Chapter 15
Enteroviruses in Blood

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Abstract At the time of clinical diagnosis, the majority of children with type 1 diabetes carry EVs of different species in their blood. Controls rarely carry EVs in blood. In the blood, these viruses are present at very low titers and are minimally able to replicate in cell culture. At the time of clinical diagnosis, the presence of asymptomatic enterovirus infections is common among family members. The enterovirus types involved remain to be defined, but enteroviruses belonging to the B species appear particularly prevalent. Geographic and temporal clusters of enterovirus infection and type 1 diabetes have been documented in Northern Italy. It will be important to determine the length of persistence of enteroviruses in the blood of diabetic children. The results do not provide direct evidence for a causal relationship between enterovirus infection and diabetes, but strongly suggest that the association is not fortuitous.

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Introduction

In just a few cases scientists have been able to isolate viruses from patients with type 1 diabetes (reviewed in: Notkins 1979; Toniolo et al. 1988; Hober and Sauter 2010; Roivainen and Klingel 2010; Tauriainen et al. 2011). Overt hyperglycemia, in fact, is the final consequence of a prolonged pathologic process that, if linked to a viral infection, may involve slowly replicating agents that challenge current methods of virus isolation in vitro.

A recent meta-analysis of molecular studies aimed at virus detection in newly diagnosed cases of type 1 diabetes lends support to the idea that RNA agents (enteroviruses) are present at varying but remarkable frequencies in the early phase of the disease (Yeung et al. 2011). It remains obscure whether these correlations were merely coincidental with the "failing" of an altered immune system or had more profound implications.

However, pathologic studies initiated over 30 years ago (Jenson et al. 1980), have demonstrated enterovirus proteins and/or genome in pancreatic islets, particularly in beta cells (Richardson et al. 2009; Willcox et al. 2011). Indirect signs of an ongoing viral infection in the pancreas of diabetic individuals have been also found. Among these, the local expression of interferon and the detection of double-stranded RNA (indicating replicating viruses) in islet cells (Richardson et al. 2010, 2011).

EVs have been considered agents capable of causing acute clinical conditions, either asymptomatic or symptomatic. However, evidence has been accumulating for these viruses being able of both producing slow pathology in vivo and persisting in the host in spite of a perceptible immune response. Examples include evolution of myocarditis into dilated myocardiopathy (Fujioka et al. 2004; Chapman and Kim 2008; Gorbea et al. 2010) as well as progressive disorders of the central nervous system (Baj et al. 2007; Feuer et al. 2009; Cavalcante et al. 2010; Rhoades et al. 2011). These clinical studies were stimulated by the results of experiments showing that—as in the Theiler virus model of demyelinating disease—a continuous low-level enterovirus replication can be established in a variety of cultured cell types (Conaldi et al. 1997; Kelly et al. 2010) and in animal models (Destombes et al. 1997; Rahnefeld et al. 2011).

It has also been recognized that viruses belonging to the enterovirus genus undergo remarkable genetic variation and evolution (McWilliam Leitch et al. 2010; Harvala et al. 2011). These events are linked to the high mutation rates proper of RNA viruses, recombination among genomes of different enterovirus types (based on little understood mechanisms), and "codon deoptimization," i.e., the accumulation of "silent mutations" that—beyond a certain threshold—slow down virus replication (Coleman et al. 2008). The end result of these multiple events is that the genetic structure of current enterovirus isolates is often different from that of enterovirus prototype strains that were collected 30–60 years ago (Tracy et al. 2010; Hu et al. 2011). In addition to that, inclusion of over 100 virus types in the enterovirus genus (Yozwiak et al. 2010) makes identification and detection of all these agents extremely complex. Difficulties not only apply to tissue culture and serology. Gene amplification methods and genome sequencing have revolutionized our ability to detect viruses both in infected hosts and in the environment (Foxman and Iwasaki

2011), but these procedures require the targeting of stable sequences (Nix et al. 2006; Pallansch and Oberste 2010). The conserved 5′ untranslated region (5′UTR) has been chosen most frequently for enterovirus investigations, although, unfortunately, it carries little or no information for identifying the type of the infecting virus.

