Enterovirus Infection and Progression from Islet Autoimmunity to Type 1 Diabetes: The Diabetes and Autoimmunity Study in the Young (DAISY)

*Short title:* Enterovirus and progression to type 1 diabetes

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

**Methods:** Since 1993, DAISY has followed 2,365 genetically predisposed children for islet autoimmunity and type 1 diabetes. Venous blood and rectal swabs were collected every 3-6 months after seroconversion for islet autoantibodies (against glutamic acid decarboxylase, insulin or IA-2), until diagnosis of diabetes. Enteroviral RNA in serum or rectal swabs was detected using reverse transcriptase PCR with primers specific for the conserved 5’ non-coding region, detecting essentially all enterovirus serotypes.

**Results:** Of 140 children who seroconverted to repeated positivity for islet autoantibodies at a median age of 4.0 years, 50 progressed to type 1 diabetes during a median follow-up of 4.2 years. The risk of progression to clinical type 1 diabetes in the sample interval following detection of enteroviral RNA in serum (3 diabetes cases diagnosed among 17 intervals) was significantly increased compared to that in intervals following a negative serum enteroviral RNA test (33 cases diagnosed among 1064 intervals; hazard ratio: 7.02, 95% CI: 1.95-25.3, after adjusting for number of autoantibodies). Results remained significant after adjustment for ZnT8-autoantibodies, and after restriction to various subgroups. Enteroviral RNA in rectal swabs was not predictive of progression to type 1 diabetes. No evidence for viral persistence was found.

**Conclusion:** This novel observation suggests that progression from islet autoimmunity to type 1 diabetes may increase after an enterovirus infection characterized by the presence of viral RNA in blood.

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Type 1 diabetes (T1D) results from destruction of the insulin producing beta-cells in the pancreatic islets (1). The majority of patients carry the human leukocyte antigen (HLA) DRB1*03-DQB1*0201 or DRB1*04-DQB1*0302 susceptibility haplotypes or both, but these are not sufficient for development of disease. For many years, viral infections have been suspected to play a role, but the specific etiologic agent(s) in human T1D remains elusive. While several viruses have been linked to T1D, seroepidemiology, histopathology, animal studies, and in vitro experiments have provided the strongest overall evidence for enteroviruses, although results have been somewhat conflicting and not conclusive (2-4).

Autoantibodies to islet autoantigens are present for years prior to diagnosis of T1D (1), and prospective studies testing whether enterovirus could predict islet autoantibodies have yielded conflicting results with positive results in Finnish studies (5-7) and no association found elsewhere (8,9). Results from animal models gave suggested that viral infections can usually not initiate the autoimmune disease process leading to diabetes, but may accelerate an already initiated disease process. Studies in various strains of NOD mice have shown that enteroviral infections may accelerate the progression to diabetes only if they occur after autoreactive T-cells have already accumulated in the islets (10-13). In an attempt to evaluate for the first time whether such a general model of disease progression rather than initiation by enteroviruses applies to human T1D, we tested whether enteroviral infections predict progression to T1D in
children repeatedly positive for islet autoantibodies.

RESEARCH DESIGN AND METHODS

Subjects and design. From 1993 to 2004, children born at St. Joseph’s Hospital in Denver carrying HLA genotypes conferring increased risk for T1D and siblings or offspring of people with T1D (regardless of their genotype) identified from the Barbara Davis Center for Childhood Diabetes have been enrolled in the Diabetes Autoimmunity Study in the Young (DAISY). Informed consent was obtained from parents of all children, and the study was approved by the Colorado Multiple Institutional Review Board. Children were followed longitudinally from soon after birth and screened for islet autoantibodies at ages 9, 15, and 24 months of age, and annually thereafter. Siblings or offspring of persons with T1D were enrolled after nine months of age (median age 1.33 years, range 0.02 - 7.9 years). Children who tested positive for islet autoantibodies were scheduled for more frequent follow-up, with visits at 3-6 month intervals.

