Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology
doi:10.1111/j.1365-2249.2011.04555.x
Clinical and Experimental Immunology

Immunology in the clinic review series; focus on type 1 diabetes and viruses: the enterovirus link to type 1 diabetes: critical review of human studies

OTHER THEMES PUBLISHED IN THIS IMMUNOLOGY IN THE CLINIC REVIEW SERIES
Metabolic diseases, host responses, cancer, autoinflammatory diseases, allergy.

L. C. Stene* and M. Rewers†
Division of Epidemiology, Norwegian Institute of Public Health, Oslo, Norway, and †Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine, Aurora, CO, USA

Accepted for publication 19 December 2011
Correspondence: L. C. Stene, Division of Epidemiology, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway.
E-mail: lars.christian.stene@fhi.no

Summary

The hypothesis that under some circumstances enteroviral infections can lead to type 1 diabetes (T1D) was proposed several decades ago, based initially on evidence from animal studies and sero-epidemiology. Subsequently, enterovirus RNA has been detected more frequently in serum of patients than in control subjects, but such studies are susceptible to selection bias and reverse causality. Here, we review critically recent evidence from human studies, focusing on longitudinal studies with potential to demonstrate temporal association. Among seven longitudinal birth cohort studies, the evidence that enterovirus infections predict islet autoimmunity is quite inconsistent in our interpretation, due partially, perhaps, to heterogeneity in study design and a limited number of subjects studied. An association between enterovirus and rapid progression from autoimmunity to T1D was reported by one longitudinal study, but although consistent with evidence from animal models, this novel observation awaits replication. It is possible that a potential association with initiation and/or progression of islet autoimmunity can be ascribed to a subgroup of the many enterovirus serotypes, but this has still not been investigated properly. There is a need for larger studies with frequent sample intervals and collection of specimens of sufficient quality and quantity for detailed characterization of enterovirus. More research into the molecular epidemiology of enteroviruses and enterovirus immunity in human populations is also warranted. Ultimately, this knowledge may be used to devise strategies to reduce the risk of T1D in humans.

Keywords: aetiology, autoimmunity, enterovirus, infection, T1D

Introduction

Type 1 diabetes (T1D) results from a selective immune-mediated destruction of the pancreatic beta cells in subjects carrying permissive human leucocyte antigen (HLA) genotypes. Rapidly increasing incidence, sometimes with epidemic patterns, and other evidence suggest that non-genetic factors are involved in the aetiology [1]. Enteroviruses have been the primary candidate since Gamble reported high titres of neutralizing antibodies to Coxsackie B virus in recent-onset T1D patients [2], and Yoon isolated a Coxsackie B4 virus from a child with T1D and established several lines of evidence for causality [3]. Despite a number of impressive investigations using different approaches, the nature of the relationship between enterovirus and T1D remains controversial. In most cases, diagnosis of T1D follows a long period of preclinical islet autoimmunity [4]. The latter is essential for interpretation of aetiological studies, as viruses present at diagnosis may have infected the host late in the disease process, or aetiological infections may have been cleared at the time of diagnosis. Enterovirus infections may contribute potentially to initiation of

© 2011 The Authors
Clinical and Experimental Immunology © 2011 British Society for Immunology,

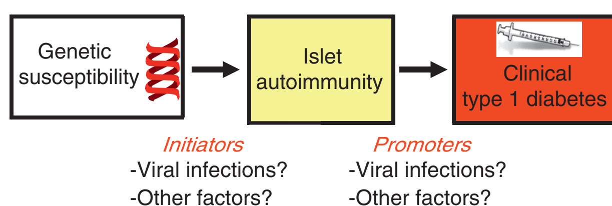

Fig. 1. Illustration of the two-stage process leading to type 1 diabetes. Enterovirus may influence initiation of autoimmunity, progression from islet autoimmunity to clinical disease, or both. It is also possible that enterovirus influences the risk of developing atypical type 1 diabetes not preceded by islet autoimmunity, such as fulminant type 1 diabetes seen in some cases in Japan [5].

autoimmunity, modulating progression from islet autoimmunity to clinical T1D or both (Fig. 1) [6,7]. Enterovirus infections can probably also precipitate diagnosis non-specifically in those with advanced preclinical disease.

Experiments in rodent models suggest that enteroviral infection accelerates the disease process when occurring after the accumulation of immune cells in the pancreatic islets [8–11]. A recent report has suggested a similar effect in humans [12]. Prospective studies in humans pose a number of challenges in study design and interpretation, which we will discuss in this review. Potential mechanisms and other aspects of importance for understanding the potential link between enterovirus and T1D are covered by others in this issue (see reviews by Lind *et al.*, Jaïdane *et al.*, Hober *et al.* and Grieco *et al.*), or have been reviewed elsewhere (e.g. [13–19]).

Enteroviruses: epidemiology and methods of detection

Enteroviruses are ubiquitous, single-stranded non-enveloped RNA viruses, transmitted generally through the faecal–oral route, and replicating primarily in the gut. There are more than 100 defined human enterovirus serotypes (http://www.picornaviridae.com), and many more strains have been defined by sequencing. Much of the knowledge about the biology of enteroviruses is derived from poliovirus and a few prototypic strains of other enteroviruses. Many properties may differ across serotypes, and even across isolates within serotypes [20,21]. Most enterovirus infections are asymptomatic, but some serotypes are associated with severe clinical symptoms in a small proportion of those infected [20,22–24]. While most children encounter an enterovirus by the age of 2 years, infection with a given serotype is obviously not as common [25,26]. Co-infections with two serotypes may occur [25,26]. Epidemic outbreaks often follow natural circulation of the same serotype, and it is not entirely clear why outbreaks of enterovirus-associated disease sometimes occur. As spontaneous mutations and recombination are common among enteroviruses [27], it is speculated that virulent strains may emerge spontaneously during an infection.

For clinical or epidemiological studies, enterovirus can be detected in various types of biological specimens from humans. Traditional cell culture methods are quite sensitive for detection, except for some species A serotypes which cannot be grown in culture [24,26]. Enterovirus is detectable most readily in faeces, where it is usually detectable for 3–4 weeks, but rarely more than 2–3 months [12,25,26,28]. In a proportion of gut infections, enterovirus is also detectable in blood, usually for a few days in immunocompetent hosts [24]. It has been suggested that enterovirus is detectable more readily in peripheral blood mononuclear cells (PBMC) than in serum, but few studies have evaluated this systematically [29,30].

Systemic infection may, in some instances, lead to dissemination to other target organs, and enterovirus RNA or protein can sometimes be detected in intestinal, heart or pancreatic tissue by reverse transcription–polymerase chain reaction (RT–PCR), immunohistochemistry or *in-situ* hybridization. Interesting studies of pancreatic tissue from T1D patients and controls have appeared in recent years [31–33]. Other studies of pancreatic tissue have not found enterovirus or found a similar proportion of positives in controls [34,35]. It appears that a number of methodological factors may profoundly influence the results of such studies, as discussed in [33,36]. This was also suggested in an as yet unpublished study presented in abstract form by Tauriainen and co-workers, including the Network for Pancreatic Organ Donors (nPOD) group (http://www.jdrfnpod.org). This and similar projects focusing on optimizing specimen handling and standardizing and validating methodology is likely to bring this field forward. Below, we focus on methods of enterovirus detection that are more feasible in large-scale prospective studies.

RT–PCR for enterovirus RNA detection

RT–PCR is a relatively simple and very sensitive method of detection and a number of different assay formulations have been used, including conventional, nested or seminested and real-time RT–PCR. Most RT–PCR primer sets used target the highly conserved 5′ non-coding region (NCR) of the enterovirus genome, which should detect essentially all serotypes. Even among primer sets targeting conserved regions of the 5′ NCR, the exact primer sequences have varied between studies. For instance, two related primer sets used in a single study produced very different results [37], suggesting that validity varied by primer sequence. Continued optimization and validation seem to improve the methodology but each assay has certain advantages and some drawbacks, depending on the application [38–41]. Detailed characterization of positive samples requires sequencing of variable parts of the genome, particularly the VP1 region.