Seeking Viruses in Pediatric Patients at the Clinical Onset of Type 1 Diabetes

There is now a very large body of evidence on this subject. It is not our intention to review this in any detail. The complexities of this area of investigation may be best illustrated by referring to our own experience of researching the problem locally, in Italy. Two studies (DAISY and MIDIA that have been carried out in the USA and Norway, respectively) have shown that detection of enterovirus in feces does not predict the development of type 1 diabetes, whereas enterovirus detection in blood is associated with an increased risk of developing diabetes (Stene et al. 2010; Tapia et al. 2011).

Based on our previous experience, a search for enteroviruses in diabetes was initiated by setting up methods capable of detecting viruses in blood under conditions in which the agents had extremely low titers and little or no ability of replicating in cultured cells. Basically, a double approach was followed: direct detection in plasma by gene amplification (reverse transcriptase polymerase chain reaction) and co-culture of peripheral blood leukocytes (PBLs) with a panel of EV-susceptible cell lines. Enterovirus genomes were then detected in RNA extracted from culture medium and/or cultured cells using RT-PCR. Viral antigens were detected by antibody assays to conserved capsid epitopes of different enterovirus types.

As shown in Fig. 15.1a, non-degenerated primers were produced to cover different genomic regions of approximately 100 different enterovirus types. The variability of genome sequences of different enterovirus types is reported in the lower panel (Fig. 15.1b). To enhance the sensitivity of tests, non-degenerated primers have been used throughout. Samples were considered EV-positive when a signal could
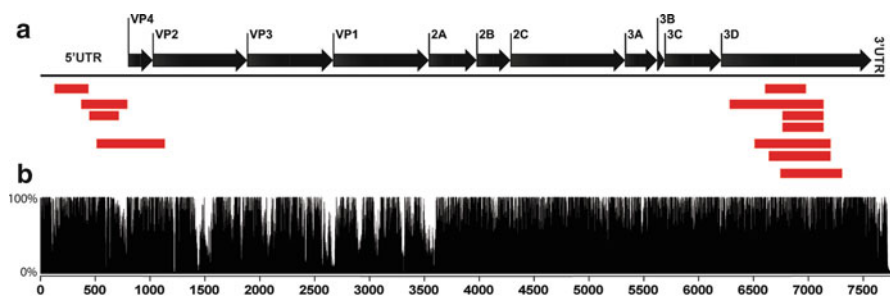
Fig. 15.1 Diagram of the enterovirus genome that is approximately 7.5 kb in length. Panel (**a**): the genome regions that have been amplified by molecular tests are represented in *red*. To this end, primers were designed to target sequences that are conserved among 96 enterovirus types. Panel (**b**): full-genome similarity plot showing the percent identity of nucleotide sequences among 96 enterovirus types (gap open cost = 10; gap extension cost = 1)

be obtained from at least two different genome regions. Results presented here refer to blood samples analyzed with primers directed to three genomic regions: 5′UTR, 5′UTR-VP2 (a region comprising the VP4 capsid gene), and 3D (RNA polymerase gene). With regard to the 3D gene, seven primer pairs had to be used, since this region is not markedly conserved. This extra effort, however, provided partial information with regard to the identification of the A–D species (and of subgroups within the B and C species). However, due to possible recombination events, identification of enterovirus types based on 3D sequences alone is not always possible. Reliable identification of the exact type of enterovirus, in fact, is based on their typing by hyperimmune sera which can neutralize their infectivity in cell culture in a serotype specific manner or on the measurement of increases in neutralizing antibodies in serum [for those agents able to attain sufficient titers and produce detectable cytopathic effects (CPE) in vitro]. Alternatively, identification can be obtained using gene sequence analysis of the highly variable capsid proteins (Nix et al. 2006; Yozwiak et al. 2010).

