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ARTICLE

No evidence of enteroviruses in the intestine of patients with type 1 diabetes

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Abstract
Aims/hypothesis The purpose of this study was to investigate whether the gut mucosa is a reservoir for enterovirus persistence in patients with type 1 diabetes.
Methods Small intestine biopsy samples from 25 individuals at different stages of type 1 diabetes, 21 control individuals and 27 individuals with coeliac disease were analysed for the presence of enterovirus RNA by using both radioactive in-situ hybridisation and real-time RT-PCR and for the presence of enterovirus proteins by immunostaining with antibodies against VP1 and VP4-2-3 capsid proteins and virus polymerase. Lymphocytic enteropathy and serum anti-VP1 antibodies were also evaluated at the time of biopsy. Moreover, high-throughput sequencing was performed to identify viral transcripts or genomes.
Results Enterovirus was not detected by in-situ hybridisation or RT-PCR in any of the individuals tested. Immunohistology revealed a few stained cells in the intestinal epithelium in a low number of individuals, with no difference between diabetic and non-diabetic individuals.

L. Piemonti and E. Bosi contributed equally to this study.

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Levels of serum IgG against VP1 did not differ between control individuals and those with diabetes or coeliac disease and no evidence of diabetes-related lymphocytic enteropathy was detected. High-throughput sequencing did not reveal specific enterovirus sequences in the gut mucosa of individuals with type 1 diabetes.
Conclusions/interpretation Prolonged/persistent enterovirus infections in gut mucosa are not common in patients with type 1 diabetes.

Keywords Enterovirus · Intestine · Type 1 diabetes

Abbreviations

EIA	Enzyme immunoassay
HEV	Human enterovirus
HPV	Human papillomavirus
IEL	Intra-epithelial lymphocyte
ISH	In-situ hybridisation
LIPS	Luminescent immunoprecipitation system
qRT-PCR	Quantitative RT-PCR

Introduction

Several viral infections have been associated with human type 1 diabetes, including those caused by human enterovirus (HEV) [1–7], rubella [8, 9], mumps [10, 11], rotavirus [12–14] and cytomegalovirus [15–17]. The mechanisms by which viruses might trigger type 1 diabetes remain unclear, but various pathogenetic models, not mutually exclusive, have been advocated [7, 18]. First, viruses may directly infect beta cells in the pancreatic islets, resulting in direct beta cell cytolysis. Second, viruses may infect beta cells without inducing cytolysis but may break self-tolerance owing to the expression of viral antigens in the beta cells, exposure of the immune system to altered beta cell antigens and/or increased expression of MHC antigens or cytokines and chemokines. Third, a viral infection may enhance autoimmunity by increasing autoreactive T cell responses by either bystander activation (including activation of autoreactive T cells) or selective deletion/impairment of regulatory T cells. Fourth, epitopes of viral proteins may have structural homology with beta cell autoantigens (molecular mimicry) and activation of cross-reacting T or B cells may induce beta cell destruction.

Recently Oikarinen et al. have suggested a new hypothesis on how viruses could trigger type 1 diabetes [1, 19]: they propose that in patients with type 1 diabetes the gut mucosa could act as an enterovirus reservoir from which viruses can spread to the pancreas. Moreover, viral persistence in the gut mucosa could maintain a chronic inflammatory milieu responsible for the promotion of islet autoreactivity by a bystander activation mechanism. In agreement with this hypothesis, they have reported the presence of enterovirus RNA in the small-bowel mucosa more frequently in patients with type 1 diabetes than in control individuals [1]. This finding could open new opportunities for studying the viral aetiology of type 1 diabetes and confirmatory observations are needed to validate its consistency and relevance. The aim of this study was to look for the presence of enteroviruses in small-intestine biopsies of patients with type 1 diabetes.