© 2011 The Authors

Enterovirus serology

Many different formulations of serological assays have been used in studies of T1D, including neutralization tests and various forms of immunoassay [42]. There is a general problem with cross-reactivity between serotypes, which may be exploited when aiming to cover all serotypes. Using mixes of different heat-treated antigens and synthetic peptides based on consensus sequences are strategies employed towards this end [43,44]. Serological assays were developed and validated with appropriately timed acute and convalescent sera from patients with aseptic meningitis confirmed by enterovirus isolation (see, e.g. [43] and references therein). There may thus be some bias towards serotypes prevalent in aseptic meningitis and which grows in culture. An important drawback is that it is difficult to define an appropriate cut-off for positivity when appropriately timed paired samples are not available. Some immunoglobulin (Ig)M enzyme assays have demonstrated high sensitivity with paired sera, usually at the cost of some reduction in specificity [45]. When applied in prospective studies with approximately 3–6-month sample intervals, the frequency of serologically defined infections are several-fold higher than the frequency of enterovirus detected by RT–PCT in serum [12,46,47].

How common are enterovirus infections in the population?

It should be clear from the above that the reported occurrence of enterovirus infections depends critically on methodology. Figure 2 shows examples of prevalence of enterovirus infections in serial samples from the Diabetes and Autoimmunity Study in the Young (DAISY) and MIDIA (Diabetes Autoimmunity Study in the Young, described below) by season, age and method of detection. In addition to variation over these established factors, there appears to be substantial random variation even when several hundreds of samples are analysed (Fig. 2a).

Regardless of the definition of enterovirus infection, human studies attempting to capture the infection history are limited by low sampling frequency. The age and

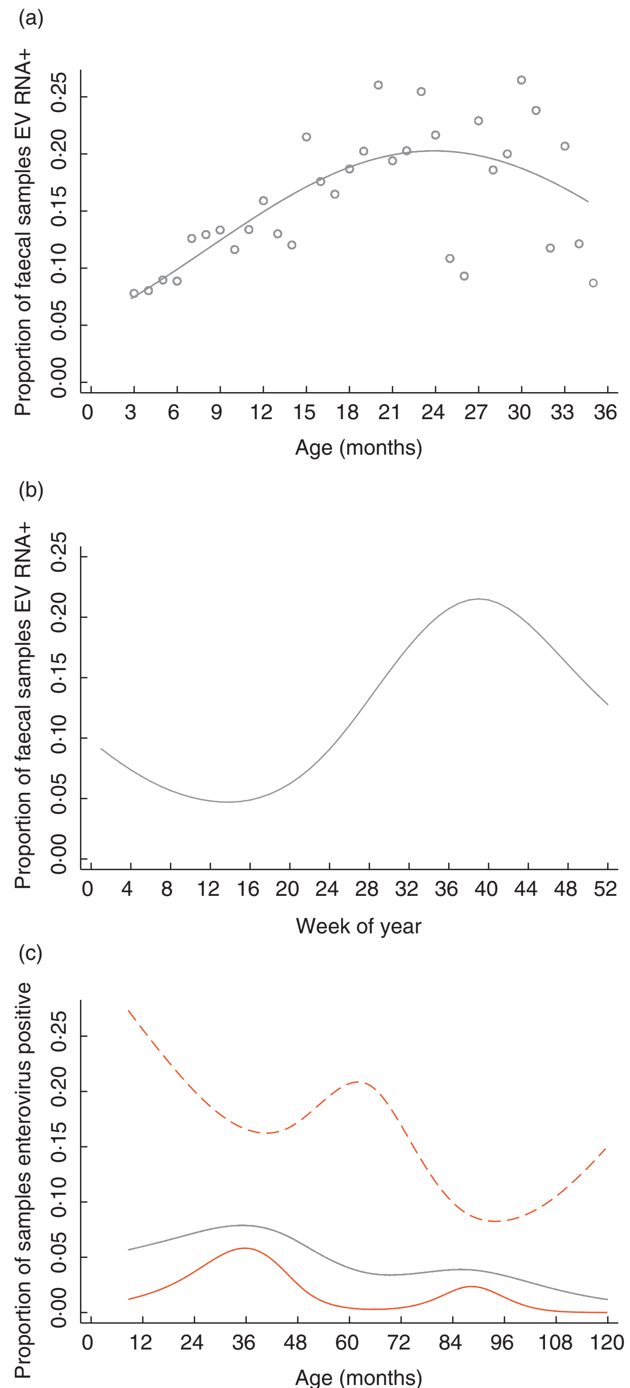

Fig. 2. Variation of enterovirus prevalence by age, season and method of detection in two longitudinal birth cohort studies [Environmental Triggers of Type 1 Diabetes (MIDIA) and Diabetes and Autoimmunity Study in the Young (DAISY)]. (a) Prevalence of enterovirus RNA by age in nearly 8000 monthly faecal samples from nearly 800 children in MIDIA (unpublished data from ref. [48]). Circles are observed prevalence in age groups, and the line represent smoothed predictions from logistic regression model with a second-degree polynomial. (b) Prevalence of enterovirus RNA in monthly faecal samples from MIDIA children aged 3–36 months, by season [smoothed as in (a), but with restricted cubic splines]. (c) Prevalence of enterovirus infection by age and type of sample in DAISY (data from ref. [12]). Subjects with autoimmunity ($n = 140$) were tested for enterovirus RNA in rectal swabs (grey line) and serum (red solid line), and enterovirus serology (red dashed line) in samples collected every 3–6 months (serology only in subset of samples, all enterovirus assays conducted in Heikki Hyöty's laboratory). Infections were defined serologically as a doubling or more in optical density in one or more of five immunoglobulin (Ig)M, IgG and IgG enzyme immunoassays (see [12] for details). All curves were smoothed using restricted cubic splines in logistic regression models. Curves must be interpreted with caution because of relatively few positive samples and substantial random variation, cf. (a).

© 2011 The Authors
Clinical and Experimental Immunology © 2011 British Society for Immunology,

frequency of sample collection, type of samples and assays will influence the ability to detect an association between enterovirus and risk of islet autoimmunity or T1D.

Enterovirus in T1D patients and healthy control subjects

Enterovirus serology

Green [42] systematically reviewed studies of Coxsackie B serology and T1D up to 2002, some of which included patients who were not recent-onset cases. Among 13 studies using positivity for any Coxsackie B antibody as a marker of infection, there were indications of a possible relationship, but the authors concluded that the heterogeneity in assays, study design and results did not allow a conclusion or calculation of a pooled estimate [42]. Also, the smaller studies tended to have larger estimated odds ratios, suggestive of publication bias [42]. Separate analyses were also performed for antibodies specific for Coxsackieviruses B3, 4 and 5 (for 11, 17 and 11 studies, respectively), with little or no suggestion of any relationship overall, and with similar heterogeneity. Few serology studies have been published since. A few studies HLA-typed cases but no study genotyped controls, and statistical adjustment for the HLA genotype as a potential confounder was thus not possible. The latter is also the case for similar studies using RT–PCR for enterovirus detection, discussed below.