The current study is a cohort analysis of all children who tested positive for one or more islet autoantibody on two or more consecutive clinic visits, and provided at least one sample for enterovirus testing during follow-up for T1D. Figure 1 shows a flow chart illustrating how the study cohort was formed. A batch of samples collected 1993-2004 were sent for enterovirus testing in 2005 and another batch of samples collected 2005-June 2007 were sent in 2008. The children were further followed for diagnosis of T1D, and the current analysis includes information on autoantibody status and diabetes up to April 2009. T1D was clinically diagnosed based on American Diabetes Association criteria (14), and details of procedures and clinical characteristics have been described elsewhere (15,16).

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

At each clinic visit, venous blood and rectal swabs were collected. Blood samples were immediately processed, aliquoted and stored at -70°C until testing. Rectal swabs were immediately placed in 1 ml of transport medium (veal infusion broth or M4-3 medium) and stored at -70°C, as previously described (8). Radio-immunoassays were used to measure serum autoantibodies to insulin, GAD65, and IA-2 (BDC512) in George Eisenbarth’s laboratory, as previously described (18-21), with rigorous duplicate testing and confirmation of all positive and a subset of negative results (22). ZnT8 autoantibodies were measured in John Hutton’s laboratory, as previously described using a dimeric construct incorporating monomeric forms of the C-terminus with the polymorphic 325 Arg and Trp variants joined by a flexible linker (23,24). This autoantibody was measured in stored, available samples (81% of samples with valid serum enterovirus RNA measurements).

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

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

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

**Statistical analysis.** Using Cox regression, we compared the rate of progression to T1D under two different models, which we have called the rapid effect model and the cumulative effect model. Both treat enterovirus infection as a time-dependent variable. In the rapid effect model, we estimated the rate of progression to diabetes in the sample interval following detection of enterovirus (median 4 months) compared to sample intervals where enterovirus was not detected. The exposure status returned to zero at the next clinic visit unless enterovirus was found also here. In the cumulative effect model, we estimated the rate of progression to diabetes according to the cumulative number of infections acquired during follow-up, which also allows for detection of delayed effects. Each individual first contributed follow-up time with zero infections, and the exposure variable increased by one at each visit when a new infection was detected. Because few individuals had repeated infections, the cumulative exposure variable had to be grouped (0 vs. ≥1 for serum RNA; 0, 1 and ≥2 for rectal swab RNA). The main time variable was time from the first clinic visit at which a child tested positive for islet autoantibodies to T1D diagnosis or to the most recent visit (up to April 9, 2009) at which the child was known not to have diabetes. Because enteroviral RNA was relatively rarely detected in serum, and consequently, the number of events during the exposed periods limited, we also carried out a Monte Carlo permutation test with 10000 repeated permutations of the enterovirus variable, to assess the validity of the standard inference based on the Cox regression model.
All analyses were done using Stata, version 11 (StataCorp LP, College Station, TX, USA). A 95% confidence interval for the hazard ratio excluding the value 1.00 or a p-value <0.05 was regarded as statistically significant.

RESULTS
A total of 140 children seroconverted for islet autoantibodies at a median age of 4.0 years. Of those, 50 developed T1D at a median age of 8.7 years, after a median follow-up of 4.1 years from the initial appearance of islet autoantibodies (Table 1). The samples tested for enterovirus were collected prior to June 2007 and 41 of the 50 children had developed T1D by that time, while another 9 progressed to T1D between June 2007 and April 2009 (Figure 1).

Positivity for two or more islet autoantibodies at the first or second positive visit strongly predicted progression to T1D, independent of other factors (Table 1). Those who progressed to T1D tended to more often carry the high risk HLA genotype, to have a first degree relative with T1D and to seroconvert for islet autoantibodies at an earlier age, but these factors did not significantly predict progression to T1D independently of positivity for multiple islet autoantibodies in at least one of the two first positive visits (Table 1). The number of positive islet autoantibodies treated as a time-dependent variable was also highly predictive of progression to T1D, and positivity for ZnT8-autoantibodies significantly predicted progression, both before and after adjusting for the other three islet autoantibodies (Online Supplemental Table 1 available at http://diabetes.diabetesjournals.org).