Methods

Study population Study participants included 73 individuals who underwent gastroduodenal endoscopy and biopsy from the distal duodenum at the Gastroenterology Unit, San Raffaele Scientific Institute, Milan. Biopsy samples were taken between 31 January 2005 and 25 October 2006. Indications for the diagnostic procedure were based on suspicion of some gastrointestinal disorders, including coeliac disease, or volunteer enrolment in the study (see Table 1). For the purpose of this investigation, participants were divided into three groups: 25 individuals with type 1 diabetes, 27 individuals with coeliac disease and 21 healthy individuals. Participants considered to have type 1 diabetes were further classified according to disease duration: two were in the pre-clinical phase, having one or more diabetes-specific autoantibodies, but not yet on insulin therapy; the remainder were diagnosed according to ADA criteria (12 with short disease duration [median 1.5 years, range 6 months to 5 years] and 11 with long-term disease duration [median 17 years, range 6–43 years]). Seven of the 25 participants with type 1 diabetes also had coeliac disease and three were positive for transglutaminase autoantibody without histological evidence of coeliac disease (in all cases type 1 diabetes was diagnosed first); for the purpose of this study, these individuals were included in the type 1 diabetes group. Individuals with coeliac disease were all investigated at the time of diagnosis, based on histological findings of intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy on duodenal biopsy, mostly associated with transglutaminase IgA antibodies; all participants lately diagnosed as having coeliac disease were on a normal gluten-containing diet at the time of the biopsy. Healthy participants, who did not have diabetes or coeliac disease, were negative for diabetes-associated and coeliac disease-associated autoantibodies and were considered as controls in this study. Demographic and clinical characteristics of study participants are summarised in Table 1. The study protocol was approved by the Ethics Committee of the San Raffaele Scientific Institute and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. Written informed consent was obtained from all adult participants (or from childrens' parents) before biopsy collection.

Table 1 Characteristics of study participants

Characteristic	Control individuals	Individuals with type 1 diabetes[a]			Individuals with coeliac disease
		Anti-islet Abs	Duration ≤5 years	Duration >5 years	
No. of participants	21	2	12	11	27
Age (years)	16 (2–67)	40 (39–41)	12 (3–37)	26 (11–77)	7 (1–20)
Males/females (*n*/*n*)	9/12	0/2	7/5	6/5	9/18
Diabetes duration (years)	–	–	1.5 (0.5–5)	17 (6–43)	–
Age at onset (years)	–	–	11.5 (1–32)	8 (1–57)	–
HbA_{1c} (%)	–	–	7.7 (6.2–13.9)	9.2 (7.3–11.4)	–
HbA_{1c} (mmol/mol)			60.65 (44.26–128.42)	77.05 (56.28–101.09)	–
Autoimmunity (*n*/*n*)					
GADA	0/21	2/2	11/12	7/11	0/27
IA-2A	0/21	1/2	7/12	3/11	0/27
IAA	0/21	0/2	6/12	7/11	1/27
TGG	0/21	0/2	6/12	2/11	23/27
TGA	0/21	0/2	7/12	2/11	24/27
TMA	0/21	1/2	2/12	2/11	3/27
Reason for biopsy (*n*/*n*)					
Volunteer	0/21	0/2	2/12	4/11	0/27
Suspected coeliac disease	1/21	2/2	8/12	3/11	27/27
Suspected food allergy	3/21	0/2	0/12	0/11	0/27
Dyspepsia	6/21	0/2	0/12	1/11	0/27
Emesis	4/21	0/2	0/12	0/11	0/27
Epigastralgia	2/21	0/2	0/12	2/11	0/27
Dysphagia	2/21	0/2	1/12	0/11	0/27
Gastro-oesophageal reflux	3/21	0/2	1/12	1/11	0/27

Data are median (range) unless otherwise indicated

[a] Seven of the individuals with type 1 diabetes also had a diagnosis of coeliac disease and three were positive for transglutaminase autoantibody without histological evidence of coeliac disease

Abs, antibodies; GADA, glutamic acid decarboxylase antibodies; IA-2A, protein-tyrosine-phosphatase-2 autoantibodies; IAA, insulin autoantibodies; TGA, anti-tissue transglutaminase IgA antibodies; TGG, anti-tissue transglutaminase IgG antibodies; TMA, thyroid microsomal autoantibodies

Enterovirus RT-PCR Unfixed biopsy samples upon collection were immediately frozen on dry ice and stored frozen at −80°C. Total RNA was extracted using the method of Chomczynski and Sacchi [20]. Detection of enteroviral RNA was performed by quantitative real-time RT-PCR using the ENTEROVIRUS R-gene kit (Argene, Varilhes, France) [21] that amplifies the highly conserved 5′ noncoding region of HEV-A (coxsackievirus A4, A6–A8, A10, A14, A16, A16V, enterovirus 71, 76), HEV-B (coxsackievirus A9, B1–B6, echovirus 1–7, 9, 11–21, 24–27, 29–33, enterovirus 69, 74, 75, 77, 78, 93), HEV-C (coxsackievirus A11, A13, A17, A20, A21, A24, A24V, poliovirus 1, 2, 3) and HEV-D (enterovirus 68, 70, 94). All analyses were performed blind to the case-control status of the study participants. Samples were available from 70/73 study participants.