Subjects Investigated: Children at the Clinical Onset of Type 1 Diabetes, Consenting Family Members, Non-diabetic Controls

Two Pediatric Endocrine Units participated in this study which involved 112 consecutive patients diagnosed from January 2007 to January 2010 (61 boys and 51 girls). The median age of diabetic children was 9.0 years (range 2–16 years). Forty-one consenting family members of 16 diabetic probands were also investigated. Non-diabetic controls ($n=69$) were composed of adult blood donors ($n=34$) and children ($n=35$; matched by age, time, and location) who had been diagnosed with either short stature or overweight/obesity. At the time of writing, the subjects participating to the study have been followed for at least 16 months. On the day of diagnosis, blood samples (K_2-EDTA and serum) were obtained from each patient and from his/her consenting family members. Plasma and serum were stored at −80°C for further analysis. EDTA blood samples were processed immediately for separating PBLs. After washing, PBLs were co-cultured with enterovirus-susceptible cell lines (RD, HeLa, AV3, CaCo) for at least 1 month. During this time, each culture underwent four to six passages by trypsinization. Primers shown in Fig. 15.1 were used for RT-PCR assays that were run on RNA extracted from plasma and from tissue culture medium of cell lines exposed to the patients' PBLs.

Routine clinical methods were used to measure the levels of blood glucose, glycosylated hemoglobin (HbA1c), and, 1 year after diagnosis, the individual insulin requirement (IU/kg/day). C-peptide levels (time 0 and 6 min after stimulation with 1 mg glucagon i.v.) were measured by radioimmunoassay. Fasting C-peptide values in the undetectable range (<0.2 ng/ml) were assigned a value of 0.1 ng/ml for the analyses. The Class-II human leukocyte antigen (HLA) genotype was determined by molecular methods. Titers of autoantibodies to glutamic acid decarboxylase (GAD), tyrosine phosphatase-like molecule (IA-2), zinc transporter-8 (ZnT8), and insulin (IA) were measured by radiobinding assays (Bonifacio et al. 1995a, b; Lampasona

et al. 2010; Naserke et al. 1998). Levels of 12 cytokines/chemokines were measured by ELISA in the supernatant of HeLa cell monolayers co-cultured for at least 1 month with PBL of patients or controls. In the same cultures, expression of the viral VP1 capsid protein was evaluated by immunofluorescence using monoclonal antibodies directed to conserved epitopes of this antigen (Miao et al. 2009).

Clinical Data and Virology

At the time of diagnosis, high glucose levels were present in the group of children investigated (median 386 mg/dl; SD 175). As compared to normal ranges (Fig. 15.2a), fasting serum levels of C-peptide were reduced significantly (median 0.30 ng/ml; SD 0.61) and were increasing modestly 6 min after glucagon stimulation (median 0.75 ng/ml; SD 1.34). The substantial reduction of C-peptide levels as compared to expected values [range 0.7–2.1 ng/ml, basal; 1.7–6.0 ng/ml, after glucagon stimulation (Sosenko et al. 2008)] indicates that a sizeable loss of the beta cell reserve had already occurred at the time of clinical onset. As shown in Fig. 15.2b, levels of HbA1c were increased markedly at the time of diagnosis [median 11.20%; SD 2.70 (normal upper level=6.1%)], indicating that, before diagnosis, elevated glucose levels had been present in newly diabetic children for considerable periods of time (i.e., ≥2–3 months). As shown in Fig. 15.2b, 1 year after diagnosis insulin therapy brought HbA1c levels to near-normal levels (median 6.95%; SD 1.02).

All diabetic children were carrying high-risk HLA alleles and were positive for at least one diabetes-related autoantibody. Significant titers of GAD, IA-2, ZnT8, IA autoantibodies were present in 53%, 66%, 51%, and 88% of cases, respectively. These autoantibodies could not be detected in blood donors, non-diabetic control children, and non-diabetic family members of children with type 1 diabetes. Thus, the children investigated could not be classified as cases of "fulminant diabetes" (a form of insulin-dependent diabetes characterized by shorter duration from onset of hyperglycemic symptoms to first hospital visit, near-normal levels of HbA1c at onset, negativity for islet autoantibodies and ketosis; Shibasaki et al. 2010; see Chap. 22).

In our cohort, the diagnosis of slowly progressing autoimmune type 1 diabetes was made on the basis of increased levels of HbA1c at the time of diagnosis, low C-peptide levels, and positivity for diabetes-related autoantibodies.

