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Molecular analysis of persistent enterovirus infections

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Abstract

Human enteroviruses (HEVs) are etiological agents of different clinical entities varying from asymptomatic to mild, severe, or fatal diseases. There is now growing evidence that HEVs can cause or contribute to common chronic diseases. Even though a specific therapies are not yet available, a correct diagnosis is necessary both for diagnostic and epidemiologic purposes. Gene amplification methods and sequencing greatly facilitates the laboratory diagnosis of these conditions and allows discovering previously unsuspected etiopathogenetic associations.

In this work, three clinical conditions possibly associated with persistent HEV infections have been investigated: type 1 diabetes in children, the post-polio syndrome in adult patients, as well as clinical cases of inflammatory cardiomyopathy. New methods for HEVs detection have been developed based on bioinformatic studies of HEV sequence databases. These methods have been tested with reference virus strains as well as a wide range of clinical specimens. Our most sensitive assay is based on the detection and sequencing of the viral polymerase (3Dpol) coding region.

Different cohorts of patients have been investigated using classical virology and molecular methods. The studies allowed to conclude that HEVs can persist for long periods in humans and that these agents can be associated with endocrine, neurological, and cardiovascular conditions.

1 - Introduction

Enteroviruses belong to genus *Enterovirus* of the *Picornaviridae* family and are associated with different human diseases. Enteroviruses were initially classified based on neutralization using type-specific antisera and antisera pools. Eighty-nine serotypes are identified on the basis of neutralization tests. Sixty-six serotypes have been found to be infectious for humans. There are both human and non-human species under the Enterovirus genus. Human enteroviruses (HEVs) were originally grouped basing on human disease manifestations, replication and pathogenesis in newborn mice.

Although most enterovirus infections are mild and asymptomatic, various fatal diseases such as respiratory illness, myocarditis, aseptic meningitis, encephalitis and acute flaccid paralysis may occur (Palacios and Oberste, 2005). Typing of viruses is required primarily to provide information on the relationship between virus type and disease, to identify variants with increased virulence or specific disease properties, for epidemiological investigations, and to allow correlation with viral immunity. New molecular technologies have spawned the development of molecular typing methods for several virus groups that are not amenable to other means of investigation. These methods have recently raised the possibility of simple genotyping methods for other virus groups that have been typed traditionally based on phenotypic traits. There is growing evidence that HEVs cause or contribute to common chronic pathologies, including dilated cardiomyopathy, insulin-dependent diabetes or type 1 diabetes (T1D), and chronic fatigue syndrome (Roivanen and Klingel, 2009). Persistent enterovirus infection has been demonstrated both in agammaglobulinemic humans and animals, as well as in immunocompetent subjects. A role of the immune system has been considered among mechanisms of HEV persistence. Molecular mimicry, bystander damage and inflammatory response have been proposed in virus-induced autoimmune diseases.

The aim of this study was to investigate the involvement of HEVs in different clinical conditions. In particular, we focused on three main pathologies: the

T1D, the post-polio syndrome, and inflammatory cardiomyopathy. In order to study these infections characterized by particularly low virus titers, new PCR methods based on the region coding for the viral RNA polymerase have been developed. These methods are designed to cover almost all known HEV types.

2 – The Enterovirus genus

On the basis of their ability to replicate in human or animal cell types, pathogenicity in humans and animals, as well as antigenic differences, the Enteroviruses were originally classified into four groups: polioviruses, Coxsackie A viruses (CVA), Coxsackie B viruses (CVB), and echoviruses. It was quickly realized that there were significant overlaps in the biological properties of viruses in the different groups. New enteroviruses isolated are after 1970 have been designed by consecutive numbers e.g., EV68, EV69, EV70, EV71, etc. (Oberste MS et al., 1999). Recently Enterovirus classification has considerably changed. Polioviruses have become part of the HEVC species, and Rhinovirus gender has been inserted into the enterovirus genus (Table 1). Even though the two classes are quite similar in their genomic structure, their clinical manifestations are vastly different. Enteroviruses infect the enteric tract, which is reflected in their name. On the other hand, rhinoviruses infect primarily the nose and the throat. Enteroviruses replicate at 37°C, whereas rhinoviruses grow better at 33-34°C (lower temperature of the upper respiratory tract). Enteroviruses are stable under acidic conditions and are able to survive exposure to gastric acid. In contrast, rhinoviruses are acid-labile (inactivated by low pH conditions). This is possibly one reason why rhinovirus infections are restricted to respiratory tract.

Table 1 Picornavirus classification (Adapted from the Picornaviridae database http://www.picornaviridae.com/; November 2010).

Genus	Species	Serotypes
Enterovirus	*Bovine enterovirus*	2 types: bovine enterovirus (BEV) 1-2
	Human enterovirus A	21 types including some coxsackie A viruses and enteroviruses
	Human enterovirus B	59 types including enteroviruses, coxsackie B viruses, echoviruses, and swine vesicular disease virus

	Human enterovirus C	19 types including poliovirus (PV) 1-3, some coxsackie A viruses and enteroviruses
	Human enterovirus D	3 types: EV-68, EV-70, EV-94
	Porcine Enterovirus B	2 types: porcine enterovirus (PEV) 9-10
	Simian Enterovirus A	3 types: simian enterovirus (SEV) V4, SV28 and SA4
	Human rhinovirus A	74 types
	Human rhinovirus B	25 types
	Human rhinovirus C	7+ types
Cardiovirus	*Encephalomyocarditis virus*	1 type: encephalomyocarditis virus (EMCV).
	Theilovirus	12 types: Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), Thera virus (TRV), Saffold virus (SAFV) 1-9
Aphthovirus	*Foot-and-mouth disease virus*	7 types: O, A, C, Southern African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1
	Equine rhinitis A virus	1 type: equine rhinitis A virus (ERAV)
	Bovine rhinitis B virus	1 type: bovine rhinitis B virus (BRBV)
Hepatovirus	*Hepatitis A virus*	1 type: Hepatitis A virus (HAV)
Parechovirus	*Human parechovirus*	14 types: Human parechovirus (HPeV) 1-14
	Ljungan virus	4 types: Ljungan virus (LV) 1-4
Erbovirus	*Equine rhinitis B virus*	3 types: equine rhinitis B virus (ERBV) 1-3
Kobuvirus	*Aichi virus*	1 type: Aichi virus (AiV)
	Bovine kobuvirus	1 type: bovine kobuvirus (BKV)
Teschovirus	*Porcine teschovirus*	11 serotypes: porcine teschovirus (PTV) 1 to 11
Sapelovirus	*Porcine sapelovirus*	1 type: porcine sapelovirus (PSV) (formerly PEV-8)
	Simian sapelovirus	3 types: simian sapleovirus (SSV) 1-3
	Avian sapelovirus	1 type: avian sapelovirus (ASV)

Senecavirus	*Seneca Valley virus* *	1 type: Seneca Valley virus (SVV)
Tremovirus	*Avian encephalomyelitis virus*	1 type: avian encephalomyelitis virus (AEV)
Avihepatovirus	*Duck hepatitis A virus*	3 types: duck hepatitis A virus (DHAV) 1-3

2.1 – Enterovirus structure

The enterovirus particle consists of a non-enveloped capsid of about 30 nm diameter with icosahedral symmetry. The capsid is composed of 60 copies of four nonidentical proteins (VP1, VP2, VP3, and VP4), arranged in sixty repeating protomeric units of an icosahedron (Figure 1). Even though the basic organization of the capsid is shared by all picornaviruses, enteroviruses are distinguished on the basis of physical properties, such as buoyant density in cesium chloride, and stability at low pH. Many aspects of enteroviral pathology, transmission, and general epidemiology are directly related to the biophysical properties and their cytolytic life cycle.

In 1985, the atomic-structure resolution of poliovirus 1 and rhinovirus 14 have been reported (Hogle et al.1985; Rossmann et al., 1985) and our understanding of *Picornaviridae* has greatly increased. To date, x-ray crystallographic data of more than seven enteroviruses have been obtained and interpreted. These studies have included poliovirus 1 (PV1), poliovirus 3 (PV3) (Filman et al. 1989), HRV3 (Zhao et al. 1993), coxsackievirus B3 (CVB3) (Muckelbauer et al.1995), bovine enterovirus (BEV) (Smyth et al. 1995), PV2 (Lentz et al. 1997), coxsackievirus A9 (CVA9), (Hendry et al. 1999) and HRV16 (Hadfield et al. 1997). All these structures share a remarkable degree of overall similarity, while variation is seen on the surface and on the inner structures of the capsid. The major capsid proteins VP1 to VP3 are each folded into eight-stranded antiparallel β-sheets with a jelly-roll topology. These β-barrels of five copies of VP1 are located around the fivefold axis, while VP2 and VP3 are around the threefold axis. VP4 is much smaller than the other three structural proteins, having a less ordered structure, and being located on the inner surface of the capsid, facing the

RNA. One of the most striking features of Picornavirus surface is the circular canyon around the fivefold axis, which has been first seen in human rhinovirus 14 (HRV14) (Rossmann et al. 1985). The enterovirus family has a deep depression or ‘canyon’ (12-15 Å depending of the serotype) on the capsid surface structure, running around each fivefold vertex. In the inner side of the canyon there are conserved amino acid residues involved in the attachment to cell receptors (Rossmann et al., 1985). This site is protected from the immune surveillance by the inability of neutralizing antibodies to penetrate into the canyon (due to their larger size), but can still be accessed by cell surface receptors. The canyon is located roughly between VP1 on the ’north’ side (the side closer to the fivefold axis), and VP2/VP3 on the ‘south’ side. This organization leaves five copies of VP1 as a protrusion at the fivefold axis. All known enterovirus and rhinovirus structures contain a hydrophobic pocket at the base of the canyon, covered by loops of VP1 (Rossmann et al., 2002). This space is assumed to be normally filled by a natural pocket factor, a fatty acid with an aliphatic chain of variable length (Figure 2). These pocket factors, including sphingosine (PV1, PV3), palmitate (CVB3), and myristic acid (BEV), are believed to be inserted into the virion upon release from the infected cell. Binding of the receptor into the canyon probably competes with the binding of the pocket factor into the hydrophobic pocket. Release of the pocket factor destabilizes the virus and thereby initiates uncoating. Replacement of the pocket factor by antiviral compounds can inhibit both attachment and uncoating (Salvati et al., 2004).

Neutralization studies with escape mutants have shown a conserved antigenic structure in the HEV capsid organization. Four major epitopes were localized, named antigenic sites 1 to 4 (AgS1-AgS4) (Minor, 2004). Antigenic site 1 consists chiefly of a continuous sequence of 12 amino acids in VP1, together with additional sequences from VP1 in regions around amino acids 140, 165 and 255 of PV-3. Site 2 is a complex site composed of a continuous sequence from VP2, in the region of amino acids 160 to 170, the C terminus of VP2 and a short continuous sequence from VP1 in the region of amino

acid 220. Site 3 consists of several elements from VP3, including residues in the region of amino acids 60 and 70, and a sequence from VP1 in the region of residues 280 to 290. Site 4, which bridges adjacent pentamers and is therefore expressed only on the assembled capsid, includes elements from VP3, in the region of amino acids 77 to 80, and VP2, in the region of amino acid 72.

2.2 – Enterovirus genome

Enteroviruses have a small single-stranded RNA genome that is positive sense and infectious. The genome, 7.5 kb long, is constituted by a single open reading frame flanked by two untranslated regions (UTR) (Figure 3). The 5'UTR region presents a highly folded secondary structure, named IRES (internal ribosome entry site) that act as a primer for RNA translation. The 3'UTR encodes a cis-acting replication element required for negative strand RNA synthesis (Brown et al., 2005). The coding region is translated into a single polyprotein of approximately 2,200 amino acids. During the replication phase, the polyprotein is processed to generate the four structural proteins (VP1 to VP4) and seven nonstructural proteins (2A to 2C and 3A to 3D). The four structural proteins are assembled to constitute the icosahedral capsid, where are located the neutralizing epitopes. Nonstructural proteins are associated with membranous replication complexes and play an essential role in virus replication (Teterina et al, 2006). Proteins 2A and 3C are proteases, 3D is the RNA-dependent RNA polymerase, 2C is a helicase used during RNA encapsidation. Proteins 2B and 3A have been associated with various functions in the replication of viral RNA. Protein 3B (VPg) is covalently linked to the 5'UTR region of viral genome and acts as a primer for RNA chain initiation and full length RNA synthesis (Pathak et al, 2008).

An important property of RNA viruses' genome is represented by its high frequency mutation and recombination rates. RNA replication is extremely error-prone, due to the lack of proofreading activity of the viral RNA-dependent RNA polymerase, taking the error rate to approximately one per

genome replication (Bouslama et al. 2007). Consequently, enteroviruses exist as a dynamic mutant population termed quasispecies, near to a mutational meltdown or error catastrophe (Koonin et al, 2008). This high variability within RNA virus families acts like an evolutionary force (Domingo et al, 2006). In addition, an intratypic and intertypic form of homologous recombination between quasispecies ensures adaptability in the environment, contributing to viral pathogenesis and to the continuous emerging of new viruses (Arbiza et al, 2010).

2.2.1 – The RNA dependent RNA polymerase

The viral RNA-dependent RNA polymerase (RdRp) coded by the 3D region is the core of the replication process. This enzyme is assumed to be an ancient protein common to the entire positive-strand RNA virus superclass (Koonin E et al., 2008). In early comparative genomic analyses, positive strand RNA viruses of eukaryotes were classified into three superfamilies: picorna-like, alpha-like and flavi-like. These three superfamilies include most known positive-strand RNA viruses. The superfamilies were delineated through a combination of phylogenetic analysis of conserved protein sequences, primarily those of RdRps, and comparison of diagnostic features of genome organization that are linked to replication and expression strategies. In fact, comparative analysis of putative RNA polymerases of positive-strand RNA agents showed that the polymerase is the only viral protein containing conserved motifs through this entire class of viruses (Koonin E, 1993).

2.3 – Enterovirus receptors and cell entry

Capsid proteins play an important part in receptor binding and, thereby, in defining picornaviral tropism and pathogenesis (Rossmann et al., 2002). As a vector of the infecting genome, the capsid is exposed to the extracellular environment. In particular, the VP1 capsid protein plays an important role as the major protagonist in the virion-cell interplay. Therefore, it contains most of the epitopes involved in cell entry. As shown in Table 2, picornaviruses

exploit several different surface molecules as receptors, including immunoglobulin-like molecules and adhesion proteins.

Table 2. Summary of enterovirus receptors.

Serotype	Receptor	Coreceptor	References
IgSF receptors			
Polioviruses 1-3	PVR (CD155)	Ig-like	Mendelsohn et al., 1989
Coxsackieviruses A13, A18, A21	ICAM-1	Ig-like	
Coxsackievirus A9	$\alpha_v\beta_3$ integrin	Integrin	Berinstein et al., 1995
Echoviruses 1, 8	$\alpha_2\beta_1$-Integrin (VIa-2)	Integrin	Bergelson et al., 1992
Major group HRV (90 serotypes)	ICAM-1		Bella et al.m 1998
Coxsackieviruses B1-B6	CAR	Ig-like	
Other receptor types			
Coxsackievirus A21	DAF (CD55)	SCR-like (complement cascade)	
Coxsackieviruses B1, B3, B5	DAF (CD55)	SCR-like (complement cascade)	He et al., 2001; Bergelson et al., 1994; Bergelson et al., 1997
Echoviruses 3, 6, 7, 11-13, 20, 21, 24, 29, 33	DAF (CD55)	SCR-like (complement cascade)	
Enterovirus 70	DAF (CD55), Sialic acid	SCR-like (complement cascade) Carbohydrate	
Minor group HRV (10 serotypes)	LDL-R		Hewat et al., 2000

Abbreviations: PVR, poliovirus receptor; DAF, decay-accelerating factor; CAR, coxsackievirus-adenovirus receptor; ICAM, intercellular adhesion molecule; IgSF, Immunoglobulin super family; LDL-R, low-density lipoprotein receptor.

Different types of cell surface molecules serve as cellular receptors for picornaviruses, and some are shared among picornaviruses and members of other virus families. For some enteroviruses (e.g., poliovirus), a single type of receptor is sufficient for entry of viruses into cells. For other viruses, a second molecule, or coreceptor, is needed for virus entry into cells. For example, coxsackievirus A21, which binds to CD55, requires also the intercellular adhesion molecule 1 (ICAM-1) for entry into cells (Figure 4).

Most of the enterovirus cell receptors belong to the immunoglobulin superfamily domains (IgSF). These molecules usually consist of tandem repeats of between two and five Ig-like domains: the amino-terminal domains D1 interact with viruses, whereas their carboxy-terminal terminus consists of a transmembrane and a short cytoplasmic region. Besides, the canyon offers another advantage for receptor attachment, namely that the binding of the receptor triggers the uncoating process. When the receptor is bound to the capsid canyon, the virion becomes unstable and is ready for the internalization.

There are, however, other molecules that do not belong to the Ig superfamily, used as enterovirus receptors. These molecules bind outside the capsid canyon and are called *low-density lipoprotein receptors* (LDL-R) (Hofer et al., 1994). The minor-group HRV serotype use the very-low-density lipoprotein receptor (VLDL-R), whereas some of the HEVA and HEVB group serotypes use the DAF (CD55) receptor. VLDL-R binds north of the canyon close to the icosahedral fivefold vertex, whereas DAF binds south of the canyon around the icosahedral twofold axes (Shafren et al., 1997). These receptors do not cause viral instability upon binding. Thus do not, by themselves, trigger uncoating. However, their recruitment can trigger the aggregation of other receptor molecules or they could trigger endocytosis followed by lowering of pH in endosomal vesicles (for instance, FMDV; Carrillo et al., 1984).