In-situ hybridisation In-situ hybridisation (ISH) for the detection of enteroviral RNA was carried out on formalin-fixed and paraffin-embedded biopsy samples (5 μm sections) as previously described [22]. Positive-strand RNA of group A and B coxsackieviruses and echoviruses were detected by a highly specific and sensitive (20 viral copies/per cell) radioactive ISH technique using single-stranded ^{35}S-labelled RNA probes (length 100–400 bp), which were synthesised from the dual-promoter plasmid pCVB3-R1 covering the complete length of the CVB3 genome. Control RNA probes were obtained from the vector pSPT18 [22]. All analyses were performed blind to the case-control status of the study participants. Samples were available from 56/73 study participants.

Immunohistochemical staining Formalin-fixed paraffin-embedded biopsy samples (3 μm sections) were stained

with anti-VP1 antibody (clone 5-D8/1, 1:1000; Novocastra, Newcastle, UK), polyclonal rabbit sera anti-VP1 capsid protein (1:500), anti-VP4-2-3 capsid proteins (1:500) and anti-virus polymerase (1:600) (these sera were kindly provided by K. Klingel, University Hospital of Tübingen, Tübingen, Germany) using En Vision+System-HRP Labelled polymer Anti-Rabbit (K4002; Dako, Carpinteria, CA, USA) and DAB (K3468; Dako) as a detection system. All these non-commercial sera were obtained after immunisation of rabbits with coxsackievirus B3 and were cross-reactive with many coxsackieviruses and other enteroviruses [23]. Known virus-positive tissue (murine) and cell culture samples were used to confirm the staining reliability of all separate staining batches (Fig. 1). The positivity was evaluated in epithelial cells, entero-endocrine cells, Brunner's gland cells (compound tubular submucosal glands found in the duodenum above the hepatopancreatic sphincter) and muscularis mucosae cells. Samples were available from 64/73 study participants. To facilitate quantitative analysis, a case was deemed positive for enterovirus when at least one intensely stained cell was present in any cell compartment within any given section. $CD3^+$ intra-epithelial lymphocytes (IELs) were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA, USA). Positive IELs were counted with a 3,100 objective throughout the surface epithelium and biopsies scored as normal (<25 IEL/100 enterocytes), mild lymphocytic enteropathy (25–40 IEL/100 enterocytes) or strong lymphocytic enteropathy (>40 IEL/100 enterocytes). Samples were available from 66/73 study participants. All evaluations were carried out by two independent pathologists blind to disease history or laboratory findings.

Serum antibodies (IgG) against VP1 A luminescent immunoprecipitation system (LIPS) assay [24] for the measurement of antibody responses to the enteroviral capsid protein VP1 was developed by cloning the VP1 coding sequence of the echovirus 11D207 into the pTnT plasmid vector (Promega, Milan, Italy) in frame with the coding sequence of a modified *Renilla* luciferase. A recombinant Rluc/VP1 fusion protein was then produced in vitro using the pTnT-quick SP6 rabbit reticulocyte lysate cell-free system (Promega). The luciferase activity of the produced Rluc/VP1 protein was determined using the *Renilla* Luciferase Assay System (Promega) according to the manufacturer's instruction on a Centro xS3 luminometer (Berthold, Bad Wildbad, Germany). For anti-VP1 antibody measurement, serum samples (1 μl each) were then incubated with Rluc/VP1 (4×10^6 light unit equivalents per sample) for 2 h at room temperature followed by recovery of immune complexes by addition of protein-A-sepharose (G.E. Healthcare, Milan, Italy) and 1 h incubation at 4°C, followed by