Enterovirus RNA in blood samples

Yeung provided a very useful overview of studies using molecular methods of enterovirus detection [34], but calculated pooled estimates across all studies despite recommendations not to do so in the presence of large heterogeneity in results and study design [49]. For instance, results based on *in-situ* hybridization or immunohistochemistry on pancreatic tissue were pooled with those based on RT–PCR on blood samples or combined methods of detection [34]. In our judgement, a joint analysis of studies of enterovirus RNA by RT–PCR on serum, plasma or whole blood among newly diagnosed T1D patients and matched controls would be reasonable (Fig. 3). The overall result in Fig. 3a is consistent with an odds ratio of approximately 10–12, which was also obtained by Yeung *et al.* [34]. Note, however, that the *I*-square estimate of statistical heterogeneity was drastically lower (0·0%) compared to that among studies of newly diagnosed T1D patients and controls presented by Yeung *et al.* (*I*-square = 59%). While there was limited variation between studies in the frequency of enterovirus RNA in serum from healthy controls (Fig. 3b), there was wide variation between studies in the frequency of enterovirus RNA in serum from newly diagnosed T1D patients (Fig. 3c). There is an impression that the earliest studies showed a higher frequency among patients than did the more recent ones. Conversely, data so far presented only as conference abstracts in 2010 and 2011 by Antonio Toniolo's group from Italy have shown enterovirus positivity in the large majority of T1D patients.

It is notable (Fig. 3c) that one laboratory reported no enterovirus RNA in any sample from T1D patients at or near diagnosis in three independent data sets [12,54,58]. This is the Finnish laboratory that has reported many positive samples from prediabetic individuals in longitudinal studies [12,58]. Thus, lack of assay sensitivity would be unlikely. Finally, a recent study larger than all previous ones reported a threefold higher proportion of enterovirus RNA in serum from T1D patients compared to controls, but the majority of patients were unfortunately not recent-onset cases [61].

Longitudinal studies of enterovirus and islet autoimmunity

Virus detected in patients at or after diagnosis may well have infected the host after disease onset, whether the virus is detected in tissues, blood or faeces. Furthermore, lack of virus at diagnosis does not exclude a role of virus in the aetiology, as 'hit-and-run'-type mechanisms may have been involved. Prospective studies with frequent sampling of biological specimens and a sufficient number of cases with end-point are necessary to document statistically significant associations between infections and later risk of islet autoimmunity or T1D. The available longitudinal studies investigating the potential link between serial postnatal measures of enterovirus infections and islet autoimmunity (or T1D) are presented in Table 1.

They include the three Finnish studies DIPP (Diabetes Prediction and Prevention Study), DiMe (Childhood Diabetes In Finland) and TRIGR (Trial to Reduce IDDM in the Genetically at Risk), the DAISY in Colorado, MIDIA in Norway (Environmental Triggers of Type 1 Diabetes) and the German BABYDIAB and Babydiet studies. Preliminary data from a study in Australia called Viral Etiology of type 1 Diabetes (VIGR) have been presented only in abstract form at the time of writing, and results shown in the review by Yeung *et al.* [34], but details on methodology have not yet been published in full. All these studies include children with increased risk of T1D, defined by a first-degree family history, HLA susceptibility genes or both. Seven studies have published data from a total of 176 cases of islet autoimmunity, and one study (DiMe) followed subjects with islet autoimmunity for T1D as end-point. Sample frequency and method of detection varied between these studies (Table 1).

Challenges in statistical analysis of longitudinal data

Available studies varied in how data were presented and analysed. There is always a trade-off between carrying out the different types of analyses needed to detect relevant patterns, and 'data dredging'. The latter can potentially lead to false-positive associations due to multiple testing. Samples

© 2011 The Authors

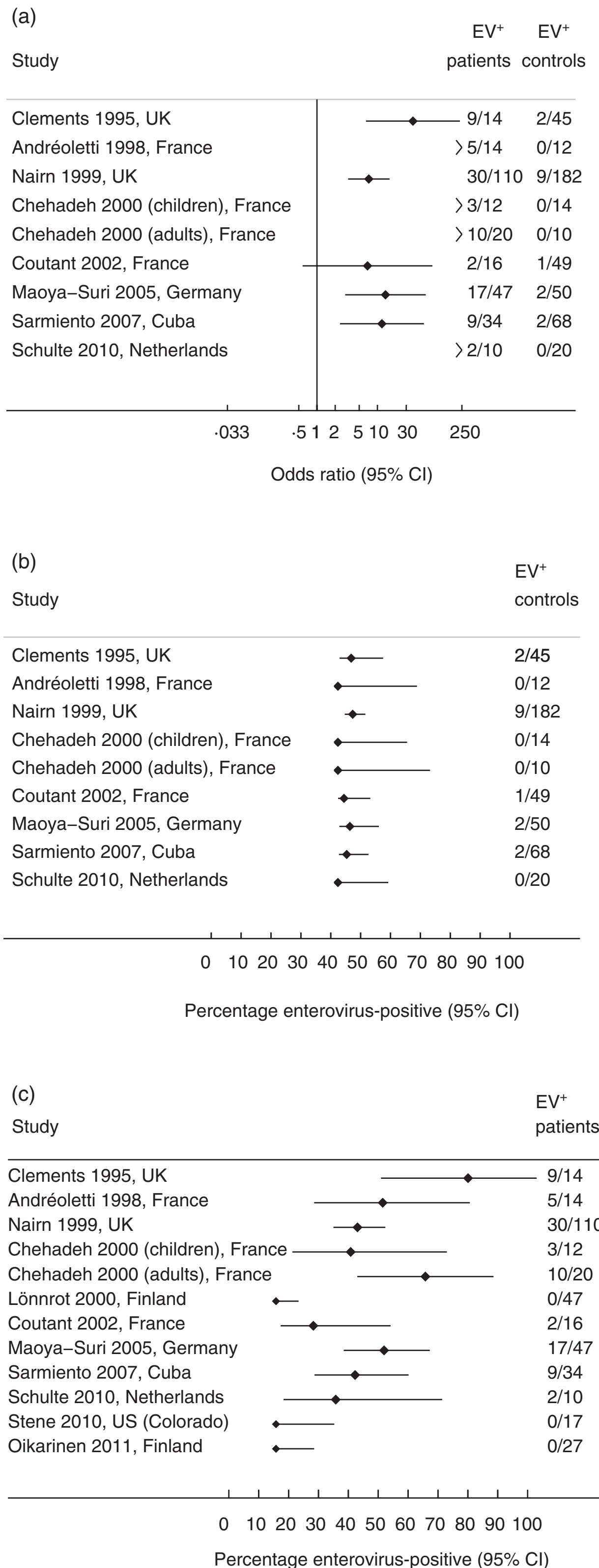

Fig. 3. Studies of enterovirus RNA in serum or plasma from patients with type 1 diabetes diagnosed within 1 month and from healthy controls. (a) Odds ratio for association between enterovirus and type 1 diabetes. Odds ratio estimates had to be calculated using Woolf's formula, as information for matched analysis was not provided in original publications. The *I*-squared estimate of statistical between study heterogeneity was 0·0%. Odds ratio estimates cannot be calculated from studies with zero observed controls with enterovirus. Overall results (association and heterogeneity) were similar after adding 0·5 to all four cells in the 2 × 2 table for studies with zero observed controls with enterovirus RNA-positive serum (data not shown). (b) Percentage of enterovirus-positive age-matched healthy controls (with exact 95% confidence intervals). (c) Percentage of enterovirus positive type 1 diabetes (T1D) patients. Note that three studies of patients with newly diagnosed T1D did not include matched controls, and are thus not included in panels (a) and (b). References cited (first author and publication year indicated) are [12,30,50–58]. Data from Oikarinen 2011 [58] include data not presented in original publication, obtained by personal communication from H. Hyöty and S. Oikarinen, Tampere, Finland. Not included are data based on enterovirus detection in peripheral blood mononuclear cells, which were available from Schulte [30], and also by two other studies [37,59]. Other studies not included were Craig [60], who did not provide separate data on results based on serum samples (only for positivity in serum and/or faecal samples), and a few studies of type 1 diabetes patients who were not newly diagnosed.

collected before islet autoimmunity (or T1D) and during the corresponding period in matched controls should be distinguished from other samples, but this has not always been carried out. If an infection is detected in the sample interval when islet autoantibodies first appear the exact time order cannot be determined, as both occur in an interval, usually of 3–12 months. The potential effect of enterovirus infections could, theoretically, be cumulative, reflecting multiple 'hits' over time. Alternatively, it could be argued that infections should occur just before islet autoimmunity to be implicated plausibly in the aetiology. The latter can be investigated by restricting analysis to narrow time-intervals [46,64], or the frequency of infections just before islet autoimmunity can be compared with other time-intervals in the same individual in so-called case 'cross-over analysis' [72]. The latter is appealing because each subject is his/her own control, but our experience from MIDIA suggests that seasonal variation in enterovirus infection makes it difficult to obtain unbiased results (unpublished observation). Intra-individual correlation among repeated samples within subjects must also be taken into account in the analysis when enterovirus frequency is the dependent variable. This is frequently ignored (see Table 2). 'Per-subject analysis' (see Table 2) is simpler, but important information in the repeated samples is lost.

