Picornavirus infections in Infancy, Risk for Islet Autoimmunity and Type 1 Diabetes

Doctoral Thesis By

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1. Preface

1.1 Acknowledgement

This work was performed at the Department of Genes and Environment, Division of Epidemiology and at the Department of Virology, Division of Infectious Disease Control, Norwegian Institute of Public Health (NIPH), Oslo, in the period 2006-2009. The main experimental work was done in collaboration with the Laboratory for Molecular Genetics, Department of Pediatrics, University Hospital Motol, Prague. I would like to thank the Laboratory for Molecular Genetics for excellent working conditions and support during this period.

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Prague, October 2009.

1.2 Summary

Background

Most previous studies on Picornaviruses come from hospital settings, and most data come from diseased individuals. The natural circulation of Parechoviruses is unclear, as there are not many studies in healthy populations. While more data on the natural circulation of enteroviruses has recently been reported, the question of their role in development of islet autoimmunity is still unclear after decades of research even if several animal models are available for testing of these theories.

Objectives

The objectives for this work were to investigate the natural circulation and molecular epidemiology of parechoviruses in Norwegian infants; and based on these data and other earlier studies, to investigate any association parechoviruses or human enteroviruses might have with development of Type 1 Diabetes.

Subjects and Methods

Newborn children were recruited, on the basis of their HLA genotype, into a prospective cohort study in Norway with the aim to identify environmental risk factors for Type 1 Diabetes. They were followed from 3 months of age with monthly stool samples and regular blood samples to monitor for the development of islet autoimmunity and with questionnaires to record data on symptoms and predictors of infection. In Paper I 1941 samples from 102 children were included, half of the

children carried a high-risk genotype for Type 1 Diabetes and were followed up to 35 months and half of the children carried other genotypes and were followed-up to 12 months of age. In Papers II-IV, up to 2044 samples from 80 children are included. All children carry the high-risk genotype and 27 of them developed islet autoimmunity during the study.

The viruses were detected and quantified using real-time reverse transcriptase PCR. Positive samples were typed by nucleotide sequencing of the VP1 region.

Main Results

Human parechovirus infections were common in infancy and asymptomatic. Ljungan virus was not detected. Neither human enterovirus nor human parechovirus RNA detected in stool had any significant association with development of islet autoimmunity. Human parechoviruses and human enteroviruses were not associated with symptoms. The most common types of these viruses were detected and no correlation with islet autoimmunity was observed.

Conclusions

This is the first study to use quantitative methods, and is the most genetically homogenous cohort study. Ljungan virus seems to be rare, and is not likely to play a major role in the aetiology of Type 1 Diabetes. Any diabetogenic strain, or any association a human enterovirus or human parechovirus might have with islet autoimmunity, is unlikely to be distinguishable from the mostly harmless strains

detected in stool. Our findings suggest that any properties that enable a virus to break immune tolerance or host-virus factors should be investigated to elucidate the role of these viruses in the development of Type 1 Diabetes.

1.3 Selected abbreviations

T1D	Type 1 Diabetes
HLA	Human leukocyte antigen
INS	Insulin gene region
PTPN22	Protein tyrosine phosphatase, non-receptor type 2
TLRA2/CD25	Alpha chain of the IL-2 receptor
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
IFIH1	Interferon induced with helicase C domain 1
HEV	Human Enterovirus
HPeV	Human Parechovirus
LjV	Ljungan Virus
RT	Reverse transcriptase
PCR	Polymerase Chain Reaction
CI	Confidence Interval
OR	Odds ratio

1.4 List of papers

I. Tapia G, Cinek O, Witsø E, Kulich M, Rasmussen T, Grinde B, Rønningen KS. Longitudinal observation of parechovirus in stool samples from Norwegian infants J Med Virol. 2008 Oct;80(10):1835-42.

II. Tapia G, Cinek O, Rasmussen T, Grinde B, Stene LC, Rønningen KS. Human parechovirus infections in infancy – no association with autoimmunity or type 1 diabetes. Submitted.

III. Tapia G, Cinek O, Rasmussen T, Witsø E, Grinde B, Stene LC, Rønningen KS. Enterovirus infections and the risk for autoimmunity and type 1 diabetes in a cohort of Norwegian children with the highest-risk HLA genotype. Submitted.

IV. Tapia G, Cinek O, Rasmussen T, Grinde B, Rønningen KS. No Ljungan virus RNA in stool samples from infants in the Norwegian MIDIA cohort. Submitted.

The papers are referred to in the text with their Roman numbers.

2. Introduction

2.1 Type 1 Diabetes

Type 1 Diabetes (T1D) is a common, chronic autoimmune disease which usually manifests in childhood or adolescence. The direct cause of the condition is the immunological destruction of the insulin-producing beta-cells in the islets of Langerhans in the pancreas. Afflicted individuals are assumed to have an autoimmune condition for months or years [1], where the pancreatic islets of Langerhans are slowly attacked and destroyed. Eventually the loss of beta-cells leads to the clinical manifestation of T1D and dependence of exogenous insulin. The autoimmune condition leading to T1D will hereafter be referred to as islet autoimmunity.

The development of islet autoimmunity can be detected and monitored by measuring autoantibodies in peripheral blood [2-4], with the more commonly used antibodies directed against glutamatic acid decarboxylase (GAD) [5], protein tyrosine phosphatase IA2 (IA2) [6] and insulin (IAA) [7]. In addition, zinc transporter antibodies (ZnT8) [8] might be used. The presence of two or more autoantibodies has a very high predictive value for T1D, and is often used as a surrogate endpoint when investigating the disease. It should be noted that T1D is considered to be a T cell mediated disease [9], thus the autoantibodies are not responsible for beta-cell destruction, but reflect the ongoing autoimmune process.

There has been a noticeable increase in the incidence of T1D over the last decades, especially in developed countries and in countries experiencing an increase in living standard. Norway has one of the highest incidences in the world [10], as shown in Figure 1, and a marked increase in the age group under five years has been reported [11]. The autoimmune process is believed to be initiated early in life, with autoantibodies being detected by 5 years of age in most of the cases where there is a family history of T1D [12]. The incidence seems to increase the mostly in younger age groups [10, 13], and the autoimmune process appears to develop faster in younger subjects [14, 15].

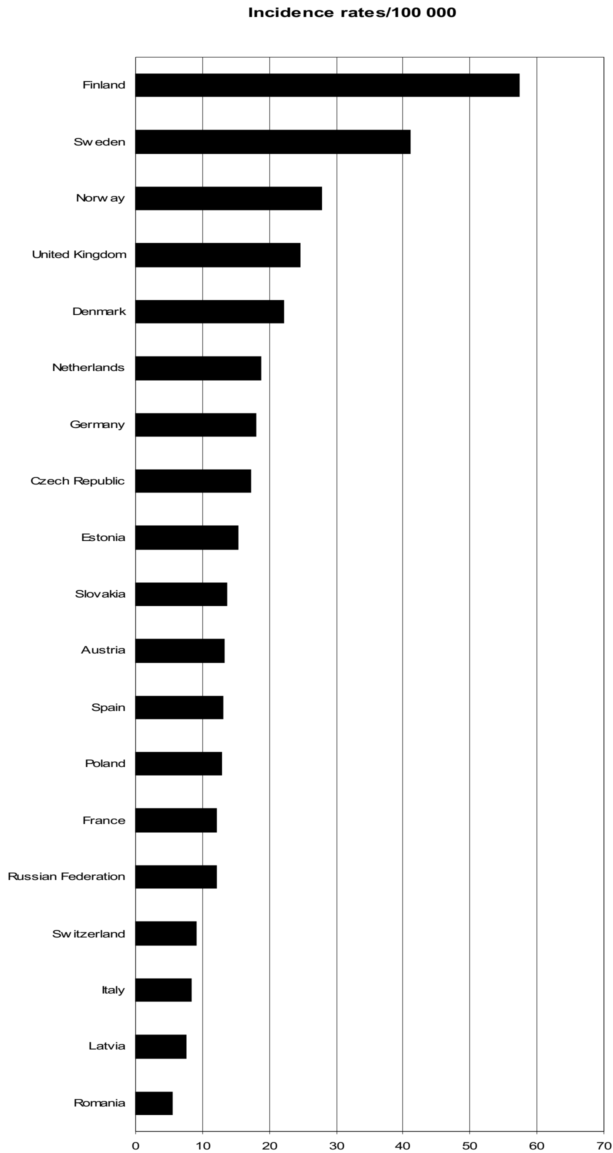

Figure 1: The estimated incidence rates in children (cases/100 000 population per year) of T1D in selected European countries in 2010. Data from the international diabetes federation Diabetes Atlas, available at http://www.diabetesatlas.org/content/estimates-type-1-diabetes-children-2010

There are several well-documented genetic risk factors for T1D. The human leukocyte antigen (HLA) genes, which are highly polymorphic and responsible for presenting epitopes to the immune system, contribute the highest genetic risk for the disease. The HLA-genes are also associated with autoimmunity [16, 17]. Other well-known and noteworthy predisposing loci are the insulin (INS) [18], protein tyrosin phosphatase non-receptor type 22 (PTPN22) [19], interleukin-2 receptor alpha chain (ILRA2 or CD25) [20], cytotoxic T-lymphocyte antigen 4 (CTLA4) [21, 22] and IFIH1 (interferon induced with helicase C domain 1) [23-25]. Many of these genes have central roles in the immune system. The odds ratios (OR) for T1D associated genes are displayed in Figure 2, which shows that HLA-genes are the most important determinants of genetic risk. In addition, several other polymorphisms have been discovered recently in genome-wide association studies [26], but their contribution to the overall risk is low.

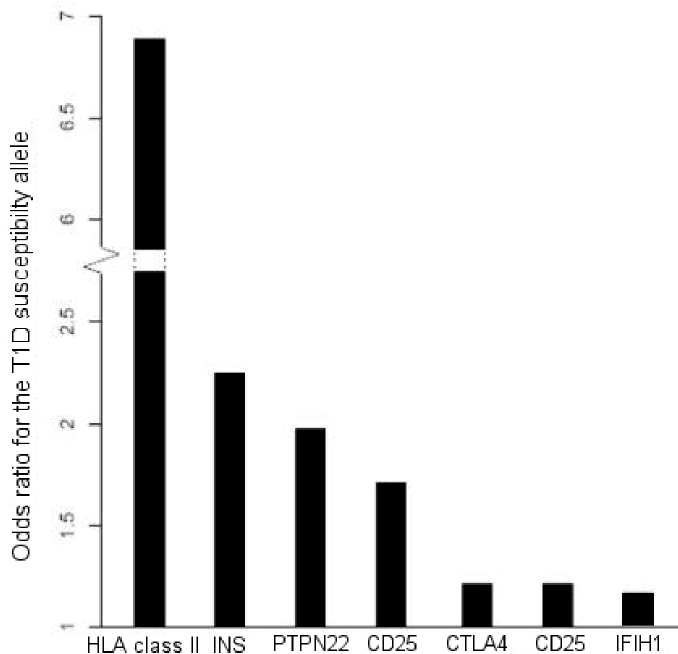

Figure 2: Odds ratio for susceptibility allele for individual T1D genes for T1D, modified from Todd et al. [24]. Note that there are two independent groups of SNPs of CD25 with different OR.

The rapid rise in T1D incidence [11], differences in geography [27], the rise in incidence for certain ethnic groups that have migrated from low-risk geographical areas [28, 29], reports of local epidemics of T1D [30] and twin studies [31] makes it unlikely that genetic factors alone can account for the risk of T1D. Genetics are believed to accounts for 40-50% of the risk [32, 33]. The remaining risk for getting the disease, as well as the recent changes in incidence, are most likely due to environmental factors and changes in these. When comparing the genotypes of recent T1D cases with older T1D cases, it seems that a much higher fraction of the cases are now individuals carrying what was presumed to be less genetic

susceptibility for developing T1D [34-36]. This observation makes it likely to think that the genetic effects on T1D risk are to a high degree dependent on the environment. Thus, environmental factors are likely to play a major role in the aetiology of T1D [31, 37].

There are several risk factors that have been proposed and investigated for T1D. Amongst these are dietary, perinatal and infectious factors, but so far these factors have not been shown to have a causal relationship [38-44]. Shedding light on the environmental factors of T1D should ultimately make intervention and prevention feasible. The high cost of the disease, monetary and otherwise, to the affected individuals, families and society, implies that preventing even a fraction of cases, or a delay in disease onset, would be of considerable value.

2.2 The immune system and development of autoimmunity.

The immune system struggles constantly to identify and eliminate pathogens, cancerous cells and foreign organisms. The immune system is divided into innate (non-specific) and adaptive (specific) immunity. The innate system is the first line of defence, is non-specific, and gives a generic response to pathogens. It senses pathogens through a number of pattern recognition receptors that bind conserved molecules such as lipopolysaccharides. The innate immune system might not be enough to stop all pathogens, but may give the adaptive (specific) immune system the time it needs to recognize foreign antigens and respond.

The adaptive immune system allows for a stronger immune response, but needs time to mount an effective response the first time a pathogen is encountered. It is specific to a certain agent, and highly adaptable B and T cells are the main components of the adaptive immune system. They are all specific for a particular antigen. This is achieved by recombination of the different parts of the DNA coding for the antigen receptor. T cells are divided into $CD4^+$ and $CD8^+$ based on different receptors on their surface. There are several subgroups of these, with the most known $CD8^+$ T cells being the cytotoxic T lymphocytes, which secrete molecules that destroy the cell they bind to. $CD8^+$ T cells recognize and bind to epitopes presented by HLA class I molecules that are expressed by almost all cells in the body. In the case of a viral infection, viral peptides would be presented on the surface of the native cells, which would be detected by $CD8^+$ T cells. $CD4^+$cells recognize and bind to antigens

presented on HLA class II molecules. If the $CD4^+$ cell binds to an antigen-presenting cell, like a macrophage or dendritic cell, the $CD4^+$ T cell releases signal molecules that attract other cells to the area. The result of this activation is inflammation. Some $CD4^+$ T cells, known as helper T cells, bind to antigens presented by B cells.

The human body makes millions of different B cells daily. Each of these has a receptor which is specific for one antigen. These B cells circulate until they become activated by binding of their specific antigen, which they engulf, digest and display the fragments of on a HLA class II molecule. This can bind a helper T cell, which upon binding will activate the B cell. This leads to the proliferation of cells secreting antibodies against the presented molecule. When activated, they differentiate into plasma B cells or a memory B cells. In short, the plasma B cells produce antibodies against the specific antigens, while the memory B cells are long-lived, and thus allows for a quick response to a second exposure of the same antigen. In addition, there are several subclasses of T and B cells, as well as other cell types, involved in the immune response. A particularly interesting subset of the T cells is the T regulatory cells, which act to suppress immune responses from other cells. They are thought to develop in the thymus, and are involved in turning off the immune reaction after the antigen is cleared from the body, and in regulating immune responses which might target the body's own cells. There is a balance between auto-reactive T cells (as some will have receptors which bind own antigens) and T regulatory cells.

Occasionally the immune system reacts too strongly to harmless antigens, causing

allergic reactions, or to self-antigens, causing autoimmunity. A low level of autoimmunity may be beneficial for keeping immune cells activated in the absence of foreign antigens [45]. The term autoimmunity is therefore usually reserved for higher levels of activity. Autoimmunity is responsible for several conditions, depending on what antigen the immune system reacts to. In the process leading to T1D, it is the insulin-producing beta-cells in the pancreas that are targeted by the immune system.

As might be expected, many of the genetic risk factors of T1D play a role in the immune system. The HLA genes, responsible for presenting epitopes to the immune system, give the highest genetic risk for T1D. PTPN22 is thought to be a negative regulator of T cell signalling [46]. CTLA-4 is important in the regulation of T cell antigen receptor [47], and acts negatively on T cell activation. IL2RA/CD25 is the receptor for interleukin 2, which is critical in T regulatory cell maintenance and function [48]. IFIH1 which is involved in inducing apoptosis in virally infected cells is especially interesting as it is critical for detection of picornaviral RNA [49]. The genetic variants that protects against T1D seem to be loss-of function mutations; truncating the protein, affecting splicing positions or highly conserved amino acids [25].

Thus, there are several observations suggesting that infectious agents, such as viruses and bacteria, could trigger the autoimmunity that leads to T1D.

Viral infections will lead to an immune response, which includes activation of B and T

cells, production of antibodies, inflammation and the development of memory cells. This might or might not be noticed by the host. Cells that show viral antigens will be recognized and destroyed, as will cells that do not show self-antigens (some viruses stop the presentation of antigens in infected cells). Most symptoms of viral infections come from inflammation and tissue damage by the immune system trying to get rid of the viruses. However, there are some viruses that are able to persistently stay in circulation, by having evolved several different mechanisms to escape detection and elimination by the immune system. There can be said to be a never-ending battle between the immune system and viruses; the immune system dominates a certain viral strain, leading to the rapid evolution of new strains, some of which will escape the immune system for some time until the immune system gets the upper hand, only to start the a new cycle. In the light of the genetic factors, it is not unlikely that viral infection, or viral infection of the beta-cells themselves, could trigger autoimmunity.

2.3 Hypotheses pertaining to T1D

The fertile field hypothesis

The fertile field hypothesis postulates that an infection gives rise to an immunological state which is "fertile" for the development of autoimmunity. A conceptual threshold for autoimmunity can be thought to exist, which can be exceeded by several factors working in conjunction. Viral infections can induce or expand a pool of autoreactive cells, leading to a transient, local "fertile field" that might react with viral antigens (crossreactivity) or with host antigens (bystander activation). These two mechanisms can interact and work in concert to induce autoimmunity. For example, autoreactive cells could be induced by molecular mimicry, and be activated later by bystander activation, or the whole process could be driven by bystander activation. Timing of infections is therefore regarded as an important factor. Thus, microorganisms should be seen as factors contributing to autoimmune disease rather than causal agents. In T1D, viruses that replicate in the pancreas could make an organ-specific "fertile field" for beta-cells [50].

The hygiene hypothesis

In addition, there is the hygiene hypothesis, which state that the lack of exposure to parasites, symbiotic organisms and infectious agents in early childhood increases the susceptibility to allergic and autoimmune diseases [51]. Humans evolved in an environment with microbial agents, and thus the immune system is adapted to take part in a continuous process of restraining the activity of bacteria and viruses. Some agents would have a permanent seat in the body, but at the same time there would

be a constant pressure of exposure. This is the natural state for which we have evolved, and consequently the state that is more likely to result in a balanced and well functioning immune activity [52]. The decline in microbial exposure in many populations is proposed to be the cause of the concomitant increase in atopic disorder over the past few decades [53], and this hypothesis has been extended to autoimmune diseases such as T1D (reviewed in [54]). In other words, T1D may result from a discord between the natural microbial load for which the immune system is adapted, and the present, presumed more sterile environment.

The hygiene hypothesis is supported by epidemiological studies that show higher prevalence of autoimmune diseases in North America and Europe compared to South America and Africa, higher incidence associated with increased material wealth and higher risk for autoimmune diseases for third world immigrants to the industrialized countries (reviewed in [55]). There are also many studies showing that some infections and microbial agents reduce the incidence of autoimmune diabetes in experimental animals (reviewed in [56, 57]). There are fewer studies in man suggesting protective effect of childhood infections against T1D. A study by Gibbon *et al*. [58] demonstrated that infection during the first year of life gives a lower risk of T1D, but the effect was weak and only 16 cases were included.

Population mixing (based on the number and diversity of incoming migrants to an area) was used as a proxy for exposure to infections in a study by Parslow et al., with the data showing that areas with higher population mixing have less risk for diabetes [59]. Additionally, in a study from Lithuania, Pundziute-Lycka showed that the incidence of T1D is lower in rural areas as opposed to urban centers and seems to

increase with higher socioeconomic status [60]. However, these are only three relatively small studies.