Highly sensitive molecular methods and attempts at isolating viruses in cell cultures demonstrated that enterovirus detection in blood is a rare event in non-diabetic subjects. In our patients, only 2/69 (3%) non-diabetic controls carried the enterovirus genome in blood. In contrast, at the time of diagnosis, 89/112 (79%) children with type 1 diabetes were positive for enterovirus genomes and had low-level infectivity in leukocytes (Toniolo et al. 2010). The data are summarized in Fig. 15.3 together with the detected enterovirus species. It should be borne in mind that species identification has been achieved through characterization of the 3D genome region. Thus, as stated previously, identification cannot be held as conclusive, mainly due to recombination events possibly occurring among different enterovirus types.

The results indicate that a single enterovirus species is not involved in all the cases investigated. However, most of the enteroviruses were associated with the B
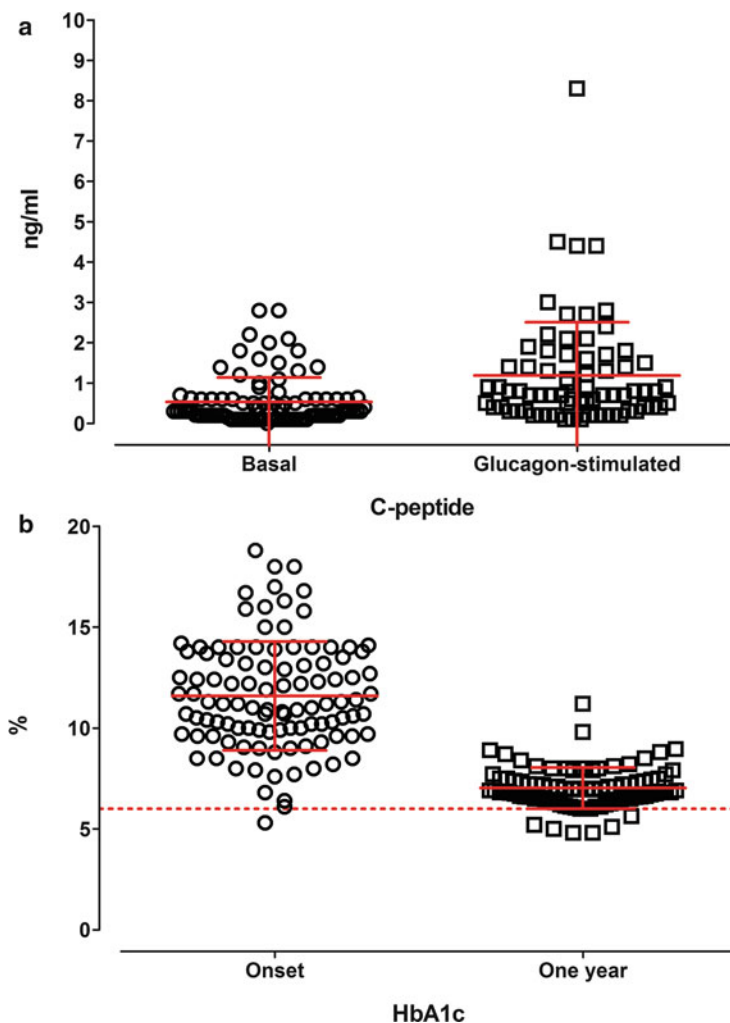
Fig. 15.2 Levels of C-peptide and glycosylated hemoglobin (HbA1c) in newly diagnosed children with type 1 diabetes (*n* = 112). Each *dot* represents a value of a single patient. Panel (**a**): C-peptide levels were measured at the time of diagnosis (basal and 6 min after stimulation with 1 mg glucagon i.v.). The median ± SD is shown. Most values are well below the expected ranges (see text). Panel (**b**): the percentage of HbA1c was measured both at the time of diagnosis (strongly elevated levels) and after 1 year of insulin therapy (levels slightly above upper reference values). The median ± SD is also shown. The upper level in healthy controls is represented by the *red dotted line*

species. It should be noted that the B species is the largest one of the enterovirus genus, containing at least 58 serotypes. Molecular tests based on the 3D region allow identifying three subgroups within this species (B1–B3).