The time-varying nature of enterovirus infection history in each individual can be modelled with time to event (autoimmunity or T1D) analysis. The type of mechanism operating may profoundly influence the ability to detect an association, and the interpretation of such analyses is complex [73], but ignoring the truly time-varying nature of enterovirus infection history is probably not a better option.

© 2011 The Authors

Table 1. Longitudinal studies of enterovirus and islet autoimmunity or type 1 diabetes (T1D).

Study	Subjects included	Age (month) at sample collection	Definition of islet autoimmunity/no. of case subjects tested for enterovirus	Type of sample and enterovirus assay	Relevant publications
DiMe	Siblings of newly diagnosed children with T1D in Finland	6-month intervals from T1D in sib	≥1 of ICA, IAA, GADA, IA2A*/11 cases progressed to T1D	Serum, serology and RT–PCR†	[54,74]
DIPP	Newborns with moderate- or high-risk HLA genes in Finland	Cord,(3),6,(9),12,(15),18,(21), 24 (30),36, . . .‡	ICA (IAA, GADA, IA2A measured if ICA-positive)/up to 41 cases§	Serum (plus faecal samples‡), serology and RT–PCR†	[28,46,58,75,76]
Second pilot of TRIGR	Newborns with family history of T1D in Finland	Cord, 3,6,9,12,18,24	≥1 of IAA, GADA, IA2A, ICA*/19 cases	Serum, serology and RT–PCR†	[47]
German BABYDIAB	Offspring of patients with T1D in Germany	Cord, 9, 24,36,60,96	≥1 of IAA, GADA, IA2A, ICA*/28 cases	Serum, Coxsackievirus serology‖	[65]
DAISY	Relatives of T1D and newborns with moderate or high-risk HLA genes in Colorado¶	9,12,15,24,36,48, ...	≥1 of IAA, GADA, IA2A on ≥2 consecutive occasions/up to 26 cases	Serum, rectal swabs, saliva, RT–PCR**	[66]
DAISY ('progression cohort')	As above	3–6-month intervals from islet autoimmunity	140 cases of islet autoimmunity tested, of which up to 50 progressed to T1D	Serum, rectal swabs, RT–PCR† (serology in subsample)	[12]
MIDIA	Newborns with the high-risk HLA genes in Norway	3,4,5,6,7,8,9, . . .,36††	≥2 of IAA, GADA, IA2-A on ≥2 consecutive occasions/27 cases	Faecal samples, RT–PCR‡‡	[64]
Babydiet	Relatives of T1D and newborns with moderate or high-risk HLA genes in Germany	3,6,9,12§§	≥1 of IAA, GADA, IA2A, Zn-T8A/22 cases	Faecal samples, RT–PCR‖‖, cell culture	[41]
VIGR	Newborns with family history of T1D in Australia	6, 12, 18, 24, 30, . . .	≥2 of IAA, GADA, IA2-A/13 cases¶¶	Serum, RT–PCR***	***

*'Persistently positive', but no explicit operational definition in publications. †Reverse transcription–polymerase chain reaction (RT–PCR) [conserved 5′ non-coding region (NCR) of enterovirus] in H. Hyöty's laboratory, details described in [67]. ‡Not all centres in Diabetes Prediction and Prevention Study (DIPP) included blood samples at ages in parenthesis. Faecal samples were collected monthly from ages 3 to 22 months; results reported for 12 cases in Salminen *et al.* 2004 [28]. §A recent publication from DIPP [62] presented data on enterovirus serology [immunoglobulin (Ig)A and IgG enzyme immunoassay (EIA) with heat-treated Coxsackie B4 virus as antigen] in serial samples at ages 3–24 months from 107 cases with islet cell antibodies (ICA) plus at least one of insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA) or insulinoma antigen 2 (IA2) autoantibodies (IA2A) on at least two consecutive occasions; see text. ‖Enzyme-linked immunosorbent assay (ELISA) IgG antibodies against a panel of heat-treated antigens from different Coxsackievirus serotypes. Not paired samples. ¶Offspring or siblings of patients with T1D were included regardless of human leucocyte antigen (HLA) genotype, while general population newborns were included if they carried at least one T1D susceptibility haplotype (DR4–DQ8, DR3–DQ2, or both). **RT–PCR in H. A. Rotbart's laboratory, details described in [68]. This study also tested for enterovirus IgM using an ELISA method described by Helfand [63] against 14 non-polio enterovirus serotypes (Coxsackie B1-6, Coxsackie A9, echo 4, 6, 9, 11, 30 and 34, and enterovirus 71), but serology data were not presented in the publication by Graves *et al*. (see text). ††While faecal samples were collected monthly as indicated, blood for islet autoantibody testing were collected at ages 3, 6, 9 and 12 months, then annually. ‡‡Real-time RT–PCR (conserved 5′ NCR of enterovirus) in O. Cinek's laboratory, details described in [69], based primarily on primer sequences from [70]. §§Faecal samples also collected later, but enterovirus only tested up to 12 months of age. Blood samples collected every 3 months up to 3 years, then annually, tested for islet autoantibodies. ‖‖Real-time RT–PCR with (panEV) primers and probes described in [39]. Positive samples partial VP1 sequenced to determine serotype. ¶¶According to Yeung *et al.* [34], 13 subjects were positive for two or three islet autoantibodies (requirement of persistence not mentioned explicitly). ***In the review by Yeung *et al*. [34], only serum enterovirus RNA results were reported, while according to the cited conference abstract by Al-Shabeeb other types of specimens were also tested for presence of enterovirus RNA. Apparently, a multiplex PCR method described in [71], with nested primers targeting the conserved 5′ NCR of enterovirus, was used for detection of enterovirus. Details of methodology for enterovirus detection and data analysis applied in viral aetiology of type 1 diabetes (VIGR) have not yet been published. ZnT8A: zinc transporter 8 autoantibody; DiMe: Childhood Diabetes in Finland study; HLA: human leucocyte antigen.

© 2011 The Authors

Table 2. Postnatal enterovirus infections before islet autoimmunity and the corresponding period for matched controls from longitudinal birth cohort studies.