Another possibility is that the age when a certain infection is encountered for the first time is important. In a similar fashion, it has been shown that colonization of the gut and intestines in early infancy by bacteria plays a role in the development of the adaptive immune system and structural development of the gut (reviewed in [61]). It is well known that due to improved hygiene some viral infections that would normally occur in early life are encountered for the first time at a later stage. For example, mononucleosis is associated with acquiring an infection by Epstein-Barr virus (EBV) at puberty rather than infancy [62]. Mononucleosis is rare in third world countries. Similarly, hepatitis A and B are less likely to cause disease if the first exposure is at an early age, and chickenpox (caused by varicella-zoster virus) is more severe in adults [63]. Apparently, late infections typically give rise to a more severe pathology and concomitant increased activation of the immune system. The increased activation of the immune system may dispose for the establishment of an autoimmune condition. This hypothesis would explain the apparent conflict in data indicating that viral infections may confer both protection and susceptibility in mouse models [56]. In addition, viral infections could be used as a proxy for hygiene, since viral exposure is expected to correlate with exposure to a large variety of microorganisms that according to the hygiene hypothesis would develop a well-functioning immune system.

Thus, there exists a twofold reason for investigating an association between viral infections and the development of T1D: The infections might be directly involved in triggering autoimmunity, or they could play a subtler role, as postulated in the *fertile field* – and *hygiene hypothesis.*

2.4 Viruses and their putative association with autoimmunity

Viruses typically consist of a protein capsid containing nucleic acids with or without an outer membrane envelope. Viruses are obligate parasites in that they can only replicate within a host cell. They replicate by entering cells and hijacking the cellular machinery to make and assemble novel viral particles. Some viruses lyse the cell in order to release the virions, while enveloped viruses normally have the viral particles bud off from cellular membranes. Viruses might lie also dormant in cells for long periods of time, or even incorporate their genome into the host genome.

Asymptomatic viral infections

Some viruses are not known to give rise to any known symptoms or disease (i.e. orphan, or asymptomatic, viruses). It is assumed that most viruses can give symptoms, but are usually asymptomatic; only a few viruses are completely without any known disease or complication. It must be conceded that viruses can not be easily classified as asymptomatic/symptomatic, as this is not necessarily a clear definition. An asymptomatic infection usually refers to no *noticeable* symptoms, not that there are no symptoms, or refers to infections with symptoms that are unnoticeable by the host. An example of such an unnoticed symptom would, e.g., be the development of islet autoimmunity as it would not be noticed until much later. In this work the focus is on viruses that are thought to usually give asymptomatic infections, but some of them are known as important human pathogens.

2.5 Picornaviridae

The viral family *Picornaviridae* consists of small non-enveloped single-stranded RNA viruses of positive polarity. Picornavirus virions are spherical with a diameter of about 30 nm, and consist of a protein shell surrounding the RNA genome. The family takes its name from *pico* and *RNA* (literally, small RNA viruses), and has several well-known human and animal pathogens, but also many viruses without any known pathology. Picornaviruses infecting humans are known to be mostly asymptomatic and are common in infancy, with prevalences of 10-12% in stool samples for human enterovirus (HEV) [64], human parechovirus (HPeV) [65] and cardiovirus [66]. The picornaviridae family consists of 12 current genera; this work will focus on the *Enterovirus* and *Parechovirus* genera. Both these replicate mainly in the gut, are resistant to low pH and are spread by the fecal-oral route. Both types of virus are common in infancy, replicate mainly in the gut, and when they do cause symptoms, these are normally mild. These viruses can, however, give rise to serious complications. Species in both the *Parechovirus* and *Enterovirus* genus have been suggested as triggers of islet autoimmunity. A graphical representation of a Picornavirus, more specifically an enterovirus, is shown in Figure 3.

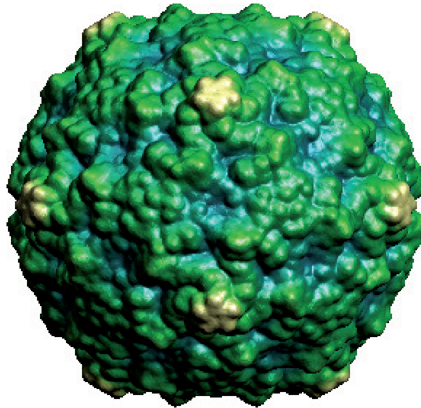

Figure 3: Graphical representation of a Coxsackie B3 virus, from the work of Muckelbauer et al. [67], downloaded from http://www.picornaviridae.com/structures/enterovirus.htm*,*

Parechoviruses

The parechoviruses were earlier classified with the enteroviruses, and have been assigned as enteroviruses in the past on the basis of cell culture [68], which is most likely not an isolated event. The parechovirus genus consists of two species, the murine virus Ljungan virus (LjV) and HPeV (HPeV). HPeVs are common in children, uncommon in adults and are present worldwide [69]. HPeV 1 and 2 have been known since 1956, and were originally classified within enteroviruses as echovirus 22 and 23. Several new parechoviruses have recently been reported, with HPeV3-8 being described from 2004 to 2009 and HPeV9-14 recently announced (for references, see [69]). The most common HPeV type is HPeV 1, followed by HPeV 3 and HPeV 6 with the remainder being rare.

LjV is a rodent virus described by Niklasson et al. [70], has been associated with a

variety of conditions in bank voles and other rodents, including T1D [71], myocarditis [72] and intrauterine death [73]. In humans, LjV has been associated with intrauterine fetal death [74], anencephaly [75], sudden infant death syndrome [74] and suggested as a risk factor for T1D [71]. A possible role of LjV in T1Dis of particular interest due to the strong association found in captive bank voles [71, 72, 76].

Enteroviruses

The enterovirus genus consists of ten species, with seven of them having human hosts – Human enteroviruses A-D and Rhinovirus A-C. HEVs are considered the most promising viral candidates in the aetiology of T1D. There are several studies support a role of HEVs, with the strongest evidence coming from Finnish studies: four important papers describe the results obtained from the "DIPP" cohort using serology or reverse transcriptase PCR for enterovirus from serum or stool [77-80]. The occurrence of enterovirus in these studies was higher in subjects with signs of autoimmune insulitis (positivity of antibody markers) than in their controls matched for time of birth, HLA, and gender. Similar results were obtained also from two other Finnish cohorts: from the "DiMe" with a considerably strong effect of enterovirus but lack of matching [81, 82], and from the second pilot phase of the dietary trial "TRIGR" [83]. In addition, an association between maternal enterovirus infection during pregnancy or enterovirus infection in children and risk for T1D [84] has been reported. In addition, enteroviruses have been shown to be more present in the sera [85], small intestine [86] and pancreatic islets [87] of recently diagnosed T1D patients. A recent study by our group suggest that there is less enterovirus infections among children with high genetic risk for T1D compared to children not carrying this

genotype, although the difference is not statistically significant due to the low number of children presently tested (Witsø E et al., J Internat Epidemiology, in press, 2009). In particular, Coxsackievirus (a member of HEV B) is suspected of having a role in the development of autoimmunity (reviewed in [43]).

Viruses as environmental factors of T1D

Viral infections have long been considered as triggers of T1D, and there are several lines of evidence implying virus infections either *in utero* or in early life in the aetiology of T1D [56]. The high frequency of T1D in children with congenital rubella syndrome was the first indication of a viral involvement, and hinted towards the importance of the intra-uterine environment [88]. However, intra-uterine rubella infection is now rare in Scandinavia due to vaccination, but the incidence of T1D is high and continues to rise. Mumps and measles were also suspected of playing a role in T1D [89], and a plateau in T1D incidence in Finland was also noted after measles, mumps and rubella vaccines were introduced [90]. Measles vaccination was also suggested to be protective in a Swedish study [91]. Another interesting observation is that acute viral infections can be associated with disease onset [92-94]. There is also a seasonal correlation between periods of viral infections and onset of T1D [95, 96].

Viral mechanism that could lead to development of autoimmunity

There are several proposed mechanisms for how viruses might be associated with T1D. Viruses can activate polyclonal cells and trigger production of autoantibodies

[97], viruses can directly infect and lyse cells [57], viral antigens might mimic self-antigens [98], inflammatory responses stemming from viral infections might trigger autoimmunity [99], or as predicted in the hygiene hypothesis, viruses might be needed for proper maturation and regulation of the immune response [54]. How these events could lead to islet autoimmunity and T1D is illustrated in Figure 4.

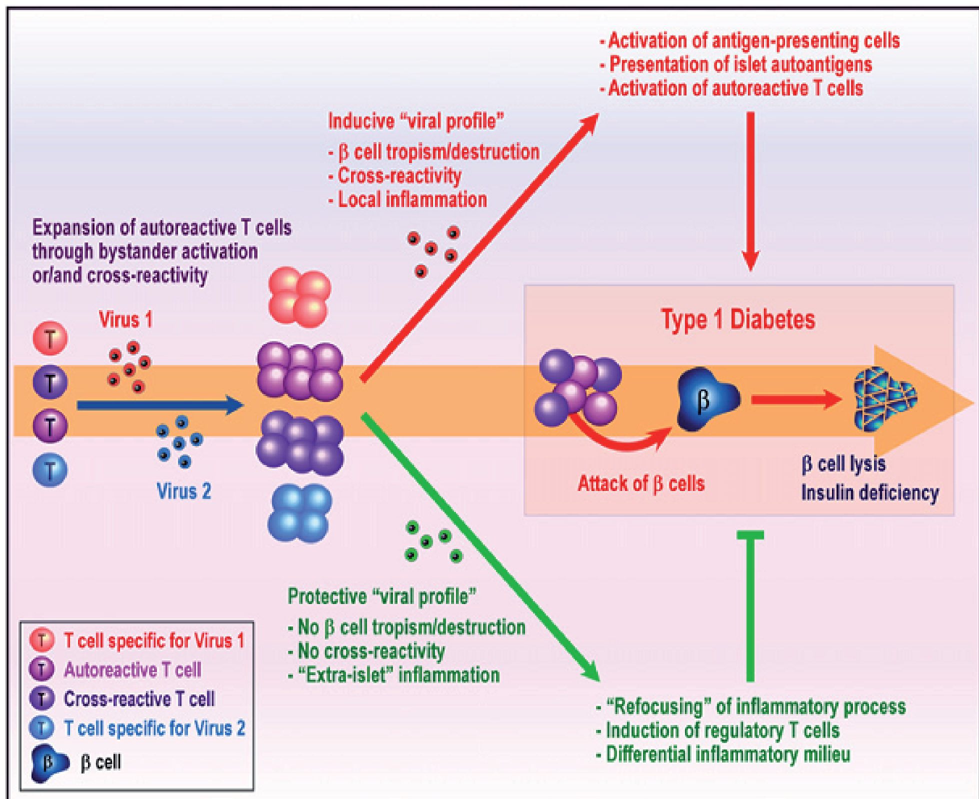

Figure 4: Possible viral mechanisms of autoimmunity, from Filipi et al *[100]. In this Figure, the red pathway denotes the pathway to autoimmunity- molecular mimicry, bystander activation and beta-cell tropism. Viral infections might also have a protective effect, shown in the green pathway, or be required to tune or mature the immune system.*

3. Subjects and methods

3.1 Subjects

In the cohort Environmental Triggers of Type 1 Diabetes: The MIDIA Study

The MIDIA study is a longitudinal cohort study with inclusion of children with the high-risk HLA genotype (DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02), with follow-up from three months of age up to 15 years of age. The original aim of the MIDIA study was to recruit and genotype 100,000 children at birth to include 2000 children with the high-risk HLA genotype for follow-up. While recruitment was taking place on a nation-wide scale in Norway all recruitment was stopped December 2007 by the Directorate of Health, who decided that performing genetic analyses in healthy infants and informing the parents about the result was in conflict with the Norwegian Biotechnology Law. The law had existed since 1994, and the MIDIA project had got all needed approvals before recruitment to the study started in the summer of 2001. However, after one mother went to the media and complained that she had not received adequate information about the study before she and her husband had given informed consent, all authorities revaluated the MIDIA study. Close to 48 000 children were genotyped, with 1047 identified with the highest genetic risk for T1D before recruitment was stopped.

Parents of the included children with high-risk for T1D have been asked send in a stool sample every months from 3-35 months of age. A questionnaire summarising

weekly diaries is filled out at 3, 6, 9 and 12 months of age. Blood samples have been asked for at the same intervals. After this period, questionnaires and blood samples are asked for annually. For more information on MIDIA, see www.fhi.no/midia.

Subjects for the study of parechovirus in Norwegian infants (Paper I)

The study described in Paper I involved two matched groups from the MIDIA cohort differing in their genetic risk of T1D: the "high-risk group" (HR) included 51 children (24 males and 27 females) born in 2004 who were identified at birth to carry the HLA genotype conferring the highest risk of T1D (DQB1*0302-DQA1*03-DRB1*0401/DQB1*02-DQA1*05-DRB1*03). Children carrying other HLA genotypes (27 males and 24 females) were recruited into a "non high-risk group" (non-HR). These non-HR children originated from the same newborn study as the HR group, and were matched 1:1 for date of birth (up to 30 days difference) and community of residence. They were followed up in the same manner with stool samples and questionnaires, but only until 12 months of age. Of the expected 1980 samples and 482 questionnaires, 1941 (98%) samples and 476 (98%) questionnaires were received. The median endpoint of the follow-up was 31 months for the HR children and 12 months for the non-HR children.

Subjects for the case-control studies (Paper II-IV)

Between 2001 and 2008, 911 children were included in the longitudinal observation in the MIDIA cohort. To monitor potential development of autoimmune insulitis, the subjects were tested for autoantibodies against glutamic acid decarboxylase 65

(GAD), protein tyrosine phosphatase IA2 (IA2) and insulin (IAA) with blood samples taken every 3 months until the age of 1 year, and every 12 months thereafter. The testing is described in detail by Stene *et al* [15]. If a sample is found positive for one autoantibody, the child was retested every six months; if two or three antibodies were positive, the child was retested every three months. The case children were defined as having at least two autoantibodies positive in two consecutive samplings. The endpoint used in the analysis was set as the development of autoimmunity, e.g. the time for positivity for the first autoantibody. Further samples were, however, collected beyond the defined case criteria until the development of T1D to investigate disease progression.

By December 2008, 27 (10 boys and 17 girls) of the 911 children in the cohort had reached the endpoint, being confirmed positive for two or three autoantibodies, and were assigned as cases. Ten of them have so far been diagnosed with T1D. Two control subjects were randomly assigned per case, in total 54 control children, matched for county of residence and age (thus also for the follow-up time, with a tolerance of 30 days). Children that dropped out of the study during an early phase, or had developed one autoantibody were ineligible as controls. Parents of one control child withdrew at a later time, decreasing the number of control subjects in the analysis to 53.

In total, 80 children (27 case and 53 control subjects, 40 males and 40 females, born between 2001-2006, were analyzed, contributing to 3555 person-months of

observation until December 2008. All subjects were followed up with stool samples, blood samples for autoantibody testing and structured questionnaires. Of 704 planned blood samples, 637 (91%); 2173 of 2482 scheduled stool samples (88%) and 492 of 547 questionnaires were received (90%). The median duration of follow-up with stool samples was 32 months (range 9-37 months), with the median age at endpoint in cases 20.5 months (range 6-43 months). To test for viral infections, stool samples taken by parents where tested for the presence of viral RNA.

Calculation of statistical power.

The power of this study was calculated based on relevant estimates from a pilot study [101]: the use of all monthly samples tested for HEV until the development of autoimmunity gives us an estimated power of 93% to detect an effect equivalent or greater than an odds ratio of 2.0, using a two-sided test with the alpha-level of 5%. The same calculation can be used for parechovirus, as they have similar prevalences.

3.2 Methods

Collection and processing of the stool samples

Parents were instructed to obtain a stool sample from the diaper using containers with a spoon attached under the lid. The containers were mailed using the national post service in pre-paid envelopes to the central laboratory, which meant 1–3 days in

transit. If a stool sample did not arrive in time, the parents were reminded by a phone call. The stool samples were suspended (one part stool to four parts buffer, w/v) in phosphate buffered saline with 0.5% bovine serum albumin, 50 IU/ml penicillin, 50μg/ml streptomycin and 2.5μg/ml fungizone, and subjected to vortexing and centrifugation at 4000×g for 30 min. The faecal supernatant was divided into aliquots and frozen at −80°C until further processing.

RNA isolation

In paper I, the DNA and RNA were co-purified from 140μl of the supernatant using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. A low amount of West Nile Virus (WNV) Armored RNA (Ambion Diagnostics, Austin, TX, USA) was added into each lysis buffer vial of the kit to serve as an exogenous internal control for the extraction, reverse transcription and amplification. The nucleic acid was eluted into 100 μl sterile nuclease-free water. Positive and negative controls were extracted with each batch of samples, with the negative control being normal saline. In papers II-IV, the protocol was modified for the use of vacuum processed 96-well plates, and the samples were subjected to a second elution step with 70 μl.

Methods for detection of human enterovirus

Enterovirus was detected using a one-step real-time RT-PCR reaction with an exogenous internal control. Two microliters of the template were pipetted in duplicate to a 10μl reaction containing 1xQuantiTect Probe RT-PCR master mix, 1xQuantiTect RT mix (both components of the QuantiTect Probe RT-PCR Kit, Qiagen. The reverse

transcription and amplification were performed on an ABI 7300 machine with the Sequence Detector Software version 1.4. The amplification profile consisted of 30 min reverse transcription at 50°C, 15 min denaturation at 95°C, and 45 cycles of 15 seconds denaturation at 94°C and 1 minute combined annealing–extension at 60 °C. The tubes were discarded unopened. Fluorescence data were collected during the annealing–extension phase, and evaluated using the Spectral Compensation option of the software, which improves the discrimination between the two reporters. First, the FAM-dye layer with the enterovirus probe was evaluated; then the VIC-dye layer was inspected for WNV amplification in enterovirus-negative samples. The control WNV RNA gave a threshold cycle value of 29–31. If the WNV threshold cycle was more than 3.5 cycles above the average of the other enterovirus-negative samples in the run, the extraction and RT-PCR were repeated. After the first screening, samples were retested in duplicate, in a 20 µl volume together with a seven-point standard curve from 10^5 to 24 copies/µl made from Enterovirus Armored RNA (Asuragen, USA). The threshold of positivity used in this study was set to 100 copies/µl RNA, a quantity that could be consistently and reliably detected.

Methods for detection of parechoviruses

In paper I, parechoviruses were detected from extracted RNA using a two-step real-time RT-PCR reaction. The samples were reverse-transcribed with specific primers using the Improm II RT kit (Promega). One tube real-time PCR, using the HotStar Taq Polymerase chemistry (Qiagen, Hilden, Germany), was used to detect the 5’-UTR region of HPeV as well as the exogenous control. The amplification was carried out

on an ABI 7300 (Applied Biosystems) with the following thermal profile: 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 1 minute at 60°C (where fluorescence data were collected). If the WNV threshold cycle was too high, or the curve was not exponential, the sample was retested and re-extracted if necessary. For the detection of LjV, the method of Donoso Mantke *et al.* [102] was used. Dilutions of a sample with known quantity of HPeV1 from the QCMD (Quality Control in Molecular Diagnostics, www.qcmd.org) were used as a standard for HPeV. For LjV, the standard was based on a transcript of a plasmid containing a cDNA clone of LjV prototype strain 87-012, kindly provided by Professor Lindberg (University of Kalmar, Sweden). The positive controls were consistently detected down to 10 copies/µl, and this level was therefore set as a positivity threshold. Negative controls (water) were included in the extraction procedure, and additional negative controls were included in the individual RT-PCR reactions. In paper II the protocol was modified to use 95°C instead of 94°C in the thermal profile. In paper IV the protocol was modified for the use of the antisense primer designed by Donose Mantke *et al*. [102] as RT primer.

4. Aims

The aim of this work was to elucidate how common HEV, HPeV and LjV infections are in early childhood and if there is any association between infections in early infancy and later development of autoimmunity.

Paper I had as aim to investigate the presence of parechovirus RNA in stool samples from healthy children, to test the methods and investigate how common these viral infections were in early childhood.

Paper II had as aim to investigate if there was any association between HPeV infections in early infancy and development of autoimmunity/T1D.

Paper III had as aim to investigate whether there was any association between HEV infections in early infancy and development of autoimmunity/T1D.