The next step of cell infection is the cell entry. Depending on the receptor type and attachment site, there seems to be different mechanisms of cell

entry. The interaction of the virion with receptor initiates major structural changes in the particle, leading to release of the genome. The resulting particles (called altered, or A-particles), have lost the internal capsid protein VP4. In addition, the N-terminus of VP1 is exposed outside of the canyon, on the surface of the A-particle (Fricks CE and Hogle JM, 1990). In this entry method, the A-particle has an increased affinity for membranes because of the VP1 hydrophobic exposed sequence. A gap is formed on the virion surface, in the area where VP1, VP2 and VP3 meet, allowing the emergence of VP4 and the N-terminus of VP1. With this conformation, the N-termini of VP1 can insert into the plasma membrane. A pore is formed and the RNA is released into the cytoplasm.

Some enteroviruses (e.g., rhinovirus) enter cells by receptor-mediated endocytosis. Uncoating may be triggered by acidification of the endosomal, pH-dependent pathway. Infection of cells by these viruses is inhibited by weak bases and ionophores that block the acidification of endosomes (Knipe et al., 1997). For these viruses, interaction with a cell receptor serves only to concentrate virus on the cell surface. The virion-receptor interaction does not induce uncoating-related changes in the virus particle, but rather serves only to tether the virus to the cell and bring it into the endocytic pathway.

2.5 – Enterovirus life cycle

Once the viral RNA is in the cytoplasm, viral replication begins. The first step in this phase is viral RNA translation, because it cannot be copied by any cellular RNA polymerase and the virion is devoid of endogenous enzymes (Figure 5). In fact, there’s no 5’ capping on the viral genome, nor viral enzymes in the capsid. The 5’ untranslated region contains a highly-folded RNA secondary structure, capable to bind to the 40S ribosomal subunit (Jang et al., 1988).

The viral RNA is translated by the cellular machinery into a single, long polyprotein. During the translation phase, when the ribosomes are attached and the polyprotein is produced, the translation's product is cleaved by virus-

encoded proteinases. This strategy allows solving the mRNA polycistronic limitation in viral replication, enabling the synthesis of multiple protein products from a single RNA genome.

Enterovirus genome encodes two proteinases: $2A^{pro}$ and $3C^{pro}$ (or $3CD^{pro}$), which carry out cleavage of the polyprotein. The cleavage process occurs in two phases. The primary cleavage between P1 and P2 is mediated by $2A^{pro}$. Several cellular proteins are cleaved by $2A^{pro}$, including eIF4GI, eIF4GII, Pabp, and heart muscle dystrophin (Glaser and Skern, 2000; Kundu et al., 2005). In the same time, $3C^{Pro}$ cleaves between P2 and P3. An intermediate cleavage produces both $3C^{pro}$ and $3CD^{pro}$ proteinases, which have different activities. $3CD^{pro}$ is released from the P3 precursor by autocatalytic cleavage and is capable to carry out secondary cleavage of glutamine-glycine dipeptides in P1 intermediate more efficiently that $3C^{pro}$. All the primary cleavages occurs *in cis*, while after the proteinases have been released, they cleave the polyprotein *in trans*. During the secondary cleavages, all the viral proteins are produced. Both $3C^{pro}$ and $3CD^{pro}$ process proteins of the P2 and P3 regions with similar efficiency. The viral 3D protein works like a RNA dependent RNA polymerase. The RNA polymerase $3D^{pol}$ is produced by cleavage of a precursor protein, $3CD^{pro}$, which is highly active as a proteinase but has no polymerase activity.

The same RNA molecule that has been used in translation, is then used as a template for synthesis of negative-stranded RNA molecules by the virus-encoded polymerase. These strands are then used in synthesizing large amounts of positive-stranded copies that are used in translation of viral proteins, as well as being encapsidated into assembling particles.

At the end of the infectious process, capsid proteins are synthesized and assembled into immature subunits, containing VP1, VP3 and VP0, which at the final stage of virion assembly is cleaved into VP2 and VP4. Infectious virus particles are produced in one infected cell that is finally destroyed allowing the viruses to be released and infect new target cells. Empty capsids (defective) are common in the Picornavirus life cycle.

A single infected cell can typically produce 10^4 to 10^5 virus particles that are released by lysis of the cell. The lysis has been proposed to occur due to increased permeability of cell membranes, which results from increasing concentrations of nonstructural proteins 3A and 3AB (Lacal, Carrasco., Lama & Carrasco 1992), 2BC (Aldabe et al. 1997), and 2B (van Kuppeveld et al. 1997). The production of viral proteins (especially 2A and 2B) inhibits the cellular proteins secretion, even in absence of the viral RNA replication (Doedens and Kirkegaard, 1995).

2.6 – Clinical diseases

Enteroviruses are present in the environment and vehicles for their dissemination include stools, skin lesions, nasal and bronchial secretions. They can be isolated both from the upper and lower alimentary tract and can be transmitted by the fecal-oral and respiratory routes. Fecal-oral transmission is the primary mode of spread, especially in poor sanity conditions: contaminated water and wastewater represent the main sources of this virus (Ali et al., 2003). Clinical outcome ranges from unapparent infection to severe and life threatening disease. Enteroviruses that cause a vesicular exanthem (like FMDV, CVA-10, 16, 8; EV-71) presumably can be spread by direct or indirect contact with vesicular fluid, which contains infectious virus.

In particular, EVs are among the most commonly identified etiologies of myocarditis, causing between 25 and 35% of cases for which an etiology is found (Martino et al., 1994). Neonates and young infants are particularly susceptible to EV myocarditis, but most cases occur in young adults between the age of 20 and 39 years.

Meningitis is a frequent manifestation of EV infection which, in turn, is the most common cause of meningitis in the US. The severity of EV meningitis varies with host age and immune status (Rotbart et al., 1997). EV Encephalitis is well documented, but relatively uncommon (Tyler, 2009) and generally linked to immunodeficiency diseases. Unlike aseptic meningitis,

which may have prolonged morbidity but from which recovery is generally the rule, encephalitis due to the EV may have long-term sequelae.

Neonatal infection with EVs poses the greatest risk for severe disease when illness develops in the first days of life; this pattern suggests possible transplacental acquisition (Hawkes and Vaudry, 2005). Mortality is typically due to hepatic failure or myocarditis.

By the end of the 70s, CVBs has been implicated as an etiologic agents of various diseases, both acute (i.e. myocarditis, aseptic meningitis, pancreatitis) and chronic (i.e. dilated cardiomyopathy, insulin dependent diabetes mellitus, chronic meningoencephalitis). Nowadays there are no vaccines against non-polio enteroviruses and treatment of infection is only symptomatic.

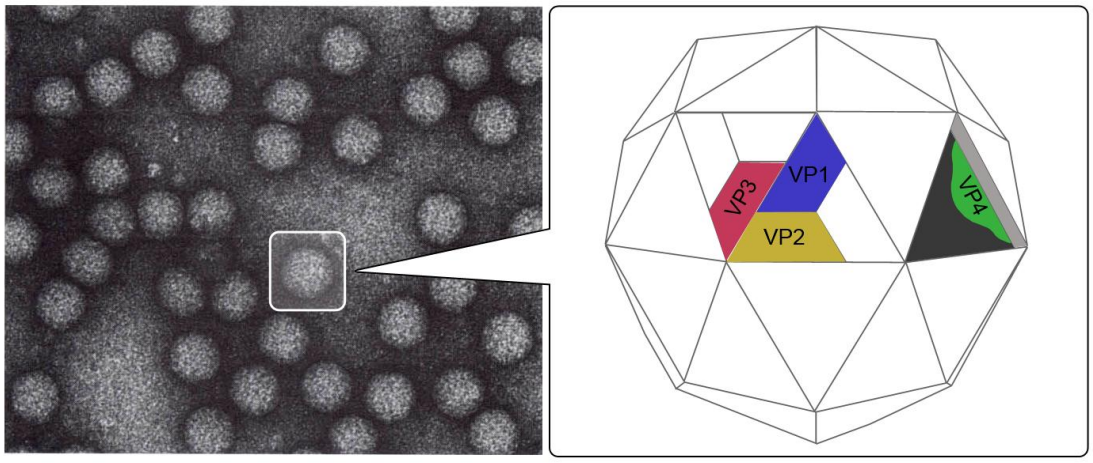
Table 3. Clinical syndromes associated with infections by enteroviruses.

Polioviruses, types 1-3
Paralysis (complete to slight muscle weakness)
Aseptic meningitis
Undifferentiated febrile illness, particularly during the summer
Coxsackieviruses group A, types 1-24
Herpangina
Acute lymphatic or nodular pharingitis
Aseptic meningitis
Paralysis
Exanthema
Hand-foot-and mouth disease (A10, A16)
Pneumonitis of infants
Common cold
Hepatitis
Infantile diarrhea
Acute hemorrhagic conjunctivitis (type A24 variant)
Coxsackieviruses group B, types 1-6
Pleurodynia
Aseptic meningitis
Paralysis (infrequently)
Severe systemic infection in infants, meningoencephalitis, and myocarditis

Pericarditis, myocarditis
Upper respiratory illness and pneumonia
Rash
Hepatitis
Undifferentiated febrile illness
Echoviruses, types 1-33
Aseptic meningitis
Paralysis
Encephalitis, ataxia, or Guillan-Barré syndrome
Exanthema
Respiratory disease
Others: diarrhea, epidemic myalgia, pericarditis, myocarditis, hepatic disturbance
Enterovirus, types 68-71
Pneumonia and brionchiolitis
Acute hemorrhagic conjunctivitis (type 70)
Paralysis (types 70-71)
Meningoencephalitis (types 70-71)
Hand-foot-and-mouth-disease (type 71)

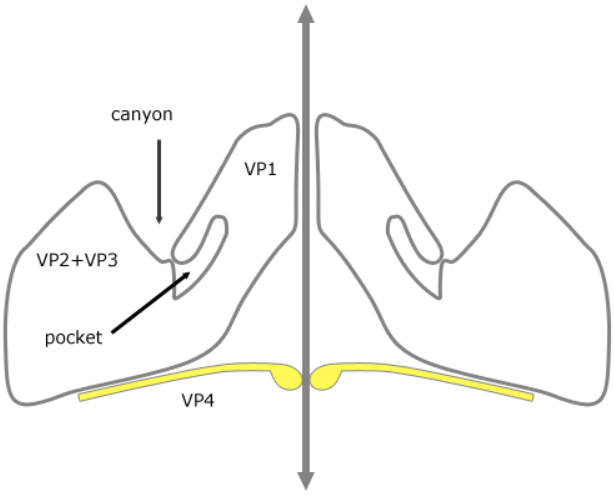
2.7 – Figures

Figure 1. Poliovirus capsid structure.



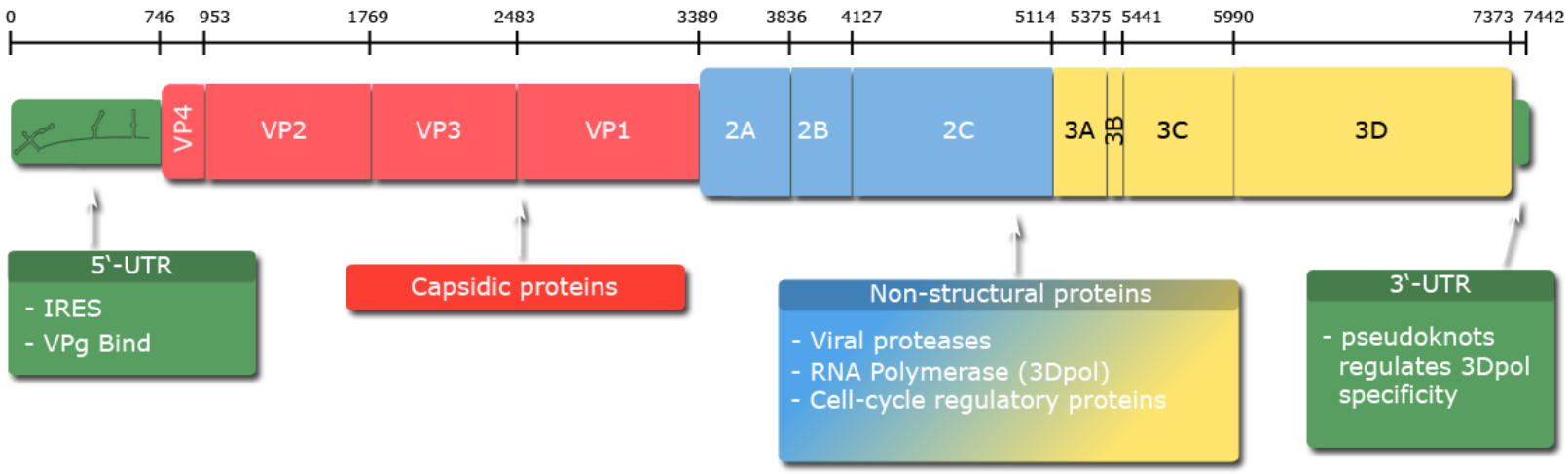
Electronic micrograph of negatively stained poliovirus (left). Diagram of the virion, showing the location of capsidic protein and genomic RNA (right). The capsid consist of 60 structural units, each made up of a single copy of VP1, VP2, VP3, and VP4, arranged in 12 pentamers.

Figure 2. Enterovirus capsidic pocket factor.



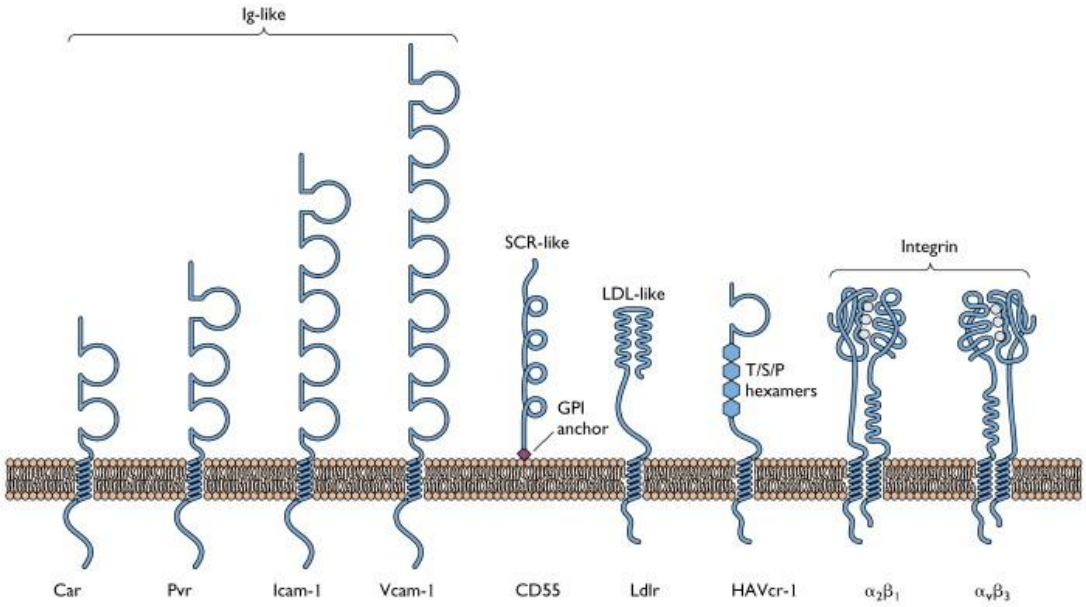
All known enterovirus structures contain a hydrophobic pocket at the base of the canyon, covered by loops of VP1 normally filled by a natural pocket factor, a fatty acid with an aliphatic chain of variable length. Binding of the receptor into the canyon probably competes with the binding of the pocket factor into the hydrophobic pocket. Release of the pocket factor destabilizes the virus and thereby initiates uncoating.

Figure 3. Enterovirus genome organization.



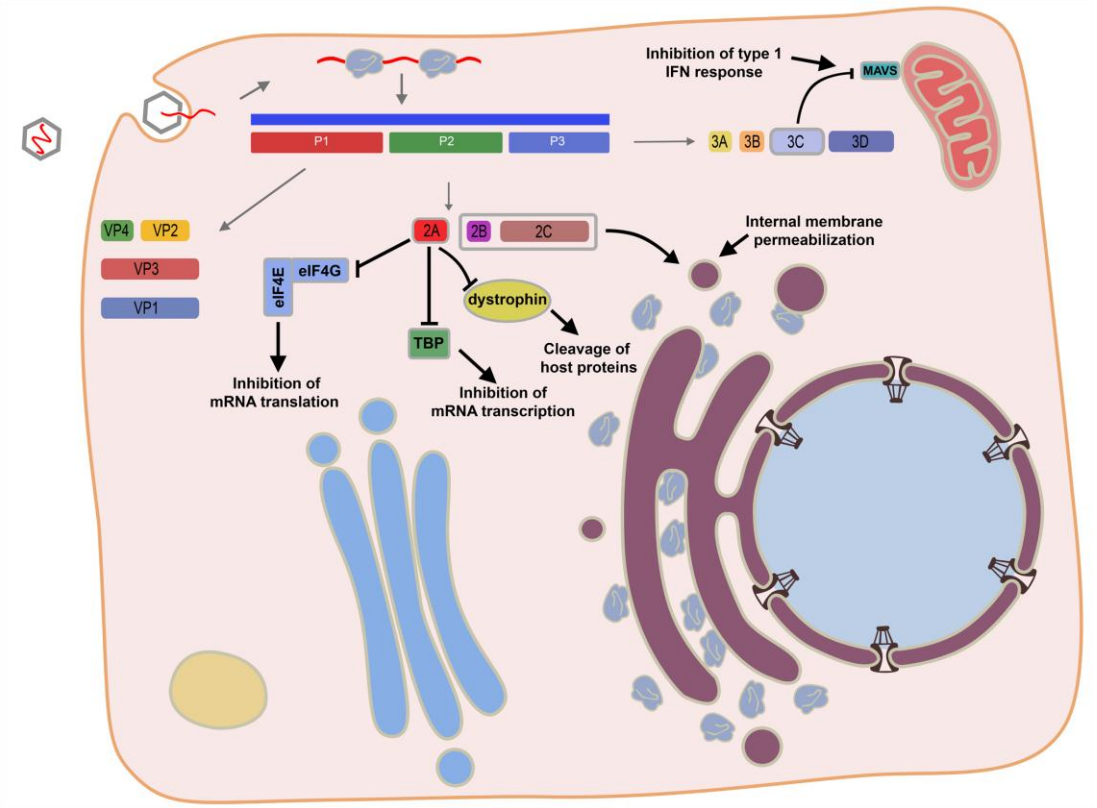
The genome is a positive stranded RNA particle and has a single ORF that encodes a polyprotein precursor. The ORF is flanked by two untranslated regions. The polyprotein is cleaved during translation by two virus-encoded proteases to produce structural and non-structural proteins. The P1 protein is cleaved into the virion capsid proteins, while the P2 and P3 proteins are cleaved to form the proteases and the proteins that participate in viral RNA synthesis.