Study and type of enterovirus (EV) Assay/sample	Per sample results EV+ case samples *versus* EV+ control samples Odds ratio (95% CI)*	Per subject results Case subjects EV+ at least once *versus* controls Odds ratio (95% CI)*
RNA in faeces		
DIPP [28]	Not reported	5/12 (42%) *versus* 15/53 (28%), OR = 1·8 (0·5–6·6)
MIDIA [64]§	43/339 (13%) *versus* 94/692 (14%), OR = 1·0 (0·6–1·7)	18/27 (67%) *versus* 30/53 (57%), OR = 1·5 (0·6–4·0)
Babydiet [41]	5/72 (7%) *versus* 27/267 (10%), OR = 0·7 (0·2–2·2)	4/22 (18%) *versus* 20/82 (24%), OR = 0·7 (0·2–2·3)
RNA in serum		
DIPP [46]	Not reported†	Not reported
TRIGR [47]	?/? (14%) *versus* ?/? (8·4%), OR = 1·8 (?–?)	Not reported
VIGR‡	Not reported	5/13 (38%) *versus* 28/198 (14%), OR = 3·8 (1·2–12)‡
Serology‖		
BABYDIAB [65]	0/62 (0%) *versus* not reported¶	0/28 (0%) *versus* not reported¶
Combination of methods		
DIPP serology and/or serum RNA [46]	33/152 (22%) *versus* 105/751 (14%), OR = 1·7 (1·1–2·6)	Not reported
TRIGR serology and/or serum RNA [47]	Not reported (but 0·83 *versus* 0·29 infections per child reported significantly different)	Not reported
DAISY (GP) RNA in rectal swab or saliva [66]**	0/17 (0%) *versus* 3/35 (9%)	0/10 (0%) *versus* 3/21 (14%)
DAISY (relatives) EV RNA in serum, rectal swab or saliva [66]**	1/10 (10%) *versus* 2/8 (25%), OR = 0·3 (0·02–4·6)	1/6 (17%) *versus* 2/6 (33%), OR = 0·4 (0·03–6·2)

*EV+: positivity for enterovirus detectable with given assay (positives/number tested). Unless stated otherwise, odds ratios (ORs) with confidence intervals (CI) were calculated based on Woolf's formula, because information for/from appropriate matched analysis was not presented in most publications. If anything, this tends to slightly underestimate ORs. For 'per sample analysis', this analysis ignores correlation among repeated measurements, and tends to result in too narrow CIs/too low *P*-values. Yeung's reporting [34] of per-subject results from several studies [28,47,66] were inconsistent with our interpretation of the original publications. †Results reported for samples including before and after islet autoimmunity [10/248 (4%) *versus* 33/1113 (3%), OR = 1·4, 95% CI: (0·7–2·8)]. ‡Preliminary result reported in the review by Yeung *et al.* [34], with no details on methodology. Controls were apparently all non-cases in the cohort. It was not stated explicitly that infections were counted only before islet autoimmunity in cases and a corresponding period in controls. Results apparently based on serum enterovirus RNA only. §OR for per-subject analysis from random intercept model to account for repeated measurements within individuals. 'Naive' analysis (ignoring repeated measurements), as in the other results provided here, give OR = 0·9 (95% CI: 0·6–1·4). Per-subject results presented here were calculated using Woolf's formula for comparison with other studies, based on raw data available in Supplementary Fig. 1 in [64]. ‖Several other studies tested for enterovirus antibodies in serum, but separate results were not reported separately in the publications. A recent publication from Diabetes Prediction and Prevention Study (DIPP) reported no overall association between signs of enterovirus infection detected by serial increase in immunoglobulin (IgA) or IgG enzyme immunoassay (EIA) (with heat-treated Coxsackievirus B4 as antigen) at ages 3–24 months and later risk of islet autoimmunity in 107 cases and 446 islet autoantibody-negative controls [62]. Frequencies of serologically defined infections were not reported, however. Graves *et al.* /Diabetes and Autoimmunity Study in the Young (DAISY) [66] also tested for enterovirus IgM and found no significant differences between the percentage of sera positive for IgM in cases and controls for each of the serotypes examined separately (all *P*-values >0·31), no significant differences between cases and controls in median optical density for each serotype, and no significant difference in proportion of cases and controls with positive IgM for at least one serotype (M. Rewers, *et al.*, unpublished). ¶Two cases positive for islet autoantibodies at ages 1 and 2 years, respectively, were positive for Coxsackievirus IgG in samples after islet autoimmunity, but one of the two cases was positive for one of the 'secondary' enterovirus antibody assays (CVB4IgG) in a sample collected before islet autoimmunity. **GP: general population newborns with human leucocyte antigen (HLA) susceptibility risk haplotypes; relatives: siblings or offspring of patient with type 1 diabetes, included regardless of HLA genotype. Enterovirus RNA was not tested in serum from the GP cohort because of apparently redundant information in serum, rectal swab and saliva, and some unavailable serum samples. MIDIA: Diabetes Autoimmunity Study in the Young; TRIGR: Trial to Reduce IDDM in the Genetically at Risk; VIGR: viral aetiology of type 1 diabetes.

© 2011 The Authors
Clinical and Experimental Immunology © 2011 British Society for Immunology,

Systematic analysis of the longitudinal studies of islet autoimmunity

We have attempted to present results from the available studies in a comparable manner across studies, but appropriate information was unfortunately not often available from the original publications (Table 2).

Three studies (DIPP, MIDIA and Babydiet) reported separate data from stool samples, and none showed any significant association with islet autoimmunity [28,41,64]. In MIDIA, the lack of association also remained when restricting to periods just prior to seroconversion, and there was also no difference when counting samples after seroconversion for islet autoantibodies [64]. In DIPP, there was a tendency towards an association, but the result was significant only when combined with enterovirus serology, which rather suggests an association with serology [28]. In the Babydiet study, some infections may have been missed because of sampling every 3 months rather than monthly, and a possible close temporal association could not be investigated as enterovirus was tested only up to 12 months, while most cases seroconverted at a later age (mean 2·6 years, up to 7·9 years) [41].

Separate results for serum enterovirus RNA in the period before islet autoimmunity and the corresponding period in matched controls has not been reported explicitly in any publication we are aware of. Abstracted information suggests no significant association in DIPP or TRIGR [46,47], and only modest differences if anything. There was an apparent association in the new study from Australia (preliminary results from VIGR presented in review by Yeung *et al.* [34]), but detailed information for appropriate interpretation has not yet been published (Table 2). Results from studies of serum enterovirus RNA restricted to cases progressing to T1D are discussed in the next section.

DIPP and TRIGR reported significant associations when combining enterovirus RNA in serum with infections defined serologically based on serial increase in at least one of several assays. The fact that large majority of infections were detected with serology suggests that serology was driving this association [46,47]. BABYDIAB analysed only enterovirus serology [65], but infrequent sampling and in many instances only one or no sample available from before islet autoimmunity suggest limited power to detect any relationship. A recent publication from DIPP describes no significant association between serologically defined infections in serial samples from ages 3–24 months and later risk of islet autoimmunity in 107 cases of autoimmunity and 446 matched controls, but the frequencies of serologically defined infections were not reported [62]. Graves *et al.* [66] also tested for enterovirus serology in DAISY, and found no significant association with islet autoimmunity in DAISY (unpublished observation, M. Rewers). Notably, a uniform finding in the longitudinal studies was that enterovirus RNA was detected rarely, if ever, continuously in the same individual for more than about 3 months.

Does enterovirus influence progression from islet autoimmunity to T1D in humans?

The hypotheses that enterovirus infections can initiate islet autoimmunity or enhance progression from islet autoimmunity to T1D are not mutually exclusive, but testing of the two hypotheses requires different study designs. We investigated recently in DAISY whether enterovirus infections can contribute to increased rate of progression from islet autoimmunity to clinical T1D [12], in an attempt to mimic results from mouse models [8,19]. The rate of progression to T1D was significantly higher in sample intervals after detection of enterovirus RNA in serum [12]. The observed association may be interpreted in various ways, but it was remarkable that none of the samples available from the day of T1D diagnosis were positive for enterovirus RNA. This suggests that the observed association was not due to reverse causality.

Recently, Oikarinen reported the frequency of serum enterovirus RNA during follow-up of 38 cases of islet autoimmunity who progressed to T1D and in controls who remained negative for islet autoantibodies [58]. The difference was largest around the time of seroconversion, which suggests a role in initiating islet autoimmunity, as reported previously from the same study. However, interpretation in relation to the hypothesis that enterovirus may accelerate progression is hampered by the fact that this study did not include follow-up samples from individuals with islet autoimmunity who did not progress to T1D [58]. DiMe is the only other longitudinal study of enterovirus with T1D as end-point, and significant associations with enterovirus RNA and serology have been reported [54,74]. This study also included control subjects who were negative for islet autoimmunity throughout follow-up, and no subjects with islet autoimmunity who did not progress.