Paper IV had as aim to investigate whether Ljungan virus had any association with development of autoimmunity or T1D

5. Brief summary of results

Paper I: Longitudinal Observation of Parechovirus in Stool Samples from Norwegian Infants

The aim of this paper was to assess the prevalence and molecular epidemiology of the genus *Parechovirus* in Norwegian infants, and investigate whether the presence of virus correlated with symptoms of infection. A group of 102 infants were followed - 51 infants carrying the high-risk T1D genotype (aged 3 to 35 months), and 51 children without this genotype (aged 3 to 12 months). Stool samples were obtained each month, and symptoms of infection were regularly recorded onto questionnaires.

HPeV was detected in 220 of 1941 samples (11.3%), examined by real-time RT-PCR. No LjV RNA was detected using a separate real-time RT-PCR. The HPeV quantity ranged from a single copy to more than 10^5 copies /µl RNA and the 220 positive samples were assigned to 119 discrete infection episodes. There was a distinct and highly significant ($p < 0.0001$) seasonality, peaking from September to December while a trough was noted between April and June. By twelve months of age, 43% of the infants had had at least one infection, while 86% of the infants had encountered the virus by the end of the second year, with the cumulative incidence of infections increasing steeply between 6 and 18 months of age. Children carrying the high-risk (HR) genotype for T1D had a tendency towards fewer initial infections during the first year compared to children carrying other genotypes (non-HR), but the difference was

not statistically significant ($p = 0.07$).

The estimated median duration of an infection episode was 51 days, with about 10% of the episodes lasting more than 3 months. The first sample typically contained more virus than subsequent samples from the same episode, and the duration of infection episodes correlated with the peak virus quantity. A previous infection decreased the future risk of reinfections by 75% (RR = 0.25, 95%CI: 0.16 –0.39, $p < 0.0001$). Neither the gender of the subject, nor the number of their siblings affected the risk of infection. Symptoms were reported slightly more often in months with virus present in faeces, but none of the symptoms were significantly associated to neither parechovirus infections as a whole or by quantity. Sequences from samples with quantities exceeding 1000 copies/µl showed that HPeV 1 was the most prevalent type (76%), followed by HPeV 3 (13%), HPeV 6 (9 %) and HPeV 2 (1%). There was no significant association between infections and the following symptoms: coughing, sneezing, fever, diarrhoea or vomiting. In conclusion, HPeV infections are frequent in early childhood, without usually causing symptoms. LjV was not found in Norwegian infants.

Paper II: Human parechovirus infections in infancy – no association with islet autoimmunity.

The aim of this paper was to investigate a possible association between HPeV infections in early infancy and the development of islet autoimmunity. The study was

done in a cohort of Norwegian infants carrying the highest genetic risk for T1D. A nested case-control design was used, selecting 27 children that developed islet autoimmunity (repeatedly positive for 2 or 3 autoantibodies) as cases and 53 children matched for age and county of residence as controls. Monthly stool samples from these children were analysed for HPeV using a semi-quantitative real-time PCR.

HPeV was detected in 195 of 1747 samples (11.2%). No significant difference was found in the prevalence of HPeV in stool samples comparing cases and controls; 12.2% and 10.7%, respectively (OR=1.1, 95% CI: 0.74 – 1.62). By restricting the analysis to samples taken prior to the development of islet autoimmunity (and the corresponding time point in the matched controls), the frequencies were 12.5% for cases and 11.0% for controls (OR=1.18, 95% CI: 0.74 – 1.85). Interestingly, restricting the analysis to samples collected 3, 6 or 12 months prior to seroconversion for islet autoantibodies, gave a suggestive association in the shortest time window (11.7 vs. 6.8%, OR=3.06, P=0.083).

The 195 positive samples were assigned to 128 infection episodes, with no significant differences between the cases and the controls before development of autoimmunity or when looking at the total dataset. No symptoms were associated with HPeV infection in any of the two groups. A subset of the positive samples (n=31) were sequenced, suggested that HPeV 1 was the dominant genotype

In conclusion, no support for any association between HPeV infections and the

development of T1D was found. However, the non-significant higher frequency of infection in case children in the interval 3 months before seroconversion for islet autoantibodies could warrant replication in larger studies.

Paper III – No association of human enterovirus RNA in monthly faecal samples and repeated positivity for multiple islet autoantibodies in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study

The aim of this paper was to test whether the frequency of HEV RNA in faecal samples collected monthly from early infancy was associated with development of multiple islet autoantibodies in children. The study was implemented in a cohort of Norwegian infants carrying the highest genetic risk for T1D A nested case-control design was used, selecting 27 children that developed islet autoimmunity (repeatedly positive for 2 or 3 autoantibodies) as cases and 53 children matched for age and county of residence as controls. Monthly stool samples from these children were analysed for HEV using a semi-quantitative real-time RT-PCR.

HEV was detected in 290 of 2 044 stool samples (14.2%). No significant difference was found in the prevalence of HEV in stool samples comparing cases and controls; 12.8% and 14.8%, respectively (OR = 0.84, 95% CI: 0.58 - 1.22). By restricting the analysis to samples taken prior to the development of islet autoimmunity, the frequencies were 12.7% for cases and 13.6% for controls (OR=1.01, 95% CI: 0.59 - 1.72). There was no difference in average quantity of enterovirus RNA, or any

tendency of repeatedly positive samples, in any of the groups. There was a pronounced seasonality with a significant association ($p<0.001$) for infections with a peak in autumn and a smaller peak in July while a through was observed in March. The occurrence of infections was also age-dependent. A rise was noted from the fifth to ninth month of age, and during the first half of the second year of life. Only 11 subjects did not shed enterovirus in their stool during their entire observation period (4 cases and 7 controls).

No significant differences were found by restricting the analysis to various time windows before islet autoimmunity. Prior to the development of islet autoimmunity there were 137 positive samples, assigned to 95 infection episodes, with no significant difference in infection episodes per child between cases and controls (1.1 vs. 1.2, respectively). These results remained essentially unchanged after adjustment for potential confounders. Selected positive samples were genotyped by determination of their VP1 region. A wide repertoire of serotypes was observed, with the samples clustering after time and geographical location without appreciable connections to islet autoimmunity. While these were not representative for the whole dataset, several suspected diabetogenic strains from other reports were detected in both the cases and the controls.

In conclusion, the faecal shedding of enteroviral RNA does not predict advanced islet autoimmunity. Further research should therefore be focused on host-virus interactions and the ability of an enterovirus to invade the target pancreatic tissues.

Paper IV – No Ljungan virus RNA in stool samples from infants in the Norwegian MIDIA cohort

The aim of this paper was to test for the presence of LjV in young children that developed autoimmune insulitis and for any association LjV might have with T1D.

A nested case-control design including 27 infants carrying the highest genetic risk for T1D who developed autoimmune insulitis and 53 matched controls was employed to test for the presence of LjV and the development of autoimmune insulitis. Testing of the monthly stool samples from the subjects detected no LjV RNA. By merging this dataset with an earlier dataset, the dataset from Paper I, a total of 175 children and 3803 samples were tested and found negative. Considering the long follow-up time, the number of tested samples and that both children with prediabetic insulitis and healthy children were tested, these results were taken as an indication that LjV is very rare in the stool samples of Norwegian infants

In conclusion a possible association between infection with LjV and development of islet autoimmunity could not be ruled out, but the virus is seemingly very rare in young Norwegian children, suggesting that LjV is not a common risk factor in the aetiology of the disease.

6. Discussion of main Results

The primary aim of this work was to study asymptomatic viral infections in infancy and if any association with islet autoimmunity T1D could be found. The main work has thus been focused on the parechoviruses and enteroviruses in their primary replication site, the gut. Shedding of LjV RNA in stool seems rare in early childhood, and was not detected in this work. Shedding of HEV RNA and HPeV RNA in stool seems common, and they appear to be ubiquitous viruses, infecting infants from an early age and do not appear to be associated with symptoms or development of islet autoimmunity.

6.1 Methodological considerations

Study design and the Cohort

The subjects in this work are participants in "Environmental Triggers of Type 1 Diabetes: The MIDIA study" (see www.fhi.no/midia for details). The subjects from the general population were identified at birth based on genetic testing for the HLA genotype conferring the highest genetic risk of T1D, DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02, and a group of children carrying other genotypes were also recruited. Considering the children with the highest genetic risk for T1D, the MIDIA study is the most genetically restricted cohort of all reported studies. The high risk genotype is carried by 2.1% of Norwegian newborn children

and gives a 7% risk for developing T1D before 15 years of age and a 20% lifetime risk [103-107] . Selection strategies are a compromise between the level of risk, the homogeneity of the cohort, and the time and costs needed to recruit the children. The genotype chosen gave a cohort with a very high risk of diabetes within a reasonable time scale, so that matching for selected confounders as place and time of birth was feasible.

Definition of endpoint

The definition of a case in Paper II-IV was positivity for 2 autoantibodies on 2 consecutive samplings at least 3 months apart. This strict classification was chosen since children with only one autoantibody might revert and a single positive sample might be a false positive. Still, the number of cases, control and samples from these in this study are comparable or larger than previously published ones. For the purposes of analysis, however, the endpoint was set to the time point when the child developed one autoantibody as this was taken to reflect the start of islet autoimmunity.

Confounding and matching strategies

The children in this study were tightly matched to reduce the effects of confounding variables. Matching for some variables, such as age, is necessary as it has a big effect on viral infections. The downsides to matching are that too fine matching might reduce the number of available controls. In Papers II-IV, up to 3 months in age difference or follow-up time was tolerated, as was the use of neighbouring communities if there were not a sufficient number of available controls. Several

putative confounders, such as siblings, were included in the statistical analyses, described in detail in the respective papers.

Selection bias

The criteria or procedures for selection of participants might introduce systemic errors in the study, which will make the observed relation between exposures and outcomes different from the “true” values. In Paper II-IV, such a bias may occur if the selected genotype has an influence on viral infections, which cannot be ruled out, and a non-significant trend for the HLA genotype having an effect on the number of infections was observed in Paper I. In addition, factors influencing study participation might also introduce systemic errors. Any serious diseases, chronic conditions or symptoms can be presumed to exclude children from participation in the study. Thus, the study has a bias for healthy children and a bias against any children who get seriously ill from viral infections or other factors that make the parents unable to send in the scheduled stool sample, thus the symptoms might be underreported. However, the observed frequencies and incidence are congruent with other studies in genetically heterogeneous subjects, and other studies also show enterovirus and parechovirus infections to be mostly asymptomatic and self-limiting.

Are the results valid for the general population?

As the children studied are not a random sample from the general population, the question might be asked if the results can be generalized to the general population. Not only the HLA-genotype, but also the geographical location and relatively short

period of time the children are from could present a bias as viral strains have differences in prevalence both on a time- and geographical scale. Thus, both the virus types detected and observed prevalences could be different from the true values, but this effect is expected to be minimal as the results from Paper I-III show similar seasonality pattern and frequencies as earlier reports from other countries.

Sampling of monthly stools

While it cannot be ruled out that a viral infection might last less than a months and thus not be detected in our monthly samples, a comprehensive review aimed to determine optimal timing of stool specimen collection in poliovirus infections found 3-4 weeks as the usual duration of poliovirus excretion in unvaccinated infants [108], a situation that may be analogical to natural infection with an enterovirus or parechovirus not encountered previously. Thus, the monthly sampling schedule, with samples taken to represent the viral load in the primary replication site, should be sufficient for detecting most viral infections. Several long infections were observed in Paper I-III, but only around 10% lasted more than three months (Paper I), and a study on enterovirus found only 6% of infections lasting 2 months or more [101], so studies using sampling intervals of three or more months are likely to underestimate the true prevalence. Detection of viral RNA in blood is likely to further underestimate the prevalence of infection as the viraemia has much shorter duration (1-3 days) than viral replication in the gut. A weakness with our study design is that the use of stool samples means we cannot expose any properties of the viruses unless they are linked to the primary replication of the virus.

Power calculations for Paper II-IV

Using relevant estimates from an earlier pilot study [101] and Paper I, the lowest odds ratio for association between virus infections and islet autoimmunity that could be detected was calculated with a two-sided test with alpha-level of 5% and 80-95% power under various scenarios. On assuming the total prevalence of virus infections in the cohort (12% used for power calculations), assuming a true odds ratio and intra-individual correlation for virus infections (0.1 was used for conservative estimations) the "effective sample size" was set to n(1/(0.1+1)), i.e. 90% of the actual number of samples [109]. Different comparisons are shown in Table I.

Table I: Statistical power considerations for association between islet autoimmunity and prevalence of enterovirus RNA in samples. To illustrate, with 900 pre autoimmunity samples (300 from cases and 600 from controls), the study had at least 93% power if the true odds ratio was 2.0 or greater.

Comparison	Assumed true OR	Prevalence, controls	Prevalence, cases	Samples from cases	Samples from controls	Power[1]
All samples	1.6	11.8%	17.7%	600	1200	88%
All samples prior to autoimmunity	2.0	11.7%	21.0%	300	600	93%
The three last samples prior to autoimmunity	3.0	11.5%	28.1%	81	162	87%

[1] For each case, the corresponding matched samples from controls were used in the analysis.

Experimental procedures

This study is - to the best of our knowledge - the first study to use a quantitative molecular approach. The ability to distinguish between low-quantity and high-quantity positivity and follow the dynamics of the viral load give this work a new dimension. However, the analysis showed no appreciable differences in the average viral load among cases and controls for neither HEV nor HPeV, and it presumably even very low quantities of virus might lead to islet immunity. An important point is that the obtained data must be treated as semiquantitative rather than quantitative due to the nature of the source of RNA - stool content is impossible to standardise, both from a quantitative and qualitative point of view. The chief obstacle was the variable degree of inhibition seen in a proportion of the samples, which was controlled for using the signal from the exogenous internal control, which safeguards against the presence of inhibitors and RNA degradation.

Virus detection

The PCR methods used had a very high specificity for their respective viruses, and a high sensitivity. The sensitivity should not have a major influence, as we detect the virus from the primary replication site. However, PCR methods are vulnerable to contamination and carryover of PCR products. An advantage of the methods used was that the tubes were discarded unopened after testing and not processed further. The genotyping assays were more exposed for a possible contamination, but the sequencing data would exposed any possible contamination. A weakness with PCR based methods is that it might introduce a bias for certain strains or types of viruses. The genotype distribution might be biased if the assay works much better, or worse, on a particular type of virus.

Measurement errors

All observations are prone to systematic and random errors, which may influence the results of this work. The effect of any random errors should be reduced by using a larger sample set. As the papers in this work all have close to 2000 observations from 80 to 100 children, the influence of random errors is presumed to be negligible. Even if there might be e.g. host genetic factors present that influence viral infections or experimental errors, the majority of observations will be unchanged and the effect will be negligible. Systematic measurement errors are harder to safeguard against, and might present a bias. A known error (not at the time of publication) is the primers used for the VP1 genotyping in Paper 1 are suboptimal for the amplification of the

HPeV5 VP1 region [110], which would underestimate the frequency of this type. However, the updated primers used in Paper II do not have this problem, and HPeV5 is considered rare. Viral strains might have different properties of shedding in stool, which might also influence the results.

6.2 Theoretical considerations

Shared characteristics and natural circulation of HEV and HPeV

HEV and HPeV had similar prevalence; 14.2% vs 11.2%, and a number of other similarities. Both had similar seasonality, with a peak in autumn. However, HEVs had a through in March and a second peak in summer, while the HPeVs had through during the summer. The occurrence of infections was age-dependent for both, with a rise from 5-6 months until 18 months of age. This might be explained by the loss of transplacental maternal antibodies by the age of six months, or by the fact that Norwegian children are often introduced to kindergartens at 12 months of age. The seasonality and prevalence was very similar over the years the samples were obtained for both viruses and most infections started with a high viral load that subsequently decreased to lower values, which suggests an active immunological response to the viruses. Almost all children had a history of infection by the third year of life, indicating that HEV and HPeV are both common and normally harmless.

A slight trend towards fewer HPeV infections was observed in children with the highest genetic risk for T1D (Paper I, P=0.07). More data is required to investigate such an association, but it is interesting to note that the same HLA genotype seems

to protect against HEV infection (E.Witsø, J Internat Epidemiol, in press, 2009). Picornaviruses may also replicate outside the intestinal tract and may cause viraemia or respiratory infections.

Important differences in results with other studies

Despite testing of around 2000 monthly samples collected in children with the HLA genotype conferring the highest risk for T1D, and a high statistical power to detect associations of moderate magnitude, no association was detected between the presence, quantity, infection length, symptomatic infections or number of infections of these viruses, as measured by shed RNA in stool, and the risk of islet autoimmunity. The sequencing data does not indicate that any certain groups of enterovirus or parechovirus are associated with autoimmunity.

Ljungan virus

Although data supporting LjV infections in humans have been published, there is so far no conclusive evidence, and LjV was not identified in stool samples from healthy children in our studies, see Paper I, or children who developed islet autoimmunity, see Paper IV. The long follow-up time, high number of tested samples, high number of children involved, different geographical areas covered and the use of a PCR method that has been successful in detecting the virus in other studies indicate that LjV is rare in the stool of Norwegian infants. The typical stool quantities of HEV and HPeV in samples from the MIDIA cohort study were two to five orders of magnitude

higher than the detection limit for LjV (Paper II and III); any appreciable replication of LjV in the gut would presumably be detected. This does not mean that the virus does not infect humans at all – there were also several known enterovirus serotypes and HPeV types that were not detected in our studies. The possibility that the infants investigated were never exposed cannot be ruled out, as this may depend on unknown temporal and geographical factors. Other studies on LjV indicate that infection is an uncommon event and may be primarily during the perinatal period. Thus, although the data in Paper IV do not rule out the possibility that LjV can cause T1D, but do suggest that this virus is not a common risk factor in the aetiology of the disease in Norway.

Human parechovirus

Unlike most studies on HPeVs, the data in Paper I and II comes from mostly healthy children. Although in some cases mild symptoms, (e.g. fever, cold or diarrhoea) was reported for the same month that the infant shed HPeV RNA in the faeces, there was no significant correlations between symptoms and the presence of virus neither in healthy children or children that developed islet autoimmunity (Paper I and II). This is in contrast to other studies reporting HPeVs as human pathogens, but this discrepancy can be explained by our detection of the virus in the primary replication site, while other studies have detected it other tissues. As such, these observations do not exclude that HPeVs may cause symptoms, or serious complications [111-113] in individual cases, but the very high prevalence in healthy children should be kept in mind when evaluating causality based on findings from stool samples.

The HPeVs found in Paper I and II, consisting of samples taken from relatively few communities in Norway, displayed diversity close to what is observed worldwide. The different strains observed did not cluster in time or locality as strongly as the HEVs (as seen in Paper III). These observations suggest that HPeV is efficiently disseminated worldwide, a conclusion that is also supported by the high prevalence. HPeV1, HPeV3 and HPeV6 seem to be the more common viruses, while the other types are considered rare. Since the larger part of infections are HPeV1 it is difficult to conclude anything about the other genotypes.

HPeV infection during infancy does not seem to significantly predict islet autoimmunity, and there was no different frequency of infection after seroconversion for islet autoantibodies. Thus, Paper II supports and supplements the previously published data by Tauriainen *et al*. [114], which found no association between HPeV 1 and T1D. However, a suggestive higher frequency of infection was observed in case children in the interval 3 months before seroconversion for islet autoantibodies (P=0.083), which could warrant replication in larger studies.

Human enterovirus

There are few prospective studies investigating enterovirus and its association with the risk of islet autoimmunity, and they tend to have limited size or suboptimal case - control matching. Few previous studies have tested for enterovirus in faecal samples, and only some indices of infections were significantly associated with islet

autoimmunity.

Interestingly, positive findings of an association between enterovirus and the subsequent islet autoimmunity seem to be restricted to Finland, with three independent cohorts reporting an association between enterovirus and islet autoimmunity: DiMe [81, 82], DIPP [77-80], and the second pilot phase of TRIGR [83].

In the DiMe cohort non-diabetic siblings of children with T1D were investigated. Enterovirus antibody frequencies were higher (78%) in sample intervals where islet cell antibodies were detected compared to other sample intervals (33%) or samples from control subjects (29%) [82]. The association was corroborated using reverse transcriptase PCR from sera [81], resulting in 22% positivity in children with islet autoimmunity, compared to 2% in controls. In the TRIGR study [83] serology and enterovirus RNA detection from serum was used, demonstrating an increased prevalence in infections before the appearance of autoantibodies. However, it should be noted that the DiMe and TRIGR papers give little information on the matching employed.