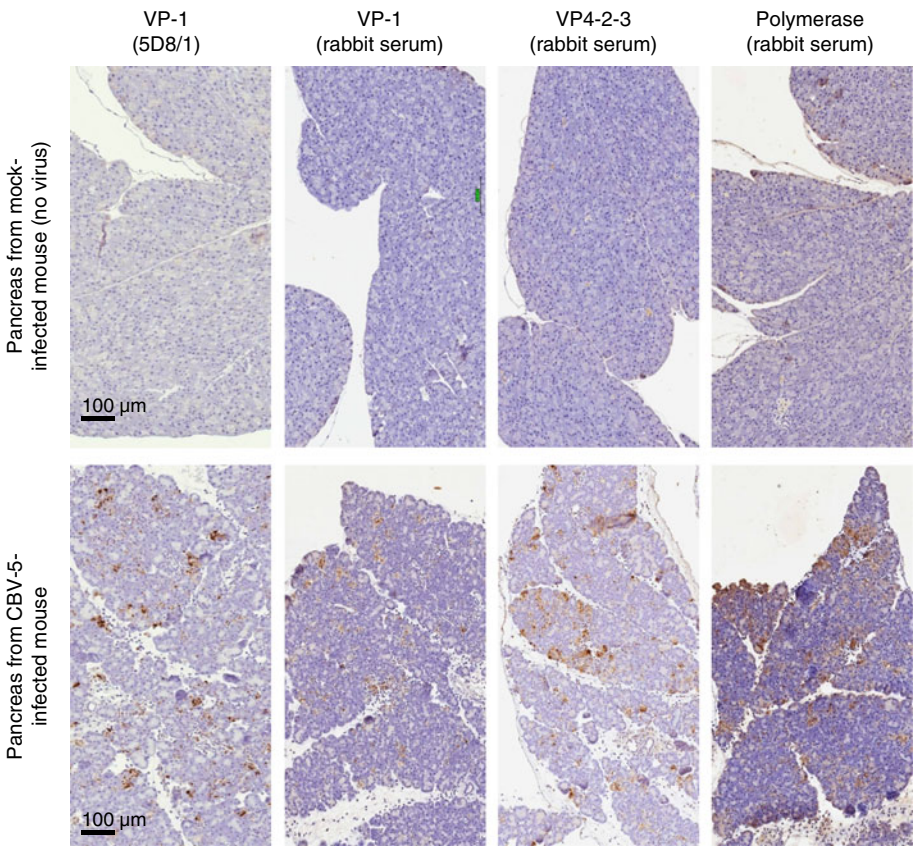

Fig. 1 Immunohistochemical analysis of anti-enterovirus antibody staining in pancreas from mock-infected or coxsackie B virus 5 (CBV-5)-infected mice. To confirm the ability of antibodies used to identify enterovirus immunoreactivity in the intestine of patients with type 1 diabetes, we initially examined their reactivity in formalin-fixed, paraffin-embedded pancreas samples taken from CBV-5-infected mice. Pancreatic samples from CBV-5-infected mice contained many cells that were strongly immunopositive for enterovirus. By contrast, none of the samples examined from mock-infected mice were immunopositive for enterovirus

washing in 96-well Costar 3504 filter plates. After washing recovered luciferase, activity was measured in a luminometer using the *Renilla* Luciferase Assay System substrate. Results were expressed as arbitrary units using an antibody index relative to anti-VP1 positive rabbit polyclonal serum obtained by immunisation with the EAIPALTAVETGHTSQVC coxsackievirus peptide. As further controls, polyclonal sera were tested from rabbits immunised either with whole viruses (echovirus 11 D207, coxsackieviruses CVA-18, CVB-5) or peptide sequences from the VP1 enteroviral capsid protein (EAIPALTAVETGHTSQVC, KEVPALTAVETGATC). Results obtained in LIPS for VP1 antibodies showed a significant degree of correlation with those obtained in a conventional enzyme immunoassay (EIA) for the enterovirus group-reactive antigen peptide B995 (amino acid sequence KEVPALTAVETGATC) coupled to BSA (Spearman R= 0.66, p<0.0001) (data not shown). All analyses were performed blind to the case-control status of the study participants. Samples were available from 49 of the 73 study participants.

Presence and characterisation of viral transcripts or genomes via deep sequencing and homology search in sequence databases DNA was extracted from three sections of 5 μm formalin-fixed paraffin-embedded biopsy samples as previously described using xylene and proteinase K [25]. The procedure of deep sequencing is described in the electronic supplementary material (ESM) Methods.

Statistical analysis Statistical analyses were performed using SPSS 13.0 for Windows (SPSS, Chicago, IL, USA). Frequency comparison was performed with the Pearson χ^2 and Fisher exact tests, and continuous variables were analysed by independent sample t test or by the Kruskal–Wallis test.

Results

In-situ hybridisation and RT-PCR Small-intestine biopsy samples were screened for the presence of enterovirus genome (Table 2). In none of the individuals tested was enterovirus RNA detected either by radioactive ISH (Fig. 2) or by real-time RT-PCR (ESM Fig. 1).

Immunohistochemical study The lack of viral RNA detection was confirmed by the absence of staining for viral proteins, including VP1 and VP4-2-3 capsid proteins and virus polymerase (Fig. 3 and Table 3). By using monoclonal anti-VP1 clone 5-D8/1 antibody, a few positive cells in the intestinal epithelium were stained in three study participants (two controls and one with type 1 diabetes). Two other participants (one control, one with coeliac disease) showed a few positive cells in Brunner's glands. Positive staining of smooth muscle cells in the muscularis mucosae was seen in nine study participants (two controls, three with coeliac disease, four with type 1 diabetes), but was considered to be non-specific, since this type of cell is known to be refractory to enteroviral infection [26, 27]. As a whole, the immunohistochemical analysis with VP1 clone 5-D8/1 antibody suggested enteroviral infection in five (7.5%) study participants: three controls, one with coeliac disease and one with type 1 diabetes (p=NS). To confirm the data obtained with the VP1 clone 5-D8/1 antibody, the staining pattern was compared with that obtained using a polyclonal antiserum raised against VP1 (designated VP1AK) and additional pan-enteroviral polyclonal antibodies not specifically directed against VP1, namely anti-VP4-2-3 and anti-virus