Early studies of viruses in type 1 diabetes and the few cases in which virus isolation/typing has been achieved (e.g., Yoon et al. 1979; Champsaur et al. 1980; Dotta et al. 2007) indicate members of the B species as those most probably implicated in
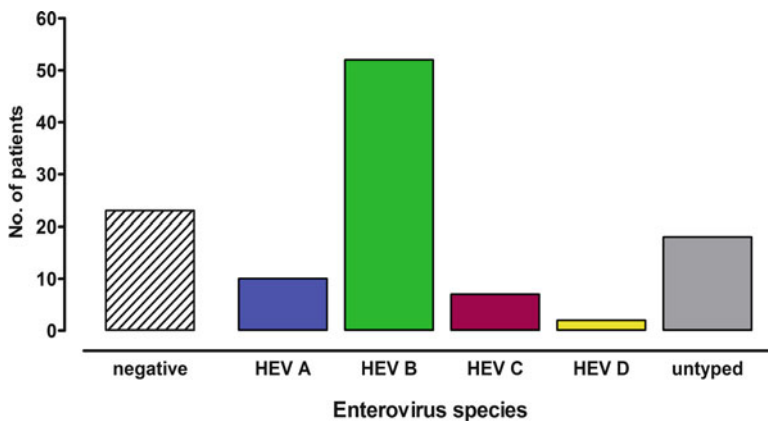
Fig. 15.3 Detection and identification of enterovirus species in the blood of children with newly diagnosed type 1 diabetes. Among 112 probands, 23 children were enterovirus negative (21%), 89 were enterovirus positive (79%). Among enterovirus-positive patients, analysis of the 3D genome region allowed to assign the majority of cases to enteroviruses of the B species. Approximately 20% of cases could not be typed

diabetes (Coxsackievirus B4 in particular). It should be considered, however, that involvement of the "B1 subgroup" of the B species has been particularly frequent in our patients. Based on the 3D amplification method developed by us, the B1 sub-group contains 20 echovirus types together with coxsackieviruses B1–B3. This is in agreement with reports from Finland and the tropics that have shown an association of echoviruses with type 1 diabetes (Vreugdenhil et al. 2000; Otonkoski et al. 2000; Díaz-Horta et al. 2001; Paananen et al. 2003; Cabrera-Rode et al. 2003, 2005; Williams et al. 2006; Al-Hello et al. 2008).

When cultured for prolonged periods with human cell lines, the infectious agents derived from PBLs of type 1 diabetes patients may produce weak but perceptible CPE. Figure 15.4 shows phase-contrast microscopy of HeLa cells exposed to PBLs obtained from four members of a single family (panel a) and the expression of entero-virus antigens (immunofluorescence) in cultures of corresponding samples (panel b). As shown in panel a, co-culture of cell lines with samples of the mother and father of the type 1 diabetes proband failed to produce CPE, whereas samples from the diabetic proband and his brother produced faint CPE. CPE production was in agreement with the expression of the enteroviral VP1 capsid protein: fluorescent dots are seen in the cytoplasm of HeLa cells co-cultured with PBLs of the diabetic proband and his brother, but not in cells co-cultured with PBLs of their parents. RT-PCR confirmed the findings: the type 1 diabetes proband and his brother were carrying an enterovirus of the B1 subgroup of the B species. This, and extensive observations made in addi-tional families, indicate that, at the time of clinical diagnosis, enteroviruses are pres-ent often not only in type 1 diabetes probands but also in their family members.