In contrast, no differences were reported between cases and controls in enterovirus RNA in serum before autoimmunity in the largest sample of children from the DIPP study tested for enterovirus so far [79]. The children used in this cohort have increased genetic risk and are carefully matched, similarly to children in the MIDIA

study, but with a broader repertoire of HLA genotypes eligible for participation. However, by comparing the frequency of serum enterovirus RNA and/or increases in enterovirus antibodies for the whole dataset or within a six month window prior to islet autoimmunity a significant relation was reported [79, 81].

The most recent report on enterovirus from the DIPP cohort uses reverse transcriptase PCR on monthly stool samples from a carefully matched nested case-control study [80]. No difference in HEV occurrence in stool samples before autoantibodies was reported; but more frequent repeated positivity in consecutive stool samples from cases than in controls was found, and the authors suggest that the clearance of the virus may be retarded. In DAISY in Colorado, 26 cases with at least one autoantibody were compared to 39 controls using enterovirus PCR from serum, saliva and rectal swabs; the materials were taken at clinic visits at ages 9, 12, 15 and 24 months and then annually. There was no significant difference between cases and controls in the occurrence of enterovirus either before or after seroconversion in cases, but notably, the cases had a trend for multiple infections to be more frequent than in controls [115]. Both these findings – more frequent positivity in consecutive stool samples and more multiple infections in cases – is in contrast to our data in Paper III.

As our study (Paper III) has not tested for HEV in serum, our data neither support nor contrast the positive findings in serum, but any infection would presumably be reflected by shedding of viral RNA in the stool. The lack of any clear association in

stool samples is, however, supported by our data in Paper III. The shorter shedding duration compared to faecal infection would make testing only for serum RNA likely to miss some infections, particularly with the longer sampling intervals used in several other studies.

In addition, no preponderance of a strain, serotype or particular groups in either cases or controls was observed. Several serotypes that have previously been reported as possibly diabetogenic (e.g. Coxsackie B), were observed in both cases and controls, but the viruses seem to cluster according to time and geographical location without appreciable connections to islet autoimmunity.

A rather unlikely explanation of the discrepancies between our negative results in Paper III and earlier studies would be that the observed associations were population-specific for the Finns, either due to a special enterovirus strain circulating there, or to a unique genetic background of the population. On the other hand, it is not unlikely that some properties of certain virus strains or of the host must be present for a virus to break the immune defence, cause significant viraemia or invade the target organ – in short become diabetogenic.

Unanswered questions

Considering the negative findings in Paper IV, the question still remains if LjV infects humans, what could be the site of infection and which populations are at risk. However, these question are likely not answerable within the MIDIA study.

The suggestive increase in HPeV infections in the 3 month window before islet autoimmunity in Paper II should be explored further, as should any effects of HLA genotype on HPeV infections. Considering the data on HPeV in Paper I and II, it is natural to ask what factors could differentiate between asymptomatic infections and the serious conditions reported in other studies. This is also true for HEVs, which seem to have similar characteristics. Considering the earlier studies on enterovirus and Paper III, the explanation of the putative association between enterovirus and islet autoimmunity must lie outside the primary replication site.

It appears unlikely that any presumed diabetogenic strain of *Parechovirus* or *Enterovirus* can be distinguished from the natural circulation of the viruses, as no parameters dependent on the replication in the gut could be associated with islet autoimmunity. Testing serotypes may be of limited value for any diabetogenic effect of a virus, as any such properties may be encoded elsewhere than the capsid protein VP1 and shared across several types. Other factors may be necessary for a virus to have diabetogenic properties.

In addition, it is not unlikely that in complex diseases like T1D a single factor and its biological effects are just one of many contributing in the pathogenesis. The possibility exists that T1D is a result from several different pathways leading to autoimmunity, each with its own aetiology and thus lumping together all children that develop T1D will obscure the effects relevant environmental factors (as each would

just be present in a small subset of the subjects, where it might have a different effects depending on other necessary factors). However, studies on viral infections can at least provide a hint on factors and expose important mechanisms. Thus, further research should be focused on host-virus interactions, the character of a possible viraemia and the ability of viruses to invade the target pancreatic tissues.

7. Conclusions and future work

This work has focused on enteroviruses and parechoviruses as environmental factors in T1D. While no association was found, earlier results should not be discounted. There could be other factors that determine the diabetogenic properties of a virus, and viral infections cannot be seen as isolated events. Rather, they should be seen as parts of a whole in the development of the immune system, so further studies are needed. Testing of maternal antibodies is also needed to fill out the picture of childhood infections.

The hygiene hypothesis must be more thoroughly tested, as must the fertile field hypothesis; thus infections in early childhood should be investigated further. As more tests are done in the same material, and in similar materials, a meta-analysis of the data will show whether virus infections show any association with T1D.

Viral infections may appear to be just one factor among others causing islet autoimmunity and T1D. Just considering the viruses, whether the child got maternal antibodies, the time of first infection, in which order the different infections were encountered, prior infections *in utero* and host factors could influence the outcome of a viral infection. Thus more data are needed to elucidate the causes behind T1D.

8. References

[1] Achenbach P, Bonifacio E, Koczwara K, Ziegler AG (2005) Natural history of type 1 diabetes. Diabetes 54 Suppl 2: S25-31

[2] Atkinson MA, Eisenbarth GS (2001) Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 358: 221-229

[3] Achenbach P, Warncke K, Reiter J, et al. (2004) Stratification of type 1 diabetes risk on the basis of islet autoantibody characteristics. Diabetes 53: 384-392

[4] Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA (1997) Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. Diabetes 46: 1701-1710

[5] Baekkeskov S, Aanstoot HJ, Christgau S, et al. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347: 151-156

[6] Solimena M, Dirkx R, Jr., Hermel JM, et al. (1996) ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules. Embo J 15: 2102-2114

[7] Palmer JP, Asplin CM, Clemons P, et al. (1983) Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 222: 1337-1339

[8] Wenzlau JM, Juhl K, Yu L, et al. (2007) The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 104: 17040-17045

[9] Dotta F, Censini S, van Halteren AG, et al. (2007) Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A 104: 5115-5120

[10] Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G (2009) Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. Lancet 373: 2027-2033

[11] Gale EA (2002) The rise of childhood type 1 diabetes in the 20th century. Diabetes 51: 3353-3361

[12] Bonifacio E, Hummel M, Walter M, Schmid S, Ziegler AG (2004) IDDM1 and multiple family history of type 1 diabetes combine to identify neonates at high risk for type 1 diabetes. Diabetes Care 27: 2695-2700

[13] Karvonen M, Pitkaniemi J, Tuomilehto J (1999) The onset age of type 1 diabetes in Finnish children has become younger. The Finnish Childhood Diabetes Registry Group. Diabetes Care 22: 1066-1070

[14] Rasmussen T, Stene LC, Samuelsen SO, et al. (2009) Maternal BMI before pregnancy, maternal weight gain during pregnancy, and risk of persistent positivity for multiple diabetes-associated autoantibodies in children with the high-risk HLA genotype: the MIDIA study. Diabetes Care 32: 1904-1906

[15] Stene LC, Witsøo E, Torjesen PA, et al. (2007) Islet autoantibody development during follow-up of high-risk children from the general Norwegian population from three months of age: design and early results from the MIDIA study. J Autoimmun 29: 44-51

[16] Achenbach P, Koczwara K, Knopff A, Naserke H, Ziegler AG, Bonifacio E (2004) Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. J Clin Invest 114: 589-597

[17] Williams AJ, Aitken RJ, Chandler MA, Gillespie KM, Lampasona V, Bingley PJ (2008) Autoantibodies to islet antigen-2 are associated with HLA-DRB1*07 and DRB1*09 haplotypes as well as DRB1*04 at onset of type 1 diabetes: the possible role of HLA-DQA in autoimmunity to IA-2. Diabetologia 51: 1444-1448

[18] Bell GI, Horita S, Karam JH (1984) A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. Diabetes 33: 176-183

[19] Bottini N, Musumeci L, Alonso A, et al. (2004) A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat Genet 36: 337-338

[20] Vella A, Cooper JD, Lowe CE, et al. (2005) Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms. Am J Hum Genet 76: 773-779

[21] Nistico L, Buzzetti R, Pritchard LE, et al. (1996) The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry. Hum Mol Genet 5: 1075-1080

[22] Ueda H, Howson JM, Esposito L, et al. (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 423: 506-511

[23] Smyth DJ, Cooper JD, Bailey R, et al. (2006) A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. Nat Genet 38: 617-619

[24] Todd JA, Walker NM, Cooper JD, et al. (2007) Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. Nat Genet 39: 857-864

[25] Nejentsev S, Walker N, Riches D, Egholm M, Todd JA (2009) Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science 324: 387-389

[26] Barrett JC, Clayton DG, Concannon P, et al. (2009) Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat Genet

[27] Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J (2000) Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care 23: 1516-1526

[28] Bodansky HJ, Staines A, Stephenson C, Haigh D, Cartwright R (1992) Evidence for an environmental effect in the aetiology of insulin dependent diabetes in a transmigratory population. Bmj 304: 1020-1022

[29] Cataldo F (2005) Early onset of Type 1 diabetes mellitus in immigrant children from developing countries to Western Europe: the role of environmental factors? J Endocrinol Invest 28: 574-575

[30] Rewers M, LaPorte RE, Walczak M, Dmochowski K, Bogaczynska E (1987) Apparent epidemic of insulin-dependent diabetes mellitus in Midwestern Poland. Diabetes 36: 106-113

[31] Kyvik KO, Green A, Beck-Nielsen H (1995) Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins. Bmj 311: 913-917

[32] Hyttinen V, Kaprio J, Kinnunen L, Koskenvuo M, Tuomilehto J (2003) Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study. Diabetes 52: 1052-1055

[33] Davies JL, Kawaguchi Y, Bennett ST, et al. (1994) A genome-wide search for human type 1 diabetes susceptibility genes. Nature 371: 130-136

[34] Hermann R, Knip M, Veijola R, et al. (2003) Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes--indication of an increased environmental pressure? Diabetologia 46: 420-425

[35] Fourlanos S, Varney MD, Tait BD, et al. (2008) The rising incidence of type 1 diabetes is accounted for by cases with lower-risk human leukocyte antigen genotypes. Diabetes Care 31: 1546-1549

[36] Gillespie KM, Bain SC, Barnett AH, et al. (2004) The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet 364: 1699-1700

[37] Redondo MJ, Yu L, Hawa M, et al. (2001) Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. Diabetologia 44: 354-362

[38] Rosenbauer J, Herzig P, Giani G (2008) Early infant feeding and risk of type 1 diabetes mellitus-a nationwide population-based case-control study in pre-school children. Diabetes Metab Res Rev 24: 211-222

[39] Karjalainen J, Martin JM, Knip M, et al. (1992) A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med 327: 302-307

[40] Lamb MM, Myers MA, Barriga K, Zimmet PZ, Rewers M, Norris JM (2008) Maternal diet during pregnancy and islet autoimmunity in offspring. Pediatr Diabetes 9: 135-141

[41] Haynes A, Bower C, Bulsara MK, Finn J, Jones TW, Davis EA (2007) Perinatal risk factors for childhood Type 1 diabetes in Western Australia--a population-based study (1980-2002). Diabet Med 24: 564-570

[42] Waldhoer T, Rami B, Schober E (2008) Perinatal risk factors for early childhood onset type 1 diabetes in Austria - a population-based study (1989-2005). Pediatr Diabetes 9: 178-181

[43] Richer MJ, Horwitz MS (2009) Coxsackievirus Infection as an Environmental Factor in the Etiology of Type 1 Diabetes. Autoimmun Rev

[44] Goldberg E, Krause I (2009) Infection and type 1 diabetes mellitus - a two edged sword? Autoimmun Rev 8: 682-686

[45] Stefanova I, Dorfman JR, Germain RN (2002) Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. Nature 420: 429-434

[46] Bottini N, Vang T, Cucca F, Mustelin T (2006) Role of PTPN22 in type 1 diabetes and other autoimmune diseases. Semin Immunol 18: 207-213

[47] Chikuma S, Imboden JB, Bluestone JA (2003) Negative regulation of T cell receptor-lipid raft interaction by cytotoxic T lymphocyte-associated antigen 4. J Exp Med 197: 129-135

[48] Wang J, Wicker LS, Santamaria P (2009) IL-2 and its high-affinity receptor: Genetic control of immunoregulation and autoimmunity. Semin Immunol

[49] Kato H, Takeuchi O, Sato S, et al. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441: 101-105

[50] von Herrath MG, Fujinami RS, Whitton JL (2003) Microorganisms and autoimmunity: making the barren field fertile? Nat Rev Microbiol 1: 151-157

[51] Strachan DP (1989) Hay fever, hygiene, and household size. Bmj 299: 1259-1260

[52] Jones PD, Gibson PG, Henry RL (2000) The prevalence of asthma appears to be inversely related to the incidence of typhoid and tuberculosis: hypothesis to explain the variation in asthma prevalence around the world. Med Hypotheses 55: 40-42

[53] Yazdanbakhsh M, Kremsner PG, van Ree R (2002) Allergy, parasites, and the hygiene hypothesis. Science 296: 490-494

[54] Cooke A (2009) Infection and autoimmunity. Blood Cells Mol Dis 42: 105-107

[55] Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 347: 911-920

[56] Honeyman M (2005) How robust is the evidence for viruses in the induction of type 1 diabetes? Curr Opin Immunol 17: 616-623

[57] van der Werf N, Kroese FG, Rozing J, Hillebrands JL (2007) Viral infections as potential

triggers of type 1 diabetes. Diabetes Metab Res Rev 23: 169-183

[58] Gibbon C, Smith T, Egger P, Betts P, Phillips D (1997) Early infection and subsequent insulin dependent diabetes. Arch Dis Child 77: 384-385

[59] Parslow RC, McKinney PA, Law GR, Bodansky HJ (2001) Population mixing and childhood diabetes. Int J Epidemiol 30: 533-538; discussion 538-539

[60] Pundziute-Lycka A, Urbonaite B, Ostrauskas R, Zalinkevicius R, Dahlquist GG (2003) Incidence of type 1 diabetes in Lithuanians aged 0-39 years varies by the urban-rural setting, and the time change differs for men and women during 1991-2000. Diabetes Care 26: 671-676

[61] Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9: 313-323

[62] Pohl D (2009) Epstein-Barr virus and multiple sclerosis. J Neurol Sci

[63] Limb J, Binning A (2009) Thrombosis associated with varicella zoster in an adult. Int J Infect Dis

[64] Cinek O, Witsø E, Jeansson S, et al. (2006) Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. J Clin Virol 35: 33-40

[65] Tapia G, Cinek O, Witsø E, et al. (2008) Longitudinal observation of parechovirus in stool samples from Norwegian infants. J Med Virol 80: 1835-1842

[66] Blinkova O, Kapoor A, Victoria J, et al. (2009) Cardioviruses are genetically diverse and cause common enteric infections in South Asian children. J Virol 83: 4631-4641

[67] Muckelbauer JK, Kremer M, Minor I, et al. (1995) The structure of coxsackievirus B3 at 3.5 A resolution. Structure 3: 653-667

[68] Benschop KS, Schinkel J, Minnaar RP, et al. (2006) Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin Infect Dis 42: 204-210

[69] Harvala H, Simmonds P (2009) Human parechoviruses: biology, epidemiology and clinical significance. J Clin Virol 45: 1-9

[70] Niklasson B, Kinnunen L, Hornfeldt B, et al. (1999) A new picornavirus isolated from bank voles (Clethrionomys glareolus). Virology 255: 86-93

[71] Niklasson B, Heller KE, Schonecker B, et al. (2003) Development of type 1 diabetes in wild bank voles associated with islet autoantibodies and the novel ljungan virus. Int J Exp Diabesity Res 4: 35-44

[72] Niklasson B, Nyholm E, Feinstein RE, Samsioe A, Hornfeldt B (2006) Diabetes and myocarditis in voles and lemmings at cyclic peak densities--induced by Ljungan virus? Oecologia 150: 1-7

[73] Samsioe A, Sjoholm A, Niklasson B, Klitz W (2008) Fetal death persists through recurrent pregnancies in mice following Ljungan virus infection. Birth Defects Res B Dev Reprod Toxicol 83: 507-510

[74] Samsioe A, Papadogiannakis N, Hultman T, Sjoholm A, Klitz W, Niklasson B (2009) Ljungan virus present in intrauterine fetal death diagnosed by both immunohistochemistry and PCR. Birth Defects Res A Clin Mol Teratol 85: 227-229

[75] Niklasson B, Samsioe A, Papadogiannakis N, Gustafsson S, Klitz W (2009) Zoonotic Ljungan virus associated with central nervous system malformations in terminated pregnancy. Birth Defects Res A Clin Mol Teratol

[76] Niklasson B, Samsioe A, Blixt M, et al. (2006) Prenatal viral exposure followed by adult stress produces glucose intolerance in a mouse model. Diabetologia 49: 2192-2199

[77] Sadeharju K, Lonnrot M, Kimpimaki T, et al. (2001) Enterovirus antibody levels during the first two years of life in prediabetic autoantibody-positive children. Diabetologia 44: 818-823

[78] Lonnrot M, Korpela K, Knip M, et al. (2000) Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. Diabetes 49: 1314-1318

[79] Salminen K, Sadeharju K, Lonnrot M, et al. (2003) Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. J Med Virol 69: 91-98

[80] Salminen KK, Vuorinen T, Oikarinen S, et al. (2004) Isolation of enterovirus strains from children with preclinical Type 1 diabetes. Diabet Med 21: 156-164

[81] Lonnrot M, Salminen K, Knip M, et al. (2000) Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: a prospective study. Childhood Diabetes in Finland (DiMe) Study Group. J Med Virol 61: 214-220

[82] Hiltunen M, Hyoty H, Knip M, et al. (1997) Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. J Infect Dis 175: 554-560

[83] Sadeharju K, Hamalainen AM, Knip M, et al. (2003) Enterovirus infections as a risk factor for type I diabetes: virus analyses in a dietary intervention trial. Clin Exp Immunol 132: 271-277

[84] Elfving M, Svensson J, Oikarinen S, et al. (2008) Maternal enterovirus infection during pregnancy as a risk factor in offspring diagnosed with type 1 diabetes between 15 and 30 years of age. Exp Diabetes Res 2008: 271958

[85] Clements GB, Galbraith DN, Taylor KW (1995) Coxsackie B virus infection and onset of childhood diabetes. Lancet 346: 221-223

[86] Oikarinen M, Tauriainen S, Honkanen T, et al. (2008) Detection of enteroviruses in the intestine

of type 1 diabetic patients. Clin Exp Immunol 151: 71-75

[87] Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG (2009) The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. Diabetologia 52: 1143-1151

[88] Menser MA, Forrest JM, Bransby RD (1978) Rubella infection and diabetes mellitus. Lancet 1: 57-60

[89] Vuorinen T, Nikolakaros G, Simell O, Hyypia T, Vainionpaa R (1992) Mumps and Coxsackie B3 virus infection of human fetal pancreatic islet-like cell clusters. Pancreas 7: 460-464

[90] Hyoty H, Hiltunen M, Reunanen A, et al. (1993) Decline of mumps antibodies in type 1 (insulin-dependent) diabetic children and a plateau in the rising incidence of type 1 diabetes after introduction of the mumps-measles-rubella vaccine in Finland. Childhood Diabetes in Finland Study Group. Diabetologia 36: 1303-1308

[91] Dahlquist G, Blom L, Lonnberg G (1991) The Swedish Childhood Diabetes Study--a multivariate analysis of risk determinants for diabetes in different age groups. Diabetologia 34: 757-762

[92] Elfaitouri A, Berg AK, Frisk G, Yin H, Tuvemo T, Blomberg J (2007) Recent enterovirus infection in type 1 diabetes: evidence with a novel IgM method. J Med Virol 79: 1861-1867

[93] Frisk G, Friman G, Tuvemo T, Fohlman J, Diderholm H (1992) Coxsackie B virus IgM in children at onset of type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection. Diabetologia 35: 249-253

