Professor Mikael Knip and Docent Jorma Ilonen are warmly thanked for collaboration and for allowing me to use DIPP material. I also wish to express my gratitude to the EPIVIR study group for valuable collaboration and contribution to these studies: Professor Heikki Hyöty, Professor Raivo Uibo, Professor Mikael Knip, Docent Jorma Ilonen, Professor Antti Reunanen, Dr. Lina Salur, Professor Johnny Ludvigsson, Dr. Dalia Marciulionyte, Dr. Robert Hermann, Professor Gyula Soltesz, Dr. Martin Füchtenbusch, Professor Annette Ziegler, Dr. Anita Kondrashova and Professor Anatolij Romanov. Professor Pentti Koskela is thanked for collaboration and for allowing me to use the National Health Institute’s Finnish maternity cohort invaluable to these studies. My warm thanks are also due to all personnel of these study settings. Heini Huhtala is thanked for her kind help with statistical problems. I owe my sincere thanks to all the people and families participating in these study projects.
I owe my thanks to everyone in the virology laboratory: Thank you for hilarious moments during some serious work. Marja Lönnrot, Karita Sadeharju, Kimmo Salminen, Sami Oikarinen, Sisko Tauriainen, Anita Kondrashova and Kaisa Kankaapää are thanked not only for their expert help and discussions but also fun companionship inside and outside the lab. Thanks also to the next generation of scientists in our lab: Tapio Seiskari, Kati Vuori, Maarit Oikarinen and Mika Martiskainen. I wish to thank the superlative team in the lab for their expertise in technical assistance: Eveliina Jalonen, Eeva Jokela, Miia Jääntti, Anne Karjalainen, Mervi Kekäläinen, Katri Koivumäki, Inkeri Lehtimäki, Jussi-Petteri Lehtonen, Maarit Patrikainen, Eeva Tolvanen and Sari Valorinta. I have truly enjoyed working with you.

I wish to thank my family, especially my mother Hannele and my father Heikki who have always been there for me and believed in me. My friends spread around Finland deserve my sincere thanks: Thank you for your friendship! Finally, I wish to express my special thanks to my soulmate and companion, Jukka.

These study projects were financially supported by grants from the European Commission (INCO-Copernicus Program), Päivikki and Sakari Sohlberg’s Foundation, Juvenile Diabetes Research Foundation International and Academy of Finland. This study was also financially supported by personal grants from the Yrjö Jahnsson Foundation, the Finnish Medical Foundation, the Tampere City Funds and Medical Research Funds of the Tampere University Hospital.

Tampere July 2005
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