Figure 4. Cell receptors for Picornaviridae.



A schematic diagram of the cell proteins that function as receptors for different picornaviruses is shown. The different domains (Ig-like, short consensus repeat-like [SCR-like], low-density lipoprotein-like [LDL-like], and threonine/serine/proline [T/S/P] are labeled. CAR, coxsackievirus and adenovirus receptor; PVR, Poliovirus receptor; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule 1; CD55 or DAF, Decay Accelerating Factor; LDLR, low-density lipoprotein receptor.

Figure 5. Enterovirus life cycle.



The viral RNA is translated by the cellular machinery into a single, long polyprotein. Enterovirus genome encodes two proteinases: $2A^{pro}$ and $3C^{pro}$ (or $3CD^{pro}$), which carry out cleavage of the polyprotein. The cleavage process occurs in two phases. The primary cleavage between P1 and P2 - mediated by $2A^{pro}$ – and the secondary cleavage of P2 and P3 proteins. Several cellular proteins are cleaved by $2A^{pro}$, including eIF4GI, eIF4GII, Pabp, and heart muscle dystrophin. The same RNA molecule that has been used in translation, is then used as a template for synthesis of negative-stranded RNA molecules by the virus-encoded polymerase. At the end of the infectious process, capsid proteins are synthesized and assembled into immature subunits.

3 – Persistent enterovirus infections

To establish persistent infection, a virus must be able to reduce its cytopathic effects (i.e., its ability to kill or damage the infected cell), maintain its genome within host cells over time, and avoid elimination by the host immune system. In vitro, persistent PV infection has been studied in neuroblastoma cells (Gosselin et al., 2003) and cultured fetal neuronal cells (Pavio et al., 1996). Persistence is a rather common event in enteroviral infections. In vitro, human glomerular mesangial and vascular endothelial cells may support chronic enteroviral infection; in vivo, coxsackievirus infection has been documented in endomyocardial biopsies of individuals progressing from viral myocarditis to dilated cardiomyopathy. In vitro, persistence is associated to cellular secretion of a variety of cytokines and growth factors that modulate cell behavior (e.g., motility, adhesion, proliferation) and stimulate the inflammatory response (Zanone et al., 2003). Nowadays, it is becoming evident that these viruses can be implicated in several chronic illnesses (Roivanen and Klingel, 2009) including juvenile onset diabetes mellitus, chronic fatigue syndrome, dermatomyositis and polymyositis, congenital hydrocephalus, amyotrophic lateral sclerosis. Persistent EV infections occur most often in agammaglobulinemic patients; a common clinical manifestation is represented by meningoencephalitis (McKinney et al., 1987); half patients with persistent EV meningoencephalitis have concomitant dermatomyositis or polymyositis. These observations confirm the important role of antibody in EV clearance, an unusual phenomenon because many other viruses are contained largely by cell-mediated immunity.

There have also been reports demonstrating persistence of enterovirus or the enterovirus genome in immunocompetent humans and animals with several diseases, including chronic dilated myocardiopathy, inflammatory muscle disease (myositis), and diabetes.

Although the B- and T-cell response in enterovirus infections has a role in virus clearance, there are indications that it may also lead to disease. There

are several reasons for considering a role for immunopathology in some enterovirus-induced diseases:

a. The enterovirus-induced diseases are inflammatory in nature;
b. The experimental animal models for enterovirus-induced diseases demonstrate the importance of genetic susceptibility in their causation, and the efficacy of treatment with immunosuppressive drugs;
c. The enterovirus-induced diseases are related to human diseases that are considered to have an autoimmune contribution. An immune-mediated disease process has been vigorously proposed in the case of enterovirus-induced chronic dilated cardiomyopathy, chronic myositis, and diabetes.

A role for both molecular mimicry and bystander damage has been proposed in virus-induced autoimmune disease. In the former, an immune response directed against the virus cross-reacts with a cellular protein and causes disease. In the case of bystander damage, a virus up-regulates nonspecific immune responses that lead to pathology. Recent data regarding coxsackievirus-induced T1D and coxsackievirus-induced myocarditis suggest the importance of bystander damage rather than molecular mimicry (Fujinami et al., 2006). The presence of proinflammatory cytokines would then activate Th1 cells or macrophages that foster immunopathology. Once damage occurs, new cellular epitopes that are not normally exposed and that are antigenic may trigger additional reactive epitopes, a process called epitope spreading.

3.1 – Type 1 Diabetes

T1D is a particular form of diabetes mellitus due to the loss of pancreatic beta cells and to the consequent deficit of insulin production. Classical symptoms are *polyuria* (frequent urination), *polydipsia* (increased thirst), *polyphagia* (increased hunger), and weight loss. T1D is a chronic immune-mediated disease with a sublinical prodromic period of variable duration, which follows a defective function or a selective and massive destruction (80–90%) of β-

cells (Notkins, 2002). The progression of the autoimmune process is slow, with a highly variable duration. Selective β-cells distruction may take from a few months to several years, before the eventual presentation of overt disease. The subclinical phase is characterized by the circulation of autoantibodies targeting β-cells. There are several disease-related autoantibodies that have shown to predict clinical T1D, the most important are: *insulin autoantibodies* (IAAs), the *65kDa GAD isoform autoantibodies* (GAD65), the *transmembrane protein tyrosine phosphatase (TPT)-related IA-2* molecule, and the *Zinc transporter 8 protein autoantibodies* (ZnT8).

GAD65 is encoded by a gene on chromosome 10p11. It is expressed in neuroendocrine cells, including pancreatic islets, and is located within neuron-like small vesicles. However, the function of GAD65 in the islets is not known. Between 60 and 80% of newly diagnosed T1D patients have autoantibodies to GAD65. These antibodies are directed primarily to the middle and C-terminal portions of the molecule and recognize conformational epitopes. The second major autoantigen, IA-2 (or ICA512), is encoded by a gene on chromosome 2q35. Because of a critical amino acid replacement at position 911 (Asp for Ala), IA-2 is catalytically inactive. IA-2 is a transmembrane protein found in the secretory vesicles of both endocrine and neuronal cells that seems to play a role in insulin secretion (Saeki et al., 2002). Nearly the same percentage (60–70%) of newly diagnosed T1D patients have autoantibodies against IA-2 and GAD. These autoantibodies are directed exclusively to the intracellular domain of IA-2 and recognize primarily conformational epitopes. The third major autoantigen is insulin. This protein is only 51 amino acids in length, and its gene is on chromosome 11p15. As with IA-2 and GAD65, the majority of autoantibodies recognize conformational epitopes, mainly on the B chain of insulin. Autoantibodies against insulin are among the first autoantibodies to appear in the prediabetic state and are usually found in very young children. Between 30 and 50% of children with T1D have autoantibodies against insulin (Atkinson and Eisenbarth, 2001).

Individuals must be genetically predisposed to develop T1D. The most important genes contributing to disease susceptibility are located in the HLA class II locus on the short arm of chromosome 6 (Pociot et al., 2010). Nevertheless, only a small amount of genetically susceptible individuals - less than 5% - progress to clinical disease, and the concordance range is only 30-50% between monozygotic twins (Redondo et al., 1999). This implies that additional factors are needed to trigger and drive cell destruction in genetically predisposed subjects. It is thus strongly suspected that environmental factors play a role in the pathogenesis of the disease in genetically characterized subset of the population. The surprising increase in T1D incidence during recent decades, its incidence variation from one country to another and seasonality are factors that lead to the hypothesis of viral involvement in this pathology. Besides rubella virus, mumps virus, cytomegalovirus, retroviruses and rotaviruses, the most suspected viruses are enteroviruses, especially CVB4 (Goldberg and Krause, 2009).

3.1.1 – Enteroviruses and T1D

There are many studies that suggest a possible association of T1D disease with enterovirus infections. Many of these are based on serological data, including some studies on virus isolation or detection in pancreas autopsy samples. In recent years, the introduction and development of the molecular methods have opened up many new possibilities to assess the role of viral infections.

Studies undertaken in several countries showed that anti-enterovirus antibodies as well as enterovirus RNA are found more frequently in the blood of recently diagnosed diabetic patients than in healthy controls.

Table 4. Detection of EVs in T1D patients.

Country	Patients	Sample	No. Patients	Positive	Controls	Reference
France	Children & Adults	Plasma	39	50	0	Chehadeh et al., 2000

Finland	Prediabetic Children	Serum	49	22	2	Lönnrot M et al., 2000
Sweden	Children	PBMC	24	46	29	Yin et al., 2002
Australia	Children	Plasma	206	30	4	Craig ME et al., 2003
Japan	Children	Serum	61	38	3	Kawashima H et al., 2004
Germany	Children	Serum	47	36	4	Moya-Suri et al., 2005
Norway	Children	Feces	113	51,3	-	Witsø E et al., 2006
Finland	Children & Adults	Intestine	12	9	10	Oikarinen et al., 2007

3.1.2 – Possible mechanisms of HEV pathogenesis in T1D

There are several mechanisms, not mutually exclusive, through which enteroviruses can play a role in β-cells destruction and T1D onset.

- *Pancreas and β-cells infection:* Various enterovirus serotypes can infect and replicate in human islets in vivo as well as in vitro. This can results in β-cells destruction (Klemola et al., 2008) which induces the production of cytokines (like IFN-α, β). This event stimulates the expression of molecules (like IL-15 and ICAM-1) that intervene in the pathogenic processes. The assumption that enteroviruses cause β-cell cytolysis is supported by observations describing the presence of viral antigens in human β-cells as well as the destruction of islet cells in patients deceased from systemic enterovirus infections (Ujevich et al., 1980). The presence of enterovirus RNA and/or of infectious virus, especially CVB4, in pancreatic islets of T1D patients was also reported (for literature, see Table 4**Error! Reference source not found.**).
- *Persistence of enteroviruses*: Enteroviruses are able to establish persistent infections, a characteristic that can contribute or trigger the

chains of events that lead to T1D. A particular CVB4 strain has been characterized. It is able to establish persistent infections in human pancreatic islet cells in vitro (Frisk and Diderholm, 2000) due to a series of mutation on the canyon portion of the VP1 capsid protein. There are several possible mechanisms that can induce β-cells destruction by persistent infection. EV infection can disturb the release of insulin in response to high glucose levels. A persistent infection can result in the induction of an autoimmune response directed towards β-cells through presentation of viral antigens and self antigens to T lymphocytes and activation of the immune system (Hyöty and Taylor, 2002). Enterovirus infections, and re-infections can initiate or accelerate β-cell impairment through the activation of anti-enteroviral T lymphocytes which, by cross-reactivity, participate in aggravation of the impairment of β-cells persistently infected by another enterovirus serotype (Hyöty and Taylor, 2002). The local inflammatory reaction which accompanies the persistent viral replication can also take part in recruitment of autoreactive cytotoxic T lymphocytes (CTL) in the islets. The infection of β-cells causes an inflammation which provokes cell damages and release of sequestered antigens. Through bystander cell activation, the local inflammation results in recruitment and activation of autoreactive T lymphocytes directed against these self-antigens (Horwitz et al., 2002).

- *Enteroviruses and molecular mimicry:* A partial sequence homology (i.e., molecular mimicry) has been detected between enteroviral proteins and β-cell self-antigens: 1) 2C viral protease and the GAD65; 2) VP1 viral capsid protein (plus the VP0 precursor) and IAR/IA-2 protein on one hand and the HSP60 protein on the other hand. In a mouse model designed for testing molecular mimicry between P2C and GAD65, the infection with CVB4 did not accelerate diabetes. However serum collected from patients with an enterovirus infection can react with IA-2/IAR (Harkonen et al., 2003).

- *Antibody-mediated enhancement of enterovirus infection:* Non-neutralizing CVB4 antibodies are able to enhance the replication of CVB4 in monocytes/macrophages, and hence the CVB4-induced IFN-α production by these cells (Hober et al., 2002). The enhancing effect was higher with plasma or IgG from patients with T1D than from healthy individuals and it was higher with peripheral blood cells from patients, due to IgG bound to their cells (Chehadeh et al., 2005). The target protein of antibodies that increased the CVB4-induced IFN-α production by PBMCs and CVB4 infection of PBMCs was identified as the capsid protein VP4, which indicates that this protein is accessible to antibodies at physiological temperatures. The levels and the prevalence of anti-VP4 antibodies were significantly higher in patients with T1D than in healthy subjects (Sauter et al., 2008). The antibody-dependent enhancement of CVB4 infection of circulating blood cells could play a role in virus dissemination in the host, and consequently in the pathogenesis of diseases induced by this virus.
- *Enteroviruses and thymus:* T1D is an autoimmune disease that is the result of a defect in tolerance towards β-cell antigens at the peripheral level through anomalies of regulatory T lymphocytes (Treg) and/or at the central level through disturbances of thymus functions. It has been hypothesized that the infection of thymus by an enterovirus could disturb thymic function thus playing a role in T1D pathogenesis. In the human system in vitro, CVB4 replicates and persists in human thymic epithelial cells and infects human fetal thymus fragments, resulting in disturbances of T lymphocyte maturation (Brilot et al., 2002). In the mouse system, CVB4 can infect the thymus in vivo and, in vitro, it replicates in thymic cell primary cultures and in fetal thymus organ cultures, resulting in a disturbance of the maturation/differentiation of T lymphocytes (Jaïdane et al., 2006). The possibility that virus-induced disturbances of thymus play a role in the pathogenesis of T1D can thus be taken into consideration.

3.3 – Post polio syndrome (PPS)

After the acute episode, polio survivors experience a period of neurological and functional recovery, followed by a phase of almost complete stability (Farbu et al., 2006). Since 1980, there has been an increasing acceptance of PPS. The condition may develop 15 to 40 years after acute paralytic and nonparalytic disease in 20 to 78% of polio survivors. (Trojan and Cashman, 2005). Common manifestations include generalized, central and peripheral fatigue, muscle weakness, musculoskeletal pain, and new disabilities that sometimes involve the respiratory and the alimentary tracts. Worldwide, there are over 20 million polio survivors, thus PPS represents the most prevalent motor neuron disease today (www.post-polio.org). In spite of the numbers of affected patients, the etiology and pathogenesis of this syndrome are obscure and no effective therapy is available (Farbu et al., 2006). Current treatments are based on a conservative approach consisting of exercise, avoidance of muscular overuse, orthoses, and assistive devices. Diagnosis is based on medical history and clinical-instrumental examination, since no specific biomarkers are available. A number of hypotheses have been proposed to explain the pathological changes associated with PPS. The most granted conjecture is the distal degeneration hypothesis (Wiechers and Hubbell, 1981) (Figure 6). In a normal subject's spinal cord, a single anterior horn motor neuron does innervate numerous skeletal muscle myofibers (e.g., 200 to 4,000 myofibers per neuron). When PV infects motor neurons, the cells are killed and do not regenerate. As a consequence of axonal degeneration, innervation of myofibers is lost and paralytic disease ensues. This event marks the onset of paralytic poliomyelitis. After the acute episode, during a period of several years, a neurogical and functional recovery occurs. This phenomenon is attributed to a compensatory axonal sprouting of residual anterior horn motor neurons, which markedly increases the number of muscle fibers innervated by residual motor neurons in the affected area. Extreme enlargement occurs in these motor units, reaching 7-8 times the normal

innervation ratio of individual motor neurons. Functional stability goes on for 8-35 years, with little variation in strength and physical performance. After years of stability, the late effects of polio develop and are interpreted as due to the degeneration of axonal sprouts in the enlarged motor units and /or to the loss of entire motor units.

Different factors may contribute to the distal degeneration years after the acute episode:

- *Aging, overuse myopathy and disuse muscular atrophy*. With the normal aging process, the physiological loss of motor neuron is particularly felt in patients with reduced physical activity. Overuse contributes to skeletal muscles weakness in partially denerved muscles (Gordon et al., 2004). Disuse is associated in normal individuals with muscular isotropy and weakness (Thomas and Zijdewind, 2006).
- *Autoimmune reaction.* No motor unit antibodies have been detected in PPS patients.
- *Viral infections.* A viral persistent infection in the central nervous system is plausible. Persistent infections have been observed in animal models and cell cultures, as well as immunocompromised patients. Muscular biopsy of PPS patients reveals small perivascular and perimisial lymphocytic infiltrates. This observation led to the chronic myositis hypothesis (Dalakas et al., 1986). The presence of lymphocytic infiltrates, loss of neurons and gliosis has been observed in deceased PPS patients (Kaminski et al., 1995). Finally, some studies report the intrathecal production of anti-PV antibody as a sign of active intrathecal infection (Sharief et al., 1991). This observation has not been confirmed (Julien et al., 1999).

3.3.1 – Viral persistence in PPS patients

In support of a persisting PV infection, several authors have detected portions of PV genomes in cerebrospinal fluid (CSF) samples taken from PPS patients, with a prevalence of positives ranging from 10% to 65% in different studies. Sequencing of amplified fragments allowed the presumptive identification of the three PV serotypes in different patients. In few cases, the presence of different enterovirus serotypes has been reported.

So far, serum and muscle tissue samples were consistently negative for PV infection. Mononuclear cells of the peripheral blood resulted positive in seven patients out of 37 (19%). Taken together, the above results report the possibility of persisting PV genome fragments in the CNS of PPS patients. These data do not clarify the role of genome fragments in PPS pathology.