[94] Osame K, Takahashi Y, Takasawa H, et al. (2007) Rapid-onset type 1 diabetes associated with cytomegalovirus infection and islet autoantibody synthesis. Intern Med 46: 873-877

[95] Jun HS, Yoon JW (2003) A new look at viruses in type 1 diabetes. Diabetes Metab Res Rev 19: 8-31

[96] Richer MJ, Horwitz MS (2008) Viral infections in the pathogenesis of autoimmune diseases: focus on type 1 diabetes. Front Biosci 13: 4241-4257

[97] Schatz DA, Barrett DJ, Maclaren NK, Riley WJ (1988) Polyclonal nature of islet cell antibodies in insulin-dependent diabetes. Autoimmunity 1: 45-50

[98] Hiemstra HS, Schloot NC, van Veelen PA, et al. (2001) Cytomegalovirus in autoimmunity: T cell crossreactivity to viral antigen and autoantigen glutamic acid decarboxylase. Proc Natl Acad Sci U S A 98: 3988-3991

[99] Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N (1998) Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 4: 781-785

[100] Filippi C, von Herrath M (2005) How viral infections affect the autoimmune process leading to

type 1 diabetes. Cell Immunol 233: 125-132

[101] Witsø E, Palacios G, Cinek O, et al. (2006) High prevalence of human enterovirus a infections in natural circulation of human enteroviruses. J Clin Microbiol 44: 4095-4100

[102] Donoso Mantke O, Kallies R, Niklasson B, Nitsche A, Niedrig M (2007) A new quantitative real-time reverse transcriptase PCR assay and melting curve analysis for detection and genotyping of Ljungan virus strains. J Virol Methods 141: 71-77

[103] Joner G, Sovik O (1989) Increasing incidence of diabetes mellitus in Norwegian children 0-14 years of age 1973-1982. Diabetologia 32: 79-83

[104] Rønningen KS, Spurkland A, Iwe T, Vartdal F, Thorsby E (1991) Distribution of HLA-DRB1, -DQA1 and -DQB1 alleles and DQA1-DQB1 genotypes among Norwegian patients with insulin-dependent diabetes mellitus. Tissue Antigens 37: 105-111

[105] Undlien DE, Friede T, Rammensee HG, et al. (1997) HLA-encoded genetic predisposition in IDDM: DR4 subtypes may be associated with different degrees of protection. Diabetes 46: 143-149

[106] Joner G, Sovik O (1991) The incidence of type 1 (insulin-dependent) diabetes mellitus 15-29 years in Norway 1978-1982. Diabetologia 34: 271-274

[107] Molbak AG, Christau B, Marner B, Borch-Johnsen K, Nerup J (1994) Incidence of insulin-dependent diabetes mellitus in age groups over 30 years in Denmark. Diabet Med 11: 650-655

[108] Alexander JP, Jr., Gary HE, Jr., Pallansch MA (1997) Duration of poliovirus excretion and its implications for acute flaccid paralysis surveillance: a review of the literature. J Infect Dis 175 Suppl 1: S176-182

[109] Rosner B, Milton RC (1988) Significance testing for correlated binary outcome data. Biometrics 44: 505-512

[110] de Vries M, Pyrc K, Berkhout R, et al. (2008) Human parechovirus type 1, 3, 4, 5, and 6 detection in picornavirus cultures. J Clin Microbiol 46: 759-762

[111] Verboon-Maciolek MA, Krediet TG, Gerards LJ, de Vries LS, Groenendaal F, van Loon AM (2008) Severe neonatal parechovirus infection and similarity with enterovirus infection. Pediatr Infect Dis J 27: 241-245

[112] Abed Y, Boivin G (2006) Human parechovirus types 1, 2 and 3 infections in Canada. Emerg Infect Dis 12: 969-975

[113] Ito M, Yamashita T, Tsuzuki H, Takeda N, Sakae K (2004) Isolation and identification of a novel human parechovirus. J Gen Virol 85: 391-398

[114] Tauriainen S, Martiskainen M, Oikarinen S, et al. (2007) Human parechovirus 1 infections in young children--no association with type 1 diabetes. J Med Virol 79: 457-462

[115] Graves PM, Rotbart HA, Nix WA, et al. (2003) Prospective study of enteroviral infections and

development of beta-cell autoimmunity. Diabetes autoimmunity study in the young (DAISY). Diabetes Res Clin Pract 59: 51-61

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II

Running Title page

Running title: Human parechovirus and islet autoimmunity.

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Human parechovirus infections in infancy – no association with islet autoimmunity

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Abstract

Objective

A nested case-control study of Norwegian infants was set up to investigate a possible association between human parechovirus infections in early infancy, diagnosed in faecal samples, and the development of islet autoimmunity.

Subjects and methods

In the "Environmental triggers of type 1 diabetes - the MIDIA study" newborns with the highest genetic risk for type 1 diabetes were identified and followed with regular faecal sampling and questionnaires. A nested case-control study, including 27 children that developed islet autoimmunity (repeatedly positive for 2 or 3 autoantibodies) and 53 children matched for age and community of residence was used. Monthly stool samples from these children were analysed for human parechovirus using a semi-quantitative real-time PCR.

Results

There was no significant difference in the prevalence of human parechovirus in stool samples comparing cases and controls; 12.2% and 10.7%, respectively. There was neither any difference as to the number of infection episodes. Restricting analysis to samples collected 3, 6 or 12 months prior to seroconversion for islet autoantibodies, there was a suggestive association in the shortest time window (11.7 vs. 6.8%, odds ratio=3.06, P=0.083). No symptoms were associated with human parechovirus infection in any of the two groups. A subset of the positive samples (n=31) were sequenced, suggesting that human parechovirus 1 was the dominant genotype.

Conclusions

The present study does not support any association between human parechovirus infections and the development of type 1 diabetes.

KEY WORDS: Human parechovirus; Diabetes Mellitus, Type 1; Nested case-control study; Epidemiology; Polymerase Chain Reaction

Introduction

Environmental factor(s) presumably contribute to the development of islet autoimmunity, and ultimately type 1 diabetes. Viral infections are considered to be likely candidates for causing islet autoimmunity (reviewed in (1)). The *Picornaviridae* are among the most frequently studied viruses in relation to type 1 diabetes in humans and animal models. They are single stranded RNA viruses that replicate mainly in the gut and are transmitted by the faecal-oral route. Human parechoviruses belong to the relatively new genus *Parechovirus* within the family of *Picornaviridae*, and are closely related to the enteroviruses, which have been implicated in type 1 diabetes (2). The *Parechovirus* genus includes eight genotypes of human parechovirus as well as the rodent parechovirus Ljungan virus, which has been proposed as having an association with type 1 diabetes in bank voles (3). Human parechovirus 1 and 2 were described in the 1950s, while genotypes 3-8 have been described quite recently (4-9). There are also VP1 sequences of five undescribed human parechoviruses assigned as human parechovirus 9-14 (10). The most common human parechovirus is genotype 1, followed by human parechovirus 3 and 6, with the remaining genotypes being rare.

Human parechoviruses are common in infancy, and while the presence of virus in stool samples seems mostly asymptomatic, viral infections are also associated with various diseases (10). A previous report (11) did not find any association between human parechovirus 1 and type 1 diabetes by measuring specific antibodies against human parechovirus 1 in a nested case control-study in a cohort similar to the one used in the present study.

There are two reasons for investigating an association between parechovirus infections and the development of type 1 diabetes: First, the infections might be directly involved in triggering autoimmunity. Second, infection with common viruses can be used as a proxy for hygiene, since the presence of virus is expected to correlate with exposure to a large variety of microorganisms, which, according to the hygiene hypothesis, is the preferred situation in order to develop a well-functioning immune system. Thus, the aim of the present study was to establish if there is any association between the development of islet autoimmunity and human parechovirus infections in infancy, as well as the viral load and associated symptoms of infection as indicators of infection severity.

Methods

Subjects and study design

The children followed in this study participate in “Environmental Triggers of Type 1 diabetes: The MIDIA study”. They were identified at birth to carry the HLA genotype conferring the highest genetic risk of type 1 diabetes: DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02 (12). The parents were asked to submit monthly stool samples from their infants from the 3rd to the 35th month, and to record information on the type and dates of symptoms of infection (coughing and sneezing, diarrhoea, vomiting or fever), and some indicators of diet, in questionnaires. In order to monitor for the development of islet autoimmunity the children are tested for autoantibodies against glutamic acid decarboxylase$_{65}$ (GAD), protein tyrosine phosphatase IA2 (IA2) and insulin (IAA) with blood samples taken every 3 months until the age of 1 year, and every 12 months thereafter. The testing is described in detail by Stene *et al*. (12). If a blood sample is found positive for one autoantibody, the child is retested every six months; if two or three antibodies are positive, the child is retested every three months. The endpoint for this study was set as the development of autoimmunity defined as at least two autoantibodies positive in at least two consecutive samples. The study was approved by The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

By December 2008, 27 children (10 boys and 17 girls) were confirmed positive for 2 or 3 autoantibodies and were assigned as cases. In cases who at first tested positive for a single autoantibody, this first occurrence of autoantibody positivity was regarded as the onset of autoimmunity, and was used as the endpoint. Two children per case were randomly selected from the cohort using a computer program, matched for duration of follow-up, date of birth (+/- 1 month, tolerating up to 3 months in a few cases where necessary) and county of residence The family of one control child later withdrew and asked us to delete their data. In total, 80 children were included in the analysis (40 males and 40 females, born between 2001-2006). Of the scheduled samples and questionnaires, 637 of 704 blood samples (91%), 2173 of 2482 stool samples (88%) and 492 of 547 questionnaires (90%) were received. Of these, 1747 stool samples were tested for human parechovirus. The median age at onset of islet autoimmunity was 12.0 months (range 5.4-37.4) and the the median duration of follow-up with stool samples was 28 months (range 7-36 months).

Statistical analysis and power calcuations

The odds ratio for association between parechovirus infections and autoimmunity that could be detected with a two-sided test with alpha-level of 5% and 80-95% power under various scenarios was calculated. Assuming a total human parechovirus prevalence of 12% based on earlier data (13), 1800 samples, a true

odds ratio of 1.6, and comparing all samples, a power of 88% would be expected. Assuming a true odds ratio of 2.0, and comparing samples prior to development of autoimmunity, gives an expected power of 93%. The association of parechovirus frequency with islet autoimmunity was tested and estimated using logistic regression with random intercept to take into account repeated measures within individuals. This was done using the xtmelogit procedure in Stata 11.

Virus detection

Methods for the collection, processing and RNA extraction of the monthly stool samples are described elsewhere (14). The extraction protocol described therein was modified for the use of 96-well vacuum-processed QIAAmp plates (Qiagen) instead of single columns. To avoid false negative results, West Nile Virus Armored RNA (Asuragen, USA) was added to the sample in the first step of extraction and used as an exogenous internal control for RNA extraction, reverse transcription (RT) and real-time PCR. Briefly, the samples were reverse-transcribed with specific primers for human parechovirus, using the Improm II RT kit (Promega), and a real-time RT-PCR reaction was used to detect human parechovirus RNA from the extracted samples, described in detail in (13). The amplification was carried out on an ABI 7300 (Applied Biosystems) with the following thermal profile: 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C (where fluorescence data were collected). If the threshold cycle of the internal control was too high, or the amplification curve was not exponential, the sample was re-extracted and retested. Positive and negative controls were included in the extraction procedure. Dilutions of a mix of positive samples with known quantities were used to quantify the human parechovirus present in the reactions. All samples were subjected to blind testing. The positive controls were consistently detected down to 10^1 copies/μl, and this level was therefore set as a positivity threshold. The real time PCR method used is theoretically able to detect all the eight genotypes of human parechoviruses.

Genotyping of positive samples

A subset of the positive samples (n=31) were genotyped by sequencing and comparison of the VP1 polymerase region, using primers designed by Benschop et al. (15). The products were obtained by a RT-step using the Improm II RT system, followed by a PCR with the VP1 region as the target, using GoTaq chemistry (Promega). Detailed protocols are available from the authors. Products were cleaned using AMPure chemistry run on an Biomek 3000 robot (both Beckman Coulter, Beverly, MA), sequenced using BigDye Terminator v3.1 (Applied Biosystems) with the same primers as used in the PCR, purified using the CleanSEQ chemistry on the Biomek 3000 robot, and analysed on an ABI3130XL (Applied Biosystems)

capillary sequencer.

Results

A total of 1747 samples from 27 cases and 53 matched controls were analysed, of which 11.2% were positive. The frequency of human parechovirus infections did not differ significantly between cases (12.2%) and controls (10.7%) when all samples were included (OR=1.16, 95% CI 0.74 – 1.62). Restricting the analysis to samples taken prior to the development of islet autoimmunity (and the corresponding time point in the matched controls), the frequencies were 12.5% for cases and 11.0% for controls (OR=1.18, 95% CI 0.74 – 1.85), summarized in Table I. Interestingly, if using a time window 3 months before development of autoimmunity, 11/70 samples (15.7%) from cases had an infection as opposed to 11/161 (6.8%) samples from controls (OR=3.06, 95% CI 0.86 –10.8, P=0.083).

Counting only the first in a series of two or more consecutive positive samples from an infant (assuming they were part of the same infectious episode) gave no significant differences in the occurrence of human parechovirus episodes between cases and controls, and neither were there any significant difference in number of infections, or infections with symptoms (Table I). The median number of viral genomes was 3054 RNA copies per microliter in the positive samples obtained from cases prior to the development of islet autoimmunity, compared to 585 RNA copies per microliter in controls; the difference was not significant. Similar numbers were found when including all the positive samples from both groups.

A subset of the viral samples (n=31, 26 infectious episodes) were sequenced in order to identify the genotypes present. The following genotypes were found (number of infectious episodes in parenthesis): human parechovirus 1 (15), human parechovirus 2 (1), human parechovirus 3 (7) and human parechovirus 6 (3). The sequencing of samples taken in consecutive months supported the notion that these samples reflected a single infectious episode.

Discussion

Human parechovirus infection during infancy does not seem to significantly predict islet autoimmunity, and there was no different frequency of infection after seroconversion for islet autoantibodies. The present study supports and supplements the previously published data by Tauriainen *et a*l. (11), which found no association of human parechovirus 1 and type 1 diabetes. However, we did find a suggestive higher frequency of infection in case shildren in the interval 3 months before seroconversion for islet autoantibodies that was not statistically significant (P=0.083), but which could warrant replication in larger studies.

Although the data indicate that human parechoviruses are not a major cause of type 1 diabetes, it is still conceivable that these viruses may be involved in rare cases due to individual vulnerability or qualities of particular strains of virus. A possible correlation between one of the rarer parechovirus genotypes and type 1 diabetes cannot be ruled out, but seems unlikely. In addition, qualities that might make a human parechovirus diabetogenic do not necessarily correlate with genotype, viral load, symptoms or other parameters presently investigated.

Although in some cases mild symptoms, (e.g. fever, cold or diarrhoea) was reported for the same month that the infant shed human parechovirus RNA in the faeces, there was no significant correlations between symptoms and the presence of virus neither in cases nor in controls. Moreover, there was no correlation between infections associated with symptoms and the development of islet autoimmunity; and the viral load observed was similar in both cases and controls. These observations further point against any involvement of human parechoviruses. The stool samples were taken monthly, and the possibility of having an infection between two samples cannot be dismissed, however, in 50 of the 128 infectious episodes viral RNA was detected in two or more consecutive months, suggesting that infections are likely to be detected by monthly sampling. The lack of difference between cases and controls in length of infections further supports the conclusion that human parechoviruses are unlikely to be an important factor for the development of type 1 diabetes. In conclusion the role of human parechoviruses as environmental factors of type 1 diabetes seems to be very small.

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References

1. van der Werf N, Kroese FG, Rozing J, Hillebrands JL. Viral infections as potential triggers of type 1 diabetes. Diabetes Metab Res Rev. 2007; 23:169-83.
2. Hyoty H. Enterovirus infections and type 1 diabetes. Ann Med. 2002; 34:138-47.
3. Niklasson B, Heller KE, Schonecker B, Bildsoe M, Daniels T, Hampe CS, et al. Development of type 1 diabetes in wild bank voles associated with islet autoantibodies and the novel ljungan virus. Int J Exp Diabesity Res. 2003; 4:35-44.
4. Ito M, Yamashita T, Tsuzuki H, Takeda N, Sakae K. Isolation and identification of a novel human parechovirus. J Gen Virol. 2004; 85:391-8.
5. Al-Sunaidi M, Williams CH, Hughes PJ, Schnurr DP, Stanway G. Analysis of a new human parechovirus allows the definition of parechovirus types and the identification of RNA structural domains. J Virol. 2007; 81:1013-21.
6. Benschop KS, Schinkel J, Luken ME, van den Broek PJ, Beersma MF, Menelik N, et al. Fourth human parechovirus serotype. Emerg Infect Dis. 2006; 12:1572-5.
7. Watanabe K, Oie M, Higuchi M, Nishikawa M, Fujii M. Isolation and characterization of novel human parechovirus from clinical samples. Emerg Infect Dis. 2007; 13:889-95.
8. Li L, Victoria J, Kapoor A, Naeem A, Shaukat S, Sharif S, et al. Genomic characterization of novel human parechovirus type. Emerg Infect Dis. 2009; 15:288-91.
9. Drexler JF, Grywna K, Stocker A, Almeida PS, Medrado-Ribeiro TC, Eschbach-Bludau M, et al. Novel human parechovirus from Brazil. Emerg Infect Dis. 2009; 15:310-3.
10. Harvala H, Simmonds P. Human parechoviruses: biology, epidemiology and clinical significance. J Clin Virol. 2009; 45:1-9.
11. Tauriainen S, Martiskainen M, Oikarinen S, Lonnrot M, Viskari H, Ilonen J, et al. Human parechovirus 1 infections in young children--no association with type 1 diabetes. J Med Virol. 2007; 79:457-62.
12. Stene LC, Witso E, Torjesen PA, Rasmussen T, Magnus P, Cinek O, et al. Islet autoantibody development during follow-up of high-risk children from the general Norwegian population from three months of age: design and early results from the MIDIA study. J Autoimmun. 2007; 29:44-51.
13. Tapia G, Cinek O, Witso E, Kulich M, Rasmussen T, Grinde B, et al. Longitudinal observation of parechovirus in stool samples from Norwegian infants. J Med Virol. 2008; 80:1835-42.
14. Cinek O, Witso E, Jeansson S, Rasmussen T, Drevinek P, Wetlesen T, et al. Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. J Clin Virol. 2006; 35:33-40.
15. Benschop K, Thomas X, Serpenti C, Molenkamp R, Wolthers K. High prevalence of human Parechovirus (HPeV) genotypes in the Amsterdam region and identification of specific HPeV variants by direct genotyping of stool samples. J Clin Microbiol. 2008; 46:3965-70.

Tables

Table I

Comparison of human parechovirus infections in stool samples from cases and controls. The endpoint reflects the time when the cases developed autoimmunity. None of the comparisons made between cases and controls had significance, whether looking at samples taken prior to the endpoint, or all the samples.

	Positive for human parechovirus RNA	**Infection episodes (per child)**	**Infection episodes lasting >2 months (per child)**	**Positive samples with symptoms**	**Positive samples, no symptoms**
Cases before endpoint	38 (12.5%)	23 (0.85)	12 (0.44)	7/21 (33.3%)	14/21 (66.7%)
Controls before endpoint	67 (11.0%)	42 (0.79)	22 (0.42)	10/38 (26.3%)	28/38 (73.7%)
OR (95% CI)	*1.18 (0.74-1.85)*	*1.06 (0.62-1.79)*	*1.06 (0.512-2.18)*	*1.4 (0.44-4.46)*	
ses in total	68 (12.2%)	41 (1.52)	17 (0.63)	10/30	20/30
Controls in total	127 (10.7%)	87 (1.64)	33 (0.62)	17/75	58/75
OR (95% CI)	*1.16 (0.74-1.62)*	*1.01 (0.69-1.49)*	*1.12 (0.62-2.03)*	*1.7 (0.67-4.33)*	
All	**195 (11.2%)**	**1.6 (128 episodes)**	**0.63 (50 episodes)**	**27/105**	**78/105**

III

No association of human enterovirus RNA in monthly faecal samples and repeated positivity for multiple islet autoantibodies in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study

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Abstract

Aims/hypothesis

To test whether the frequency of human enterovirus RNA in faecal samples collected monthly from early infancy was associated with development of multiple islet autoantibodies in children with the highest risk HLA genotype.