Table 2 Positive findings for enterovirus RNA by ISH and RT-PCR analysis in small-intestine biopsy samples

Participant group	RNA enterovirus	
	qRT-PCR[a]	ISH[b]
Control individuals	0/21	0/16
Individuals with type 1 diabetes		
Anti-islet Abs	0/2	0/1
Duration ≤5 years	0/12	0/6
Duration >5 years	0/11	0/8
Individuals with coeliac disease	0/27	0/25

Data are no. of samples positive for enterovirus/no. of samples examined

[a] Samples were available from all 73 study participants

[b] Samples were available from 56/73 study participants

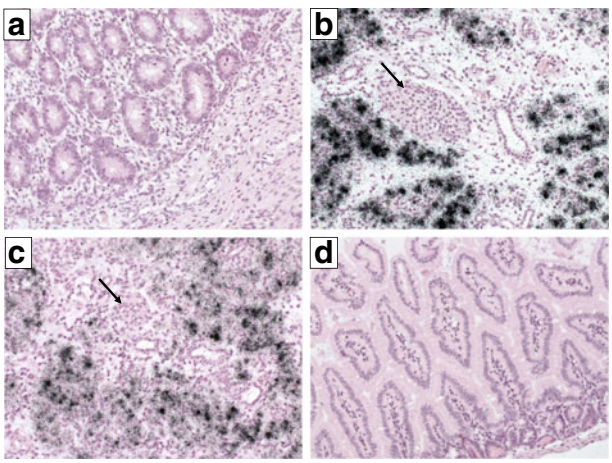

Fig. 2 Radioactive ISH for the detection of enteroviral RNA. No positive cells were visualised in gut biopsies of individuals with type 1 diabetes (**a**). In contrast, ABY/SnJ mice infected with 1×10^5 plaque-forming units of CVB3 revealed in-situ positive signals in all pancreatic exocrine cells but not in islets (black arrows) or vessels at day 4 post infection (**b**) and at day 8 post infection (**c**) (positive controls). No viral RNA was detected in the gut of these mice (**d**). Magnification: ×200 in all images

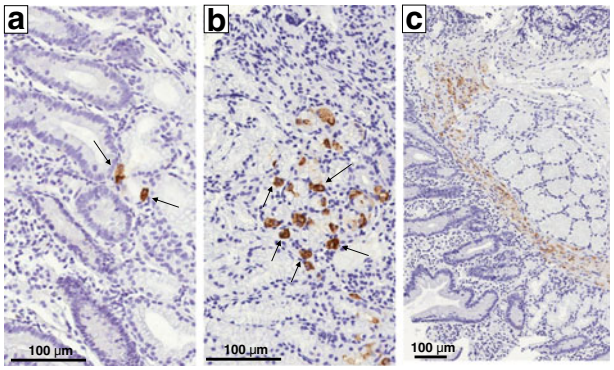

Fig. 3 Detection of enterovirus in small intestine biopsy samples. The brown colour indicates the presence of enterovirus VP1 protein (immunohistochemistry, clone 5-D8/1 antibody). **a** Crypt epithelium from a patient with diabetes (magnification: ×400). **b** Brunner's glands from a control individual (magnification: ×400). **c** Muscularis mucosae from a control individual (magnification: ×200). Some virus-positive cells are marked by arrows

polymerase 3D. Of the five biopsy samples demonstrating positivity with VP1 clone 5-D8/1 antibody, none stained positive with VP1AK, VP4-2-3 or polymerase antiserum (Table 3).

VP1 antibodies Fifteen study participants with type 1 diabetes, 14 with coeliac disease and 20 control individuals were analysed for the presence of serum IgG antibodies against VP1 at the time of the study and biopsy collection, with no differences found among the groups (Fig. 4).

Lymphocyte intestinal infiltration A total of 20 study participants with type 1 diabetes, 25 with coeliac disease and 21 control individuals were analysed for the presence of $CD3^+$ intraepithelial lymphocytes (Table 4). As expected, the prevalence of lymphocytic enteropathy was significantly higher in individuals with coeliac disease than in control individuals (88% and 0%, respectively; $p<0.001$). In individuals with type 1 diabetes, lymphocytic enteropathy was detected only in those with concomitant coeliac disease or transglutaminase autoantibodies (see Table 4).

