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ARTICLE

Coxsackievirus B1 infections are associated with the initiation of insulin-driven autoimmunity that progresses to type 1 diabetes

Amir-Babak Sioofy-Khojine[1] · Jussi Lehtonen[1] · Noora Nurminen[1] · Olli H. Laitinen[1,2] · Sami Oikarinen[1,3] · Heini Huhtala[4] · Outi Pakkanen[2] · Tanja Ruokoranta[2] · Minna M. Hankaniemi[2,5] · Jorma Toppari[6,7] · Mari Vähä-Mäkilä[6,7] · Jorma Ilonen[8,9] · Riitta Veijola[10] · Mikael Knip[11,12,13,14] · Heikki Hyöty[1,3]

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Abstract

Aims/hypothesis Islet autoimmunity usually starts with the appearance of autoantibodies against either insulin (IAA) or GAD65 (GADA). This categorises children with preclinical type 1 diabetes into two immune phenotypes, which differ in their genetic background and may have different aetiology. The aim was to study whether Coxsackievirus group B (CVB) infections, which have been linked to the initiation of islet autoimmunity, are associated with either of these two phenotypes in children with HLA-conferred susceptibility to type 1 diabetes.

Methods All samples were from children in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study. Individuals are recruited to the DIPP study from the general population of new-born infants who carry defined HLA genotypes associated with susceptibility to type 1 diabetes. Our study cohort included 91 children who developed IAA and 78 children who developed GADA as their first appearing single autoantibody and remained persistently seropositive for islet autoantibodies, along with 181 and 151 individually matched autoantibody negative control children, respectively. Seroconversion to positivity for neutralising antibodies was detected as the surrogate marker of CVB infections in serial follow-up serum samples collected before and at the appearance of islet autoantibodies in each individual.

Results CVB1 infections were associated with the appearance of IAA as the first autoantibody (OR 2.4 [95% CI 1.4, 4.2], corrected $p = 0.018$). CVB5 infection also tended to be associated with the appearance of IAA, however, this did not reach statistical significance (OR 2.3, [0.7, 7.5], $p = 0.163$); no other CVB types were associated with increased risk of IAA. Children who had signs of a CVB1 infection either alone or prior to infections by other CVBs were at the highest risk for developing IAA (OR 5.3 [95% CI 2.4, 11.7], $p < 0.001$). None of the CVBs were associated with the appearance of GADA.

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✉ Amir-Babak Sioofy-Khojine
Amirbabak.sioofy.khojine@staff.uta.fi

1 Department of Virology, Faculty of Medicine and Life Sciences, University of Tampere, PL 100, 33014 Tampereen yliopisto, Tampere, Finland

2 Vactech Ltd, Tampere, Finland

3 Fimlab laboratories, Pirkanmaa Hospital District, Tampere, Finland

4 Faculty of Social Sciences, University of Tampere, Tampere, Finland

5 Biomeditech, University of Tampere, Tampere, Finland

6 Institute of Biomedicine, Research Centre of Integrative Physiology and Pharmacology, University of Turku, Turku, Finland

7 Department of Paediatrics, Turku University Hospital, Turku, Finland

8 Immunogenetics Laboratory, Institute of Biomedicine, University of Turku, Turku, Finland

9 Department of Clinical Microbiology, Turku University Hospital, Turku, Finland

10 Department of Paediatrics, PEDEGO Research Unit, Medical Research Centre, Oulu University, Hospital and University of Oulu, Oulu, Finland

11 Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

12 Research Programs Unit, Diabetes and Obesity, University of Helsinki, Helsinki, Finland

13 Tampere Centre for Child Health Research, Tampere University Hospital, Tampere, Finland

14 Folkhälsan Research Centre, Helsinki, Finland

Research in context

What is already known about this subject?

- Coxsackievirus group B (CVB) infections have been linked to type 1 diabetes in previous studies
- Type 1 diabetes has been categorised into two major subtypes identified by the appearance of either insulin autoantibody (IAA) or GAD autoantibody (GADA) as the first islet autoantibody type at the beginning of the beta cell damaging process
- Neutralising antibodies against CVB are specific markers of past infections

What is the key question?

- Are CVB infections associated with the initiation of either insulin- or GAD-driven islet autoimmunity in a prospective birth cohort study?

What are the new findings?

- CVB1 infections are associated with the initiation of insulin-driven islet autoimmunity

How might this impact on clinical practice in the foreseeable future?

- These results suggest that CVB could contribute to the development of type 1 diabetes in a certain subgroup of individuals that are characterised by early autoimmunisation against insulin
- This finding offers new opportunities to explore the mechanisms that are involved in enterovirus-induced beta cell damage and to develop vaccines against these viruses

Conclusions/interpretation CVB1 infections may contribute to the initiation of islet autoimmunity being particularly important in the insulin-driven autoimmune process.