Methods

Individuals from the general population carrying the HLA-DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02 genotype were identified at birth and followed with monthly stool samples from age 3 to 35 months. Blood samples from these children taken at age 3, 6, 9, 12 months, and then annually, were tested for autoantibodies to insulin, glutamic acid decarboxylase 65 and IA-2. Among 911 children, 27 developed positivity for >=2 islet autoantibodies in >=2 consecutive samples (cases). Two matched controls per case were selected. Stool samples were analyzed for human enterovirus with a semiquantitative real-time reverse transcriptase PCR. The statistical power to detect association was at least 93% assuming the true odds ratio was >=2.0.

Results

The frequency of human enterovirus RNA in stool samples from cases prior to seroconversion (43/339, 12.7%) did not differ from the frequency in matched controls (94/692, 13.6%); odds ratio=1.01 (95% CI: 0.59 - 1.72), P=0.97. Results remained essentially unchanged after adjustment for potential confounders, restriction to various time windows before seroconversion, or after including samples collected after seroconversion. There was also no difference in average quantity of enterovirus RNA, or the tendency of repeatedly positive samples.

Conclusions/interpretation

The data strongly suggest that faecal shedding of enteroviral RNA does not predict advanced islet autoimmunity, as human enterovirus infections are not more frequent before or after seroconversion for autoantibodies.

Introduction

The aetiology of type 1 diabetes is multifactorial. While the genetic component is relatively well described, the presumed environmental factors are still largely unknown. Human enteroviruses have been considered to be particularly plausible candidates, being proposed as important environmental triggers or accelerators of autoimmunity leading to type 1 diabetes [1, 2]. Human enteroviruses are found more frequently in recently diagnosed type 1 diabetes patients compared to controls: the evidence includes serology, reverse-transcriptase PCR, and now also evidence of the virus in the pancreata of subjects who died shortly after the disease onset [3]. Interpretation of these results is, however, difficult with respect to type 1 diabetes pathogenesis, as the process starts months to years before diabetes onset. The infections observed at - or shortly after - the disease onset may be just an epiphenomenon to yet unknown mechanisms independent of triggering or accelerating the autoimmunity.

To assess the role of enteroviruses in pathogenesis of type 1 diabetes, the occurrence of virus must be studied before and during the development of autoimmunity. In order to achieve this, longitudinal cohorts with high genetic risk of type 1 diabetes are - or have been - followed in several populations. However, very few studies on enterovirus and islet autoimmunity are available where cases (children who developed signs of islet autoimmunity) and control subjects were from the same cohort, and/or properly matched for various potential confounders (i.e. HLA-conferred risk, time of birth or sex). The strongest evidence comes from Finnish studies: four papers describe the results obtained from the “DIPP” cohort using serology or reverse transcriptase PCR on serum or stool [4-7], with enterovirus occurrence reported higher in subjects with signs of islet autoimmunity (positivity of islet antibodies) than in their controls matched for time of birth, HLA, and gender. Similar results were obtained also from two other Finnish cohorts: from the “DiMe” with a considerably strong effect of enterovirus but lack of matching [8, 9], and from the second pilot phase of the dietary trial “TRIGR” [10].

In contrast, a nested case-control study conducted within the rather infrequently sampled German “BABYDIAB” cohort found no association of antibodies against Coxsackieviruses with islet autoimmunity [11], and a case-control study nested in two cohorts of the “DAISY” study conducted in Denver, CO, did not find any significant difference in the frequency of enterovirus RNA in serum or rectal swab samples [12].

Unfortunately, there is considerable heterogeneity between all these studies as to the character of the underlying cohorts, the definitions of islet autoimmunity, the sampling frequency, detection methods and

several other aspects. Particularly the sampling frequency of biological specimens for enterovirus testing is likely to influence the results. A comprehensive review aimed to determine optimal timing of stool specimen collection in poliovirus infections found 3-4 weeks as the usual duration of poliovirus excretion in unvaccinated infants [13] - a situation that may be analogical to natural infection with an enterovirus not encountered previously. In our recent study, excretion of one strain lasted usually less than three months [14]. Thus, studies using sampling intervals of three or more months are likely to underestimate the true prevalence, and may experience a bias. Detection of viral RNA in blood is likely to further underestimate the prevalence of infection as the viremia seems likely to be of shorter duration than viral replication in the gut.

In view of the conflicting results of previous studies, we have studied the occurrence of human enteroviruses in monthly stool samples from 27 children developing islet autoimmunity within the Norwegian MIDIA cohort, and 53 controls with the identical highest-risk HLA genotype, matched for the time of follow-up, date of birth, and county of residence. The aim was to test whether the frequency of human enterovirus RNA in serial faecal samples collected monthly from infancy was associated with repeated positivity for multiple islet autoantibodies. The regular monthly intervals of stool samples, together with reliable reverse-transcriptase PCR detection and careful matching within a genetically homogeneous cohort render us relatively high power to detect any potential effect of the virus infection.

Methods

Subjects and study design

The children prospectively observed in this study participate in the Norwegian cohort entitled "Environmental Triggers of Type 1 Diabetes: The MIDIA study". The cohort was identified at birth from the general population based on genetic testing for the HLA genotype conferring the highest genetic risk of type 1 diabetes, *DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02*. Between 2001 and 2006, 911 children were included into the cohort. All subjects were followed up with stool samples, blood samples for autoantibody screening and structured questionnaires. The study was approved by The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

To monitor potential development of islet autoimmunity, the subjects were tested for autoantibodies against glutamic acid decarboxylase$_{65}$ (GAD65), protein tyrosine phosphatase IA2 (IA2) and insulin (IAA), using radiobinding assays from blood samples taken at ages 3, 6, 9, and 12 months and every 12 months thereafter, as described in detail in [15], including the quality control results from the international Diabetes Antibody Standardization Program (DASP). Mailed questionnaires were administered at the same times. If a plasma sample was found positive for one autoantibody, the child was retested every six months; if two or three antibodies were positive, the child was retested every three months. The endpoint for this study, islet autoimmunity, was set to development of repeated positivity for two or more autoantibodies in at least two consecutive plasma samples. Type 1 diabetes was diagnosed according to the WHO criteria.

By December 2008, 27 of the 911 children in the cohort had reached the endpoint, and were assigned as cases. The median age at onset of islet autoimmunity was 12.0 months (range 5.4-37.4). Of the 27 case children, ten subjects were diagnosed with diabetes by September 1, 2009, at a median age of 23.1 months (range 8.7-54.2). Two control subjects were randomly assigned per case, matching for follow-up (at least as long as the time when the corresponding case developed multiple islet autoantibodies, tolerating up to one month difference if necessary), date of birth within +/- 1 month (tolerating up to +/- 3 months if necessary) and county of residence (tolerating closest neighbouring county if necessary – the smallest counties in Norway have 800-1000 inhabitants). Data from one control child (matching group 27) are lacking as the parents later withdrew from the study and refused any use of the collected data.

To test for enterovirus infections, we utilized stool samples obtained by the parents; they collected stool

samples from their children every month from 3 to 35 months of age, which were sent by mail to our central laboratory. They also kept records of symptoms of infection in structured questionnaires. Of 704 planned blood samples, 637 were taken (91%); 2173 of 2482 scheduled stool samples (88%) and 492 of 547 questionnaires were received (90%). The median duration of follow-up with stool samples was 28 months (range 7-36 months). The characteristics of the study participants are described in **Table 1**.

Processing and molecular testing of stool samples

The processing and testing of stool samples in this study has been described earlier [16]. Briefly, the samples were received by postal service, diluted and centrifuged. The supernatants were frozen at -80°C until co-purification of RNA and DNA. The extraction protocol utilized the vacuum-processed 96-well QIAamp plates processed under the QIAamp Viral RNA Mini protocol (Qiagen, Hilden, Germany). West Nile Virus (WNV) Armored RNA (Asuragen, USA) was added in a constant quantity to the lysis buffer which was used in the first step of the protocol. This exogenous internal control monitored the success of RNA extraction and detection. Testing for human enterovirus RNA was performed in duplicates in 20 µl volume one-step real-time reverse transcriptase PCR with primer-probe combination specific for the conserved 5'-untranslated region of human enteroviruses. This combination does not react with the *rhinovirus* species. Serial dilutions of Enterovirus Armored RNA (Asuragen, USA) were used to construct a seven-point standard curve from 24 to 10^5 copies/µl. The threshold of positivity used in this study was set to 100 copies/µl RNA, a quantity that could be consistently and reliably detected.

Genotyping of enterovirus-positive samples

To distinguish between continuous infection, and two distinct strains infecting the child in two consecutive months, enterovirus genotypes were determined in selected samples by partial sequencing of the VP1 region. First, a separate reverse transcription step was performed with a specific primer, using the Improm II chemistry (Promega, USA), followed by a nested PCR with primers published by Casas *et al* [17]. Direct product sequencing was performed using the inner amplification primer pair. Detailed protocols are available from the authors. The partial VP1 sequences were compared to published sequences in the GenBank database to determine whether the strain was genetically related to any known enterovirus type. The sequences were aligned and phylogenetic analysis performed, using the Molecular Evolutionary Genetics Analysis software, version 4.0 [18]. In the multiple alignment analysis the Kimura two-parameter model was used as a model of nucleotide substitution and the neighbour-joining method was used to reconstruct the phylogenetic tree. The statistical significance of the phylogenies constructed was estimated by bootstrap

analysis with 1,000 pseudoreplicate data sets.

Statistical analysis

The power of this study was calculated based on relevant estimates from our pilot study: the use of all monthly samples tested for enterovirus until the development of autoimmunity suggests an estimated power of at least 93% to detect a significant association if the true odds ratio is 2.0 or greater, using a two-sided test with the alpha-level of 5% (for detailed power calculations, please see **ESM Table I**). The number of samples positive for enterovirus was used for every subject in the matching group until the date of the endpoint (development of two or three autoantibodies on two following samplings in the case child; corresponding age in matched controls). In cases who at first tested positive for a single autoantibody, this first occurrence of autoantibody positivity was regarded as the onset of autoimmunity, and was used as the endpoint. The calculations were also done for other time windows prior to development of autoimmunity. In separate analyses only the first enterovirus RNA positive samples among series of two or more consecutively positive samples was counted, assuming they were part of the same infectious episode.

Statistical testing of difference in enterovirus RNA positivity in cases and controls were done using a logistic regression model with a random intercept to account for intra-individual correlation between enterovirus infections (using the xtmelogit command in Stata 11). From this model the (subject-specific) odds ratio with 95% confidence interval (CI) for association were estimated, with corresponding P-values. Potential confounders adjusted for are reported in the results section.

Results

The frequency of human enterovirus before development of autoimmunity

The frequency of human enterovirus RNA in stool samples prior to the development of islet autoimmunity did not differ between cases (12.7%) and controls (13.6%). Results were similar even after adjusting for age, sex, month of sample, year of sample, number of siblings, breastfeeding, first degree relatives with type 1 diabetes and number of siblings (**Table 2**). Similarly, no association was seen when restricting the time window before seroconversion in cases to six or twelve months: with a six months window, the frequency was 20 / 142 (14.1%) in cases versus 42 / 308 (13.6%) in controls, (OR = 1.05, 95% CI 0.54 - 2.04), and with a twelve months window the frequency was 31 / 214 (14.5%) in cases versus 62 / 454 (13.7%) in controls (OR = 1.09, 95% CI 0.62 - 1.92). Using infectious episodes rather than number of positive stool samples, i.e., consecutive positive samples were deemed as a single episode, did not appreciably alter the above figures.

The quantity of human enterovirus RNA

The effect of viral load was assessed by dividing the positivity into two categories: low to moderate (quantity of 100 - 9 999 RNA copies / µl) and high (10 000 or more RNA copies / µl). No association with islet autoimmunity was found in this type of analysis (**Table 3**). In the 43 enterovirus-positive samples from the pre-autoimmunity period among cases, the median estimated human enterovirus quantity was $18x10^3$ RNA copies / µl compared to a median of $12x10^3$ among 94 enterovirus RNA positive samples from matched controls from the corresponding periods (Mann-Whitney non-parametric test: P=0.37). Similar results were seen in the samples collected after the onset of autoimmunity. Among the 30 new enterovirus episodes during the pre-autoimmune period of cases, 13 (43.3%) were followed by at least one additional consecutive enterovirus positive sample, compared to 29 of 65 (44.6%) among the controls (chi-square test: P=0.73).

The occurrence of human enterovirus during the whole observation period

All infections and their distribution over the observational period in case and control subjects of the 27 matching groups are shown in **Figure 1**. In total, we tested 2 044 stool samples from the cases (627) and controls (1417) in the study. Human enterovirus was detected in 80 / 627 (12.8%) samples from cases, and 210 / 1417 (14.8%) samples from controls; the overall occurrence did not differ between cases and controls (OR = 0.84, 95% CI: 0.58 - 1.22, P=0.36). Only 11 subjects did not shed enterovirus in their stool during their entire observation period (4 cases and 7 controls). The remaining children had varying number of monthly samples positive, from only one (n=7) up to 8-9 (n=7).

Seasonal variation of infections

There was a pronounced seasonality of infections with a peak in autumn (October with 27% positive samples) and a smaller peak in July (with 24% positive samples), and a through in March with 3% positive samples; consequently, there was a highly significant association of positivity for enterovirus with the month the sample was taken ($p<0.001$). **Figure 1** exemplifies several episodes of increased density of infections that can be observed across the case-control matching group. The occurrence of infections was also age-dependent: a rise was noted from the fifth to ninth month of age, and during the first half of the second year of life.

Molecular typing of enterovirus strains using partial VP1 sequencing

Selected positive samples (97) had their VP1 genotypes determined in order to distinguish prolonged infections with one strain against multiple consecutive infections. The distribution of the 17 different serotypes found is shown in **Table 4**. As the sequenced samples were not representative for the whole case-control dataset, direct comparison of serotype repertoire between cases and controls was not possible. A phylogenetic tree constructed from the dataset is shown in **Figure 2**.

Discussion

The present study tested over 2000 monthly faecal samples that were collected from a tightly matched case-control set of children with a single HLA-DQ, -DR genotype conferring the highest risk of type 1 diabetes. Despite a high statistical power to detect associations of moderate magnitude, we observed no association between presence or quantity of human enterovirus in stool and the risk of islet autoimmunity.

Comparison with other related studies

Prospective studies investigating enterovirus and its association with the risk of islet autoimmunity are generally scarce. Moreover, they tend to suffer from limited size or suboptimal case - control matching. The most important studies are from Finland, Germany and the USA; interestingly, positive findings of an association between enterovirus and the subsequent islet autoimmunity seem to be restricted to Finland where three independent cohorts report an association between enterovirus and islet autoimmunity: DiMe [8, 9], DIPP [4-7], and the second pilot phase of TRIGR [10]. Of note, few previous studies have tested for enterovirus in faecal samples, and only some indices of infections were significantly associated with islet autoimmunity even in the Finnish studies.

The prospective Finnish study DiMe investigated a cohort of non-diabetic siblings of type 1 diabetic cases. First, enterovirus antibody frequencies were increased (78%) in sample intervals where islet cell antibodies were detected as compared to other samples intervals (33%) or samples from control subjects (29%) [9]. The association was corroborated using reverse transcriptase PCR from sera [8]: here, one of the most profound differences was reported with 22% positivity in 93 serum samples from 11 prediabetic children compared to 2% in 108 samples from 34 controls. Unfortunately, the paper does not report how the cases were matched to controls, or how the authors handled the known effects of seasonality, local epidemics, and age-dependent prevalence changes. In the second pilot TRIGR study [10], serology and enterovirus RNA detection from serum was tested and an increased prevalence of infections were demonstrated before the appearance of autoantibodies. Although matching was employed, neither age, time nor place of birth were among the matching variables, and no information is given on handling their influence in the analysis.

The DIPP study observes a cohort of children from the general population with increased genetic risk identified at birth. In contrast to our MIDIA cohort with the highest-risk children, the repertoire of HLA genotypes eligible for participation in the DIPP is broader. The largest sample of children from the DIPP study tested for enterovirus so far was reported by Salminen *et al* [6]. The serology consistently showed an

increased proportion of enterovirus in those who progress to islet autoimmunity as compared to carefully matched controls both for the whole dataset and for observations within a six-month window before the diagnosis of islet autoimmunity. Reverse transcriptase PCR from serum detected a rather low absolute overall frequency of enterovirus (4% in cases versus 3% in controls), but there was a highly significant difference in a six-months' window prior to islet autoimmunity (OR = 8.4, 95%CI 1.7 - 40.2) [5].

The so far last paper on enterovirus from the DIPP cohort utilizes reverse transcriptase PCR from monthly taken stool samples in 12 cases of islet autoimmunity versus 53 controls (878 stool samples) in a carefully matched nested case-control study [7]. There was no difference in enterovirus occurrence in stool samples before autoantibodies appeared, but the authors observed more frequent repeated positivity in consecutive stool samples in cases compared to controls, and suggest that the clearance of the virus may be retarded. This latter finding is in contrast to ours.

Two prospective studies outside Finland did not find an association of enterovirus and islet autoimmunity. In DAISY in Colorado, 26 cases with at least one autoantibody were compared to 39 controls using enterovirus PCR from serum, saliva and rectal swabs; the materials were taken at visits to the clinic at ages 9, 15 and 24 months and then annually. There was no significant difference between cases and controls in the occurrence of enterovirus either before or after seroconversion in cases, but there was a trend for multiple infections to be more frequent in the cases [12]. In the German BABYDIAB study antibodies against Coxsackie viruses were tested in blood samples collected at age of 9 months, and at 2, 5 and 8 years in 28 cases with persistent islet antibodies compared to 51 matched controls. No association was found, and no consistent sequence in time between infection and autoimmunity could be observed [11]. However, the rather infrequent sampling and considerable genetic heterogeneity of the cohort might be an obstacle.

Possible explanations of the observed lack of association

Our study is comparable or larger than previously published work in the size of the tested case-control dataset, utilizes a frequent sampling schedule, and the definition of a case is similar or stricter than in the above cited studies. Lack of association between islet autoimmunity and enterovirus in stool samples has been observed in three studies so far (the present one, Graves *et al* [12] and Salminen *et al* [7]). Therefore, possible explanations must be sought for this apparent phenomenon and for its discrepancy to the results obtained from blood (be it either serology, or reverse transcriptase PCR).

An insufficient power of our study to detect a moderate effect can be ruled out: the monthly sampling and the high completeness of the sample set renders the study a power of 93% to detect an effect corresponding to OR>=2.0 using all samples collected prior to islet autoimmunity. This putative effect is much weaker than e.g. the OR = 8.4 observed in the six-months window prior to islet autoimmunity in the study by Lonnrot *et al* [5], and the positive findings from other Finnish studies suggest that enterovirus infection might be a relatively strong contributor to diabetes pathogenesis.

Another rather unlikely explanation of our negative result would be to postulate that the observed associations were population-specific for the Finns, either due to a special enterovirus strain circulating there, or to a unique genetic background of the population. Rather, it is the ability to conduct adequately powered and well organized prospective studies that made the Finnish research on enteroviruses very unique - at least until the advent of the TEDDY study [19].

Given that neither the enterovirus rate of infection nor its timing or frequency before the development of islet autoimmunity differ to controls, the explanation of the putative association between enterovirus and islet autoimmunity must lie outside the primary replication site, the gut. Some properties of certain virus strains or the host are needed in order to break the lines of immune defence, cause significant viraemia, and invade the target organ - pancreas and its islets. Our study design, however, cannot find such properties unless linked to the likelihood of infection of the gut or to the primary replication of the virus, such as differences in the viral load or duration of the infection. No such differences were observed in our or two other studies utilizing stool as the source material [7, 12]; therefore we may expect that such differences are either subtle, or non-existent. If the character of virus replication in the gut (reflected by duration of enterovirus shedding and the viral load) is not responsible, what causes the observed higher occurrence of enterovirus antibodies in children with islet autoimmunity than in controls? Probably it is the level and duration of viraemia; which are most likely independent of parameters observed in our study. Not all enterovirus infections in the gut may result in viraemia, and viraemia may be more strongly associated with risk for autoimmunity than faecal shedding. Unfortunately, we did not have serum tested for enterovirus RNA in our study.