It has been suggested that the presence of the viral genome in CSF is a purely random event and there is no link between viral persistence and pathological effects on neuronal cells. However, no PV genomic fragments were found in CSF of control patients (Julien et al., 1999; Muir et al., 1995; Leon-Monzon and Dalakas, 1995; Leparc-Goffart et al., 1996; personal data).

It is hypothesized that the persistence of the genome of PV in the CNS can cause a chronic inflammatory response and pathologic changes determined by cytotoxic or immune-mediated damage. Moreover, in PPS, as well as in other degenerative motor neurons pathologies, there is a decrease in the expression of neurotrophic factors (Goodall et al., 2006; Pun et al., 2006).

At present, however there are no reliable data that allow correlating the persistence of genomic sequences of PV with the typical symptoms of PPS. It’s possible that viral persistence is established in the early stage of the disease and the viral sequences remain present at low levels in the "stable phase".

Table 5. PV detection in previous studies.

PPS Patients					Controls		Bibliography
#	Sample	Positives	Region	Serotype	PPS	Other	
24	CSF	3 (12,5%)	5'UTR	CVB-1,4	0/24	0/36	
	Serum	0 (0%)		-	0/24	0/36	
7	Bone marrow	3 (42,8%)		CVB-4	-	-	Muir P et al., 1995
	Cerebral cortex	0 (0%)		-	-	-	
40	CSF	0 (0%)	5'UTR	-	-	0/17	Leon-Monzon ME and Dalakas MC, 1995
		4 (10%)	3Dpol	PV-1	-	0/17	
37	Peripheral blood mononuclear cells	0 (0%)	5'UTR	-	-	0/5	
		7 (18,9%)	3Dpol	-	-	0/5	
10	Muscular	0 (0%)	5'UTR	-	-	-	
10	CSF	5 (50%)	5'UTR	PV-1,2,3	0/23	2/23	Leparc-Goffart I et al., 1996
		5 (50%)	VP1	-	0/23	0/23	
20	CSF	13 (65%)	5'UTR	PV-1,2,3	3/7	0/20	Julien J et al., 1999
		11 (55%)	VP1	-	0/7	0/20	
			VP1-2°	-	0/7	0/20	

3.2 – Viral cardiomyopathy

Myocarditis is a clinical condition characterized by infiltration of the heart via inflammatory cells. There can be considerable destruction to the heart tissue, which in a significant number of patients may lead to persisting cardiac abnormalities or death. Idiopathic dilated cardiomyopathy (DCM) may also occur as a late consequence of myocarditis. DCM is a pathologic process characterized grossly by dilatation and contraction of impaired ventricles. Histologically shows a marked myocyte hypertrophy with myocytes containing large bizarre nuclei, extensive interstitial fibrosis, focal replacement fibrosis, and endocardial thickening (Richardson et al., 1996). It is an end-stage process and has no known etiology. However, many investigators favor the

possibility that DCM may represent a sequelae of acute viral myocarditis, either caused by persistence of virus or as a result of an autoimmune phenomenon secondary to previous exposure to virus. Evidence in favor of this hypothesis includes the presence of foci of inflammatory cells in DCM, the difficulty in both the clinical and pathologic distinction between DCM and acute myocarditis, and the documented presence of HEVs genome fragments (Satoh at al., 2007) and proteins (Zhang et al., 2004). Since enteroviruses are thought to be one of the most common causes of acute myocarditis, several groups have sought evidence of these viruses in DCM.

Table 6. Investigated causes of cardiomyopathy

Viral causes of cardiomyopathy	Non-viral causes of cardiomyopathy
Adenovirus, Arbovirus, Arenavirus, Coxsackie virus Epstein—Barr virus, Echovirus, Encephalomyocarditis Virus, Hepatitis C, Human herpesvirus 6, Human immunodeficiency, virus-1 and 2, Human and avian, influenza viruses, Mumps virus, Parvovirus B19, Poliomyelitis virus, Rabies Respiratory syncytial, virus, Rubella virus, Rubeola virus, Vaccinia virus, Varicella virus, Variola virus	**Bacterial:** Brucellosis, Clostridia, Diphtheria, Francisella, Cytomegalovirus, Gonococcus, Haemophilus, Legionella, Meningococcus, Mycobacterium, Mycoplasma, Pneumococcus, Psittacosis, Salmonella, Staphylococcus, Streptococcus, Tropheryma whippleii, Rickettsia, Borrelia, Leptospira, Syphilis, Typhus
	Fungal: Actinomyces, Aspergillus, Blastomyces, Candida, Coccidioides, Cryptococcus, Histoplasma, Nocardia, Sporothrix
	Helminthic: Cysticercus, Echinococcus, Schistosoma, Toxocara, Trichinella
	Protozoal: Entamoeba, Leishmania, Trypanosoma, Toxoplasmosis

3.2.1 – Hypothesized viral cardiomyopathy mechanism

In order to understand the viral cardiomyopathy pathologic mechanism, acute myocarditis course have to be explained. Myocarditis is usually viewed as a chronological sequence of three pathologically distinct phases that occur after

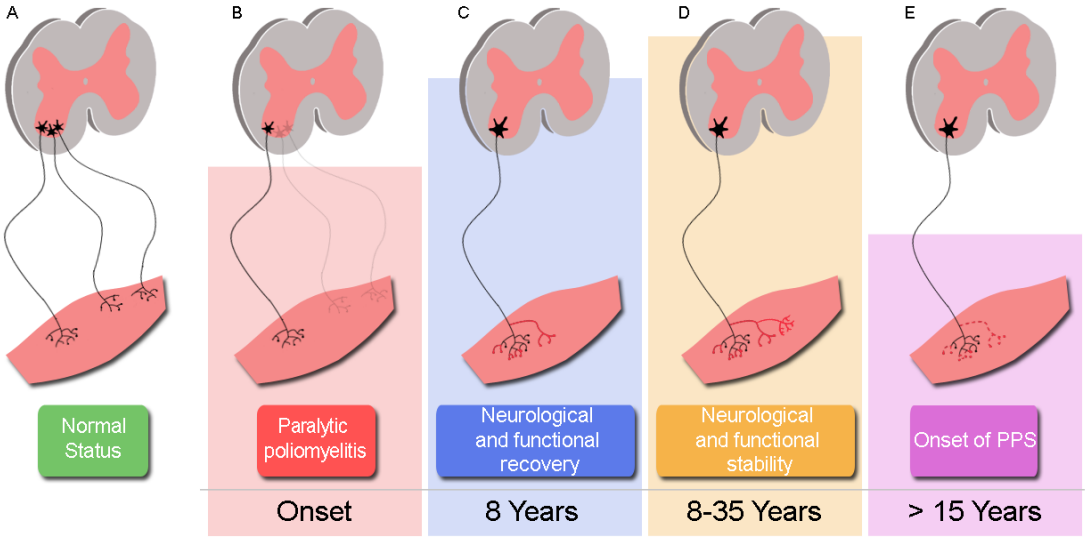
the virus comes into contact with the cardiac myocyte (Yajima and Knowlton, 2009). During the first phase, direct destruction of the cardiomyocytes occurs by virus-mediated lysis causing degradation of cell structures, which in turn facilitates entry of the virus into the cells with consequential myocyte injury and cardiac dilatation. This initial phase frequently passes unnoticed since the initial damage is often prevented by the innate immune response. The second phase develops as a result of immune dysregulation triggered by the initial cardiomyocyte injury. The initial cellular and humoral immune responses may improve the outcome during phase 1; conversely, they are responsible for the harmful effect during phase 2. In addition, some host myocardial cellular antigens can share epitopic similarities (molecular mimicry) with viral antigens, and can therefore induce an autoimmune trait that can sustain the inflammatory response and hence the chronic inflammation phase (Feldman and McNamara, 2000). Finally, in the third phase, the pathological signs of myocarditis generally disappear and the destroyed myocytes are replaced by diffuse fibrosis. A typical picture of dilated cardiomyopathy develops as a result of extensive myocardial injury. Evolutive biventricular dilatation with cardiac failure can be observed, which has been linked to a persistent virus or latent endomyocardial replication (Andréoletti et al., 2000).

While the pathological changes seen in acute myocarditis are compatible with injury caused as a consequence of infection with a lytic virus and the immune response following such infection, the structural alterations required at cellular level to cause DCM are more difficult to interpret in the context of a viral disease. Immune mediated damage to the heart as a mechanism of pathogenesis for viral cardiomyopathy is at present speculative. The interaction between persistent infection and immune response to the agent may cause sufficient cellular damage to initiate disease. Experimental studies in mice have shown that different components of the immune system play a role in the survival of the infected animals. Mice lacking CD4 positive cells are more likely to develop myocarditis but those without functional T cytotoxic

cells survive longer (Henke et al., 1995) and are less likely to develop myocarditis. Which component of the immune response is most active during a low grade infection in man and against which viral proteins is at present unknown.

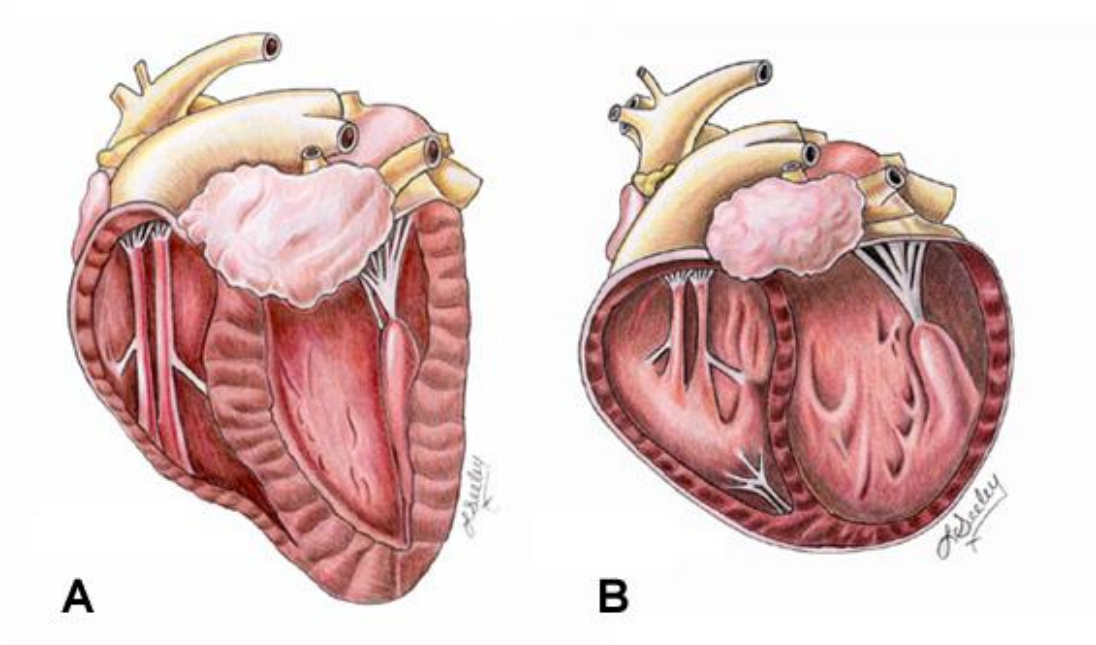
3.4 – Figures

Figure 6. Distal degeneration Hypotesis.



A) When PV infects motor neurons, the cells are killed and do not regenerate. B) As a consequence of axonal degeneration, innervation of myofibers is lost and paralytic disease ensues. C) After the acute episode, a neurogical and functional recovery occurs. This phenomenon is attributed to a compensatory axonal sprouting of residual anterior horn motor neurons. D) After years of stability, the late effects of polio develop and are interpreted as due to the degeneration of axonal sprouts in the enlarged motor units and /or to the loss of entire motor units.

Figure 7. Dilated cardiomyopathy morphology. A) Normal heart section. B) DCM heart.



4 - Enterovirus diagnosis

Serotype identification is an important aspect of Enterovirus diagnosis both for a scientific and clinical aspect. Serotype identification and sub-grouping of viruses can provide the framework for a better understanding of the biological properties of the agents thus defined. For clinical diagnosis it is often sufficient to establish a diagnosis of “enterovirus infection” without further investigations. However, in certain situations serotype identification can provide a reliable basis for medically significant differentiation which will provide clinical guidance in particular syndromes (e.g., hemorrhagic conjunctivitis, acute paralytic disease) and contribute to epidemiological knowledge and disease prevention (e.g., the recent HEV71 epidemic in Asia).
Prior to the molecular era, monoclonal antibodies and oligonucleotide fingerprinting were used to analyze serotype variations. These approaches were limited by their inability to show small differences among similar agents. Neither technique was able to readily detect any patterns among seemingly unrelated virus isolates. The introduction of molecular techniques, in particular genomic sequencing, has significantly enriched the tools available to epidemiologists and taxonomists .By analyzing the random mutations that occur in the genome of different EVs, closely related viruses were easily detected and, in addition, more distantly related viruses were clustered into distinct phylogenetic groups. Molecular epidemiologic studies have been applied to the poliovirus global eradication program. These studies were able to determinate the relations between isolates of different geographic areas, the infection trend and the recombination of vaccine strains with a variety of EVs (Nathanson N, Kew OM, 2010).

4.1 - Biological approach

The classical gold standard for virus identification is to isolate the virus in cell culture followed by neutralization test using pooled and monospecific antisera. It was shown that Enteroviruses can be propagated using several human and monkey cell lines (e.g., *Vero*, *human rhabdomyosarcoma, CaCo-*

2, WI-38 cell lines; Terletskaia-Ladwig et al., 2008). At present, there is no universal protocol on the optimal combination and number of cell lines required for enterovirus isolation, and different combinations are used in different laboratories. Virus isolation by cell culture may be affected by a number of factors. The physiological conditions of cells and whether blind passages are performed is an important aspect. Blind passages are important when the initial virus titer is extremely low and cytopathic effect is not apparent (Lipson et al., 1988). The evolution of the classical culture assay is the shell vial-assay and low speed centrifugation. This method is able to shorten detection time and enhance viral recovery (Gleaves et al., 1984). Neutralization tests using pooled antisera can give objective serotype identification.

4.1.1 – Determination of neutralizing antibodies

In neutralization assays, virus suspension (100 PFU) is incubated with antibodies of known viral specificity. After incubation for 1 hour, the mixture is inoculated into susceptible cell cultures. Cell cultures are observed for evidence of viral cytopathic effect (CPE) for 5 to 7 days. CPE indicates that the antibodies failed to neutralize the virus. Conversely, lack of CPE indicates that virus was neutralized by one of the employed the antibody. This is a cumbersome procedure that requires determining the titer of the virus prior to the start of the procedure and a lengthy incubation after inoculation of cell cultures with the mixture of antibody and virus-infected cells. Although neutralization testing may be used in identifying all types of viruses, it is used only when more rapid methods are not available.

Equine type-specific sera have been mixed to give pools containing different combination of individual antisera. Enterovirus isolates are incubated with each pool and reinoculated onto susceptible cell lines. From the neutralization pattern, the virus serotype can be inferred. The most famous intersecting pool is the Lim Benyesh-Melnick (LBM) pool scheme, divided into eight pools (A-H) containing antisera for 42 different serotypes. The antisera pool has been

recently updated with seven new pools against 19 serotypes (J-P) (Bendig and Earl, 2005). Cell culture isolation and serotyping using neutralization tests are accurate but have several disadvantages. They are expensive (Rigonan, 1998) and time-consuming since virus isolation may take up to 3 weeks' time and neutralization test can takes one week to give results. Cell culture isolation has a relatively low sensitivity and neutralization can be hindered by virus aggregation, antigenic drifts or presence of multiple viruses in clinical specimens (Muir et al., 1998).

Table 7. Susceptible EV cell lines.

Cell line	Organism	Type	Receptor	EV susceptibility
HEL	Homo sapiens	Fibroblast	CAR, CD155	Coxsackie viruses A, ECHO viruses 3-6-7-9-11-12-27; PV 1,2,3;
KB	Homo sapiens	Epithelial	CAR, DAF, CD155	PV 1,2,3; ECHO viruses 3-6-7-9-11-12-27; Coxsackie viruses A9-B1-2-3
MRC-5	Homo sapiens	Fibroblast	ICAM-1, DAF	Rhinovirus (major group); Coxsackie viruses A, B
BGMK	African green monkey	Epithelial	CAR, CD155	PV 1,2,3; ECHO viruses 3-6-7-9-11-12-27; Coxsackie viruses A9-B1-2-3
RD	Homo sapiens	Fibroblast	DAF	PV 1, enterovirus and Coxsackie A virus
CaCo-2	Homo sapiens	Epithelial	DAF	ECHO viruses 3-6-7-9-11-12-27, Coxsackie B virus
RMK	Rheus Monkey	Epithelial	CAR, CD155	PV 1,2,3; ECHO viruses 3-6-7-9-11-12-27; Coxsackie viruses A9-B1-2-3
SF	Homo sapiens	Fibroblast	DAF	Coxsackie viruses A, B
DAF-BGMK	African green monkey	Epithelial	CAR, DAF	ECHO viruses 3-6-7-9-11-12-27, Coxsackie A virus ,Coxsackie B virus
Vero	Cercopithecus	Epithelial	CD155	PV-1,2,3

	aethiops			
A549	Homo sapiens	Epithelial	CAR	Coxsackie viruses A9-B1-2-3
HEp2	Homo sapiens	Epithelial	CAR,CD155	PV-1,2,3; Coxsackie viruses B.
HeLa	Homo sapiens	Epithelial	DAF,CD155	PV-1,2,3
AV3	Homo sapiens	Epithelial	CD155	PV 1,2,3
WI-38	Homo sapiens	Fibroblast	CAR, CD155	PV 1,2,3 Coxsackie viruses A9-B1-2-3

Abbreviations: HEL, human embryonic lung fibroblasts; KB, human epidermoid carcinoma cells; MRC-5, human diploid fibroblasts; PRMK, primary rhesus monkey kidney; BGMK, buffalo green monkey kidney; RD, embryonic rhabdomyosarcoma; CaCo-2, colonic carcinoma; RMK, rhesus monkey kidney; SF, human fetal foreskin cells; DAF-BGM, genetically engineered BGM expressing human decay-accelerating factor; AV3, cervical adenocarcinoma; WI-38, Wistar Institute cell line 38.