Keywords Coxsackievirus group B · Glutamic acid decarboxylase autoantibody (GADA) · Insulin autoantibody (IAA) · Islet autoimmunity · Logistic regression · Plaque reduction assay · Type 1 Diabetes Prediction and Prevention (DIPP) · Virus neutralising antibodies

Abbreviations

CAR	Coxsackie and adenovirus receptor
CVB	Coxsackievirus group B
GADA	GAD65 autoantibody
IAA	Insulin autoantibody
ICA	Islet cell antibody
DIPP	Type 1 Diabetes Prediction and Prevention

Introduction

Recent studies have suggested that the pathogenesis of type 1 diabetes is heterogeneous. One of the most important indicators of this heterogeneity is seen during the early phases of islet autoimmunity and is characterised by the specificity of the first appearing autoantibody targeting either insulin or the 65 kD isoform of glutamic acid decarboxylase (GAD), known as IAA and GADA, respectively. Both insulin and GAD are present in beta cells together with insulin secretary granules [1]. Insulin constitutes 50% of the total protein content of the beta cells and plays a major role as an autoantigen in type 1 diabetes [2]. It is almost exclusively expressed in beta cells, although small amounts can also be found in some neuronal cells and in the thymus [3]. The expression of GAD on the other hand, is not limited to beta cells but can be seen in all islet cells including alpha and delta cells as well as in the nervous system and to a lesser extent in the lungs [4]. The IAA driven autoimmune process typically starts at a younger age than the GADA-driven process [5] and these two phenotypes of type 1 diabetes differ from each other in their genetic background since the IAA phenotype associates with the HLA-DR4-DQ8 haplotype and INS gene polymorphism, whereas the GADA phenotype is primarily associated with the HLA-DR3-DQ2 haplotype [6].

It is possible that each of these phenotypes has a different aetiology and relate to different beta cell damaging processes. The aim of the present study was to investigate whether strains of Coxsackievirus group B (CVB); one of the strongest

environmental triggering candidates for type 1 diabetes [7, 8], are associated with the initiation of islet autoimmunity in either of these two phenotypes.

Methods

Study cohort The Type 1 Diabetes Prediction and Prevention (DIPP) study is a population-based observational study that was launched in Finland in 1994. The study has been approved by the ethical committees of the hospital districts of Southwest Finland (Turku University Hospital), Pirkanmaa (Tampere University Hospital) and Northern Ostrobothnia (Oulu University Hospital) and the parents of the participating children gave their informed written consent to the participation in the study. Formal assent was not sought from children, as this was not required under Finnish law and ethical regulations. However, children were informed about the procedure at a level appropriate for their age and understanding. In this study cord blood samples from all individuals with parental consent are analysed for the presence of HLA-DR/DQ alleles and children with genotypes associated with increased risk for type 1 diabetes are recruited for follow-up [9]. HLA screening criteria were modified during the DIPP study to identify a larger repertoire of DQB1 and DQA1 alleles, along with HLA-DRB1*04 subtypes which increased the sensitivity and specificity of the risk estimation for type 1 diabetes [5, 10]. Blood samples were collected regularly at 3–12 month intervals and islet cell antibodies (ICA), IAA, GADA and islet antigen 2 autoantibody 2 (IA-2A) have been analysed [11]. Prior to 2003, ICA was used as a primary screening marker meaning that if the child turned ICA positive then biochemical autoantibodies (IAA, GADA and IA-2A) were analysed from all available samples from that child.

Study design All individuals ($N = 169$) were selected among those who became seropositive for a single autoantibody (IAA [$n = 91$] or GADA [$n = 78$]) but seroconverted persistently to any combinations of additional autoantibodies (multiple islet autoantibodies) during the follow-up period. We used all available individuals in the study from whom the serum sample sets were available. The mean (±SD) age of the 'case' children in the 'IAA first' group was 21.4 ± 14.9 months compared with 58.2 ± 34.4 months in children in the 'GADA first' group, $p < 0.001$. Altogether 31.9% of case children in the IAA first group and 39.7% of case children in the GADA first group were girls ($p = 0.37$). Five (5.5%) mothers of children in the IAA first group and two (2.6%) mothers of children in the GADA first group had type 1 diabetes at the time of the child's birth. By February 2016, 66 children in the IAA first group (72.5%) and 29 in the GADA first group (37.2%) had progressed to type 1 diabetes (diagnosed according to WHO criteria [12]). Two autoantibody negative 'control' children were matched to each case child individually according to date of birth (±1 month), sampling date (±2 months), sex, registered study centre and HLA-DQB1 risk alleles to form control groups. In total, there were 181 control children for the IAA first group and 151 control children for the GADA first group. On a few occasions only one control child for a case child met the inclusion criteria. The age at first introduction of dairy products in the child's diet did not differ between case and control children in either of these groups (data not shown).

Virus antibody measurement A standard plaque reduction assay was used to identify neutralising effect of antisera. Briefly, fourfold dilutions of sera in volumes of 3 μl were incubated with equal volume of approximately 100 PFU infectious virus for 1 h at 37°C, continued overnight at room temperature. The volume of each reaction was made up to 100 μl using Hanks' Balanced Salt solution (HBSS)-HEPES. Suspensions were incubated with 95% confluent GMK cells maintained in minimum essential medium (MEM; Sigma) in 12 well plates (Nunc) for 1 h at 37°C. Cells were then covered with semi-solid media (MEM supplemented with 0.67% carboxymethyl cellulose) after removing the virus–serum suspension and incubated for 2 days at 37°C in a humid chamber supplemented with 5% CO_2. Virus plaques were visualised and quantified after fixing the cells with formaldehyde solution containing crystal violet. A reduction in plaque number ≥80% compared with untreated virus suspension was considered positive.

Study samples, shown in Fig. 1, included cord blood serum at birth and further blood samples at the autoantibody seroconversion time, 6 months prior to the autoantibody seroconversion (−6 M) and 12 months prior to the autoantibody seroconversion (−12 M). Neutralising antibodies against all six members of the CVB group (CVB1-6) were analysed using a sensitive plaque reduction assay with blinding to the case/control status of the child. Persistent seroconversion to CVB neutralising antibody positive in consecutive samples was used as the criterion to identify virus infections. To enable this, an algorithm was developed which is shown in ESM Fig.1. The algorithm was first validated by comparing the results to those from two experts with identical diagnostic criteria and was subsequently used to identify the CVB infections. The algorithm also took into account the possible presence of maternal CVB antibodies and was used to eliminate any possible biasing effect of such maternal antibodies on the diagnosis of the child's infections. The increased serum titers for virus-specific antibodies were identified to diagnose virus infections using this algorithm.