Strengths of the study

This study is the first - to our best knowledge - to use a quantitative molecular assay for testing the viral load. Our ability to distinguish between low- and high-quantity infections, and follow the dynamics of the viral load

gives the study a new dimension. It should, however, be noted that the obtained data must still be treated semiquantitative rather than quantitative due to the nature of the source of RNA: stool content is impossible to standardise, both from the quantitative and qualitative point of view. The chief obstacle was a variable degree of inhibition seen in a proportion of the samples: this was controllable using the signal from the exogenous internal control, yet the quantitative character of the assay was hampered to a certain degree.

We follow the most genetically restricted cohort of all reported studies. The children were identified at birth to carry the highest risk genotype for type 1 diabetes, defined not only by the HLA-DQB1 and DQA1 loci, but also the presence of the HLA-DRB1*0401 subtype. This subtype further increases the risk of diabetes in the Norwegian population, and this increase is rather dramatic compared to the DRB1*0403 subtype that can be present in individuals defined only at the HLA-DQ locus. The currently selected genotype allowed us to create a relatively homogeneous cohort with a very high risk of type 1 diabetes within a reasonable time scale, so that matching for selected confounders as place and time of birth was feasible. The current selection strategy is likely to eliminate putative influence of the class II HLA genotypes on the risk of enterovirus infection, and together with careful matching it provides an unbiased insight into differences in the true frequency of infections.

Selected positive samples were genotyped in order to distinguish prolonged infections with one strain against multiple consecutive infections. Although the group of serotypes therefore was not representative for the complete repertoire of positive samples in this study, we observed no preponderance of a strain, serotype or their group in either cases or controls. **Table 4** shows the diverse repertoire of serotypes is in both groups: several serotypes that have previously been reported as possibly diabetogenic (e.g. Coxsackie B), were observed in both cases and controls, and the viruses seem to cluster according to time and geographical location without appreciable connections to islet autoimmunity. Interestingly, the ECHO25 serotype was found only in case children, but there was no clear temporal relationship between detection of the strain and seroconversion. Although some types seem to be quite prevalent (the ECHO3, CBV4, CAV9 strains all had ten or more monthly samples), these come mostly from repeatedly positive stool samples within one infection episode or taken in a small geographical area during a short period, reflecting local epidemics. Testing serotypes may, however, be irrelevant for the diabetogenicity of the virus, as the capsid protein VP1 is most likely not responsible for the presumed abilities of a diabetogenic enterovirus to infiltrate the islets. These properties may be encoded elsewhere and may be shared across serotypes. Further studies that use sequencing of other regions - or the whole genome - of enterovirus are therefore needed.

Conclusions

Our study shows in a large and frequently sampled dataset from a genetically homogeneous high-risk cohort that there is no appreciable difference in the occurrence of enterovirus infection in the gut, or in the viral load, between children who subsequently develop islet autoimmunity, and tightly matched controls. Further research should therefore be focused on the character of viraemia, and the ability of enterovirus to invade the target pancreatic tissues.

Acknowledgement

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References

[1] Hyoty H, Taylor KW (2002) The role of viruses in human diabetes. Diabetologia 45: 1353-1361.

[2] Roivainen M (2006) Enteroviruses: new findings on the role of enteroviruses in type 1 diabetes. Int J Biochem Cell Biol 38: 721-725

[3] Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG (2009) The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. Diabetologia 52: 1143-1151

[4] Sadeharju K, Lonnrot M, Kimpimaki T, et al. (2001) Enterovirus antibody levels during the first two years of life in prediabetic autoantibody-positive children. Diabetologia 44: 818-823.

[5] Lonnrot M, Korpela K, Knip M, et al. (2000) Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. Diabetes 49: 1314-1318.

[6] Salminen K, Sadeharju K, Lonnrot M, et al. (2003) Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. J Med Virol 69: 91-98.

[7] Salminen KK, Vuorinen T, Oikarinen S, et al. (2004) Isolation of enterovirus strains from children with preclinical Type 1 diabetes. Diabet Med 21: 156-164

[8] Lonnrot M, Salminen K, Knip M, et al. (2000) Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: a prospective study. Childhood Diabetes in Finland (DiMe) Study Group. J Med Virol 61: 214-220.

[9] Hiltunen M, Hyoty H, Knip M, et al. (1997) Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. J Infect Dis 175: 554-560.

[10] Sadeharju K, Hamalainen AM, Knip M, et al. (2003) Enterovirus infections as a risk factor for type I diabetes: virus analyses in a dietary intervention trial. Clin Exp Immunol 132: 271-277.

[11] Fuchtenbusch M, Irnstetter A, Jager G, Ziegler AG (2001) No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes. J Autoimmun 17: 333-340

[12] Graves PM, Rotbart HA, Nix WA, et al. (2003) Prospective study of enteroviral infections and development of beta-cell autoimmunity. Diabetes autoimmunity study in the young (DAISY). Diabetes Res Clin Pract 59: 51-61.

[13] Alexander JP, Jr., Gary HE, Jr., Pallansch MA (1997) Duration of poliovirus excretion and its implications for acute flaccid paralysis surveillance: a review of the literature. J Infect Dis 175 Suppl 1: S176-182

[14] Witso E, Palacios G, Cinek O, et al. (2006) High prevalence of human enterovirus a infections in natural circulation of human enteroviruses. J Clin Microbiol 44: 4095-4100

[15] Stene LC, Witso E, Torjesen PA, et al. (2007) Islet autoantibody development during follow-up of high-risk children from the general Norwegian population from three months of age: Design and early results from the MIDIA study. Journal of Autoimmunity 29: 44-51

[16] Cinek O, Witso E, Jeansson S, et al. (2006) Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. Journal of Clinical Virology 35: 33-40

[17] Casas I, Palacios GF, Trallero G, Cisterna D, Freire MC, Tenorio A (2001) Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. J Med Virol 65: 138-148

[18] Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599

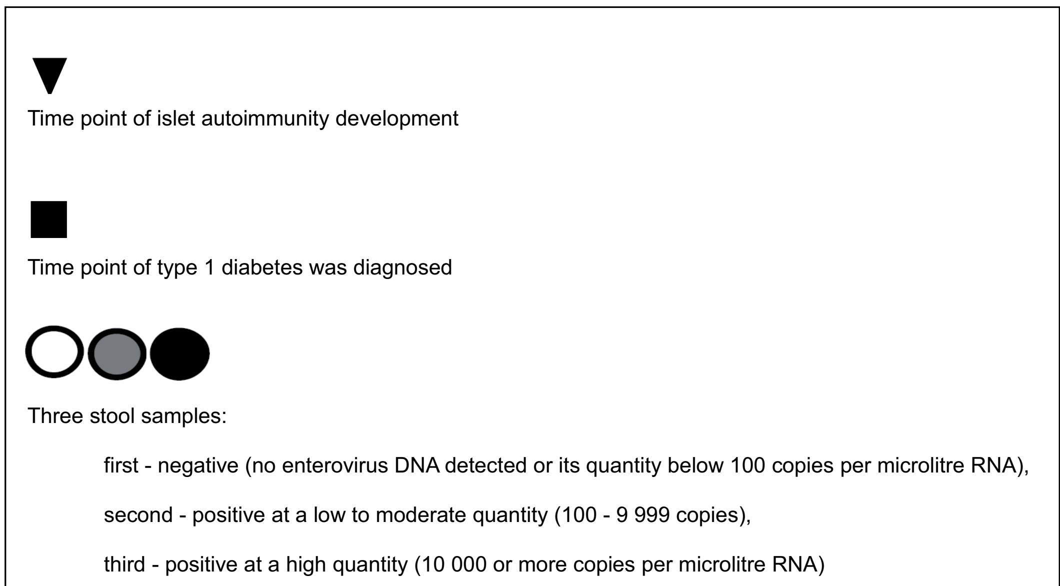
[19] (2008) The Environmental Determinants of Diabetes in the Young (TEDDY) Study. Ann N Y Acad Sci 1150: 1-13

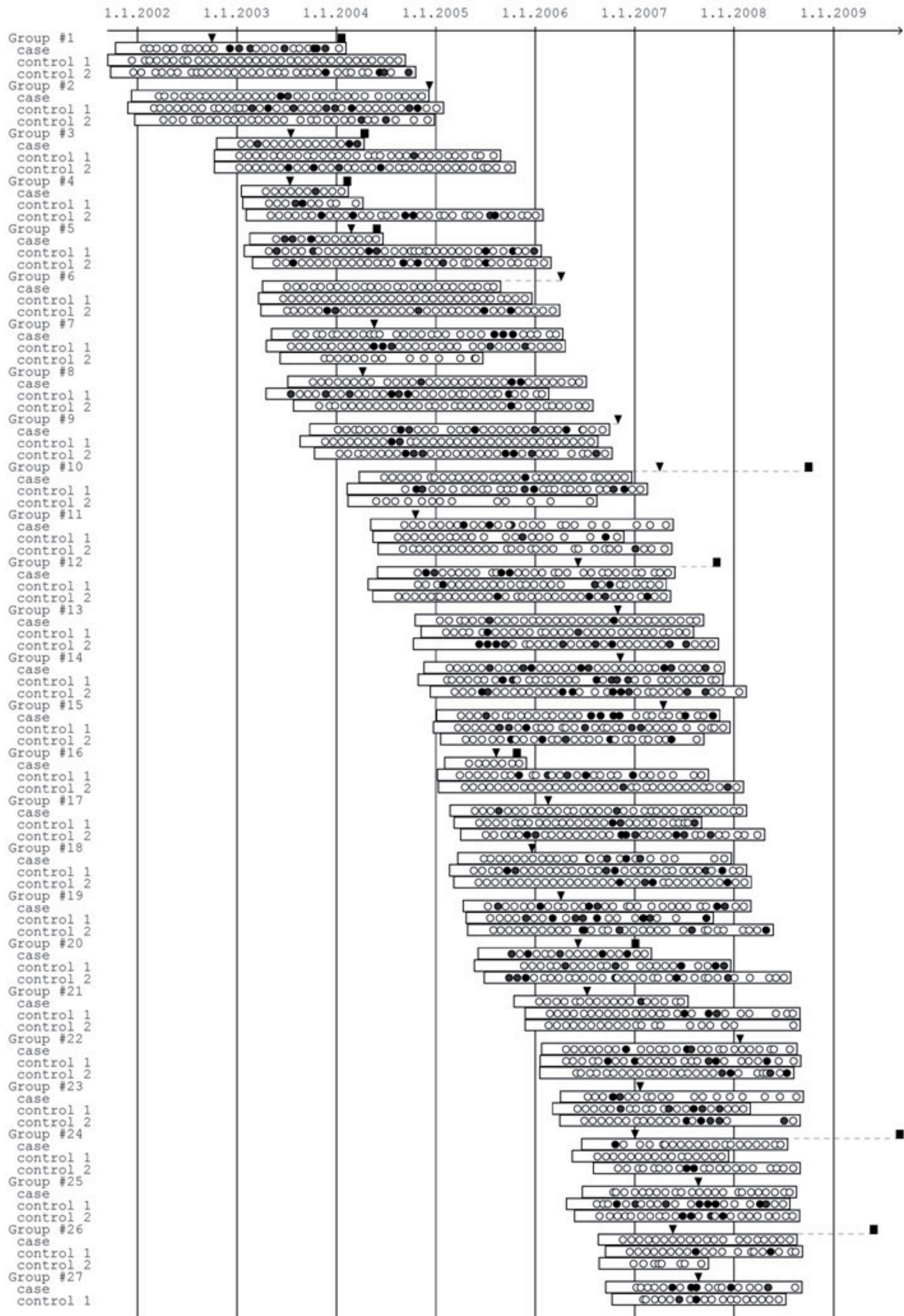
[20] Bjornvold M, Undlien DE, Joner G, et al. (2008) Joint effects of HLA, INS, PTPN22 and CTLA4 genes on the risk of type 1 diabetes. Diabetologia 51: 589-596

Figures

Figure 1. The subjects in the nested case-control study and their stool samples.

Twenty seven nested case-control trios are shown. The trios are ordered by the date of birth of the index case. The follow-up by stool samples for each child is indicated by the box framing the series of the circles depicting the stool samples obtained from each subject. The left edge of the box corresponds to the birth of the subject, the right edge to the last stool sample available (either from the 35th month of life when collecting of stool samples is terminated, or 17.8.2008, the date of the last stool sample was processed for this study). The empty circles correspond to negative stool samples, grey to low -to-moderate enterovirus RNA quantity, and black to high-quantity positivity. Triangles show the dates of diagnosis of islet autoimmunity, i.e. the first of samples with one or more autoantibodies (of GAD, IA2, IAA) that was later confirmed by repeated positivity for two or more autoantibodies. Full squares show the date of diabetes onset. Diabetes was observed in 10 children by 1 September 2009. Note that control 2 of the matching group 27 withdrew in the course of study, and the data were therefore deleted.

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1.1.2006
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1.1.2008
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Group #1
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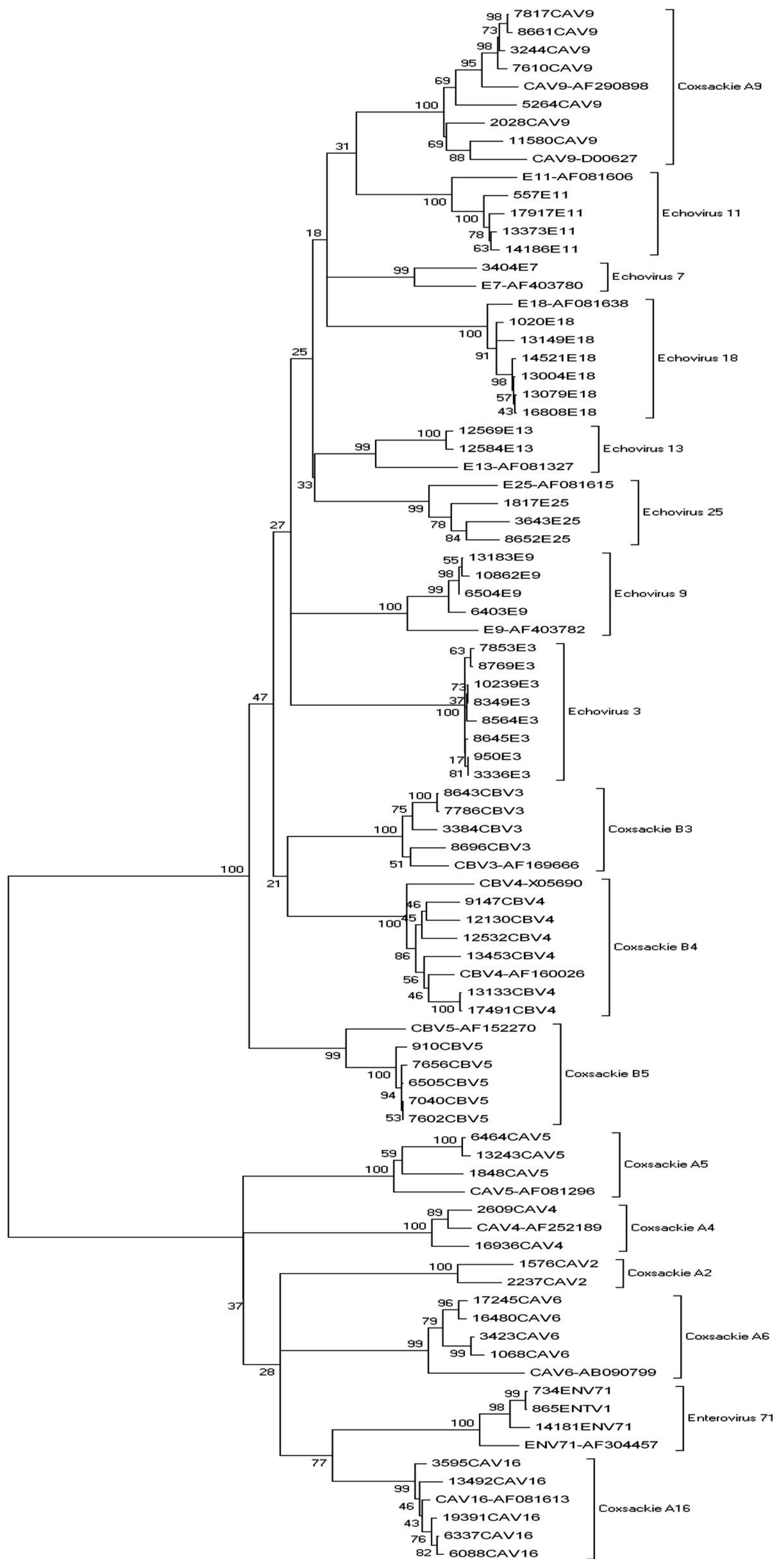
Figure 2. Phylogenetic relationships in the VP1 regions.

The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the viruses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Sequences from this study are marked with numeric sample code followed by the assigned type. Consecutive samples of the same genotype (from the same infection episode) are represented only once. Sequences from *Genbank* are marked with their type followed by the accession number. CAV: Coxsackie A virus, CBV: Coxsackie B virus, E: Echovirus, ENV: Enterovirus.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3244CAV9
7610CAV9
CAV9-AF290898
5264CAV9
2028CAV9
11580CAV9
CAV9-D00627
Coxsackie A9
E11-AF081606
557E11
17917E11
13373E11
14186E11
Echovirus 11
3404E7
E7-AF403780
Echovirus 7
E18-AF081638
1020E18
13149E18
14521E18
13004E18
13079E18
16808E18
Echovirus 18
12569E13
12584E13
E13-AF081327
Echovirus 13
E25-AF081615
1817E25
3643E25
8652E25
Echovirus 25
13183E9
10862E9
6504E9
6403E9
E9-AF403782
Echovirus 9
7853E3
8769E3
10239E3
8349E3
8564E3
8645E3
950E3
3336E3
Echovirus 3
8643CBV3
7786CBV3
3384CBV3
8696CBV3
CBV3-AF169666
Coxsackie B3
CBV4-X05690
9147CBV4
12130CBV4
12532CBV4
13453CBV4
CBV4-AF160026
13133CBV4
17491CBV4
Coxsackie B4
CBV5-AF152270
910CBV5
7656CBV5
6505CBV5
7040CBV5
7602CBV5
Coxsackie B5
6464CAV5
13243CAV5
1848CAV5
CAV5-AF081296
Coxsackie A5
2609CAV4
CAV4-AF252189
16936CAV4
Coxsackie A4
1576CAV2
2237CAV2
Coxsackie A2
17245CAV6
16480CAV6
3423CAV6
1068CAV6
CAV6-AB090799
Coxsackie A6
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865ENTV1
14181ENV71
ENV71-AF304457
Enterovirus 71
3595CAV16
13492CAV16
CAV16-AF081613
19391CAV16
6337CAV16
6088CAV16
Coxsackie A16
0,01

Tables

Table 1. Characteristics of the cases and control subjects in this study

	Cases (n = 27)	**Controls (n = 53)**
Age at onset of islet autoimmunity[a], median months (range)	12.1 (5-37)	12.3 (5-37)
Female sex	17 (63%)	23 (43%)
Number of other children in the family (siblings, half-siblings, step siblings)		
None	5 (18.5%)	16 (30.2%)
One or more	22 (81.5%)	37 (69.8%)
First degree relative with diabetes		
None	17 (63%)	50 (94.3%)
yes, of that:	10 (37%)	3 (5.7%)
- sibling only	3	0
- father only	3	2
- mother only	2	1
- multiple family members	2	0
Progression from islet autoimmunity to type 1 diabetes		
Yes	10	none
Stool samples		
total	627	1417
prior to development of islet autoimmunity*	339	692

[a] For matched controls: before the age at which the corresponding case seroconverted for islet autoantibodies.