Enterovirus infections. Enteroviral RNA results were available from serum and rectal swabs collected at 1081 and 1242 pre-diagnostic clinic visits, respectively. Results were available for either serum or rectal swab at 1295 visits, and from both types of specimens at 1028 visits. The median interval between the visits was 4 months. Enteroviral RNA was detected at a total of 54 of 1295 visits (4.2%). At 8 of these 54 visits, enteroviral RNA was detected in both serum and rectal swab. Of the 140 children in the cohort, 31 (22.1%) had at least one serum or rectal swab sample positive for enteroviral RNA. While 19 of these 31 were positive only once, some had up to six positive visits. Only two children were ever positive twice for serum enteroviral RNA.

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

Viral sequence was obtained from altogether 8 of 17 positive serum samples and from 33 of 45 positive rectal samples. The sequences were deposited in the GenBank sequence database under accession no. HM746666 to HM746706 (Online Supplemental Table 3). Sequences are shown in Online Supplemental Figure 2 together with reference strain sequences listed in Online Supplemental Table 3. Samples on which sequencing was not successful contained low concentration of viral RNA. All samples but one clustered into enterovirus genogroup II, which contains amongst others, the coxsackie B viruses (31) (Online Supplemental Figure 3). The sequence data indicated that viruses detected simultaneously in serum and rectal samples represented the same virus strain and only a single nucleotide substitution was once
observed between such strains. Viruses detected in successive samples taken from the same individual represented different enterovirus strains. Thus, no evidence of viral persistence was found.

**Progression to T1D following enterovirus infections.** The progression to T1D in the 17 intervals following detection of enteroviral RNA in serum was significantly more rapid (3 T1D cases diagnosed) than that in the 1064 intervals following negative enteroviral RNA serum test (33 T1D cases diagnosed, hazard ratio 6.36, Table 2). Further adjustment for number of positive conventional islet autoantibodies did not alter the result (Table 2). Because only 3 children were diagnosed in the interval after being positive for enteroviral RNA in serum, we employed a permutation test to make sure that the results of the standard inference based on Cox regression was valid. With 10000 permutations, the (Monte Carlo) p-value was 0.0075, thus confirming the highly significant result. After restricting the analysis to the 81% of samples with available data on ZnT8 autoantibodies, there was still a significant relation between serum enteroviral RNA and progression to T1D (hazard ratio: 6.21, 95% CI: 1.82-21.2), and this was essentially not affected by adjustment for ZnT8-autoantibody positivity in models without (hazard ratio: 8.50, 95% CI: 2.21-32.6) or with (hazard ratio: 9.08, 95% CI: 2.30-35.8) additional adjustment for the number of other islet autoantibodies.

The three children who progressed to T1D in the interval following a positive serum enteroviral RNA test all had typical characteristics of high risk for progression to T1D. They had an early age at seroconversion for multiple islet autoantibodies, an affected sibling, and two of three carried the HLA DR3/4 genotype (Online Supplemental Table 4). They also had a near average interval length between clinic visits, and all were males of non-Hispanic white ethnicity. Results were similar and remained statistically significant after restriction of the analysis to these respective subgroups (Online Supplemental Table 5). Furthermore, the results were essentially unchanged after including four children who progressed to T1D after being positive only once for islet autoantibodies (all four were negative for enteroviral RNA, adjusted hazard ratio 6.56, 95% CI: 1.84-23.5).

Presence of enteroviral RNA in rectal swabs did not predict progression to T1D in the following sample interval (adjusted hazard ratio 0.79, 95% CI: 0.10-5.92, Table 2).