Statistical analysis The conditional logistic regression method was employed since triplets (the case and two control children) were matched for age, sex, HLA and birth region using the R statistical package (version 3.2.2, www.r-project.org) to generate OR and 95% CIs with Wald's statistics

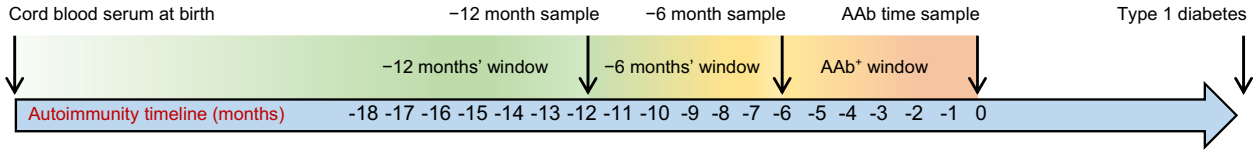

Fig. 1 Schematic representation of the sampling time and the time windows used in the statistical analysis. Serum samples taken for the analysis included the first follow-up serum that was positive for islet autoantibodies (AAb time) as well as samples taken 6 and 12 months prior to the first autoantibody positive sample and the cord blood sample. The time window between AAb time and −6 month sample was called the AAb^+ window, indicating the autoantibody seroconversion occurred in this time window. The time window between −6 month sample and −12 month sample was called −6 months' window and the time window before −12 month sample was called −12 months' window. AAb, autoantibody

(representing unadjusted two-sided p values). Bonferroni adjustment for the p value was used where appropriate in the statistical analysis.

The aim was to identify the association of infections by CVB members individually to the risk of developing subtypes of autoimmunity to islet autoantigens (IAA or GADA). Therefore, the proportion of case children who had CVB infections at any time point before autoantibody seroconversion were compared with the proportion of their matched control children who had CVB infection by the corresponding age (as pre-specified, each CVB serotype was analysed separately). Both crude p values and the p values corrected for the number of comparisons (Bonferroni correction) are presented for this primary analysis (Table 1).

The temporal profile of the associations was also established in time-window analyses (time windows are shown in Fig. 1). For this, timing of the CVB infections that occurred before the detection of predictive autoantibodies was analysed using the same statistical method as described above for IAA and GADA first groups separately.

The impact of the order of infections caused by different CVB serotypes on the risk of beta cell autoimmunity was also analysed using data from longitudinal sample sets by conditional logistic regression.

Interactions between different serotypes in exposing the children to islet autoimmunity (IAA or GADA) were also analysed using the same statistical method by studying the effect of different virus combinations.

The overall number of samples from the different time points did not differ between case and control children (average 3.6 samples per case and 3.5 samples per control child in both the IAA and in the GADA group) and conditional logistic regression analyses (by definition) were carried out using the data from only those samples that were available from the same time point from both the case and control children.

Results

CVB infections and initiation of insulin-driven islet autoimmunity CVB1 infections were associated with insulin-driven autoimmunity (IAA). The odds of being infected before the first detection of IAA was higher in case children compared with the individually matched control children (OR 2.4 [95% CI 1.4, 4.2], corrected $p = 0.02$; Table 1). This association was also detected when clinical type 1 diabetes was used as the endpoint event (OR 3.1 [95% CI 1.6, 6.2], corrected $p = 0.006$; ESM Table 1). Other CVB types were not associated with increased risk of IAA or type 1 diabetes. CVB5 also tended to have a risk association with IAA autoimmunity, however, this did not reach statistical significance (OR 2.3 [95% CI 0.7, 7.5], $p = 0.2$; Table 1).

CVB infections and initiation of GAD-driven islet autoimmunity In contrast to insulin-driven autoimmunity, the risk of GAD-driven autoimmunity was not associated with CVB1 or other CVB infections (Table 1). Since children in the GADA first group were older than those in the IAA first group, to eliminate the possibility of increasing CVB infection due to age masking the effect of earlier infections on GADA autoimmunity we performed similar analyses by restricting the age of seroconversion to 2 years (ESM Table 2). CVB1 infection was not associated with GADA in these young children (OR 0.8 [95% CI 0.2, 2.9], $p = 0.751$) while it was still associated with an increased risk of IAA autoimmunity in this age group (OR 2.3 [95% CI 1.2, 4.3], $p = 0.01$). This was also true for CVB1 infections among children who developed type 1 diabetes (OR 2.7 [95% CI 1.3, 5.5], $p = 0.01$; IAA first group).