Table 2. Frequency of human enterovirus RNA in faecal samples collected prior to islet autoimmunity

	Cases	Controls	Unadjusted	Adjusted[a]
	Cases (n=27 subjects)	**Controls** (n=53 subjects)	Odds ratio (95% CI)[b]	Odds ratio (95% CI)[b]
Enterovirus RNA **negative** samples	296	598	1.00 (reference)	1.00 (reference)
Enterovirus RNA **positive** samples	43 (12.7%)	94 (13.6%)	1.01 (0.59 - 1.72), P=0.97	1.09 (0.61, 1.96), P=0.77
Total	339	692		
New enterovirus infection episode: **No**	296	598	1.00 (reference)	1.00 (reference)
New enterovirus infection episode: **Yes**	30 (9.2%)	65 (9.8%)	0.94 (0.59 - 1.52), P=0.81	0.92 (0.54, 1.57), P=0.77
Total[c]	326	663		

[a] Adjusted for sex, calendar month of sample collection, year of sample collection (2001-3, 2004-6 or 2007-8), age (continuous), number of siblings (0 vs. >=1), breast-feeding, first degree family history of type 1 diabetes (yes/no).

[b] Odds ratio with 95% confidence interval (CI) estimated from logistic regression model with enterovirus positivity (or new infection episode) as dependent variable and case/control status (and other covariates defined above) as independent variables. Models with random intercept for each subject was used to control for intra-individual correlation (no significant random intercept in model for enterovirus episodes, but highly significant in model for enterovirus positivity). (The unadjusted odds ratio in ordinary logistic regression ignoring intra-individual correlation in infections was 0.92).

[c] Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

Table 3. Semiquantitative testing of the stool samples. Frequency of enterovirus infections with high and low viral load in the children who subsequently developed repeated positivity of multiple islet autoantibodies versus matched controls who did not develop such autoimmunity.

	Cases (n=27 subjects)	Controls (n=53 subjects)
Enterovirus RNA negative	296	598
Enterovirus RNA positive, low-mod quantity[a]	18 (5.3%)	46 (6.6%)
Enterovirus RNA positive, high quantity[a]	25 (7.4%)	48 (6.9%)
Total	339	692
Enterovirus RNA negative	296	598
New infection episode, low-mod quantity[a]	11 (3.4%)	31 (4.7%)
New infection episode, high quantity[a]	19 (5.9%)	34 (5.1%)
Total[b]	326	663

[a] Negative: <100 copies per microliter RNA, low-moderate: 100-9999, high EV RNA quantity: >=10000 copies per microliter RNA.

[b] Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

Table 4. Genotypes of human enterovirus detected.

The number of positive samples for each genotype is reported. As only a subset of samples were sequenced direct comparison of frequencies between cases and controls has limited relevance. The types are listed by their abbreviated name and number; CAV = Coxsackie A virus, CBV = Coxsackie B virus, ECHO = Echovirus, ENV = Enterovirus

Type	Cases	Controls
CAV16	0	5
CAV2	0	3
CAV4	0	4
CAV5	2	2
CAV6	2	3
CAV9	4	8
CBV3	1	4
CBV4	5	7
CBV5	2	4
ECHO11	2	2
ECHO13	1	1
ECHO18	1	5
ECHO25	5	0
ECHO3	4	10
ECHO7	0	1
ECHO9	1	4
ENV71	1	3
Total	**31**	**66**

Electronic Supplementary Material

Calculation of statistical power.

Using relevant estimates from our pilot study [1], we calculated the lowest odds ratio for association between enterovirus infections and autoimmunity that could be detected with a two-sided test with alpha-level of 5% and 80-95% power under various scenarios. On assuming total prevalence of enterovirus infections in the cohort (rounded to 12% for power calculations) and intra-individual correlation for enterovirus infections (0.1 was used for conservative estimations), the “effective sample size” was set to n (1/(0.1+1)), i.e. 90% of the actual number of samples [2]. To illustrate, with 900 pre autoimmunity samples (300 from cases and 600 from controls), the study had at least 93% power if the true odds ratio was 2.0 or greater.

ESM Table 1:

Statistical power considerations for association between islet autoimmunity and prevalence of enterovirus RNA in stool samples from 27 cases who developed repeated positivity for two or three islet autoantibodies and matched controls who did not develop islet autoimmunity during follow-up.

Type of comparison	Assumed true odds ratio	Prevalence of EV among non-autoimmunity controls[1]	Prevalence of EV among cases of autoimmunity[2]	Number of samples from cases	Number of samples from controls	Power[3]
All samples	1.6	11.8%	17.7%	600	1200	88%
All samples prior to autoimmunity‡	2.0	11.7%	21.0%	300	600	93%
The three samples closes to but prior to seroconversion‡	3.0	11.5%	28.1%	81	162	87%

EV: Enterovirus.

[1] Derived prevalence from total prevalence of enterovirus in the total cohort (of 911 individuals) rounded to 12% and assuming the given true odds ratio for association between EV and autoimmunity.

[2] Conservatively assuming within individual correlation for enterovirus of 0.1, power calculations was done with effective sample size of 0.90 times the actual number of samples.

[3] For each case, the corresponding matched samples from two controls were used in the analysis.

[1] Witso E, Palacios G, Cinek O, et al. (2006) High prevalence of human enterovirus a infections in natural circulation of human enteroviruses. J Clin Microbiol 44: 4095-4100

[2] Rosner B, Milton RC (1988) Significance testing for correlated binary outcome data. Biometrics 44: 505-512

IV

Tapia G, Cinek O, Rasmussen T, Grinde B, Rønningen KS. No Ljungan virus RNA in stool samples from the Norwegian environmental triggers of type 1 diabetes (MIDIA) cohort study. Diabetes Care. 2010 May;33(5):1069-71.

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No Ljungan virus RNA in stool samples from infants in the Norwegian MIDIA cohort

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Running title: No Ljungan virus found in stool samples

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Abstract

Objective

Ljungan virus (LjV) has been proposed as a potential environmental factor for type 1 diabetes. The objective was to test for any association of LjV with type 1 diabetes.

Research Design and Methods

A nested case-control design was employed to test for any association between the development of prediabetic autoimmunity and presence of LjV in stool samples (n=3803), obtained from 27 infants carrying the highest genetic risk for type 1 diabetes who developed prediabetic autoimmunity or type 1 diabetes during or shortly after the sampling period, 54 matched controls and 94 other children.

Results

No LjV RNA was detected.

Conclusions

The results indicate that LjV is rare in young children. Theoretically it might be present as a viremia instead of a gastrointestinal infection but this seems unlikely as LjV in rodents, and human parechoviruses, infect the gastrointestinal tract. LjV does not seem to be involved in the development of human type 1 diabetes.

Introduction

Ljungan virus (LjV), a rodent virus described by Niklasson *et al*. (1), has been associated with a variety of conditions in bank voles and other rodents, including type 1 diabetes (2), myocarditis (3) and intrauterine death (4). In humans, LjV has been associated with intrauterine fetal death (5), anencephaly (6), sudden infant death syndrome (7) and suggested as a factor in type 1 diabetes (2). LjV belongs to the viral family *Picornaviridae*, genus *Parechovirus*. The genus also includes human parechovirus (HPeV), which are common in infancy and replicate mainly in the gut. A possible role of LjV in type 1 diabetes is of particular interest due to the strong association found in captive bank voles (2; 3; 8).

The aim of the study was to investigate the presence of LjV RNA in stool samples in order to find a possible association with type 1 diabetes, in a large cohort of children carrying the highest genetic risk of type 1 diabetes.

Research design and methods.

A nested case-control study was conducted, using 2054 stool samples from 81 children (27 cases, 54 controls) all carrying the DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02 HLA genotype, which gives the highest risk for type 1 diabetes. Cases were defined on development of type 1 diabetes or of diabetes associated autoimmunity (being positive for 2 or 3 autoantibodies - GAD; IA2: IAA). Control children were matched for month of birth and geographical residence. This dataset was merged with a previously published dataset investigating parechovirus infections (9) in 1941 stool samples from 102 children - 51 with the high risk genotype and 51 without. In total, there were 3803 unique samples from 175 children, as some children participated in both studies.

Monthly stool samples, from 3-35 months of age, were collected by parents as previously described (10); the age distribution is shown in Figure 1. The median follow-up was 30 months for the stool samples (range 9-37 months), with median endpoint (autoimmunity) of the cases at 20.5 months (range 6-43 months). The presence of LjV was examined by extraction of RNA/DNA, reverse transcription (RT) and real-time PCR as described in (9) with minor modifications of the protocol due to introduction of a 96 well-format and the use of the antisense primer described by Donoso Mantke *et al* (11) as the RT primer. Two positive controls were included from extraction to PCR run (140 and 70 copies of a transcript from a plasmid containing a cDNA clone of LjV prototype strain 87-012, kindly provided by Professor Lindberg, University of Kalmar, Sweden). A fragment of West Nile Virus RNA was spiked into the samples as an exogenous internal control (see (10)). The study was approved by The Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Results

No LjV RNA was detected in any of the stool samples investigated, neither from the 27 children who had developed autoimmunity, nor from the 54 matched controls and the 94 healthy children previously tested. The positive LjV control was consistently detected, and the West Nile Virus Armored RNA used as an exogenous internal control in each sample was consistently positive.

Two samples displayed at one point a weak signal in the LjV real-time PCR, but the result could not be replicated. Still, in order to investigate whether this signal could reflect the presence of LjV, the products were run on an agarose gel; and although none of the multiple

bands corresponded to the expected size, all were cut out of the gel and sequenced. Only two readable sequences were obtained, both were identified as RNA from rectal eubacteria.

Conclusion

Considering the follow-up time, the number of tested samples and that both prediabetic and healthy children were tested, these results indicate that LjV is very rare in the stool of Norwegian infants. The typical stool quantities of human enterovirus and HPeV in samples from the MIDIA cohort study were two to five orders of magnitude higher than the detection limit for LjV (9; 12); presumably any appreciable replication of LjV in the gut would be detected. The detection of the exogenously added West Nile virus RNA safeguards against the presence of inhibitors and RNA degradation. The primers used consistently detected the positive LjV RNA controls included in each run, and are expected to detect all strains of LjV. The lack of evidence for the presence of LjV suggests that this virus rarely infects the gut of Norwegian infants, and it seems unlikely that the virus is the cause of the autoimmunity observed in the present study.

Picornaviruses may also replicate outside the intestinal tract and may cause viremia or respiratory infections. Although one would expect a gastrointestinal route of infection in the case of LjV, the virus might reach the pancreas even after very limited replication in the gut. Although data supporting LjV infections in humans have been published, there is so far no conclusive evidence. The arguably strongest evidence stems from prenatal studies (5; 6). In these studies, the presence of virus has been suggested partly by serology and partly by PCR; but, as pointed out by Bergstrom et al. (13), different methods do not seem to give congruent results. Moreover, the PCR positivity observed in the earlier studies is not reported to have been confirmed by sequencing. Although the evidence suggests possible human LjV

infections, the data also indicate that it is a rare event, and primarily during the prenatal period.

The likelihood of infection may also be geographically specific and dependent on the cycles of its natural reservoir, which presumably are bank voles in Scandinavia. Although common in Norway, their prevalence in the communities from which infants in the present study was recruited is not known. Thus, the possibility that the infants investigated were never exposed cannot be ruled out.

In conclusion, although the present data do not rule out the possibility that LjV can cause type 1 diabetes, they do suggest that this virus is not a common risk factor in the aetiology of the disease in Norway.

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References

1. Niklasson B, Kinnunen L, Hornfeldt B, Horling J, Benemar C, Hedlund KO, Matskova L, Hyypia T, Winberg G: A new picornavirus isolated from bank voles (Clethrionomys glareolus). *Virology* 255:86-93, 1999

2. Niklasson B, Heller KE, Schonecker B, Bildsoe M, Daniels T, Hampe CS, Widlund P, Simonson WT, Schaefer JB, Rutledge E, Bekris L, Lindberg AM, Johansson S, Ortqvist E, Persson B, Lernmark A: Development of type 1 diabetes in wild bank voles associated with islet autoantibodies and the novel ljungan virus. *Int J Exp Diabesity Res* 4:35-44, 2003

3. Niklasson B, Nyholm E, Feinstein RE, Samsioe A, Hornfeldt B: Diabetes and myocarditis in voles and lemmings at cyclic peak densities--induced by Ljungan virus? *Oecologia* 150:1-7, 2006

4. Samsioe A, Sjoholm A, Niklasson B, Klitz W: Fetal death persists through recurrent pregnancies in mice following Ljungan virus infection. *Birth Defects Res B Dev Reprod Toxicol* 83:507-510, 2008

5. Samsioe A, Papadogiannakis N, Hultman T, Sjoholm A, Klitz W, Niklasson B: Ljungan virus present in intrauterine fetal death diagnosed by both immunohistochemistry and PCR. *Birth Defects Res A Clin Mol Teratol* 85:227-229, 2009

6. Niklasson B, Samsioe A, Papadogiannakis N, Gustafsson S, Klitz W: Zoonotic Ljungan virus associated with central nervous system malformations in terminated pregnancy. *Birth Defects Res A Clin Mol Teratol* 85:542-545, 2009

7. Niklasson B, Almqvist PR, Hornfeldt B, Klitz W: Sudden infant death syndrome and Ljungan virus. *Forensic Sci Med Pathol*, 2009

8. Niklasson B, Samsioe A, Blixt M, Sandler S, Sjoholm A, Lagerquist E, Lernmark A, Klitz W: Prenatal viral exposure followed by adult stress produces glucose intolerance in a mouse model. *Diabetologia* 49:2192-2199, 2006

9. Tapia G, Cinek O, Witso E, Kulich M, Rasmussen T, Grinde B, Ronningen KS: Longitudinal observation of parechovirus in stool samples from Norwegian infants. *J Med Virol* 80:1835-1842, 2008

10. Cinek O, Witso E, Jeansson S, Rasmussen T, Drevinek P, Wetlesen T, Vavrinec J, Grinde B, Ronningen KS: Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. *J Clin Virol* 35:33-40, 2006

11. Donoso Mantke O, Kallies R, Niklasson B, Nitsche A, Niedrig M: A new quantitative real-time reverse transcriptase PCR assay and melting curve analysis for detection and genotyping of Ljungan virus strains. *J Virol Methods* 141:71-77, 2007

12. Witso E, Palacios G, Cinek O, Stene LC, Grinde B, Janowitz D, Lipkin WI, Ronningen KS: High prevalence of human enterovirus a infections in natural circulation of human enteroviruses. *J Clin Microbiol* 44:4095-4100, 2006

13. Bergstrom T, Liljeqvist JA: [Irrelevant about Ljungan virus]. *Lakartidningen* 106:1110; discussion 1110-1111, 2009

Figure Legends

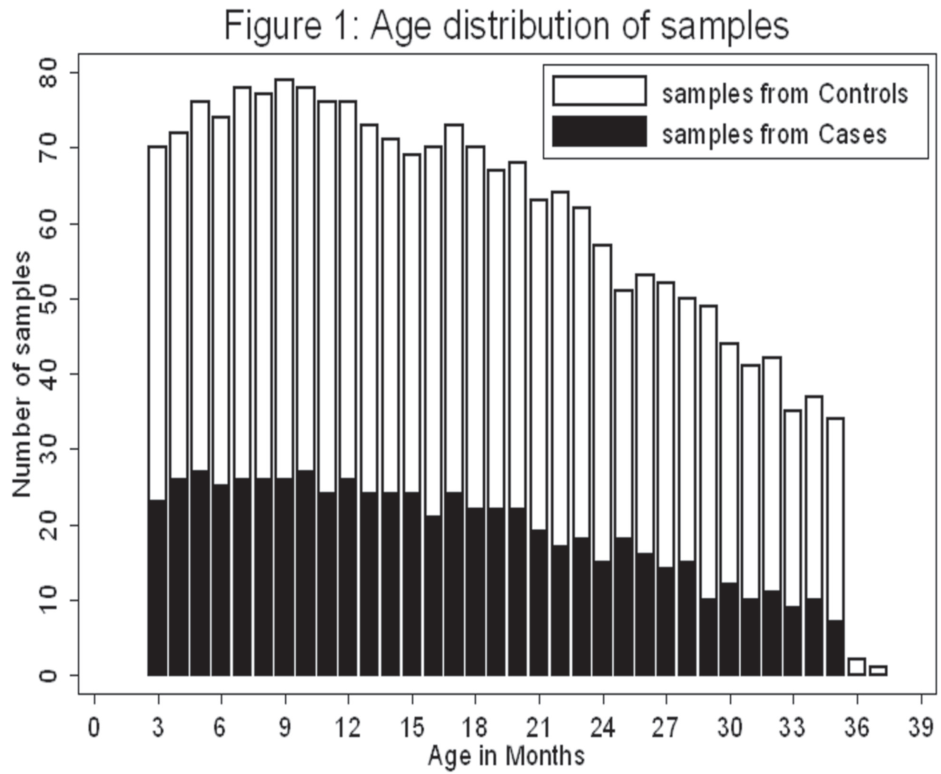

Figure 1: Age distribution of the samples. The Figure shows the number of samples from case children (black bars), and the total number of samples (white bars), distributed by month of age.

Corrections and amendments

Page 3, Chapter 1.1: The name “*Health and Rehabilitation*” was corrected to “*the Norwegian Foundation for Health and Rehabilitation”.*
Page 6, Chapter 1.2: Italicisation of *Picornaviruses* was removed,
Page 7, Chapter 1.2: Misspelling of “*aetology*” corrected to “*aetiology*”.
Page 11, Chapter 2.1: Misspelling of “*antibodied*” corrected to “*antibodies*”.
Page 13, Chapter 2.1: A superfluous full stop was removed.
Page 14, Chapter 2.1: Misspelling of “*lymphocytte*” corrected to “*lymphocyte*”.
Page 17, Chapter 2.2: Misspelling of “*defense*” corrected to “*defence*”.
Page 18, Chapter 2.2: A symbol was changed, from ` to **’** (“*body`s*” to “*body’s*”).
Page 19, Chapter 2.2: Misspelling of “*signaling*” corrected to “*signalling*”.
Page 19, Chapter 2.2: Misspelling of “*apostosis*” corrected to “*apoptosis*”.
Page 20, Chapter 2.2: The sentence “*some viruses stop the presenting of all antigens*” changed to “*some viruses stop the presentation of antigens in infected cells*” for clarity.
Page 23, Chapter 2.3: Misspelling of “*Lithuana*” corrected to “*Lithuania*”.
Page 23, Chapter 2.3: Misspelling of “*infantcy*” corrected to “*infancy*”.
Page 25, Chapter 2.4: The sentence “...*is no symptoms*,” changed to “...*are no symptoms*,”.
Page 26, Chapter 2.5: Misspelling of “*nonenveloped*” corrected to “*non-enveloped*”.
Page 27, Chapter 2.5: A superfluous space was removed.
Page 28, Chapter 2.5: Misspelling of “*aetology*” corrected to “*aetiology*”.
Page 31, Chapter 2.5: Misspelling of “*poathway*” corrected to “*pathway*”.
Page 32, Chapter 3.1: The sentence “... *Directorate of Health since it was decided that to performing genetic analyses*...” was changed to “...*Directorate of Health, who decided that performing genetic analyses*...” for purposes of clarity.
Page 32, Chapter 3.1: The sentence “...*in the summer of 2001, but after one mother went to the media and complained that she had not got good enough information...*” was changed to “*...in the summer of 2001. However, after one mother went to the media and complained that she had not received adequate information...*” for purposes of clarity.
Page 35, Chapter 3.1: Two superfluous spaces were removed.

Page 38, Chapter 3.2: The reference “*Donoso et al*.” corrected to “*Donoso Mantke* et al.” .

Page 42, Chapter 5: The sentence “*A previous infections...”* was corrected to “*A previous infection*...”

Page 45, Chapter 5: Misspelling of “*vs*” was changed to “*vs.*”.

Page 50, Chapter 6.1: Misspelling of “*neighboring*” corrected to “*neighbouring*”.

Page 52, Chapter 6.1: “*viremia*” changed to “*viraemia*” for consistency.

Page 53, Chapter 5.1: One superfluous space was removed.

Page 56, Chapter 6.2: “*viremia*” changed to “*viraemia*” for consistency.