Perinatal enterovirus infection, the polio model and potential protection by infection

Some studies have suggested a relationship between perinatal infections and risk of T1D in childhood [74,77–79], while a number of others have not found any significant relationship [46,47,65,80]. There are many methodological differences between these studies, and a detailed review of this literature is beyond the scope of this paper.

The analogy between poliomyelitis and the potential enterovirus–T1D link was pointed out a long time ago [14]. It is possible that declining proportions of pregnant women providing their infants with anti-enterovirus antibodies may explain some of the increasing incidence of T1D over time [81], although direct evidence for this in humans is lacking. Interestingly, diabetes induced with selected virus infections in LEW.1WR1 rat offspring could be prevented by infection with the same virus of the mothers prior to pregnancy, suggesting strongly that maternal antibodies could be

© 2011 The Authors

involved. Similar findings were observed for Coxsackievirus B3-induced diabetes in a transgenic NOD mouse model (P.G. Larsson and M. Flodström-Tullberg, results presented as conference abstracts in 2010 and 2011). In these models, a single strain of virus was responsible for essentially all cases of diabetes, and although the results are of interest it is questionable whether such a scenario can be extrapolated to human T1D.

A number of potential mechanisms have been proposed to explain the so-called hygiene hypothesis for type 1 diabetes [82–84]. Depending on the timing, enterovirus and other microbial agents may reduce the incidence of autoimmune diabetes in experimental animals [17,19]. Induction of regulatory T cells is among the mechanisms involved [85], but it is unknown whether a similar phenomenon operates in humans.

Some suggestions for future studies

Throughout history, different paradigms have influenced the views on criteria for evaluating causal relationships between virus or other potential aetiological factors and disease, and methodological advances have often led to new discoveries [86]. In the near future, we believe that high-throughput ('next generation') sequencing technology may contribute to human studies of viruses in causation of T1D [87,88]. Applications of this technology in virology are still scarce, and not without problems [89–92]. With proper standardization and validation, this technology has the potential to discover novel viruses and ideally combine detection with detailed characterization of genomes from potentially aetiological viruses.

With larger and higher-quality data sets in the future, such as those expected from the Environmental Determinants of Diabetes in the Young (TEDDY) study [93], we believe that the field can also benefit from more sophisticated statistical analysis and exploration of the impact of the many sources of error. No matter how refined the methods, some bias will always remain in human observational epidemiology. Conversely, the Achilles' heel of mechanistic studies based on experimental studies in rodents or *in-vitro* systems lies in generalization to humans. We hope that potential mechanisms can be discovered or corroborated in experimental models, and that this can be translated into hypotheses testable in humans. An example of a finding in a related field which may have potential relevance in T1D is the interesting data suggesting that a terminally deleted, defective form of Coxsackievirus B3 may persist in the myocardium in mice and perhaps also humans [94].

To have any impact on prevention of T1D, we have to envisage some form of randomized prevention trial in the future, based on vaccination or otherwise. If and when a human trial can be justified, it should be based on a critical and balanced summary of available evidence from human observational studies and experimental studies.

Summary and conclusion

Cross-sectional data suggest a higher prevalence of enterovirus RNA in the blood of newly diagnosed patients with T1D than that among healthy controls, although this was not confirmed by all studies. An association between enterovirus and rapid progression from autoimmunity to T1D was reported by one longitudinal study and awaits replication. Some longitudinal studies have suggested an association of enterovirus infection with the development of islet autoimmunity. On closer inspection, study designs and results were quite heterogeneous and based on a small number of subjects. Observed associations have not been investigated at the viral genotype or serotype level. Available longitudinal studies have not provided evidence for persistence of enterovirus in serum or faeces for more than a few weeks with available methods. Despite 40 years of research, the role of enteroviruses in the aetiology of T1D is far from proven. Large birth cohort studies with frequent sampling of blood and faecal samples and strict follow-up for islet autoimmunity and T1D may help, but ultimately a randomized clinical trial of vaccination or other type of intervention will be necessary.

Acknowledgement

L.C.S. was supported partly by the National Research Council of Norway (NRCN, grant 148359/330), the MIDIA study by grants 135893/330, 155300/320, 156477/730 and 166515/V50 from the NRCN (to K.S. Rønningen). M.R. and DAISY were supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (grants DK-32493 and DK-50979) and the Juvenile Diabetes Research Foundation (grant 33-2008-396). We would like to thank members of the MIDIA study team (K.S. Rønningen, O. Cinek, G. Tapia, E. Witsø) for permission to present partly unpublished data from MIDIA and for useful discussions. We thank Heikki Hyöty and Sami Oikarinen for permission to present their unpublished data on enterovirus RNA in serum from newly diagnosed patients with T1D in Fig. 3.

Disclosure

No conflict of interest reported.

References

1 Stene LC, Tuomilehto J, Rewers M. Global epidemiology of type 1 diabetes. In: Ekoé J-M, Rewers M, Williams R, Zimmet P, eds. The epidemiology of diabetes mellitus. Chichester: Wiley-Blackwell, 2008:355–83.
2 Gamble DR, Kinsley ML, FitzGerald MG, Bolton R, Taylor KW. Viral antibodies in diabetes mellitus. BMJ 1969; **3**:627–30.
3 Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 1979; **300**:1173–9.

© 2011 The Authors
Clinical and Experimental Immunology © 2011 British Society for Immunology,