Timing of CVB1 infections The potential association between CVB1 infections and the initiation of islet autoimmunity was studied further by analysing the timing of infections in relation to the first detection of islet autoantibodies, using previously defined time windows (shown in Fig. 1): autoantibody positive (AAb^+) window including a 6 month period prior to the time of autoantibody seroconversion, a 6 month period before autoantibody window (−6 M window) and the period prior to the −6 month window (−12 M window). In the IAA first group, the risk was associated with CVB1 infections that occurred within a year before the first detection of IAA (Table 2). This included both the autoantibody positive and −6 M windows (OR 2.3 [95% CI 1.1, 4.5], $p = 0.02$ and OR 2.9 [95% CI 1.3, 6.7], $p = 0.01$, respectively; Table 2). In the GADA first group, however, CVB1 infections were not associated with

Table 1 Association between CVB infections and two phenotypes of progressive islet autoimmunity (IAA first or GADA first)

Category	Virus	Infections (%)		OR (95% CI)	*p* value	[a]Corrected *p* value
		Controls	Cases			
IAA first[b]	CVB1	45.6	64.8	2.4 (1.4, 4.2)	0.003	0.018
	CVB2	61.1	48.4	0.6 (0.3, 1.0)	0.031	0.186
	CVB3	16.1	6.6	0.4 (0.2, 0.9)	0.034	0.204
	CVB4	12.8	7.7	0.5 (0.2, 1.4)	0.181	
	CVB5	3.9	7.7	2.3 (0.7, 7.5)	0.163	
	CVB6	28.9	23.1	0.7 (0.4, 1.2)	0.204	
GADA first[c]	CVB1	50.3	52.6	1.1 (0.6, 1.8)	0.850	
	CVB2	51	51.3	1.0 (0.6, 1.8)	0.923	
	CVB3	11.3	7.7	0.5 (0.2, 1.7)	0.295	
	CVB4	11.9	9	0.7 (0.3, 2.0)	0.507	
	CVB5	15.2	10.3	0.7 (0.3, 1.6)	0.335	
	CVB6	37.7	30.8	0.7 (0.4, 1.3)	0.277	

Proportion of infected individuals are presented among case and control children prior to the autoimmunity process

OR was calculated using conditional logistic regression with the control children being the reference group

[a] Bonferroni correction for six comparisons was used to generate corrected *p* values

[b] $n = 181$ for control children and 91 for case children

[c] $n = 151$ for control children and 78 for case children

The missing corrected *p* values were not significant and are, hence, not included

Table 2 Time-window analysis for CVB1

Phenotype	Time window	Infection %		OR (95% CI)	*p* value
		Control	Case		
IAA first[a]	Neg.	54.1	35.2	Ref.	
	AAb^+	21.5	29.7	2.3 (1.1, 4.5)	0.019
	−6 months	10.5	18.7	2.9 (1.3, 6.7)	0.010
	−12 months	13.8	16.5	2.0 (0.8, 4.8)	0.122
GADA first[b]	Neg.	49.7	47.4	Ref.	
	AAb^+	19.2	17.9	0.9 (0.4, 1.9)	0.797
	−6 months	7.3	5.1	0.5 (0.1, 2.3)	0.374
	−12 months	23.8	29.5	1.3 (0.4, 1.9)	0.436

A schematic presentation of the framework for time-dependent analyses

Time-dependent risk association of beta cell autoimmunity with CVB1 infection in IAA first or GADA first groups (different autoimmune phenotypes) is shown here

Children who had no CVB1 infection at any time window were used as the reference group ('Neg.' in the table)

The proportion of children who experienced CVB1 infection 'for the first time' at any time within the time windows prior to islet autoimmunity has been identified among case and control children

The analysis was performed for each autoimmune phenotype separately

[a] $n = 181$ for control children and 91 for case children

[b] $n = 151$ for control children and 78 for case children

Ref, Reference group; AAb, autoantibody

the risk of islet autoimmunity in any of these time windows (Table 2). The time-associations in the IAA first group with the autoantibody positive window was also seen in those children who progressed to clinical type 1 diabetes (OR 1.6 [95% CI 1.1, 2.5], $p = 0.025$).

Modifying the risk association of CVB1 infection with insulin-driven autoimmunity by other CVB infections Since past infections by different CVBs can influence the course of later CVB1 infection e.g. by providing some immunological cross-protection we wanted to study whether a prior history of CVB infections modulates the association between CVB1 and insulin-driven autoimmunity. For this purpose, we classified CVB into two groups: (1) CVB types that showed increased odds for IAA seroconversion and (2) CVB types that were not associated with IAA or showed decreased odds. Based on the data shown in Table 1, CVB1 and CVB5 were included in the first category and the other four CVBs were placed in the second category. Being infected by either CVB1 or CVB5 or both (CVB1/5), was associated with increased risk of insulin-driven autoimmunity (OR 2.7 [95% CI 1.4, 5.3], $p = 0.003$; ESM Table 2). In contrast, being infected by any combination of the other CVBs (CVB2/3/4/6) was associated with reduced risk of insulin-driven autoimmunity (OR 0.4 [95% CI 0.2, 0.8], $p = 0.007$; ESM Table 2). Combining CVB1–6 infections into one group did not show any association with insulin-driven autoimmunity. CVB1/5 infection was also associated with increased risk of islet autoimmunity and type 1

diabetes in the IAA first but not in the GADA first group (ESM Table 2).

To study the risk modification by CVB infections children were further grouped into four categories based on their infection history including: (1) CVB1/5$^-$ and CVB2/3/4/6$^+$; (2) CVB1/5$^-$ and CVB2/3/4/6$^-$, (3) CVB1/5$^+$ and CVB2/3/4/6$^-$ and (4) CVB1/5$^+$ and CVB2/3/4/6$^+$ (Table 3). The risk of insulin-driven autoimmunity was the highest in children who had CVB1/5 infections without any CVB2/3/4/6 infection (OR 10.5 [95% CI 3.7, 29.8], $p < 0.001$). IAA autoimmunity was also associated with CVB1/5 infections occurring with other CVB infections (OR 3.7 [95% CI 1.5, 7.2], $p = 0.002$), however, the odds were lower compared with the presence of only CVB1/5 infection. Similar results were obtained when CVB1 alone was included in the first group instead of both CVB1 and CVB5 (ESM Table 3).