Deep viral sequencing Deep sequencing investigation found ten viral sequences, but none of them were related to enteroviruses. Four were related to human papillomaviruses (HPVs). One sequence belonged to HPV9 whereas the three other sequences were new HPV sequences (here designated as HPV SE89a, SE89b and SE89c). The sequences were not

Table 3 Positive staining for enterovirus protein in immunohistochemical analysis

Tissue/participant	*n*	VP1 5D8/1	VP4-2-3 VP1AK	Polymerase	
Epithelium[a]					
Control individuals	17	2 (11.7)	0 (0)	0 (0)	0 (0)
Individuals with coeliac disease	26	0 (0)	0 (0)	0 (0)	0 (0)
Individuals with type 1 diabetes	23	1 (4.3)	0 (0)	0 (0)	0 (0)
Enteroendocrine cells[a]					
Control individuals	17	0 (0)	0 (0)	0 (0)	0 (0)
Individuals with coeliac disease	26	0 (0)	1 (3.8)	2 (7.7)	0 (0)
Individuals with type 1 diabetes	23	0 (0)	0 (0)	1 (4.3)	0 (0)
Endothelium[a]					
Control individuals	17	0 (0)	0 (0)	0 (0)	0 (0)
Individuals with coeliac disease	26	0 (0)	0 (0)	0 (0)	0 (0)
Individuals with type 1 diabetes	23	0 (0)	0 (0)	0 (0)	0 (0)
Brunner's glands[b]					
Control individuals	10	1 (10)	0 (0)	0 (0)	0 (0)
Individuals with coeliac disease	15	1 (6.6)	0 (0)	0 (0)	0 (0)
Individuals with type 1 diabetes	16	0 (0)	2 (12.5)	0 (0)	0 (0)
Muscularis mucosae[a]					
Control individuals	17	2 (11.7)	0 (0)	0 (0)	0 (0)
Individuals with coeliac disease	26	3 (11.5)	0 (0)	0 (0)	0 (0)
Individuals with type 1 diabetes	23	4 (17.3)	0 (0)	0 (0)	0 (0)
Total					
Control individuals	17	3 (17.6)	0 (0)	0 (0)	0 (0)
Individuals with coeliac disease	26	1 (3.8)	1 (3.8)	2 (7.7)	0 (0)
Individuals with type 1 diabetes	23	1 (4.3)	2 (8.7)	1 (4.3)	0 (0)

Data are number of individuals (%)

[a]Samples were available from 66/73 study participants

[b]Evaluated when present in biopsy

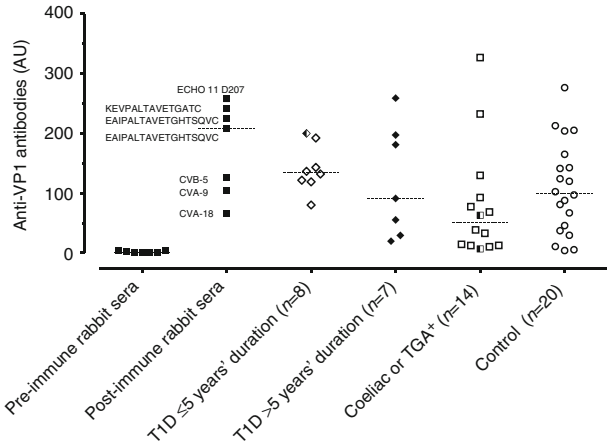

Fig. 4 Anti-VP1 antibody responses did not differ between individuals with type 1 diabetes (T1D) or coeliac disease or control individuals. Serum level of anti-VP1 IgG response in 47/73 of the study participants was measured in LIPS. Results are also shown for rabbit polyclonals, pre- and post-immunisation with whole viruses or enteroviral VP1 peptides. The dashed line indicates the median value. The split diamond symbol corresponds to an individual who has diabetes-specific autoantibodies but does not have diabetes, while the split square symbols identify individuals with anti-transglutaminase antibodies only (TGA^+). AU, arbitrary units

overlapping but as they shared an equal degree of similarity (81–86%) to the HPV isolate 915F 06 002 KN2, it is possible that they came from the same virus. Of the six remaining sequences, three were known herpesviruses and three were other known viruses (one each of entomopoxvirus, chlorella virus and *Cafeteria roenbergensis* virus). There were too few observations to enable any conclusions about possible case-control association of the viruses found.