Notably, in the families investigated, the virus-carrier status of non-diabetic family members was not associated with noticeable clinical symptoms. This obser-vation speaks openly against dramatic events being produced by low-level entero-virus infection. Analogous observations were made in the course of polio epidemics
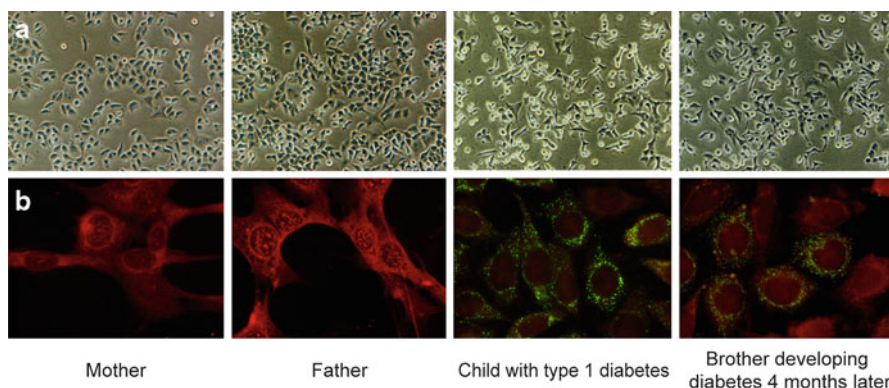
Fig. 15.4 Cytopathic effects (CPE) and expression of enterovirus capsid antigen in HeLa cell cultures exposed for 1 month to peripheral blood leukocytes (PBLs) of four members of the same family. Samples were obtained at the time of clinical diagnosis in the type 1 diabetes proband. Panel (**a**) (200× magnification): phase-contrast microscopy of subconfluent monolayers of HeLa cells co-cultured with PBL (*left* to *right*) of the mother, father, diabetic proband, and his brother. No CPE is seen in the first two images. Faint CPE is observable in the third and fourth images (diabetic proband and his brother). The virus-positive brother developed overt diabetes 5 months later. Panel (**b**) (1,000× magnification): same cultures as in panel (**a**). Indirect immunofluorescence with monoclonal antibodies to conserved capsid antigens of enteroviruses. Expression of the enterovirus capsid VP1 protein is seen as fluorescent dots in the cytoplasm of HeLa cells. *Left* to *right*: virus-negative cultures (father and mother) and virus-positive cultures (diabetic proband and his brother). The two virus-positive children were carrying an enterovirus of the B species (B1 subgroup)

(in which, generally, fewer than 1:100–1:500 infected people manifested neurologic symptoms) and seem to fit with the original definition of the echovirus genus (i.e., viruses not associated frequently with detectable symptoms or disease).

With regard to the potential pathogenetic mechanisms, examination of cell culture media derived from HeLa cells co-cultured with PBLs (either from healthy blood donors or EV-carrying diabetic children) showed that the production of the monocyte chemoattractant protein 1 (MCP1) was significantly enhanced in cultures exposed to PBLs of diabetic children. Levels of other cytokines/chemokines remained comparable. This observation suggests that the slowly replicating agent(s) detected in children at the clinical onset of type 1 diabetes can promote inflammation by attracting monocyte/macrophages. Recent data indicate that MCP1 also induces amylin expression in pancreatic beta cells (Cai et al. 2011), the main constituent of amyloid deposits in islets of patients with type 2 diabetes.