To assess the effect of the chronological order of these infections, the children were grouped into four categories: (1) Children who had experienced CVB2/3/4/6 alone or prior to CVB1/5 (the reference group); (2) children who had no CVB infection at all; (3) children infected with any combinations of CVB1/5 alone or before CVB2/3/4/6 and (4) when simultaneously infected with CVB1/5 and any combinations of CVB2/3/4/6 (Table 4). The children who experienced CVB1/5 infection alone or before any CVB2/3/4/6 had clearly the highest risk of insulin-driven autoimmunity (OR 5.3 [95% CI 2.4, 11.7], $p < 0.001$; Table 4). Simultaneous infections by CVB1/5 and CVB2/3/4/6 also increased the risk (OR 2.6 [95% CI 1.3, 5.2], $p = 0.007$). Similar results were obtained when CVB1 alone, without CVB5, was included in the analysis (ESM Table 4). When the timing of CVB1 and CVB5 infections was analysed in relation to the time of first detection of islet autoantibodies the risk association was related to CVB1/CVB5 infections that occurred within 1 year prior to IAA seroconversion (ESM Table 5).

Discussion

The present study shows that CVB infections may contribute to the initiation of islet autoimmunity characterised by IAA as the first appearing autoantibody. This particular group of children are known as one of the phenotypes of islet autoimmunity in man [6]. This recently discovered phenotype of type 1 diabetes differs from the other typical disease phenotype characterised by initiation of autoimmunity against GAD [5, 6]. The fact that the initiation of islet autoimmunity in these two phenotypes is associated with distinct HLA-DQ alleles indicates that they might have different triggers for the disease process leading to overt type 1 diabetes and different pathogenetic mechanisms [5, 6]. Our study suggests that specific CVB infections may contribute to the activation of the 'insulin-driven' pathogenetic pathway. This finding motivates further exploration of the pathogenic mechanisms underlying human type 1 diabetes related to CVB infections.

The current study supports the previous observations that enterovirus infections (including CVB) may increase the risk of islet autoimmunity and type 1 diabetes [7, 8]. In addition, the results imply that certain CVB infections might trigger the insulin-driven autoimmune process. The observation that no risk associations were seen between CVB infections and the GADA-driven pathway and that the association appears to be specific to CVB1 argues against the effect of unknown confounding factors. Additionally, to minimise any sampling bias and the effect of possible confounders, case and control children were individually matched for HLA-DQB1 genes, sex, age, sampling dates and the region of birth. The findings from the present study are also in line with those recently published by The Environmental Determinants of Diabetes in the Young (TEDDY) study [13]. In the TEDDY study parent-reported recent respiratory infections, particularly common cold, influenza-like illness, sinusitis and laryngitis/tracheitis were associated with the subsequent risk of islet autoimmunity.

Table 3 Risk of insulin-driven islet autoimmunity (IAA first) according to the history of a CVB1/5 infection vs other CVB infections

Virus positivity			Infection %		OR (95% CI)	*p* value
Group	CVB1/5[a]	CVB2/3/4/6[b]	Controls ($n = 181$)	Cases ($n = 91$)		
1	–	+	37.6	14.3	Ref.	
2	–	–	14.4	16.5	3.5 (1.3, 9.7)	0.015
3	+	–	6.6	22.0	10.5 (3.7, 29.8)	<0.001
4	+	+	41.4	47.3	3.7 (1.5, 7.2)	0.002

Children were grouped based on the history of CVB infections into four groups at any time prior to islet autoimmunity

The group with CVB infections other than CVB1/5 was used as the reference group

The OR and CI are shown with *p* values

[a] Refers to infection by any combinations of the following viruses: CVB1 or CVB5

[b] Refers to infections by any combinations of the following viruses: CVB2, 3, 4 or 6

Ref, Reference group

Table 4 Effect of the order of CVB1/5 and CVB2/3/4/6 infections on the risk of insulin-driven islet autoimmunity (IAA first group)

Group	Order of CVB infections	Infection %		OR (95% CI)	*p* value
		Controls ($n = 181$)	Cases ($n = 91$)		
1	CVB2/3/4/6 alone or before CVB1/5[a]	52.5	25.3	Ref.	
2	No infection	14.4	16.5	2.5 (1.0, 6.2)	0.044
3	CVB1/5 alone or before CVB2/3/4/6[b]	12.7	30.8	5.3 (2.4, 11.7)	<0.001
4	CVB1/5 and CVB2/3/4/6 simultaneously[c]	20.4	27.5	2.6 (1.3, 5.2)	0.007

The group who had a combination of CVB2/3/4/6 infections without or before CVB1/5 was considered as the reference group

[a] Any combination of infections by CVB2, 3, 4, or 6 without or prior to CVB1 or CVB5 infections

[b] Infected only with combinations of CVB1 and CVB5

[c] Any combination of at least one CVB1 or CVB5 infections at the same time as other CVBs

Ref, Reference group

The causative agents could not be identified since laboratory testing was not carried out. However, it is known that these symptoms are commonly caused by enterovirus infections.