4.1.2 – ELISA for enterovirus antibodies

Various Enzyme Linked Immunosorbent Assay (ELISA) have been designed for enterovirus serotyping. ELISA can be used for indirect diagnosis, can be simply automated, and may be effective in the investigation of outbreaks. ELISA methods that test for homotypical antibodies are impractical unless there is a clinical suspicion of the presence of one particular serotype, whereas heterotypical antibody assays allow detection of most enterovirus serotypes (Craig et al., 2003). Compared with classical methods ELISA assay are sensitive and specific for laboratory diagnosis (Bendig, 1996), but they have some limitations. The need to prepare large quantities of purified virions and interacting with secondary anti-human IgM in the ELISA assay made the method an expensive, laborious and lengthy process. In addition, since the whole virus is used as the capture antigen in the ELISA assay, cross-reactions with antibodies against other enteroviruses could results in false positives. Thus, the specificity of the IgM-based ELISA may be compromised by the presence of common epitopes of other enteroviruses.

4.1.3 – Immunofluorescence for enterovirus antibodies

Another conventional method used by the majority of laboratories is indirect immunofluorescence (IIF) assay. The cells are scraped from the infected monolayer and placed on a microscope slide. The preparation is fixed then flooded with MAbs of known specificity. Binding of MAbs to viral proteins is signaled by the presence of fluorescence when the preparation is viewed using the fluorescence microscope. The type of fluorescence (e.g., speckled versus confluent) and the location of the fluorescence in the cell (e.g., cytoplasmic vs. nuclear) are useful in differentiating viruses. This process takes only 1 to 2 h and overall gives a sensitive and specific viral identification. This method does not require the time-consuming neutralization test, but it still needs to isolate and propagate the virus. Since virus isolation involves a long time, it still hinders the progress of diagnosis. In addition, monoclonal antibodies are usually raised against the VP1 protein of prototype strains that may not be able to recognize mutant viruses in clinical specimens. Unfortunately, IF staining cannot be used to definitively identify all viruses. Examples of this are the coxsackieviruses, polioviruses, and echoviruses of the enterovirus group, which are closely related and have numerous serotypes. In some cases, these may be identified as to their sub-group by IF. However, the MAbs for enteroviral identification have been shown to lack sensitivity (Klespies et al., 2996), cross-react with rhinoviruses, and usually lack reactivity with enterovirus 71 (Van Doornum and De Jong, 1998).

Table 8. Advantages and disadvantages of various virus detection approaches.

Method	Advanced	Disadvantages
Cell Culture	Isolate wide variety of viruses (including unanticipated agents); provide isolate for additional studies: antiviral susceptibility testing, serotyping, and epidemiologic studies; increased sensitivity over rapid antigen tests	Technical expertise needed to read CPE; long incubation period for some viruses, need for purchasing/ maintaining a variety of cell culture types in-house
Neutralization	Provide isolate for additional studies and epidemiologic studies; increased sensitivity over rapid antigen tests. Impartial serotypic identification.	Long and impracticable job, extremelly expensive. Poor sensibility and false-positives cases.
Immuno fluorescence	Generally good sensitivity (which varies with virus detected); excellent specificity; CMV antigenemia is more sensitive than traditional or shell vial cultures for CMV in blood	Generally not as sensitive as cell cultures; requires expertise in reading; not useful for all viruses; adenovirus sensitivity especially poor
ELISA	Can be used for indirect diagnosis, can be simply automated. Sensitive and specific for laboratory diagnosis.	Need to prepare large quantities of purified virions. False positives due to cross-reactions with antibodies against other enteroviruses.
Molecular Methods	Excellent sensitivity and specificity; short turnaround with real-time PCR; useful for viruses that cannot be cultured in traditional cell cultures	Technical expertise required for developing and standardizing methods; expensive due to costs of instrumentation; may miss mutated viruses.

4.2 - Molecular approach

Development of polymerase chain reaction (PCR) greatly facilitates enterovirus diagnosis, becoming a common practice for viral identification in diagnostic laboratories. The establishment of informatics databases of published enterovirus genome sequences has allowed highly conserved sequences to be identified. These have been exploited as primer recognition sequences for reverse transcriptase PCR (RT-PCR) assays capable of detecting most or all enteroviruses, including those that cannot readily be propagated in cell culture as well as untypable isolates (Chapman et al., 1990 Hyypiä et al., 1989). It is also possible to detect enterovirus RNA by RT-PCR in specimens from which infectious virus is never or only rarely recovered by culture, such as myocardial tissue from patients with acute myocarditis or dilated cardiomyopathy (Baboonian and Treasure, 1997) and in formalin-fixed, paraffin-embedded tissue. As compared to traditional methods, viral nucleic acid detection is fast, sensitive and reliable. Different regions of the genome have different functions and the rate of their evolution reflects this condition. The noncoding regions contain sequences that are recognized by the host cell ribosomal subunits as well as by factors playing a role in the replication of the genome. These specific motifs are highly conserved. On the other hand, the external capsid proteins are subjected to the pressure caused by host antibodies and exhibit notable variation in length and sequence of the loop structures connecting the beta-sheets. This is evidently the reason why more than 100 different serotypes exist among HEVs. Nonstructural proteins have important functions in the life cycle of the virus and many of these polypeptides are highly conserved.

Since PCR technique introduction into clinical diagnosis, a plethora of molecular methods for enterovirus identification has been developed. These methods can be classified on the basis of the target region.

4.2.1 - 5’ UTR Methods

Due to the nature of Enterovirus genome, PCR primers for Enterovirus detection were firstly designed basing on the highly conserved enteroviruses 5'UTR region (Rotbart, 1990; Zoll, 1992). Pan-enterovirus primers were able to detect virtually all types of enteroviruses.

Comparison of the 5'UTR human enteroviruses nucleotide sequences has shown two main clusters with a minimum identity of 75% within each cluster (Figure 8 A). This high conservation is due to the need of maintaining the secondary structure, essential for the translation initiation (stem-loop structure). The nucleotide sequence variation seen in this region of all sequenced enteroviruses shows mainly compensatory base changes that support the proposed stem-loop structures. The 5'UTR can be divided into conserved (nucleotides 1 to 650, PV1 reference strain CHAT) and hyper variable regions (nucleotides 651 to 750) according to the nucleotide variation (Toyoda et al., 1984).

However, due to the lack of variation in the amplicon, 5'UTR-based methods can't provide serotype-specific information. Besides, 5'UTR sequences are of limited utility for molecular typing because no reliable correlation exists between 5'UTR sequence and serotype (Kubo et al., 2002).

4.2.2 - Capsid amplification methods

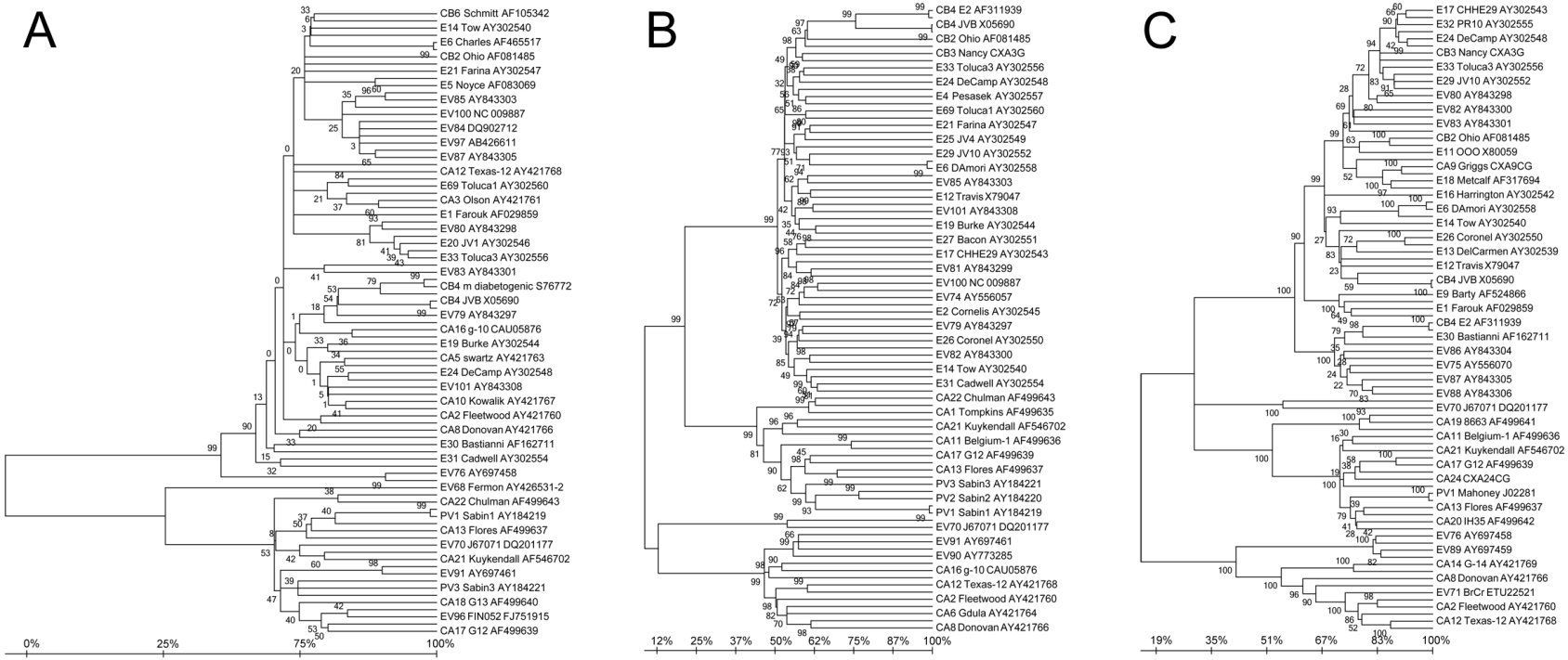
The P1 region of the enterovirus genome encodes four structural proteins (VP4, VP2, VP3 and VP1) which form the viral capsid (Figure 8 B). While VP4 is completely internalized in the infectious virion, the others are partially exposed on the virus surface. Escape mutants studies of EVs serotypes with neutralizing monoclonal antibodies had permitted to identify a number of neutralization determinants located on the three exposed viral proteins (Minor, 1992). Sequence analysis of the 'neutralizing' regions can be used to determine the virus serotype. Therefore, numerous RT-PCR assays have been developed that utilize oligonucleotide primers that anneal to sequences encoding the capsid proteins. However, primer design is crucial: primers must anneal to conserved regions, but produce a relatively divergent amplicon. The

VP4 region has been used with some success in a recent study from Japan, but is likely to suffer limitations (Ishiko et al., 2002). It is not exposed on the virion surface, therefore it doesn't feel the selective pressure and can't discriminate between different serotypes The VP2 region sequence was shown to poorly correlate with serotype since expected branch points were not observed when phylogenetic trees were constructed (Oberste et al., 1998).

The VP1 region has been used extensively for molecular typing of enteroviruses. The sequence of the VP1 region has been documented to highly correlate with serotype due to the large number of neutralization sites present on the VP1 surface. Often these assays utilize highly degenerated oligonucleotide primer sets that contain *wobbles* and deoxyinosine residues. These primer modifications allow the primer to tolerate some variations in target sequence. Subsequently, these primers are capable of amplifying many different serotypes. The deoxyinosine residues will bind to any nucleotide, but reaction kinetics involved lead to decreased sensitivity (Oberste et al., 1999), especially for clinical specimens. Thus, it is often difficult to obtain sequentiable amplicons. Most VP1 assays still require that an enterovirus be cultured in order to obtain virus concentrations significantly higher than those present in clinical specimens, giving rise to the same problems of classical virology methods listed previously.

4.3 – Figures

Figure 8. HEV molecular trees. A) 5'UTR region. B) P1 region. C) 3D region.



Dendrogram based on the nucleotide identity of the three analyzed regions. The bars show the average nucleotides identity.

5 - Methods

5.1 – Sample preparation

In this work different biological samples were used. Depending on the specimen, different approaches were employed:

- Liquor samples
  Direct PCR
- Whole blood sample
  Leukocyte separation with Sigma HISTOPAQUE-1119 and HISTOPAQUE-1077 and co-culture with cell lines (HeLa, AV3, HpL 3.4).
- Tissue sample
  Mechanic and enzymatic dissociation of primary cell for cell culture.

5.2 - Cell Culture and reference HEV types

Different EV susceptible cell types were cultivated in order to amplify the viral titer and observe a cytopatic effect. HeLa, AV3, CaCo-2 and RD cell lines were obtained from American Type Culture Collection (Promochem, Teddington, UK). Cultures of HeLa and AV3 cells were co-cultivated with $5x10^5$ peripheral blood total leukocytes obtained from patients or healthy controls and incubated at 37°C in a humidified atmosphere with 5% CO_2. To enhance the efficiency of interaction, lipofectamine was added to the medium. After five serial passages, cell supernatant was collected, clarified by low-speed centrifugation, aliquoted and frozen at –70°C (Figure 9). All the viral serotypes were obtained from American Type Culture Collection (Promochem, Teddington, UK). For a complete list, see Table 9. Viral stocks were prepared by infecting confluent HeLa monolayers using a multiplicity of infection (m.o.i.) = 3.0. Fifteen hours post-infection (p.i.), the supernatant was collected, clarified by low-speed centrifugation, aliquoted and frozen at –70°C. Infectious virus titers were determined using a microtitration assay in

96-well plates. CPE was read on day 5 p.i. Virus titers were expressed as Tissue Culture Infectious Doses 50/ml (TCID50/ml).

Table 9. Reference HEV serotypes.

Genus	Species	Serotype	Source
Enterovirus	Human Enterovirus A	CVA16	ATCC
		CVA2	NIH *
	Human Enterovirus B	CVB2 Ohio	ATCC VR-29
		CVB4 JVB	ATCC VR-184
		CVB6 Schmitt	ATCC VR-155
		CVA9 Griggs	NIH *
		E12	NIH *
	Human Enterovirus C	CVA24 DN-19	ATCC VR-1662
		CVA19 Dohi	ATCC VR-177
		PV1 Chat	ATCC VR-1562
		PV2 Sabin	NIH *
		PV3 Leon	ATCC VR-62
	Human Enterovirus D	EV94	NIH *

* National Institute for Health and Welfare (NIH), Helsinki. Thanks to Dr. Merya Roivainen.

5.3 - Indirect immunofluorescence (IIF) for capsid antigen detection

Cells infected with leukocytes of different patients were stained by IIF with EVs/PVs monoclonal antibodies (mAbs) in order to detect the expression of cytoplasmic viral antigens. Infected cell monolayers in 2-well chamber slides (Nalgene-Nunc, International PBI, Milano, Italy) were fixed with paraformaldehyde, washed in PBS containing 1% FCS, permeabilized with Triton X100 (0.05% in PBS, 10 min), briefly immersed in distilled water, and dried out. Different mAbs to capsid enterovirus antigens were used:

Table 10. Antibody table.

Antibody	Subclasses	Specificity
Pan-Enterovirus	IgG3	Enteroviruses

Poliovirus blend	IgG1 & IgG2a	PV-1,-2,-3
Poliovirus 1	IgG1	PV-1
CVB4-356.1	IgG1	CVB4

Rabbit FITC-labeled antibody to total mouse IgG (Sigma) was used as secondary antibody, upon dilution with Blue Evans in PBS. Slides were examined with a fluorescence microscope (BX-60; Olympus, Tokyo, Japan) equipped with a digital camera.

5.4 - Separation of peripheral blood leukocyte

In order to isolate and extract peripheral blood cell type(s), flow cytometry and cell sorting methods were used to identify and separate leukocyte subpopulations. Total leukocytes were obtained by centrifugation on Histopaque gradients (1.199 and 1.077 g/ml).

Table 11. Pheripheral blood leukocytes separation protocol.

Step	Action	
1	Place 4ml of whole blood into a 15ml Falcon tube with 2ml of Histopaque 1.199 and 2ml of Histopaque 1.077.	Sample preparation
2	Centrifuge for 30 minutes @ 1500 rpm.	
3	Place the two formed leukocytes rings into a new 15ml Falcon with 10ml DMEM F12 HAM + 5% FBS,r	Sample collection
4	Centrifuge for 5 minutes @ 1500 rpm, suspend the pellet in FBS + 10% DMSO, count and freeze first at -20°C for 15 minutes, then at -80°C	

5.5 – Tissue extraction

Tissue samples were first processed with FastPrep-24 automatic homogenizer (MP Biomedical LLC, Fountain Pkwy, Solon, OH) in order to

lyse the entire material. Samples were added to impact-resistant 2.0 ml tubes containing an optimized lysing matrix for RNA extraction and water. Instrument causes the lysing matrix particles to impact the sample from all directions simultaneously, releasing nucleic acids and proteins into the water. After centrifugation of the sample, normal viral RNA extraction method follows.

Table 12. Tissue extraction protocol.

Step	Action	
1	Cut approximately 3mm of tissue sample and place into Green Matrix tubes with 1ml RNAse, DNAse free water.	Preparation
2	Insert the sample in FastPrep-24 and run the cycle for 40s @ 6.5m/s.	Lysis
3	Follow the normal Viral RNA extraction protocol	Extraction

5.6 – Viral RNA extraction

Viral RNA was extracted from liquid samples (0.5-1.0 ml) using the Promega DNA and RNA extraction kits (Promega, Madison, WI) in the automated extractor Abbott M2000sp. The system makes use of magnetic particles for RNA purification. When amounts of sample <0.5 ml were available, the QIAMP Viral RNA minikit (Qiagen, Valencia, CA) was used. Viral RNA binds specifically to the vials silica membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins were removed in two wash steps with proprietary buffers, leaving pure RNA to be eluted in DNAse- and RNAse-free water. The original protocol has been optimized for low viral RNA concentrations.