Analyzing progression to T1D according to the cumulative number of enterovirus infections during follow-up, which allows for delayed effect, there was no significant relation with progression to T1D for either serum or rectal swab enteroviral RNA, or for serologically defined infections (Online Supplemental Table 6). We also ran a Cox regression model simultaneously including variables modeling enterovirus according to the rapid effect model and the cumulative effect model. The results confirmed that the non-significant tendency towards an association for the cumulative effect variable was entirely due to the rapid effect, while the rapid effect of serum enteroviral RNA was unaltered and still significant (hazard ratio 5.79, 95% CI: 1.23-27.3 for rapid effects model and hazard ratio 1.07, 95% 0.37-3.11 for cumulative effect model).

There was also no relation between infections defined as increases in enterovirus antibodies and progression to T1D according to the rapid effect model (Online Supplemental Table 7; note that antibodies were only tested in the subset of samples collected 1993-2004).

Finally, there were 19 children (61.3%) who progressed to T1D among the 31 with one or more enteroviral RNA positive serum or rectal swab sample, compared to 31 (28.4%) among the 109 children in whom
enteroviral RNA were not detected during follow-up (p=0.001). The proportion of visits where both serum and rectal swabs were positive for enteroviral RNA was higher among those who progressed to T1D (6 of 425 pre-diagnostic visits=1.4%) than among non-progressors (2 of 603 visits=0.3%), but this difference was not statistically significant.

**DISCUSSION**

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

**Strengths and limitations.** Given the amount of data available and many possible ways of analyzing data, we took great care to make all decisions regarding algorithms for defining infections and methods of analysis a priori. We used a formal cohort design and employed two main models (rapid effect and cumulative effect) to analyze two main indicators of enterovirus infections: enterovirus RNA in serum or in rectal swabs. Admittedly, our a priori defined main exposure, presence of enterovirus RNA in either serum or rectal swabs, did not significantly predict progression to type 1 diabetes (Online Supplemental Table 7). However, in pre-planned sub-analyses of serum and rectal swab enteroviral RNA examined separately, we found that the presence of enterovirus RNA in serum to be a highly significant predictor of progression. Also in the Finnish studies of enterovirus as risk factors for islet autoimmunity, enteroviral RNA in serum samples have been more predictive than enteroviral RNA in stool samples (4). The number of children who progressed in sample intervals after the detection of viral RNA in serum was limited. However, rather than relying on standard inference alone, we confirmed the highly significant result using a permutation test, which is not susceptible to bias with small sample sizes. Furthermore, the result was consistent and remained significant in subgroups defined by characteristics of those who progressed to T1D after enteroviral RNA was found in serum.

As a marker of islet autoimmunity, we used repeated presence of at least one islet autoantibody. This probably does not always reflect insulitis or activation of autoreactive T-cells, but autoantibodies are currently the best way of predicting T1D in humans (1).

**Interpretation.** About 8% of the children progressing to T1D had enteroviral RNA in their serum a few months prior to diagnosis. While our finding supports the hypothesis that infections resulting in enteroviral RNA in serum leads to a more rapid progression to clinical disease in some high risk individuals, it may also suggest that enterovirus infection is a relatively uncommon cause of progression to T1D. These observations may be explained by at least three potential scenarios.

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

Second, enterovirus may establish low-grade persistent infection in children with islet autoimmunity, but the quantity of viral RNA in serum and feces may be below the detection limit in most such cases. Some studies have indicated presence of enterovirus in pancreatic tissue in a sizeable proportion of patients dying soon after onset of T1D (35-38). Although the results varied depending on methodology and quality of specimens, detection of enterovirus in beta-cells clearly strengthen the case for its role in the pathogenesis. In addition, a recent study suggests that the virus is present in the intestinal mucosa of diabetic patients (39). Enteroviral RNA was only detected at one time point in children diagnosed with T1D during the sample interval following a positive serum enteroviral RNA test, which does not support the hypothesis of viral persistence. Sequence analysis did not give support for persistent infection, because all sequences obtained from children with multiple infections were from different genotypes. Furthermore, it is notable that none of the samples collected at the day of diabetes diagnosis were positive for enteroviral RNA. This is consistent with a previous study of serum samples from Finland (5), but apparently inconsistent with the majority of studies of enterovirus RNA in plasma or serum taken from patients soon after diagnosis, which have found approximately 30% of patients to be positive (40-42). We have no explanation for this, except a suggestion that an international laboratory standardization workshop could shed more light on these differences.