Discussion

Recently, a new hypothesis has been proposed on the possible trigger of type 1 diabetes by viral infection [1]. In fact, Oikarinen et al. [1], based on detection of signs of persistent viral infection in the gut of type 1 diabetic patients, hypothesised that the gut mucosa might act as a viral reservoir from which viruses can spread to the pancreas, maintain a chronic inflammation milieu in this district and promote islet autoreactivity via a bystander activation mechanism. This fascinating hypothesis was supported by a more frequent detection of enteroviral RNA in the small intestine of patients with type 1 diabetes compared with control individuals and by the increased inflammation activity in the gut mucosa associated with enteroviral RNA. In the present study, we tried to confirm these results. However, our findings seem to reject this hypothesis by excluding the presence of a persistent enteroviral infection in the gut mucosa of individuals with type 1 diabetes. Supporting evidence includes: (1) none of the individuals tested, irrespective of whether they had type 1 diabetes or coeliac disease, or were healthy controls, had enterovirus RNA detected by the highly sensitive ISH and RT-PCR assays; (2) in the immunohistochemistry study only a few intestinal cells in a few individuals were stained by enteroviral antigen-specific antisera, with no segregation in the group with type 1 diabetes; (3) titres of serum anti-VP1 IgG were not different between individuals with type 1 diabetes and control individuals; (4) lymphocytic enteropathy was not visible in individuals with type 1 diabetes, unless there was a histological or serological indication of coeliac disease; and (5) high-throughput sequencing did not reveal specific enterovirus sequences in the gut mucosa of individuals with type 1 diabetes. The timing and methodology of sample storage are unlikely to have introduced a bias into our results. Formalin-fixed paraffin-embedded biopsy samples were all used between 1 and 4 years after collection. The detection of enteroviral genomes by radioactive ISH is even possible in 30-year-old paraffin-embedded pancreatic and heart tissue [27, 28], demonstrating that viral RNA is rather stable even in such difficult tissues as the pancreas. Viral capsid protein is generally stable and no warning about false negative results has been reported in short-term storage. Even if viral RNA degradation cannot be ruled out completely in unfixed

Table 4 $CD3^+$ intraepithelial lymphocytes (IELs)

Participant group	Lymphocytic enteropathy		
	<25 $CD3^+$ IELs/100 enterocytes	25–40 $CD3^+$ IELs/100 enterocytes	>40 $CD3^+$ IELs/100 enterocytes
Control individuals	21 (100)	0 (0)	0 (0)
Individuals with type 1 diabetes	14 (70)	2 (10)	4 (20)
With coeliac disease or transglutaminase autoantibodies	3 (33.3)	2 (22.2)	4 (44.4)
Without coeliac disease or transglutaminase autoantibodies	11 (100)	0 (0)	0 (0)
Coeliac disease patients	3 (12)	3 (12)	19 (76)

Data are *n* (%)

biopsies, the −80°C storage temperature, the avoidance of freeze/thaw cycles and the relatively short-term storage of specimens make a false negative result unlikely. Also, high-throughput sequencing did not detect any enteroviruses, although some other viruses were detected. Although the exact sensitivity of the method is not known and low levels of enteroviruses may have escaped detection, our data do indicate that they are not among the most abundant viruses in the gut mucosa.

More than one explanation could justify the discrepancy of our findings from those of Oikarinen et al. [1]. A likely explanation is that we have studied a population with different demographic characteristics in view of the fact that in our study the participants with type 1 diabetes are younger (median age: 12 vs 43 years) with a shorter disease duration (median duration: 4 vs 20 years) and earlier onset (median age at onset: 11 vs 18 years) in comparison with the population with diabetes in Oikarinen's study. If this is the case, it can be concluded that the persistent enteroviral infection in the gut mucosa is not a pathogenetic factor for type 1 diabetes but, more probably, a consequence of the long-standing nature of the diabetes. However, in our, as well as in Oikarinen's, cohort enterovirus detection was not associated with the duration of disease or with older age, which indirectly contradicts this possibility. A second explanation could be related to the regional differences between the two cohorts of study participants. In fact, although coxsackievirus infections have worldwide distribution, we cannot exclude the possibility that their role as a pathogenetic factor for type 1 diabetes could be different depending on the genetic background of particular populations. For example, diabetes-associated polymorphisms, such as those of the interferon-induced helicase 1 gene [29], seem to be associated with a strong innate immune response, which may lead to an enhanced inflammation response during viral infection [30]. Since these genes are likely to mediate the recognition of enteroviruses by the innate immune system, different regional prevalence of their polymorphisms could modulate the outcome of virus infections in diabetes pathogenesis. Moreover, we cannot exclude an additional role of genetic polymorphisms in the development of enterovirus persistence. A third explanation could be related to the different methods used to detect enteroviruses in the gut mucosa. Reliable detection of enterovirus from tissue samples is not a trivial issue. In general, the most widely adopted detection method in tissue analysis is immunohistochemical staining with the monoclonal antibody 5D8/1 against the enterovirus VP1 protein. However, this antibody has its weaknesses, including background staining and cross-reactivity. For this reason the importance of confirming results using an alternative antibody or detection method, such as ISH and RT-PCR, is generally accepted.