CVB shares an important biological feature, which may explain their tropism to beta cells; they are the only EVs that use the Coxsackie and adenovirus receptor (CAR) to enter the cell. CAR is expressed more strongly on the surface of beta cells than other pancreatic cells [14] being selectively used by CVBs to gain entry into the beta cells hence implying that they may be responsible for the initiation of the process leading to type 1 diabetes. This also indicates that all CVB types might potentially cause beta cell damage. This feature could then be modulated by other viral characteristics and/or beta cell sensitivity to these viruses. In vitro studies have suggested that EVs infect mainly beta cells in cultured human islets [15, 16], and that CAR expression is upregulated in islets of individuals with type 1 diabetes compared with controls [17]. In fact, in vivo tropism of CVB to human pancreatic islets has also been documented in post mortem examination of children with a fatal CVB infection [18, 19]. Enterovirus capsid proteins have been detected in beta cells from individuals with type 1 diabetes as well, while other islet cells have been mostly virus negative [20–22]. A large proportion of individuals affected by type 1 diabetes were reported to carry enterovirus in a small number of pancreatic beta cells, supporting the hypothesis of a chronic/persistent viral infection being present in these individuals [14]. A chronic/persistent enterovirus infection(s), with a slower rate of virus propagation, has already been demonstrated in cell culture and mice models [23–25] and it has been shown that a deletion at the 5′UTR end of the viral genome could contribute to virus persistence in selected human tissues [26, 27]. The presence of the 5′ end deletion was also reported from a fatal case of fulminant enterovirus myocarditis in Japan [28]. Therefore, we postulate that such a persistent/chronic infection of the beta cells might be one of the mechanisms initiating the autoimmune process.

The underlying mechanisms by which certain CVBs could contribute to the autoimmune response against insulin but not GAD are yet to be identified. One possible explanation, though, would be the tropism of CVBs to beta cells where a direct infection could induce autoimmunity against the most abundant and exclusively expressed protein in these cells, namely insulin. CVB infection could potentially induce post-translational modifications of the insulin molecule rendering it highly immunogenic. Interestingly, a recent publication showed that an incomplete response to CVB is associated with insulin autoimmunity but not with GAD autoimmunity in young children [29]. Collectively, these studies suggest that further work is required to address the possible role of EVs in type 1 diabetes particularly focusing on the IAA phenotype of the disease.

One important aspect of our study is to report the existence of a possible interaction between different CVB types: CVB1-associated risk of islet autoimmunity was the highest in children who experienced mainly CVB1 infections (or CVB1/5) alone or prior to the other CVB infections. This indicates that additional infections by other CVBs may attenuate the risk association of CVB1 with islet autoimmunity. One possible explanation to this phenomenon could be immunological cross-protection between different CVB types, when earlier CVB infections could provide partial protection against subsequent CVB1 infection. In fact, an earlier study in mice has demonstrated that infection by CVB3 can attenuate later infection by CVB1 [30] supporting this hypothesis. T cell cross-reactivity can be another possible mechanism explaining a cross-protection process [31–35]. An alternative to the cross-protection hypothesis could be the presence of an immunoregulatory effect in which autoimmune responses could be down-regulated by earlier infections as reported before [36]. In this case, CVB1 could possibly lack such an immunoregulatory property due to different intrinsic properties of various CVB group viruses in vivo. It is also possible that their potential

diabetogenic effect could overcome their immunoregulatory effect.

The present observation that different CVB types can show contrasting associations with type 1 diabetes and possibly interact with each other, demonstrates the importance of the type of assays that are used to discriminate between the type of the enterovirus infections involved, i.e. virus neutralising assays which are highly specific for the serotype used in the assay. In fact, when all CVBs were analysed as a group, they showed no association with insulin-driven autoimmunity even though CVB1 was strongly associated with the appearance of IAA, thus demonstrating the importance of neutralising antibody assessments. In addition, the results demonstrate the value of prospective studies, which allows a time-ordered analysis to be conducted (e.g. IAA or GADA being the first appearing autoantibody and chronological order of different CVB infections). Therefore, the large population size and the prospective birth cohort design have clearly provided an important advantage, giving us an excellent opportunity to identify the association of CVB infections with type 1 diabetes.

It is also important to consider the limitations of the current study when interpreting the results. The study samples are representative of the Finnish population; therefore, it would be important to perform similar studies in other populations where the epidemiology of CVBs might be different. Previous studies have suggested that CVBs other than CVB1 (CVB4 in particular) could be diabetogenic [20, 37]. Hence, it is also important to keep in mind that the diabetogenic effect might not be the characteristic of a single virus serotype. In other words, other enterovirus types may also be associated with type 1 diabetes, depending on the circulation and accumulation of diabetogenic strains in different populations. In the current study CVB5 association with the risk of islet autoimmunity or type 1 diabetes was not statistically significant on its own but in combination with CVB1 there was some association. The lack of statistical significance for CVB5 could be due to its substantially lower frequency compared with CVB1. It is noteworthy that circulating strains of a single enterovirus serotype may also widely differ for their non-structural proteins, which may further affect the diabetogenicity of the viral strains [38]. Therefore, it would be important to identify recombination events in circulating EVs in different populations. In line with this, we have previously shown that the CVB1 strains have different properties encountering innate immunity [39] and causing cell death in human islet cell cultures [40], which further emphasises the possible role of strain variations. Population dynamics of enterovirus infections and levels of maternal enterovirus antibodies, which protect the offspring against enteroviral infections, may also matter. For example, the prevalence of enterovirus infections and levels of maternal enterovirus antibodies are considerably lower in Finland than in most other countries. It has been proposed that this makes Finnish children particularly susceptible to the diabetogenic effect of these viruses [41]. In fact, in the present study, 47.3–83.4% of the children lacked maternal CVB antibodies in cord blood, depending on the CVB serotype in question (data not shown).