Table 13. Viral RNA extraction protocol.

Step	Action	
1	Prepare two 1.5 ml eppendorf tubes with 560 µL of	Sample lysis

	AVL buffer, 5.6µL of RNA carrier and 140 µL of sample. Vortex and spin for 15 seconds and incubate for 10 minutes @ room temperature.	
2	Briefly centrifuge to eliminate drops inside of the lid. Add 560 µL of absolute ethanol (96%-100%). Spin for 15 seconds.	
3	Briefly centrifuge to eliminate drops inside of the lid. Add 630 µL of the resulting solution into a QIAamp Mini spin column. Centrifugate for 1 minute @ 8000 rpm and discard the eluate.	Membrane binding
4	Repeat the step 3 four times.	
5	Add 500 µL of AW1 buffer and centrifuge for 1 minute @ 8000 rpm. Discard the eluate.	
6	Add 500 µL of AW2 buffer and centrifuge for 3 minutes @ 14000 rpm. Discard the eluate.	Washing
7	Centrifuge for 1 minute @ 14000 rpm. Discard the elute and put the column into a 1.5ml eppendorf tube.	
8	Add 50 µL of AVE buffer and centrifugate for 1 minute @ 8000 rpm.	Final elution

5.7 - Viral RNA extraction from paraffin embedded specimens

Paraffin blocks were treated to extract viral RNA. In the initial phase, paraffin blocks were sectioned and deparaffinated. Afterward normal protocol for RNA extraction from tissues was performed with the QIAMP Viral RNA minikit (Qiagen, Valencia, CA).

Table 14. Viral RNA extraction from paraffin embedded specimens

Step	Action	
1	Paraffin block is trimmed to remove excess	Sectioning

	paraffin with a scalpel. Microtome blade was cleaned with acetone to eliminate contaminating RNase. 2 x 10 µm sections are cut and placed in a 1.5 ml eppendorf tube.	
2	Excess of paraffin is removed from each section with a clean scrape. Add 1 ml xylene to the sample and incubate for 20 minutes @ RT with agitation. Wash 3 times with 1 ml 100% ethanol for 3 minutes @ RT with agitation, air-dry samples under a hood.	Deparaffinization
3	Normal tissue extraction protocol followed	RNA extraction

5.8 – RNA retrotranscription

After extraction, RNA was retrotranscribed with random hexamer primers. The retro transcription process was carried out by the SuperScript® VILO cDNA synthesis kit (Invitrogen, Carlsbad, California). The original protocol has been slightly modified to improve sensitivity.

Table 15. . Viral RNA retrotranscription protocol.

Step	Action	
1	Combine the following components in a 0.2ml tube on ice. • 5X VILO™ Reaction Mix 4 µl • 10X SuperScript® Enzyme Mix 2 µl • RNA (up to 2.5 µg) 12 µl • DEPC-treated water 2 µl Gently mix tube contents.	Mix preparation
2	Set the thermocycler at: • 25°C for 10 minutes • 42°C for 120 minutes	Cycle

- 85°C for 5 minutes
- 4°C ∞

5.9 – Primer design

Reference strains sequences where chosen according to the prototype strains listed on the *Picornaviridae database* (www.picornaviridae.com). All sequenced EV serotypes were aligned for every analyzed region utilizing CLCbio Genome Workbench. Human Parechovirus (HPeVs) serotypes were used as an outgroup. Successively, alignments were analyzed with the MEGA package (Kumar et al., 2008) and a phylogenetic tree was drawn for every aligned region. A Neighbor Join algorithm was chosen for the tree prediction and a boostrap of 1,000 for statistical analysis. The complete tree was then analyzed and divided into subtrees. Every subtree was separately realigned and analyzed with the COMPASSS suite for searching conserved motifs (Maccari et al., 2010). The designed primers were analyzed for dimers, hairpins, annealing temperature with the *NetPrimer software* (Premier biosoft, www.premierbiosoft.com).

In the PPS study, an original three-primer pairs method targeted at the 3D (RNApol) region was designed. This novel highly sensitive primer set is composed of a 5 primers. This set has been developed on the basis of a pan-polio primer set originally composed of only 2 primers and unable to discriminate PV types.

For other studies, potentially involving all non-polio HEVs, a general HEV PCR-detection method has been designed targeting the 3D region. Based on a complete alignment of over 90 HEV types, a phylogenetic tree was drawn and a 7-group serotype subdivision has been defined.

For all studies, RT-PCR methods targeting the 5`UTR region have also been used. Published (Hyypiä et al., 1989; Halonen et al., 1995; Yang et al., 1992) primer sets have been employed together with an original primer set developed by our group. Primers are reported in the following Table 16.

Molecular typing of different HEVs has also been attempted by using an original real time protocol developed at the American CDC (Nix et al., 2006).

Table 16. 5'UTR primers.

Name	Sequence	Reference
PanEntero F PanEntero R	CCCCTGAATGCGGCTAAT GATTGTCACCATAAGCAGCCA	Personal data
Hyypia F Hyypia R	CATTCAGGGGCCGGAGGA AAGCACTTCTGTTTCC	Hyypiä et al., 1989
EV/PCR1 (F) EV/PCR2 (R) EV/PCR3 (F)	ATTGTCACCATAAGCAGCCA TCCTCCGGCCCCCTGAATGCG ACACGGACACCCAAAGTAGTCGGTTCC	Halonen et al., 1995 Yang et al., 1992

5.10 – PCR amplification

The retrotranscribed RNA was amplified by PCR (or real-time PCR) with primers directed to the 5`UTR, VP1, and 3D genome regions. Real time assays were performed on the *ABI Prism 7000* real-time PCR thermocycler, and end-point PCR assays were performed on the *Verity* thermocycler (Applied Biosystems, Foster City, CA) as well as the peqSTAR 96 universal gradient cycler (PeqLab, Wimington, DE).

Table 17. PCR protocol.

Step	Action	
1	Combine the following elements into a 0.2 ml tube: • GeneAmp 10X PCR Buffer II (without $MgCl_2$) 5 µl • dNTP Mix 1.25 mM 5 µl • MgCl2 1.5 mM 4 µl • AmpliTaq Gold Polymerase 0.5 µl	Sample preparation

	• Primers 10 µM 1 µl each • DMSO 2 µl • Molecular biology pure water 24.5 µl • cDNA 5 µl	
2	Set the thermocycler at: • 95°C for 10 minutes • 45-step cycle: 95°C for 30 seconds 58°C for 1 minute 70°C for 2 minutes • 70°C for 15 minutes • Store at 4°C	PCR cycle

For the real-time PCR, reaction was performed using the Sybr green system (Applied Biosystems) in 50 ml final volume. Real-time data were collected during extension steps. Serial dilutions of genomic RNA extracted from a CVB-4 stock containing $2x10^6$ $TCID_{50}$/ml were used as a quantitative reference for viral genome amplification. The threshold cycle number (Ct value) represents the cycle number at which fluorescence signals significantly different from the baseline were obtained.

Table 18. Real-time PCR protocol.

Step	Action	
1	Combine the following elements into a 96-wells microplate: • SYBR® Green PCR Master Mix • 500 nM of each primer, • Molecular biology pure water ‘till a final volume of 50 µl	Sample preparation
2	Set the thermocycler at:	PCR cycle

	• 95°C for 10 minutes • 40-step cycle: 95°C for 15 seconds 60°C for 30 seconds 72°C for 30 seconds • Real-time data are collected during extension steps.

5.11 – PCR purification

After amplification, positive PCR products were purified. Where the amplification product was poor and/or smeared, a 2% agarose gel in Tris borate EDTA (TBE) buffer was loaded with the total PCR product. The gel band was then cleaved and purified.

The liquid phase PCR product purification was carried out by the ExoSAP-IT (USB, Cleveland, OH) method, a single step enzymatic cleanup of PCR products that eliminates unincorporated primers and dNTPs.

Table 19. Liquid phase PCR product purification.

Step	Action	
1	Place 1 µl of ExoSAP-IT for each 2,5 µl of PCR product into a 0.2 ml tube.	Sample preparation
2	Set the thermocycler at: • 37°C for 15 minutes • 80°C for 15 minutes	Clean up reaction

cDNA recovery from agarose gel was carried out by TaKaRa RECOCHIP (TAKARA Bio Inc., Tokio, Japan), a disposable chip designed to recover DNA fragments from agarose gels by applying an electrical field in the absence of additional chemicals.

Table 20. Agarose gel PCR purification.

Step	Action	
1	Push the gel cutter into the agarose gel on the anode (+) side near the DNA band to create a gap. Wet the RECOCHIP in electrophoresis buffer and push it into the gap, black surface faces toward the DNA band. Start electrophoresis.	Preparation
2	Run electrophoresis until the entire band is into the RECOCHIP. Remove the RECOCHIP and centrifuge 5 seconds @ 5000 rpm into a 2 ml tube. 30-50 µl of DNA solution will be recovered.	Sample collection

5.12 – Amplicon sequencing

In order to sequence purified PCR products, sequencing reactions were performed using a *Verity* thermocycler (Applied Biosystems) and the Applied Biosystems BigDye® Terminator v1.1 Cycle Sequencing Kit. Forward and reverse primers have been used in different reaction.

Table 21. Sequencing reaction protocol.

Step	Action	
1	Combine the following elements into a 0.2 ml tube: • 5x Sequencing Buffer (5µl) • BigDye® Terminator v1.1 Ready Reaction Mix (4µl) • DMSO (2µl) • Primer 10 µM (1µl) • DMSO (2µl) • Molecular biology pure water 24.(6µl) • cDNA (2µl)	Sample preparation
2	Set the thermocycler at:	Cycling

- 95°C for 5 minutes
- 25-step cycle:
  95°C for 30 seconds
  50°C for 10 seconds
  60°C for 4 minutes
- Store at 4°C

The purified sequencing reaction product was added to formamide in a 1:1 ratio and denatured by heat shock (95°C for three minutes). The sample was ready for separation by capillary electrophoresis in an automatic sequencer ABI Prism 310 (Applied Biosystems, Foster City, CA).

5.13 - Cytokine and Chemokine ELISA assays

Twelve pro-inflammatory cytokines (Multi-Analyte ELISArray™ TLR-induced Viral Cytokines, SABiosciences, Frederick, MD) were analyzed using a conventional ELISA protocol under uniform conditions. The following cytokines and chemokines were comprised in the array: TNFα, IL1b, IL6, IL12, IL17A, IL8, MCP-1, RANTES, IP-10, MIG, TARC, and IFNa. Four additional cytokines were analyzed individually: IL10, IL4, GM-CSF, TGFß1. Incubation with liquid samples allows the capture antibodies to bind the specific protein of interest. After washing unbound proteins, cytokine-specific biotinylated antibodies added to the wells did bind the captured analyte. After washing, an avidin-horseradish peroxidase conjugate is added. Wells were washed again and a colorimetric substrate solution was added, which produced a blue color in direct proportion to the amount of analyte present in the initial sample. Color development was stopped by the stop solution. OD values were read at 450 nm. The following controls were included: a) negative control wells incubated with plain cell culture medium; b) positive control wells incubated with known amounts of reference cytokines; c) cell culture medium from uninfected HeLa cell cultures; d) cell culture medium from HeLa cell cultures exposed to PV-1 (reference Chat strain; m.o.i = 0,001

for 48 hrs); e) cell culture medium from HeLa cell cultures exposed to PV obtained from PPS patients (0.1 ml undiluted medium) or from HeLa cell cultures exposed to HEVs obtained from T1D patients (0.1 ml undiluted medium).

Table 22. ELISArray protocol.

Step	Action	
1	Prepare all reagents. Set up experimental samples, positive controls and negative control.	Samples transfer and primary antibodies conjugation.
2	Add 50 µl of assay buffer into each well of the plate. Transfer 50 µl of samples and control samples into the appropriate well and incubate for 2h.	
3	Wash three times with PBS.	
4	Add 100 µl of Detection Antibody solution and incubate for 1h.	Detection antibody conjugation
5	Wash three times with PBS.	
6	Add 100 µl of Avidin – HRP. Incubate 30 minutes.	
7	Wash four times with PBS,	
8	Add 100 µl of Development Solution. Incubate 15 minutes in the dark.	Analyze
9	Add 100 µl of Stop Solution and read OD 450nm within 30 minutes.	

5.14 – T1D patients` database

In order to organize the ever increasing data collected over the last few years, a specific database of T1D patients has been developed. Data is stored into a mySQL database, and a PHP interface allows to insert and modify data directly from an internet browser. Different levels of permission are designed for reading and writing into the database and users are organized as workgroups. Members of a workgroup can access and edit only their data.

The database has the ability to collect general data (age, sex, date of admission) as well as clinical data (pancreatic autoantibodies, glucose, C-peptide, HbA1c, HLA type). Demographic information and epidemiological data were organized and integrated into Google world maps. Sensible data is cryptographed on the client-side with a specific Java application.

Automated data analysis is performed and organized in chart, thanks to the Google chart API (Figure 14).

5.13 - Figures

Figure 9 Virus amplification by cell culture. 1, 2) Leukocytes are collected from whole blood samples and 3) are co-cultured with HeLa cells. After multiple passages, supernatant is collected. 5) RNA is extracted and retrotranscribed. Multi-cytokine ELISA assay is performed on cell-free supernatant.

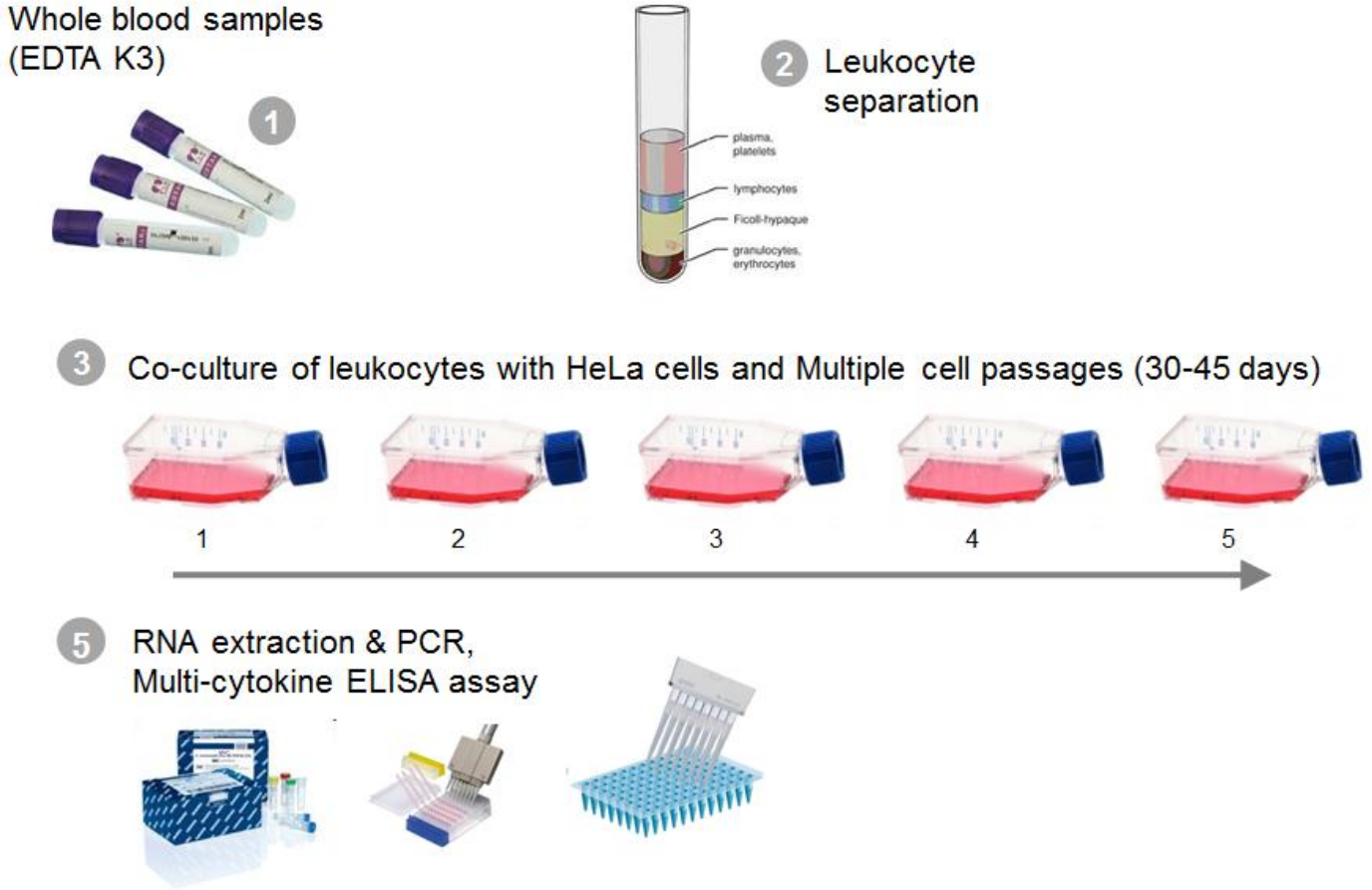


Figure 10. HEVs 3D amplification method. A) HEV 3D region tree. B) From the classical five-group organization, a seven group subdivision was designed. For each group, identified serotypes are specified.