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

Another possibility that cannot be discarded is that progression to T1D is enhanced because the viral infection induced insulin resistance sufficient to precipitate clinical disease. Non-specific febrile illness or other infectious symptoms in a period prior to diagnosis seems to be quite commonly reported, but few studies have been able to obtain comparable data in age matched controls (44), and the large majority of enterovirus infections are asymptomatic (32). Furthermore, while biopsy studies and previous cross-sectional or retrospective studies of enterovirus infections in patients with T1D cannot exclude the possibility that the disease influenced the risk of infection, our longitudinal design allowed us to draw stronger inference in this regard. The fact that none of the children who were tested on the day of diagnosis were positive for enteroviral RNA, including those who were enterovirus positive in the interval before diagnosis, shows that reverse causation were unlikely.

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

Future studies and final conclusion. Despite the huge undertaking of screening and prospectively following a large number of children for several years, the number of endpoints was still limited, and independent replication in future studies would strengthen the results. Children who progressed to T1D immediately after detection of enterovirus RNA all had clinical characteristics consistent with high risk of progression such as early development of multiple islet autoantibodies. Future studies could investigate further the potential role of additional host and viral factors in this process. Prospective studies are challenging, and up to now such studies have mainly focused on the initiation of autoimmunity as the endpoint (7-9,26), with mixed results. The Environmental Determinants of Diabetes in the Young (TEDDY) study (48) has the potential to provide answers concerning the role of enterovirus and progression to T1D with greater power and avoiding some of the limitations of the study presented here. In conclusion, the rate of progression from islet autoimmunity to type 1 diabetes was significantly increased in the approximately four month interval following detection of enteroviral RNA in serum.

Author contributors. Marian Rewers is the principal investigator, and developed the general protocol for the DAISY study with input from George S. Eisenbarth, Jill Norris, Georgeanna Klingensmith, and Henry A. Erlich. Henry A. Erlich was responsible for the HLA genotyping and George S. Eisenbarth was responsible for measuring the anti-insulin, -GAD, and –IA2 autoantibodies. John C. Hutton was responsible for measuring the ZnT8 autoantibodies. Georgeanna Klingensmith was responsible for the clinical evaluation and diagnosis of type 1 diabetes. Katherine Barriga was responsible for managing and preparing the data bases. Heikki Hyöty and Sami Oikarinen were responsible for the enterovirus testing and enteroviral sequence analysis. Lars C. Stene planned the present study with assistance from Kathy Barriga, designed the analysis strategy, did the statistical analyses, and wrote the manuscript with input from all authors.

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Conflict of interest. Professor Hyöty is a shareholder (<5%) in a company which develops vaccines against picornaviruses. Other authors do not have any conflict of interest.
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Table 1. Characteristics of the cohort and results of Cox regression survival analysis of progression from islet autoimmunity to type 1 diabetes.