4 Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001; **358**:221–9.
5 Shibasaki S, Imagawa A, Tauriainen S *et al.* Expression of toll-like receptors in the pancreas of recent-onset fulminant type 1 diabetes. Endocr J 2010; **57**:211–9.
6 Coppieters KT, Wiberg A, Tracy SM, von Herrath MG. Immunology in the clinic review series: focus on type 1 diabetes and viruses: the role of viruses in type 1 diabetes: a difficult dilemma. Clin Exp Immunol 2012; **168**:5–11.
7 Lind K, Hühn MH, Flodström-Tullberg M. Immunology in the clinic review series; focus on type 1 diabetes and viruses: the innate immune response to enteroviruses and its possible role in regulating type 1 diabetes. Clin Exp Immunol 2012; **168**:30–8.
8 Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. Diabetes 2000; **49**:708–11.
9 Horwitz MS, Fine C, Ilic A, Sarvetnick N. Requirements for viral-mediated autoimmune diabetes: β-cell damage and immune infiltration. J Autoimmun 2001; **16**:211–7.
10 Christen U, Edelmann KH, McGavern DB *et al.* A viral epitope that mimics a self antigen can accelerate but not initiate autoimmune diabetes. J Clin Invest 2004; **114**:1290–8.
11 Drescher KM, Kono K, Bopegamage S, Carson SD, Tracy S. Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: insulitis determines susceptibility of pancreatic islets to virus infection. Virology 2004; **329**:381–94.
12 Stene LC, Oikarinen S, Hyöty H *et al.* Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). Diabetes 2010; **59**:3174–80.
13 Craighead JE. The role of viruses in the pathogenesis of pancreatic disease and diabetes mellitus. Prog Med Virol 1975; **19**: 161–214.
14 Gamble DR. The epidemiology of insulin dependent diabetes, with particular reference to the relationship of virus infection to its etiology. Epidemiol Rev 1980; **2**:49–70.
15 Jun HS, Yoon JW. A new look at viruses in type 1 diabetes. Diabetes Metab Res Rev 2003; **19**:8–31.
16 Varela-Calvino R, Peakman M. Enteroviruses and type 1 diabetes. Diabetes Metab Res Rev 2003; **19**:431–41.
17 von Herrath M. Can we learn from viruses how to prevent type 1 diabetes?: the role of viral infections in the pathogenesis of type 1 diabetes and the development of novel combination therapies. Diabetes 2009; **58**:2–11.
18 Kemball CC, Alirezaei M, Whitton JL. Type B coxsackieviruses and their interactions with the innate and adaptive immune systems. Future Microbiol 2010; **5**:1329–47.
19 Tracy S, Drescher KM, Jackson JD, Kim K, Kono K. Enteroviruses, type 1 diabetes and hygiene: a complex relationship. Rev Med Virol 2010; **20**:106–16.
20 Morens DM, Pallansch MA. Epidemiology. In: Rotbart HA, ed. Human enterovirus infections. Washington, DC: ASM Press, 1995:3–23.
21 Roivainen M, Ylipaasto P, Savolainen C, Galama J, Hovi T, Otonkoski T. Functional impairment and killing of human beta cells by enteroviruses: the capacity is shared by a wide range of serotypes, but the extent is a characteristic of individual virus strains. Diabetologia 2002; **45**:693–702.
22 Melnick JL. Poliovirus and other enteroviruses. In: Evans AS, Kaslow RA, eds. Viral infections in humans: epidemiology and control. New York: Plenum Press, 1997:583–663.
23 Modlin JF. Coxsackieviruses, echoviruses, and newer enteroviruses. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell, Douglas, and Bennett's principles and practice of infectious diseases. Philadelphia, PA: Elsevier, 2005:2148–61.
24 Pallansch MA, Roos RP. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe DM, Howley PM, eds. Field's virology. Philadelphia, PA: Lippincott Williams & Wilkins, 2007:839–93.
25 Kogon A, Spigland I, Frothingham TE *et al.* The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. VII. Observations on viral excretion, seroimmunity, intrafamilial spread and illness association in coxsackie and echovirus infections. Am J Epidemiol 1969; **89**:51–61.
26 Witsø E, Palacios G, Cinek O *et al.* Natural circulation of human enteroviruses: high prevalence of human enterovirus A infections. J Clin Microbiol 2006; **44**:4095–100.
27 Oberste MS. Comparative genomics of the coxsackie B viruses and related enteroviruses. Curr Top Microbiol Immunol 2008; **323**:33–47.
28 Salminen KK, Vuorinen T, Oikarinen S *et al.* Isolation of enterovirus strains from children with preclinical Type 1 diabetes. Diabet Med 2004; **21**:156–64.
29 Prather SL, Dagan R, Jenista JA, Menegus MA. The isolation of enteroviruses from blood: a comparison of four processing methods. J Med Virol 1984; **14**:221–7.
30 Schulte BM, Bakkers J, Lanke KH *et al.* Detection of enterovirus RNA in peripheral blood mononuclear cells of type 1 diabetic patients beyond the stage of acute infection. Viral Immunol 2010; **23**:99–104.
31 Ylipaasto P, Klingel K, Lindberg AM *et al.* Enterovirus infection in human pancreatic islet cells, islet tropism *in vivo* and receptor involvement in cultured islet beta cells. Diabetologia 2004; **47**:225–39.
32 Dotta F, Censini S, van Halteren AG *et al.* Coxsackie B4 virus infection of β cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci USA 2007; **104**:5115–20.
33 Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. Diabetologia 2009; **52**:1143–51.
34 Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ 2011; **342**:d35.
35 Foy CA, Quirke P, Williams DJ *et al.* A search for candidate viruses in type 1 diabetic pancreas using the polymerase chain reaction. Diabet Med 1994; **11**:564–9.
36 Roivainen M, Klingel K. Virus infections and type 1 diabetes risk. Curr Diab Rep 2010; **10**:350–6.
37 Yin H, Berg AK, Tuvemo T, Frisk G. Enterovirus RNA is found in peripheral blood mononuclear cells in a majority of type 1 diabetic children at onset. Diabetes 2002; **51**:1964–71.
38 Piqueur MA, Verstrepen WA, Bruynseels P, Mertens AH. Improvement of a real-time RT–PCR assay for the detection of enterovirus RNA. Virol J 2009; **6**:95.
39 Kilpatrick DR, Yang CF, Ching K *et al.* Rapid group-, serotype-, and vaccine strain-specific identification of poliovirus isolates by real-time reverse transcription–PCR using degenerate primers and

probes containing deoxyinosine residues. J Clin Microbiol 2009; **47**:1939–41.

40 Oberste MS, Penaranda S, Rogers SL, Henderson E, Nix WA. Comparative evaluation of Taqman real-time PCR and semi-nested VP1 PCR for detection of enteroviruses in clinical specimens. J Clin Virol 2010; **49**:73–4.

41 Simonen-Tikka ML, Pflüeger M, Klemola P *et al*. Human enterovirus infections in children at increased risk for type 1 diabetes: the Babydiet study. Diabetologia 2011; **54**:2995–3002.

42 Green J, Casabonne D, Newton R. Coxsackie B virus serology and Type 1 diabetes mellitus: a systematic review of published case–control studies. Diabet Med 2004; **21**:507–14.

43 Samuelson A, Glimåker M, Skoog E, Cello J, Forsgren M. Diagnosis of enteroviral meningitis with IgG-EIA using heat-treated virions and synthetic peptides as antigens. J Med Virol 1993; **40**:271–7.

44 Hober D, Sane F, Jaïdane H, Riedweg K, Goffard A, Desailloud R. Immunology in the clinic review series; focus on type 1 diabetes and viruses: role of antibodies enhancing the infection with Coxsackievirus-B in the pathogenesis of type 1 diabetes. Clin Exp Immunol 2012; **168**:47–51.

45 Samuelson A, Skoog E, Forsgren M. Aspects of the serodiagnosis of enterovirus infections by ELISA. Serodiagn Immunother Infect Dis 1990; **4**:395–406.

46 Salminen K, Sadeharju K, Lönnrot M *et al*. Enterovirus infections are associated with the induction of β-cell autoimmunity in a prospective birth cohort study. J Med Virol 2003; **69**:91–8.

47 Sadeharju K, Hämäläinen AM, Knip M *et al*. Enterovirus infections as a risk factor for type I diabetes: virus analyses in a dietary intervention trial. Clin Exp Immunol 2003; **132**:271–7.

48 Witsø E, Tapia G, Cinek O, Pociot F, Stene LC, Rønningen KS. Polymorphisms in the innate immune *IFIH1* gene, frequency of enterovirus in monthly fecal samples during infancy, and islet autoimmunity. PLoS ONE 2011; **6**:e27781.

49 Egger M, Davey Smith G, Altman DG. Systematic reviews in health care: meta-analysis in context. London: BMJ Publishing Group, 2001.

50 Clements GB, Galbraith DN, Taylor KW. Coxsackie B virus infection and onset of childhood diabetes. Lancet 1995; **346**:221–3.

51 Andréoletti L, Hober D, Hober-Vandenberghe C *et al*. Coxsackie B virus infection and β cell autoantibodies in newly diagnosed IDDM adult patients. Clin Diagn Virol 1998; **9**:125–33.

52 Nairn C, Galbraith DN, Taylor KW, Clements GB. Enterovirus variants in the serum of children at the onset of Type 1 diabetes mellitus. Diabet Med 1999; **16**:509–13.

53 Chehadeh W, Weill J, Vantyghem MC *et al*. Increased level of interferon-α in blood of patients with insulin-dependent diabetes mellitus: relationship with coxsackievirus B infection. J Infect Dis 2000; **181**:1929–39.

54 Lönnrot M, Salminen K, Knip M *et al*. Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: a prospective study. Childhood Diabetes in Finland (DiMe) Study Group. J Med Virol 2000; **61**:214–20.