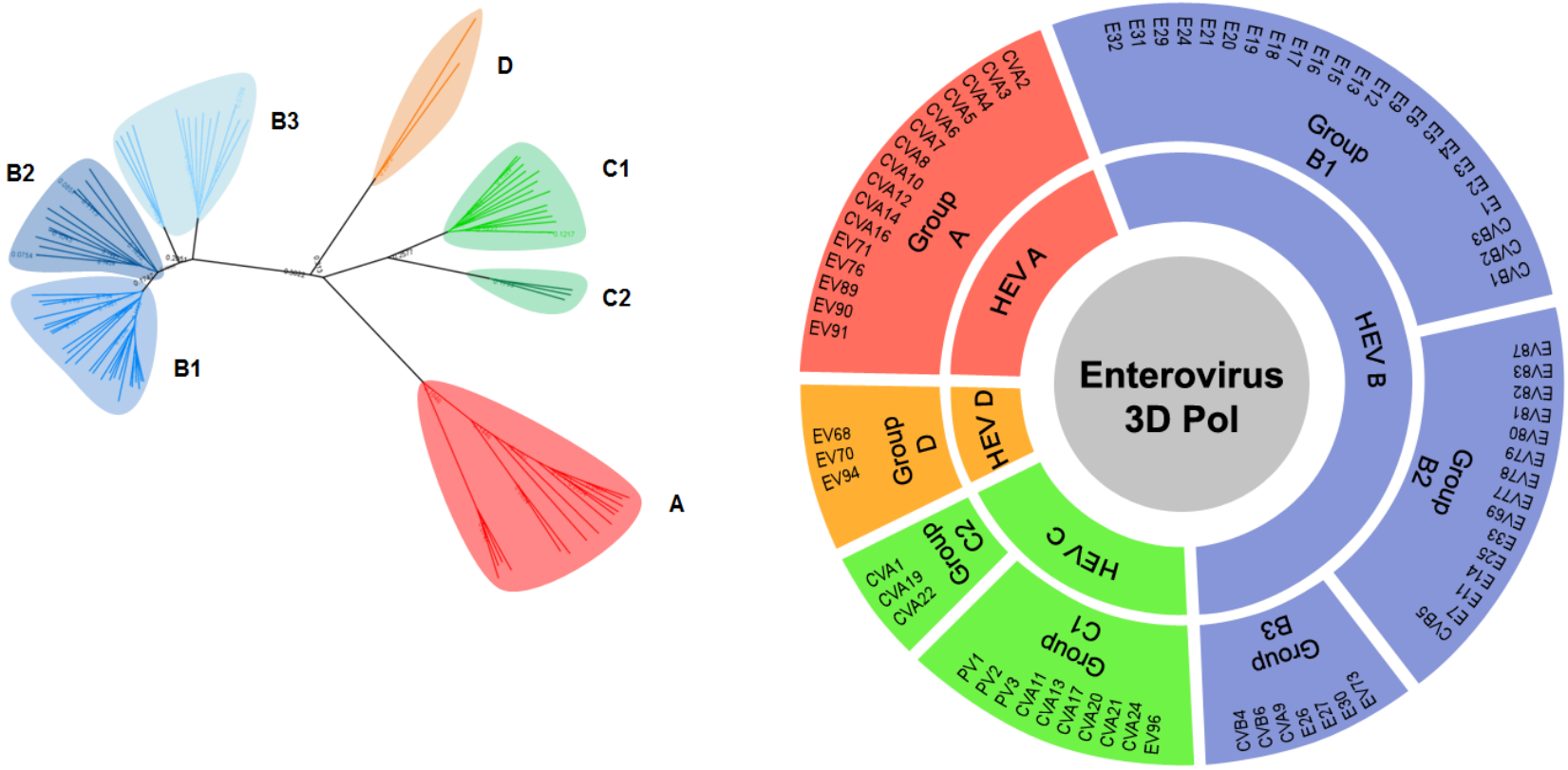
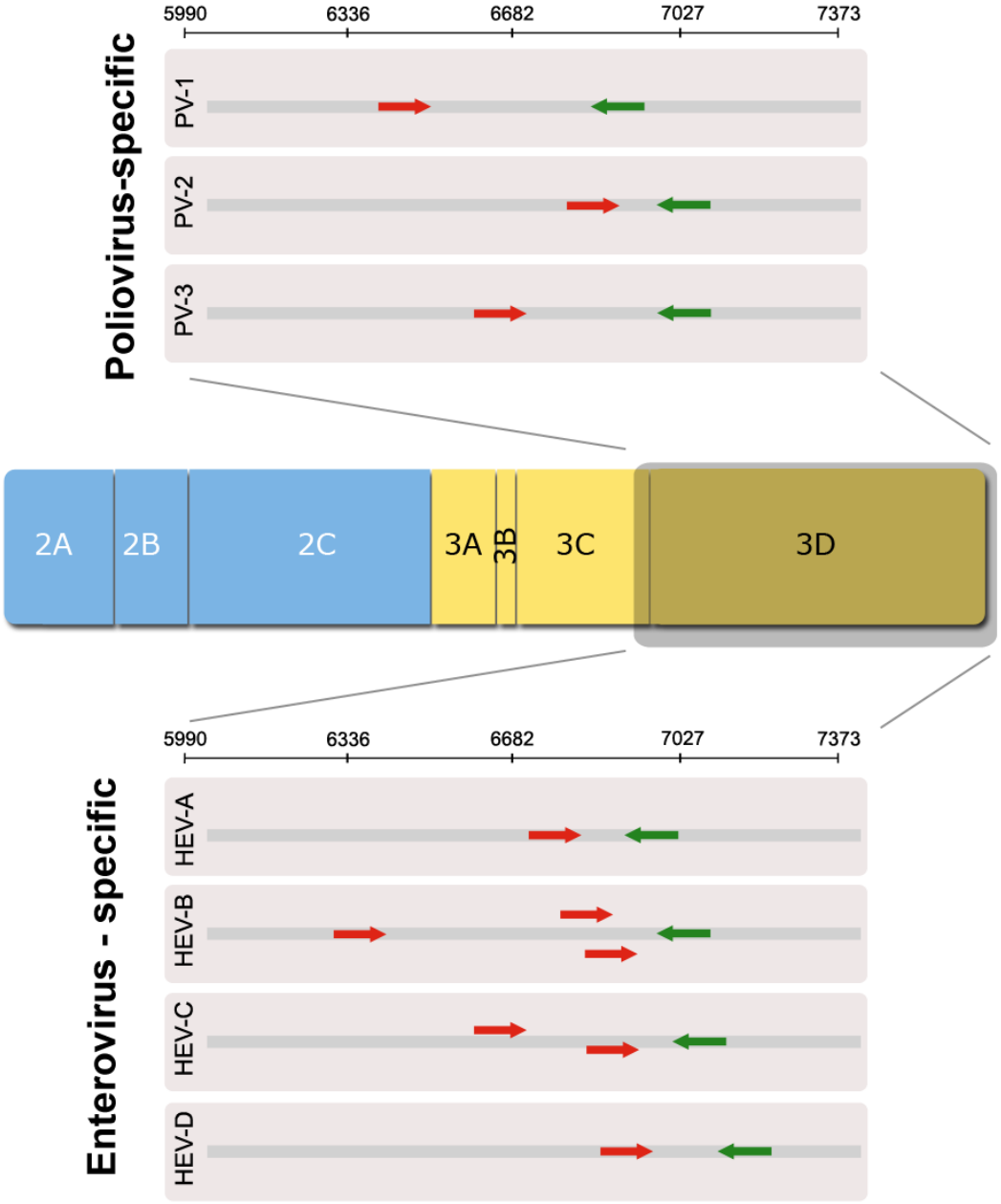
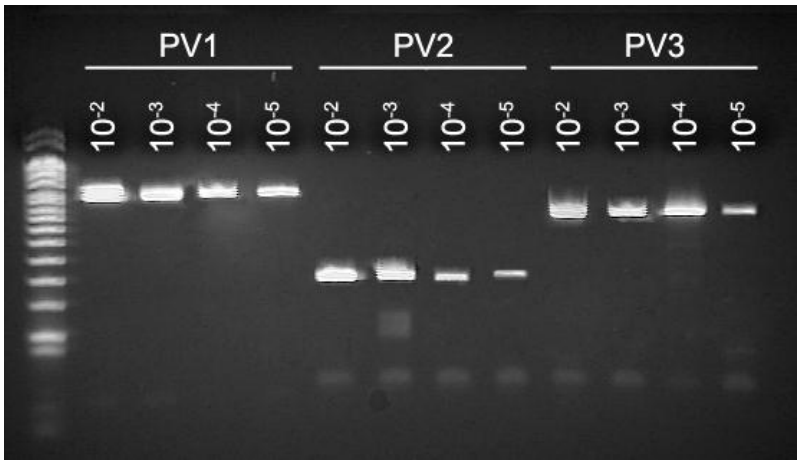


Figure 11. PCR primers.



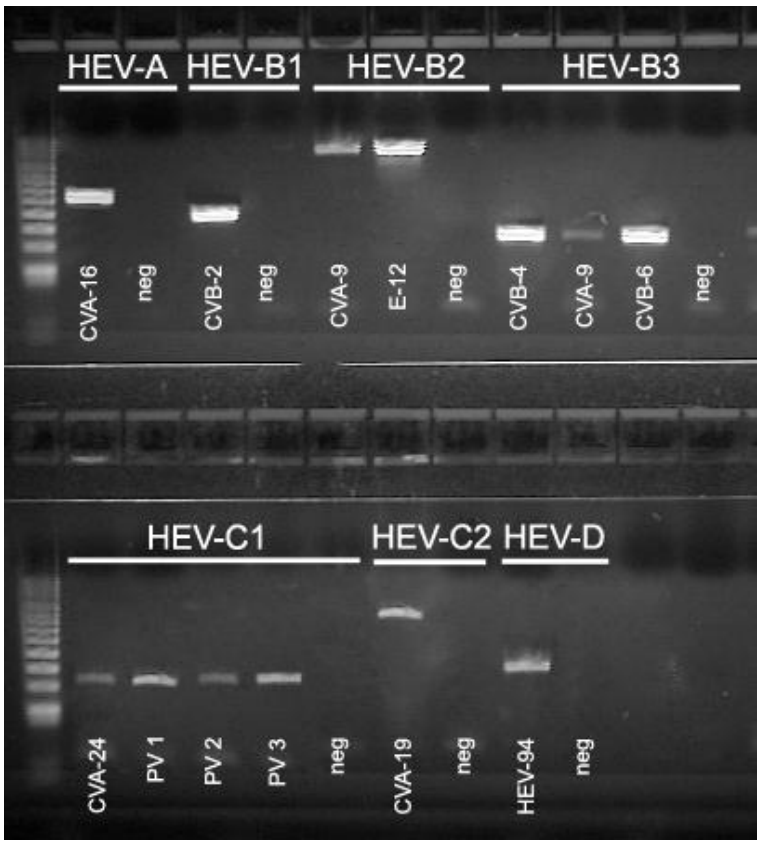
In the PPS study, an original three-primer pairs method was designed. We have added to our previous panPolio 3D primer-set three new primers with high affinity for the three serotypes 3D region. A more general HEV PCR-detection method was designed for the viral RNA polymerase amplification. Basing on a complete HEVs alignment of the 3D region, a phylogenetic three was drawn and a 7-group serotype subdivision was specified.

Figure 12. PVs-specific primer pairs against the 3D genomic region.



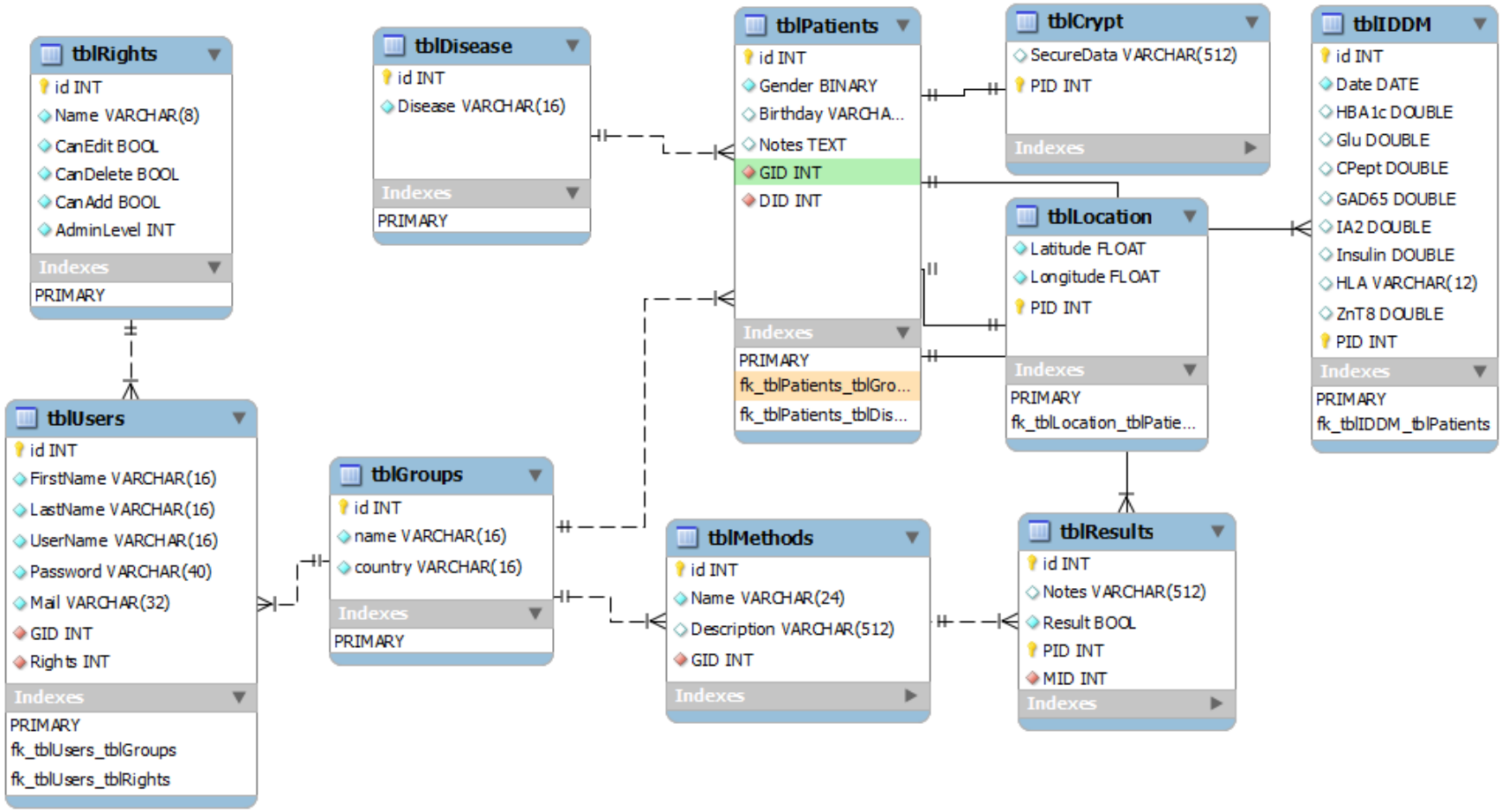
Primers were tested against the three PV serotypes, in four serial dilutions (10^{-2}, 10^{-3},10^{-4}, 10^{-5}).

Figure 13. HEVs-specific primer set against the 3D genomic region.



Different HEV reference serotypes were tested with HEV-specific primer pairs.

Figure 14. IDDM patients SQL database scheme.



6 – Results

6.1 – Type 1 Diabetes

Samples from 112 T1D children aged 2 to 18 years have been investigated for the presence of HEV genomes in blood at the time of clinical onset. HeLa cells were co-cultured with leukocytes in order to amplify the virus load. After five consecutive passages, a quota of supernatant was analyzed. Two different cohorts were investigated: a 90-children group from Varese, Italy (years 2005-2010) and a 22-children group from Pisa, Italy (years 2005-2010). To organize this increasing amount of data (demographic information, date of diagnosis, pancreatic autoantibodies, glucose, C-peptide, HbA1c, HLA type) a specific database has been developed. Tissue culture, viral protein expression, and gene amplification of conserved genomic regions were used for virus detection. Three different methods were used to investigate virus persistence:

- Amplification methods based on the 5'UTR genomic region;
- A patented and validated method based on the capsidic VP1 region;
- A novel original method based on the 3D region coding for the viral RNA polymerase.

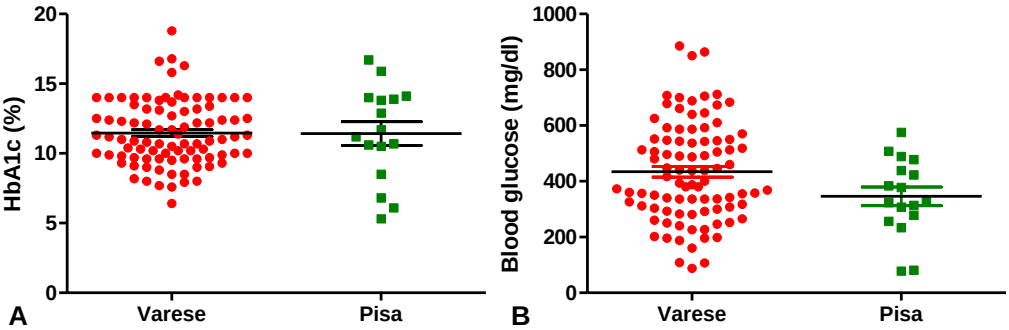
A total of 69 samples were used as controls, including relatives (n=37), blood donors (n=20) and pediatric healty controls (children admitted to our Pediatric ward for minor traumatic lesions n=12).

Temporal and geographic clusters of T1D cases were observed in different years. In 13 different cases, samples have been obtained from consenting first-degree relatives and intra-familial studies were performed.

Table 23. T1D Patients details.

Years	Country	Locality	Patients		Relatives
			No.	Mean Age	
2005-2010	Italy	Varese	90	9,32	29
2005-2010	Italy	Pisa	22	7,63	8
Total			112	8,95	37

Figure 15. T1D patients data. A) % of HbA1c B) Blood glucose.



6.1.1 – PCR and real-time PCR results

In a first phase of the study, HEVs genome fragments have been investigated with a real-time PCR approach. We chose this method because it is fast and provides quantitative information on virus levels.