Another limitation of the present study is that the identification of CVB types was based solely on neutralising antibody analyses. Therefore, further studies identifying enterovirus serotypes by direct sequencing from diabetic and prediabetic children should be conducted. However, such studies can easily be compromised by difficulties in identifying the individual enterovirus serotypes in sufficient numbers, leading to limited statistical power to address this question. For example, we have previously observed an excess of enterovirus RNA by RT-PCR in serial stool and blood samples collected prior to autoantibody seroconversion from prospectively followed children in the DIPP study [42, 43]. However, the identification of the serotype of these enteroviruses by sequencing the viral genome was successful only in a portion of samples where too few CVB viruses were identified to enable us to analyse their association with the initiation of islet autoimmunity. We also have to appreciate the general limitations of the case–control study design including the risk of selection bias. However, this study had a nested case–control design that minimises selection and recall bias compared with a conventional case–control study [44]. Nonetheless, it is difficult to completely exclude a possible contribution of unknown confounding factors to the observed association between CVBs and islet autoimmunity. However, this was minimised using strict matching criteria for the selection of control children at an individual level.

In conclusion, the present study suggests that CVB infections could contribute to the development of type 1 diabetes via a specific pathway characterised by IAA as the first appearing islet autoantibody. An early infection by CVB1 or possibly by some other CVBs such as CVB5, might lead to the induction of an autoimmune response against insulin. However, the underlying mechanism(s) remain to be identified and further studies are needed to fully characterise this phenomenon. Large prospective studies conducted in different populations are also crucial tools to achieve this goal.

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Data availability The data generated during the course of this study are available on request from the authors after the acceptance provided by the DIPP steering committee.

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Duality of interest HHy and MK are minor (5%) shareholders and members of the board of Vactech Ltd., which develops vaccines against picornaviruses. Companies owned by their families are also shareholders of Vactech Ltd. No other potential conflicts of interest relevant to this article are reported. The sponsors funded the study but did not participate in the study design or the interpretation of the data.

Contribution statement The corresponding author performed the laboratory analysis, researched the data and wrote the manuscript. HHy, JI, MK, JT, RV, MV-M provided the DIPP data and samples. NN, SO, OHL, OP, TR and MMH partially researched the data and all reviewed/edited manuscript. HHu and JL performed statistical analysis and reviewed the manuscript, MV-M, OHL, MMH, JL, JI, RV, MK, JT, OP and TR reviewed/edited the manuscript. HHy designed the study and contributed to the discussion and reviewed/edited the manuscript. HHy is the guarantor of this work and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All co-authors have approved the final version.

References

1. Arvan P, Pietropaolo M, Ostrov D, Rhodes CJ (2012) Islet autoantigens: structure, function, localization, and regulation. Cold Spring Harb Perspect Med 2:a007658
2. Eizirik DL, Colli ML, Ortis F (2009) The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol 5: 219–226
3. Kutlu B, Burdick D, Baxter D et al (2009) Detailed transcriptome atlas of the pancreatic beta cell. BMC Med Genet 2:3-8794-2-3
4. Wang C, Mao R, Van de Casteele M, Pipeleers D, Ling Z (2007) Glucagon-like peptide-1 stimulates GABA formation by pancreatic beta-cells at the level of glutamate decarboxylase. Am J Physiol Endocrinol Metab 292:E1201–E1206
5. Ilonen J, Hammais A, Laine AP et al (2013) Patterns of beta-cell autoantibody appearance and genetic associations during the first years of life. Diabetes 62:3636–3640
6. Krischer JP, Lynch KF, Schatz DA et al (2015) The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study. Diabetologia 58:980–987
7. Laitinen OH, Honkanen H, Pakkanen O et al (2014) Coxsackievirus B1 is associated with induction of beta-cell autoimmunity that portends type 1 diabetes. Diabetes 63:446–455
8. Oikarinen S, Tauriainen S, Hober D et al (2014) Virus antibody survey in different European populations indicates risk association between coxsackievirus B1 and type 1 diabetes. Diabetes 63:655–662
9. Nanto-Salonen K, Kupila A, Simell S et al (2008) Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial. Lancet 372:1746–1755
10. Ilonen J, Kiviniemi M, Lempainen J et al (2016) Genetic susceptibility to type 1 diabetes in childhood - estimation of HLA class II associated disease risk and class II effect in various phases of islet autoimmunity. Pediatr Diabetes 17(Suppl 22):8–16
11. Knip M, Virtanen SM, Seppa K et al (2010) Dietary intervention in infancy and later signs of beta-cell autoimmunity. N Engl J Med 363:1900–1908
12. World Health Organization. Department of Noncommunicable Disease Surveillance. (1999) Definition, diagnosis and classification of diabetes mellitus and its complications : report of a WHO consultation. Part 1, Diagnosis and classification of diabetes mellitus. World Health Organization, Department of Noncommunicable Disease Surveillance, Geneva
13. Lonnrot M, Lynch KF, Elding Larsson H et al (2017) Correction to: respiratory infections are temporally associated with initiation of type 1 diabetes autoimmunity: the TEDDY study. Diabetologia 61:254
14. Oikarinen M, Tauriainen S, Honkanen T et al (2008) Analysis of pancreas tissue in a child positive for islet cell antibodies. Diabetologia 51:1796–1802
15. Sarmiento L, Frisk G, Anagandula M, Cabrera-Rode E, Roivainen M, Cilio CM (2013) Expression of innate immunity genes and damage of primary human pancreatic islets by epidemic strains of Echovirus: implication for post-virus islet autoimmunity. PLoS One 8:e77850
16. Frisk G, Diderholm H (2000) Tissue culture of isolated human pancreatic islets infected with different strains of coxsackievirus B4: assessment of virus replication and effects on islet morphology and insulin release. Int J Exp Diabetes Res 1:165–175
17. Hodik M, Anagandula M, Fuxe J et al (2016) Coxsackie-adenovirus receptor expression is enhanced in pancreas from patients with type 1 diabetes. BMJ Open Diabetes Res Care 4: e000219
18. Jenson AB, Rosenberg HS, Notkins AL (1980) Pancreatic islet-cell damage in children with fatal viral infections. Lancet 2:354–358
19. Ujevich MM, Jaffe R (1980) Pancreatic islet cell damage. Its occurrence in neonatal coxsackievirus encephalomyocarditis. Arch Pathol Lab Med 104:438–441
20. 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
21. Richardson SJ, Leete P, Bone AJ, Foulis AK, Morgan NG (2013) Expression of the enteroviral capsid protein VP1 in the islet cells of patients with type 1 diabetes is associated with induction of protein kinase R and downregulation of Mcl-1. Diabetologia 56:185–193
22. Krogvold L, Edwin B, Buanes T et al (2015) Detection of a low-grade enteroviral infection in the islets of langerhans of living patients newly diagnosed with type 1 diabetes. Diabetes 64:1682–1687
23. Klingel K, Hohenadl C, Canu A et al (1992) Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: quantitative analysis of virus replication, tissue damage, and inflammation. Proc Natl Acad Sci U S A 89:314–318
24. Andreoletti L, Hober D, Becquart P et al (1997) Experimental CVB3-induced chronic myocarditis in two murine strains: evidence of interrelationships between virus replication and myocardial damage in persistent cardiac infection. J Med Virol 52:206–214
25. Klingel K, Stephan S, Sauter M et al (1996) Pathogenesis of murine enterovirus myocarditis: virus dissemination and immune cell targets. J Virol 70:8888–8895
26. Chapman NM, Kim KS, Drescher KM, Oka K, Tracy S (2008) 5′ terminal deletions in the genome of a coxsackievirus B2 strain occurred naturally in human heart. Virology 375:480–491
27. Kim KS, Tracy S, Tapprich W et al (2005) 5′-terminal deletions occur in coxsackievirus B3 during replication in murine hearts and cardiac myocyte cultures and correlate with encapsidation of negative-strand viral RNA. J Virol 79:7024–7041
28. Oka K, Oohira K, Yatabe Y et al (2005) Fulminant myocarditis demonstrating uncommon morphology—a report of two autopsy cases. Virchows Arch 446:259–264