<table>
<thead>
<tr>
<th></th>
<th>Progressed to T1D (n=50)</th>
<th>No T1D (n=90)</th>
<th>Unadjusted hazard ratio (95% CI)</th>
<th>Adjusted hazard ratio (95% CI) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr) at diagnosis of T1D (median, range)</td>
<td>8.7 (1.9-15)</td>
<td>4.6 (1.6-14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follow-up (yr) from onset of islet autoimmunity (median, range)</td>
<td>4.1 (0.2-11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive for ≥2 islet autoantibodies in the first and/or second positive visit</td>
<td>36 (72%)</td>
<td>21 (23.3%)</td>
<td>4.57 (2.46, 8.51)</td>
<td>4.24 (2.26, 7.95)</td>
</tr>
<tr>
<td>Female sex</td>
<td>26 (52%)</td>
<td>48 (53.3%)</td>
<td>1.18 (0.67, 2.06)</td>
<td>1.41 (0.79, 2.50)</td>
</tr>
<tr>
<td>1st degree relative with type 1 diabetes †</td>
<td>35 (70%)</td>
<td>53 (58.9%)</td>
<td>1.23 (0.67, 2.26)</td>
<td>1.13 (0.61, 2.10)</td>
</tr>
<tr>
<td>HLA DRB1<em>04-DQB1</em>0302/DRB1<em>03-DQB1</em>0201</td>
<td>26 (52.0%)</td>
<td>27 (30.0%)</td>
<td>1.84 (1.06, 3.21)</td>
<td>1.51 (0.86, 2.67)</td>
</tr>
<tr>
<td>Non-Hispanic white ethnicity‡</td>
<td>46 (92.0%)</td>
<td>72 (80.0%)</td>
<td>1.94 (0.70, 5.39)</td>
<td>1.45 (0.51, 4.13)</td>
</tr>
<tr>
<td>Age (yr) when first islet autoantibody positive (median, range) §</td>
<td>3.1 (0.7-12)</td>
<td>5.2 (0.7-13)</td>
<td>0.93 (0.85, 1.02)</td>
<td>1.01 (0.91, 1.11)</td>
</tr>
</tbody>
</table>

T1D: Type 1 diabetes; CI: Confidence interval.
* Estimates from Cox regression model simultaneously adjusting for multiple autoantibodies in first two visits, HLA high risk genotype, presence of first degree relative with type 1 diabetes and age when first positive for islet autoantibodies.
† Of these, 35 had an affected father only, 16 had an affected mother only, 34 had an affected sibling, and 3 had a sibling and a parent with T1D.
‡ Ethnic group was self-reported. There were 118 non-Hispanic whites, 19 Hispanic, one African American and two children of mixed ethnicity in the cohort.
§ Hazard ratios per year increase in age when first positive for islet autoantibodies.

Table 2. Progression from islet autoimmunity to clinical type 1 diabetes in sample interval (median ~4 month) following infection detected by enterovirus RNA in serum or rectal swab sample.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Enterovirus RNA in previous sample</th>
<th>Person-years of follow-up</th>
<th>Cases progressing to type 1 diabetes in interval*</th>
<th>Hazard ratio (95% CI)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unadjusted</td>
</tr>
<tr>
<td>Serum</td>
<td>No</td>
<td>494</td>
<td>33</td>
<td>1.00 (reference)</td>
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<tr>
<td></td>
<td>Yes</td>
<td>6.5</td>
<td>3</td>
<td>6.36 (1.89, 21.4)†</td>
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<tr>
<td>Rectal swab</td>
<td>No</td>
<td>537.1</td>
<td>32</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>21.2</td>
<td>1</td>
<td>0.93 (0.12, 6.90)</td>
</tr>
</tbody>
</table>

* Forty-one of 140 children in the study cohort progressed to type 1 diabetes during the period where collected samples were tested for enterovirus, of which serum enterovirus RNA results were available from the clinic visit preceding diagnosis in 36 (of which 3 were positive) and rectal swab enterovirus RNA was available in 33 (of which 1 was positive, and serum from the same visit was also positive for enterovirus RNA). Enterovirus exposure variables coded according to the rapid effect model described in the methods section.
† Cox-model: P=0.003. Permutation test based on Cox regression model with 10000 permutations of the enterovirus variable: P=0.0075.
Figure legend:

Figure 1. Flow chart illustrating formation of the study cohort.
* Samples were tested for the three islet autoantibodies: anti-GAD65, anti-insulin and anti-IA2.
† If positive for ≥1 islet autoantibody at or after 12 months of age, frequency of blood sampling was increased to every 3-6 months.
HLA: Human leukocyte antigen