We investigated the presence of enteroviral RNA by using both radioactive ISH and a commercial quantitative RT-PCR (qRT-PCR) TaqMan assay validated for diagnostic use. Unlike Oikarinen et al. [1] who had previously found enteroviral RNA in 74% of individuals with type 1 diabetes and 29% of control samples, we found no enteroviral RNA by the well-established method of radioactive ISH. It is worth noting that Okairinen et al. used a single oligonucleotide probe for ISH while our study adopted ^{35}S-labelled RNA probes covering the full enteroviral genome (7,500 bp), a strategy with the highest demonstrated sensitivity and specificity [22, 26]. In fact, with the ability to detect about 20 enteroviral genomes within a cell [31] and a sensitivity equivalent to that of RT-PCR, radioactive ISH is definitely the most sensitive method used to detect enteroviral genomes in tissues and cannot be the limiting factor for our negative results. Another discrepancy was observed between our completely negative results in qRT-PCR analysis and those of Okairinen et al., which confirmed by PCR the positivity in ISH in less than 20% of samples. In addition to these discrepancies, they presented a nuclear localisation for enteroviral RNA rather than the cytoplasm in enterocytes suggesting a technical problem of ISH specificity as enteroviruses have never before been detected in the nuclei of any cell type.

We also investigated the presence of enteroviral proteins immunohistochemically by staining using a panel of three different antibodies in addition to the anti-VP1 monoclonal antibody 5-D8/1 used by Okairinen et al. [1]. Although the 5-D8/1 monoclonal has been previously used in several studies for the detection of enteroviral proteins in different tissues, this antibody proved to react not only with virus-specific epitopes but also with unrelated cellular proteins (e.g. heat-shock protein 60/65 and tyrosine phosphatases IA-2/IAR) [32, 33]. Our immunohistochemical staining performed with multiple antibodies against different regions of the enterovirus demonstrated a few positive cells in the intestinal epithelium of a few individuals and without segregation in patients with type 1 diabetes. Moreover, there was no concordance either in the positivity or in cellular compartments between the different antibodies used. Since no enteroviral protein can be translated without viral RNA, it is likely that positive immunostaining for viruses obtained in the absence of viral RNA indicates the recognition of cellular antigens by the antibodies (unspecific staining/cross-reactivity).

In summary, in their studies Oikarinen et al. [1] detected enteroviral antigen-positive intestinal cells by immunostaining only with the anti-VP1 5-D8/1 monoclonal antibody and by non-radioactive ISH using a short oligonucleotide probe. Both these methods are known to have problems involving unspecific reactions against diverse human tissues, including the intestine, possibly explaining the discrepant results of viral RNA and protein detection in the gut of individuals with type 1 diabetes in different studies [1, 19]. In fact, in

their first published report, Oikarinen et al. suggested that enteroviruses may be present in the small intestine of 75% of individuals with type 1 diabetes and 10% of control individuals on the basis of anti-VP1 reactivity in immunohistochemical studies using the 5-D8/1 monoclonal antibody [19]. In contrast, in their most recent study, positivity for VP1 protein in the gut was noticeably reduced and the presence of enteroviruses was reported mainly at the RNA level, in the absence of infective viral particles and of capsid protein synthesis [1].

In conclusion, as recently suggested [1], it is possible that (persistent) enteroviral infection in the gut might be related to the pathogenesis of type 1 diabetes. Indeed, replication of the virus in the gut could provide a virus reservoir anatomically contiguous and connected to the pancreas, possibly representing a source of pancreatic infection. However, this observation has to be definitively proven before it may be considered to have clinical relevance. A relatively small number of gut biopsy samples, which were not collected to answer this specific question, were studied here and also in the paper by Oikarinen. Further studies, preferably based on dedicated gut mucosa sampling, are required to elucidate this phenomenon and to obtain more data about the proportion of individuals with diabetes, those who have diabetes-specific autoantibodies but do not have diabetes and control individuals who harbour the virus in the gut mucosa. In addition, the overall consistency of this evidence should be established by using well-controlled molecular and virological techniques.

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Contribution statement AMer, EB and LP led the design, analysis and interpretation of data and drafted the article. JD and MR analysed data and revised the article for important intellectual content. AMar, VL, KK, LA, CL, JE, DB, AB and VS were involved in the conception and design of the study, interpretation of data and revision of the article for important intellectual content. All authors gave final approval of the version to be published. LP 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.

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