55 Coutant R, Carel JC, Lebon P, Bougneres PF, Palmer P, Cantero-Aguilar L. Detection of enterovirus RNA sequences in serum samples from autoantibody-positive subjects at risk for diabetes. Diabet Med 2002; **19**:968–9.

56 Moya-Suri V, Schlosser M, Zimmermann K, Rjasanowski I, Gurtler L, Mentel R. Enterovirus RNA sequences in sera of schoolchildren in the general population and their association with type 1-diabetes-associated autoantibodies. J Med Microbiol 2005; **54**:879–83.

57 Sarmiento L, Cabrera-Rode E, Lekuleni L *et al*. Occurrence of enterovirus RNA in serum of children with newly diagnosed type 1 diabetes and islet cell autoantibody-positive subjects in a population with a low incidence of type 1 diabetes. Autoimmunity 2007; **40**:540–5.

58 Oikarinen S, Martiskainen M, Tauriainen S *et al*. Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes 2011; **60**:276–9.

59 Foy CA, Quirke P, Lewis FA, Futers TS, Bodansky HJ. Detection of common viruses using the polymerase chain reaction to assess levels of viral presence in type 1 (insulin-dependent) diabetic patients. Diabet Med 1995; **12**:1002–8.

60 Craig ME, Howard NJ, Silink M, Rawlinson WD. Reduced frequency of HLA DRB1*03–DQB1*02 in children with type 1 diabetes associated with enterovirus RNA. J Infect Dis 2003; **187**:1562–70.

61 Chistiakov DA, Voronova NV, Savost'Anov KV, Turakulov RI. Loss-of-function mutations E6 27X and I923V of IFIH1 are associated with lower poly(I : C)-induced interferon-beta production in peripheral blood mononuclear cells of type 1 diabetes patients. Hum Immunol 2010; **71**:1128–34.

62 Lempainen J, Tauriainen S, Vaarala O *et al*. Interaction of enterovirus infection and Cow's milk-based formula nutrition in type 1 diabetes-associated autoimmunity. Diabetes Metab Res Rev 2011 (in press).

63 Helfand RF, Gary HE, Jr, Freeman CY, Anderson LJ, Pallansch MA. Serologic evidence of an association between enteroviruses and the onset of type 1 diabetes mellitus. Pittsburgh Diabetes Research Group. J Infect Dis 1995; **172**:1206–11.

64 Tapia G, Cinek O, Rasmussen T *et al*. Human enterovirus RNA in monthly fecal samples and islet autoimmunity in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study. Diabetes Care 2011; **34**:151–5.

65 Füchtenbusch M, Irnstetter A, Jäger G, Ziegler AG. No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes. J Autoimmun 2001; **17**:333–40.

66 Graves PM, Rotbart HA, Nix WA *et al*. Prospective study of enteroviral infections and development of beta-cell autoimmunity. Diabetes Autoimmunity Study In The Young (DAISY). Diabetes Res Clin Pract 2003; **59**:51–61.

67 Lönnrot M, Sjöroos M, Salminen K, Maaronen M, Hyypiä T, Hyöty H. Diagnosis of enterovirus and rhinovirus infections by RT–PCR and time-resolved fluorometry with lanthanide chelate labeled probes. J Med Virol 1999; **59**:378–84.

68 Rotbart HA, Sawyer MH, Fast S *et al*. Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. J Clin Microbiol 1994; **32**:2590–2.

69 Cinek O, Witsø E, Jeansson S *et al*. Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. J Clin Virol 2006; **35**:33–40.

70 Verstrepen WA, Kuhn S, Kockx MM, Van De Vyvere ME, Mertens AH. Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription–PCR assay. J Clin Microbiol 2001; **39**:4093–6.

71 McIver CJ, Jacques CF, Chow SS *et al*. Development of multiplex

© 2011 The Authors
Clinical and Experimental Immunology © 2011 British Society for Immunology,

PCRs for detection of common viral pathogens and agents of congenital infections. J Clin Microbiol 2005; **43**:5102–10.
72 Stene LC, Hyöty H. A novel approach to the investigation of potential precipitating factors in type 1 diabetes. Pediatr Diabetes 2006; **7**:143–5.
73 Fisher LD, Lin DY. Time-dependent covariates in the Cox proportional-hazards regression model. Annu Rev Public Health 1999; **20**:145–57.
74 Hyöty H, Hiltunen M, Knip M *et al*. A prospective study of the role of Coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Diabetes 1995; **44**:652–7.
75 Lönnrot M, Korpela K, Knip M *et al*. Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. Diabetes 2000; **49**:1314–8.
76 Sadeharju K, Lönnrot M, Kimpimäki T *et al*. Enterovirus antibody levels during the first two years of life in prediabetic autoantibody-positive children. Diabetologia 2001; **44**:818–23.
77 Dahlquist GG, Ivarsson S, Lindberg B, Forsgren M. Maternal enteroviral infection during pregnancy as a risk factor for childhood IDDM. A population-based case–control study. Diabetes 1995; **44**:408–13.
78 Dahlquist GG, Boman JE, Juto P. Enteroviral RNA and IgM antibodies in early pregnancy and risk for childhood-onset IDDM in offspring [Letter]. Diabetes Care 1999; **22**:364–5.
79 Dahlquist GG, Forsberg J, Hagenfeldt L, Boman J, Juto P. Increased prevalence of enteroviral RNA in blood spots from newborn children who later developed type 1 diabetes: a population-based case–control study [Letter]. Diabetes Care 2004; **27**:285–6.
80 Viskari HR, Roivainen M, Reunanen A *et al*. Maternal first-trimester enterovirus infection and future risk of type 1 diabetes in the exposed fetus. Diabetes 2002; **51**:2568–71.
81 Viskari H, Ludvigsson J, Uibo R *et al*. Relationship between the incidence of type 1 diabetes and maternal enterovirus antibodies: time trends and geographical variation. Diabetologia 2005; **48**:1280–7.
82 Kolb H, Elliott RB. Increasing incidence of IDDM a consequence of improved hygiene? Diabetologia 1994; **37**:729.
83 Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002; **347**:911–20.
84 Gale EA. A missing link in the hygiene hypothesis? Diabetologia 2002; **45**:588–94.
85 Filippi CM, Estes EA, Oldham JE, von Herrath MG. Immunoregulatory mechanisms triggered by viral infections protect from type 1 diabetes in mice. J Clin Invest 2009; **119**:1515–23.
86 Evans AS. Causation and disease: the Henle–Koch postulates revisited. Yale J Biol Med 1976; **49**:175–95.
87 Kircher M, Kelso J. High-throughput DNA sequencing – concepts and limitations. Bioessays 2010; **32**:524–36.
88 Metzker ML. Sequencing technologies – the next generation. Nat Rev Genet 2010; **11**:31–46.
89 Lipkin WI. Microbe hunting. Microbiol Mol Biol Rev 2010; **74**:363–77.
90 Breitbart M, Haynes M, Kelley S *et al*. Viral diversity and dynamics in an infant gut. Res Microbiol 2008; **159**:367–73.
91 Finkbeiner SR, Allred AF, Tarr PI, Klein EJ, Kirkwood CD, Wang D. Metagenomic analysis of human diarrhea: viral detection and discovery. PLoS Pathog 2008; **4**:e1000011.
92 Svraka S, Rosario K, Duizer E, van der Avoort H, Breitbart M, Koopmans M. Metagenomic sequencing for virus identification in a public-health setting. J Gen Virol 2010; **91**:2846–56.
93 The TEDDY Study Group. The Environmental Determinants of Diabetes in the Young (TEDDY) study: study design. Pediatr Diabetes 2007; **8**:286–98.
94 Chapman NM, Kim KS. Persistent coxsackievirus infection: enterovirus persistence in chronic myocarditis and dilated cardiomyopathy. Curr Top Microbiol Immunol 2008; **323**:275–92.

© 2011 The Authors
Clinical and Experimental Immunology © 2011 British Society for Immunology,