29. Ashton MP, Eugster A, Walther D et al (2016) Incomplete immune response to coxsackie B viruses associates with early autoimmunity against insulin. Sci Rep 6:32899
30. Landau BJ, Whittier PS, Finkelstein SD et al (1990) Induction of heterotypic virus resistance in adult inbred mice immunized with a variant of Coxsackievirus B3. Microb Pathog 8:289–298
31. Kutubuddin M, Simons J, Chow M (1992) Identification of T-helper epitopes in the VP1 capsid protein of poliovirus. J Virol 66:3042–3047
32. Mahon BP, Katrak K, Mills KH (1992) Antigenic sequences of poliovirus recognized by T cells: serotype-specific epitopes on VP1 and VP3 and cross-reactive epitopes on VP4 defined by using CD4+ T-cell clones. J Virol 66:7012–7020
33. Katrak K, Mahon BP, Minor PD, Mills KH (1991) Cellular and humoral immune responses to poliovirus in mice: a role for helper T cells in heterotypic immunity to poliovirus. J Gen Virol 72(Pt 5): 1093–1098
34. Beck MA, Tracy SM (1989) Murine cell-mediated immune response recognizes an enterovirus group-specific antigen(s). J Virol 63:4148–4156
35. Wang KG, Sun LZ, Jubelt B, Waltenbaugh C (1989) Cell-mediated immune responses to poliovirus. I. Conditions for induction, characterization of effector cells, and cross-reactivity between serotypes for delayed hypersensitivity and T cell proliferative responses. Cell Immunol 119:252–262
36. Drescher KM, von Herrath M, Tracy S (2015) Enteroviruses, hygiene and type 1 diabetes: toward a preventive vaccine. Rev Med Virol 25:19–32
37. Yoon JW, Austin M, Onodera T, Notkins AL (1979) Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 300:1173–1179
38. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, Ilonen J (2005) Recombination in circulating human enterovirus B: independent evolution of structural and non-structural genome regions. J Gen Virol 86:3281–3290
39. Hamalainen S, Nurminen N, Ahlfors H et al (2014) Coxsackievirus B1 reveals strain specific differences in plasmacytoid dendritic cell mediated immunogenicity. J Med Virol 86:1412–1420
40. Anagandula M, Richardson SJ, Oberste MS et al (2014) Infection of human islets of langerhans with two strains of Coxsackie B virus serotype 1: assessment of virus replication, degree of cell death and induction of genes involved in the innate immunity pathway. J Med Virol 86:1402–1411
41. Viskari HR, Koskela P, Lonnrot M et al (2000) Can enterovirus infections explain the increasing incidence of type 1 diabetes? Diabetes Care 23:414–416
42. Honkanen H, Oikarinen S, Nurminen N et al (2017) Detection of enteroviruses in stools precedes islet autoimmunity by several months: possible evidence for slowly operating mechanisms in virus-induced autoimmunity. Diabetologia 60:424–431
43. Oikarinen S, Martiskainen M, Tauriainen S et al (2011) Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes 60:276–279
44. Sedgwick P (2014) Nested case-control studies: advantages and disadvantages. BMJ 348:g1532