Geographic and Temporal Clusters of EV-Positive Cases of Type 1 Diabetes

In the course of these studies, 76 EV-positive cases of type 1 diabetes have been detected in the Northern part of the Province of Varese, Italy (Fig. 15.5a). This area is bordered by high mountains to the North and set apart by lakes and rivers to the
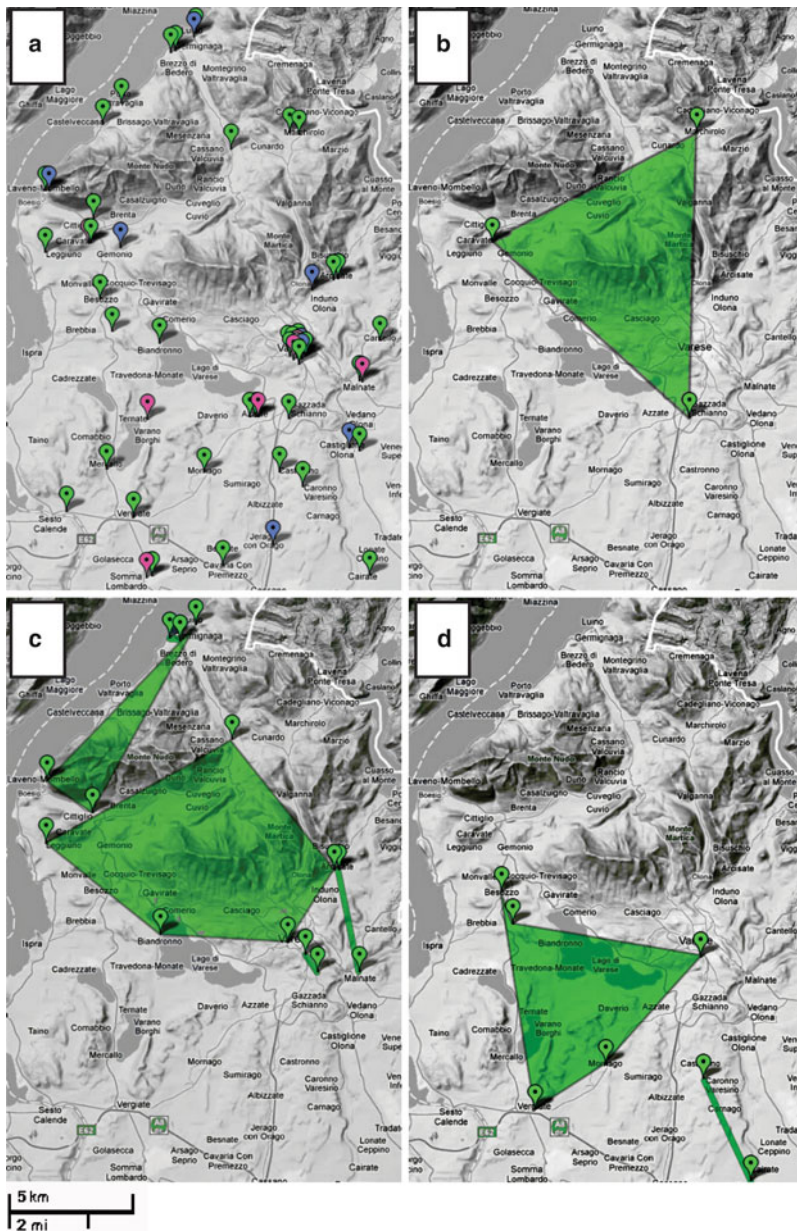
Fig. 15.5 Google® map of the Northern part of the Province of Varese, Italy, with the location of the enterovirus-positive cases of type 1 diabetes (**a**) and examples of geographic and temporal clusters of cases (**b–d**). (**a**) Location of 76 enterovirus-positive cases of type 1 diabetes that have been detected in the course of the study. Cases associated with different species of enteroviruses are represented with markers of different colors: A species (*blue*); B species (*green*); C species (*fuchsia*). (**b**) One cluster of 3 cases associated with an enterovirus of the B1 subgroup (the B1 subgroup comprises 20 echovirus types and Coxsackieviruses B1–B3). (**c**) Five clusters (total 14 cases) associated with an enterovirus of the B1 subgroup (as above). (**d**) Two clusters (total 7 cases) associated with an enterovirus of the B2 subgroup (the B2 subgroup comprises selected echoviruses, selected numbered enteroviruses, and Coxsackievirus B5)

West and the East. As a consequence, most children developing symptoms of type
1 diabetes are seen at a single hospital. Over a 4-year period, geographic and temporal
clustering of cases have been noted. Clusters were defined as those cases that
occurred with a maximum lag of 9 months within 20 km from each other. It was
found that each cluster was associated with a single enterovirus species, and, in the
case of the B and C species, each cluster was associated with a single B or C virus
subgroup. The sites of the new cases that have been investigated during the study are
shown in Fig. 15.5 (panel a). Clusters associated with enterovirus B1 (panels b and
c) and B2 (panel d) subgroups are also shown.

Conclusions

Taken together, these studies point to a significant association between early stage
type 1 diabetes and the presence of enteroviruses in blood. Whether the association
was merely casual or had a triggering/etiologic role remains to be defined. The
above observations, however, substantiate further the long-suspected implication of
these agents in juvenile type 1 diabetes.

It is well known that C-peptide levels decline rapidly in the perionset period of
type 1 diabetes (Sosenko et al. 2008) and that preservation of the residual beta cell
reserve is essential for a favorable prognosis. Thus, early postdiagnosis interventions
need to be developed for possible therapy as close to the diagnosis as possible.
Among these, antiviral drugs/antibodies may be taken into consideration (Norder
et al. 2011; Chen et al. 2011).

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