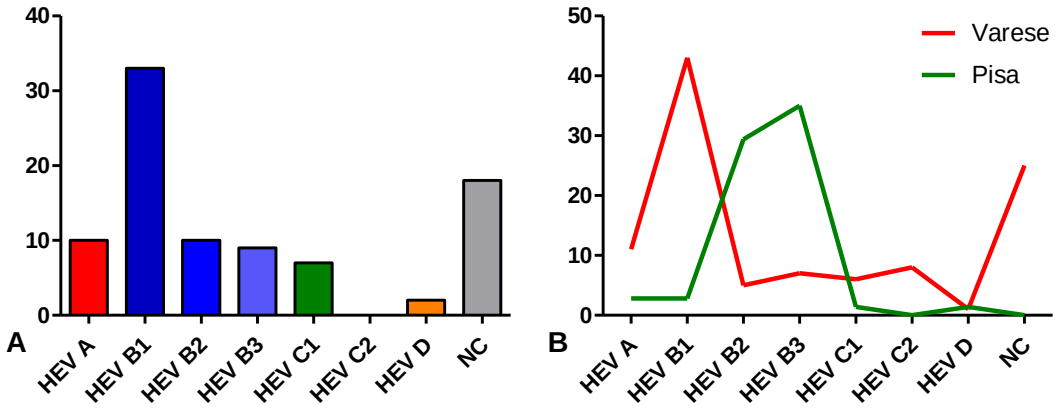
The target region of the real-time PCR is a conserved domain in the 5'UTR fragment. Then we realized that this did not give satisfactory results, since the method was not sensitive enough.

Table 24. PCR and real-time PCR results.

	5' UTR	3D polymerase
Patients (n)	28% positive (n=64) – Years 2005-2010	80% positive (n=112) – Years 2005-2010
Controls (n)	0% positive (n=69)	2.8% positive (n=69)

When patient samples were analyzed with the highly sensitive 3D method, 5'UTR results were confirmed and the percentage of positive samples was sensibly increased. Moreover, the 3D method can discriminate between seven subgroups of HEVs, providing some information about virus serotype. Thanks to these results, a temporal and geographic epidemiologic analysis can be inferred.

Figure 16. T1D positive patients HEV species and 3D group distribution. A) Overall distribution (n=112). B) Varese cohort distribution (n=90) vs. Pisa cohort distribution (n=22). NC, not classified.



6.1.2 – Family cases

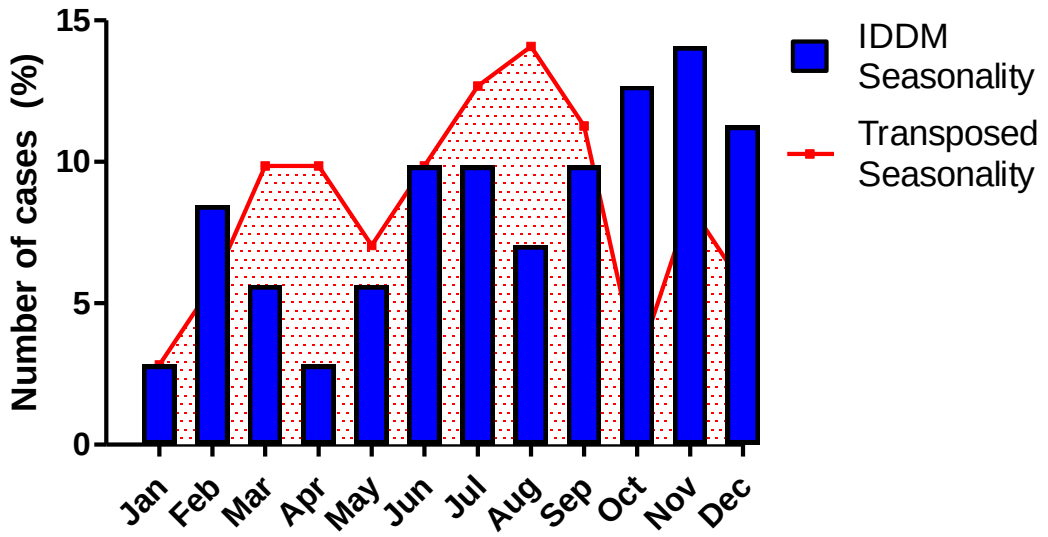
Over the past few years, a number of 13 families with at least one member with T1D were analyzed for HEV infections. A total of 51 subjects were investigated. Clinical (autoantibodies, HbA1, C-pept) and epidemiological data (temporal and geographic associations of cases) were collected. In most cases, HEVs positivity was also found in family members (first degree relatives of diabetic children). In positive-families, the same HEV type was detected. In a few cases (2 families), double positivity for two different viral species and 3D groups was detected. Interestingly, during a short period of time, the same virus group was found in different families living within 5 Km distance.

In two different families (GG and UG), two consecutive T1D cases were observed. In the UG family, the T1D patient's sister developed diabetes nine month after her brother. In the GG family, GN patient developed diabetes 14 month after his brother.

6.1.3 – Temporal and geographic epidemiologic analysis

A temporal and geographic epidemiologic study was performed on subjects from the Varese cohort (n=90).

Figure 17. T1D Seasonality in Varese cohort (n=90).



Based on the T1D onset, seasonality of the Varese cohort was deduced (Figure 17). Assuming that enterovirus infection occurred at least 3 months before clinical onset, infection seasonality was transposed of 3 months. At T1D onset, the HbA1 values were already high implying that high glucose values had been present for at least 2-3 months before onset. According to our data, cases of T1D in the Varese cohort are concentrated in the months of October-December. Supposedly, HEV infections peaked in summer months. Cases were grouped into clusters occurring within a 5 km radius in a 3-month period (Figure 22).

6.1.4 – Virus protein expression by IIF in cell lines exposed to leukocytes of T1D patients

Immunofluorescence tests were made in human HeLa and AV3 cell lines as well as in mouse HPL3.4 cells co-cultured with leukocytes obtained from peripheral blood samples of T1D patients at onset. Expression of viral proteins was evaluated using commercial monoclonal antibodies directed to capsid epitopes common to HEVs (Table 10). Immunofluorescence results obtained with HeLa, AV3, and HPL 3.4 cells were comparable. As positive controls, HeLa, AV3 and HPL 3.4 cells were infected at low m.o.i. with PV-1 CHAT strain or CVB4. Since most clear IIF results were obtained with the AV3 cell line, these cells have been used in subsequent experiments.

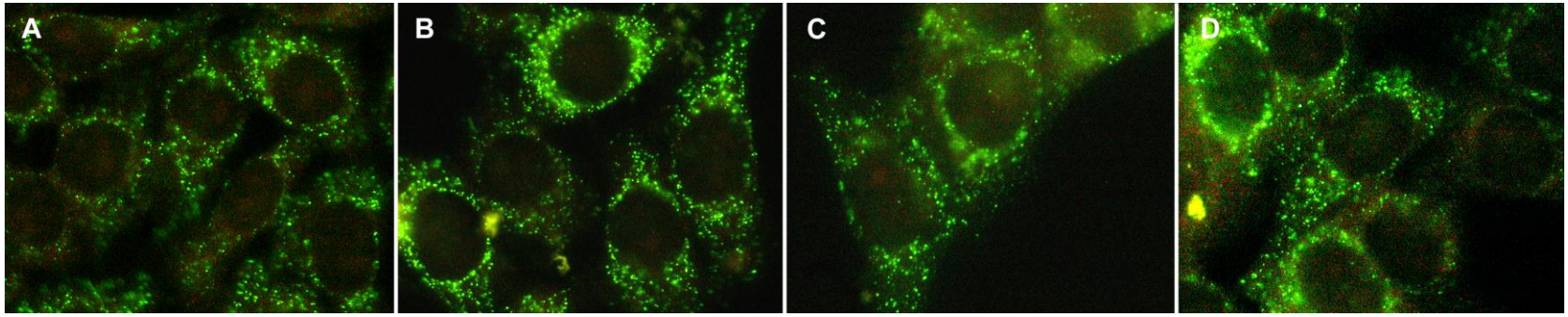
In particular, when T1D familial cases were analyzed (

Table 25) all but one samples positive by PCR for HEV genome, were also positive for capsid antigen expression. In the family GG, positive results were obtained in all family members. The GG brother, who developed T1D several months later, was already positive by IIF at the time of testing. Similar results were observed in the UG family. The T1D patient's sister developed T1D nine month later, but was already positive by IIF and PCR at the time of testing. Surprisingly, the BTS family resulted negative by IIF, but positive by PCR (Figure 20). This may be interpreted as a lack of reactivity of panenterovirus mAbs with the particular type of the infecting HEV.

Table 25 T1D Families comparative results.

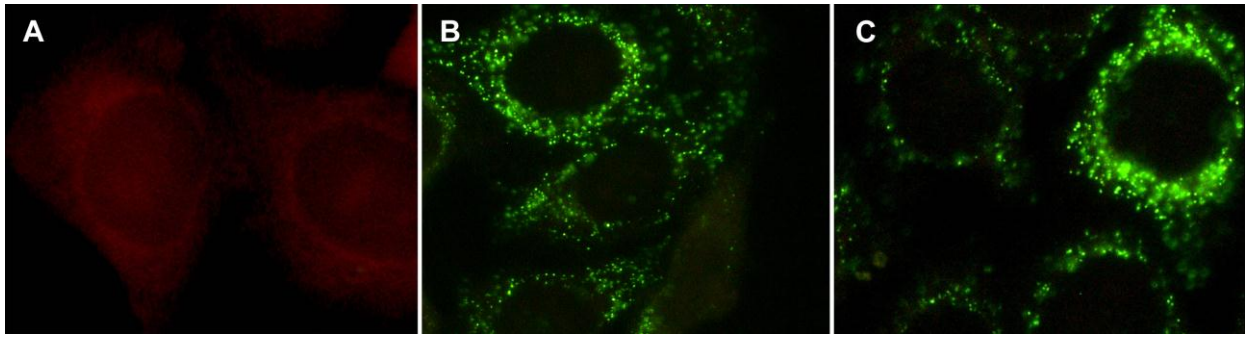
	Patient	IIF	PCR
Family GG	GG Father	+	+
	GG Mother	+	+
	GG T1D Patient	+	+
	GG Brother	+	+
Family SC	SC Father	-	-
	SC T1D Patient	+	+
	SC Brother	+	+
Family BTS	BTS Father	-	+
	BTS Mother	-	+
	BTS T1D Patient	-	+
Family UG	UG Father	-	-
	UG Mother	+	-
	UG T1D Patient	+	+
	UG Sister	+	+

Figure 18. IIF of GG Family. A) GG Father. B) GG Mother. C) T1D patient. D) GG Brother.



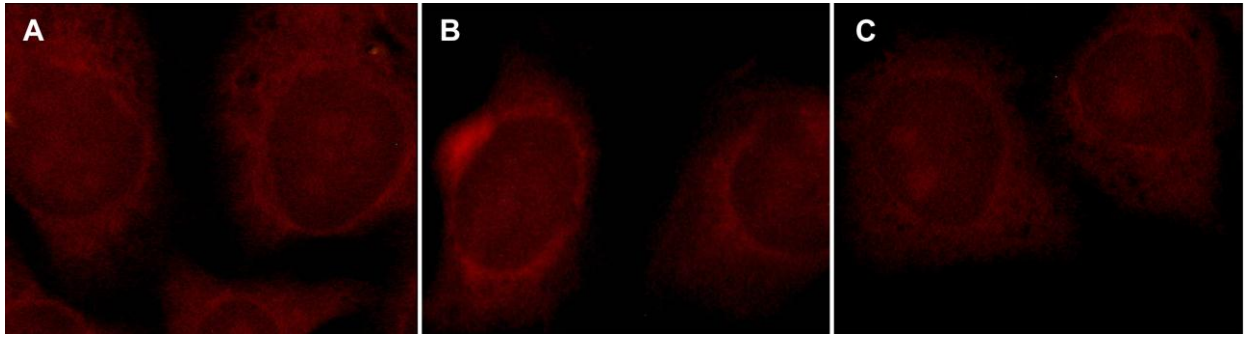
In the GG family, T1D patient's brother developed diabetes 14 month later. IIF positivity can be notice in T1D patient as well as the entire family.

Figure 19. IIF of SC Family. A) SC Father. B) T1D Patient. C) SC Brother.



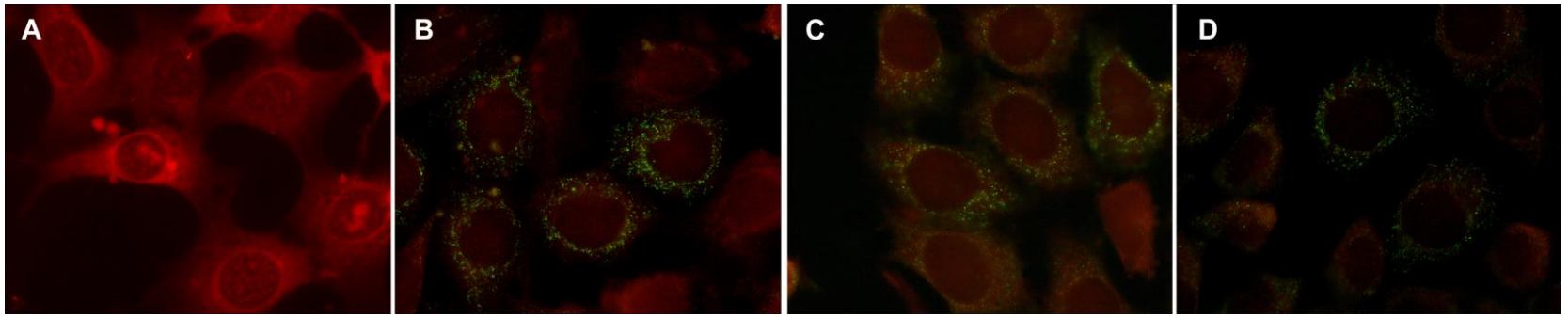
T1D patient and his mother result positive to EVs antibodies. No IIF can be notice from SC Father.

Figure 20. IIF of BTS Family. A) BTS Father. B) BTS Mother. C) T1D patient.



No IIF can be notice from BTS T1D patient, as well as in the rest of the family.

Figure 21. IIF of UG Family. A) UG Father. B) UG Mother. C) T1D Patient. D) UG Sister.



Cytoplasmic IIF was detected in UG T1D patient as well as his sister and mother. No IIF in UG Father. UG Sister developed T1D nine month later.

Table 26. T1D Family cases. T1D patients are highlighted in gray. Positive autoantibodies are written in orange.

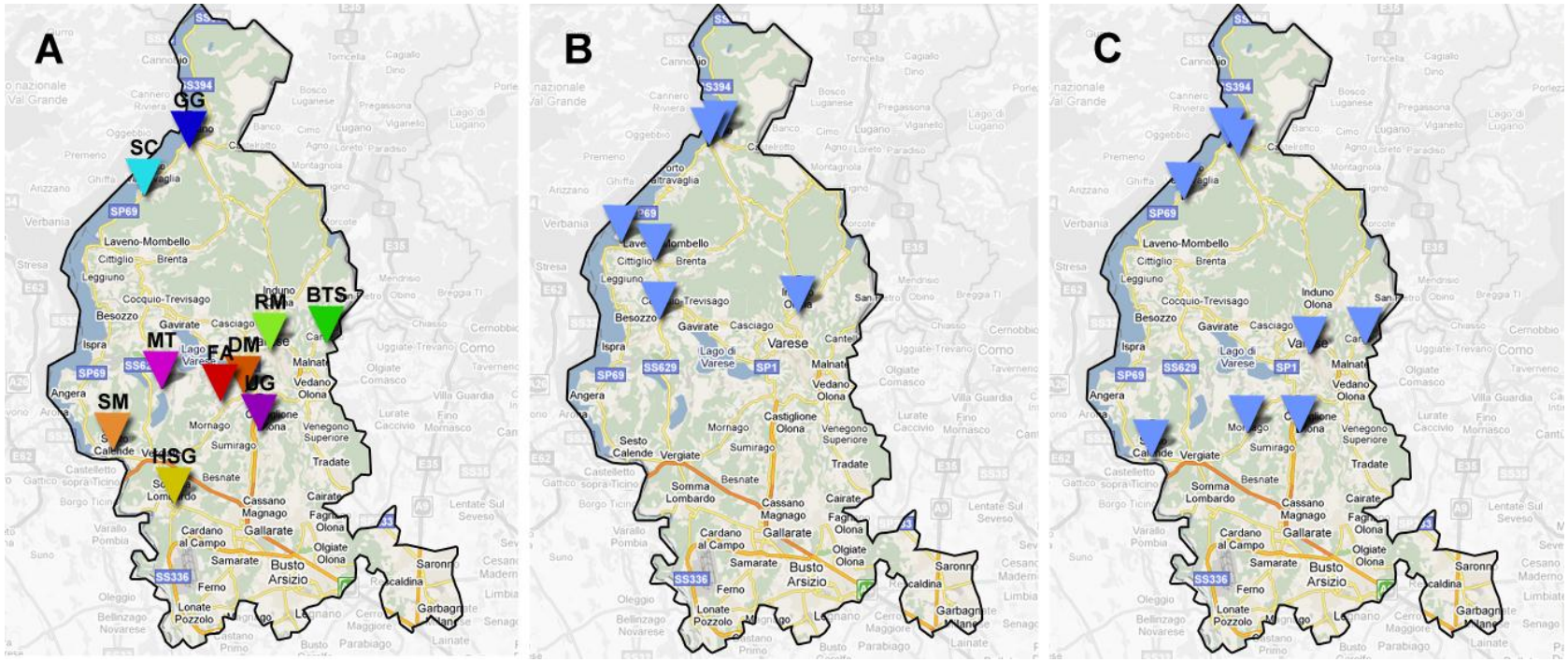
Family	ID	Gender	Age at onset	HLA	HbA1c	C pept	GAD	IA2	ZnT8	HEVA	HEVB1	HEVB2	HEVB3	HEVC1	HEVC2	HEVD
BTS	BTS (patient)	F	3,5	DR3 DR4 DQ2 DQ3	10,9	0,3-0,3	50,6	>100	21			+				
	BT (father BTS)	F		DR3 DR4 DQ2			0,1	0,5	0,7			+				
	BTA (mother BTS)	M		DR3 DQ2			0,1	0,6	1			+				
DM	DM (patient)	M	10	DR3 DQ2	14	0,3-0,4	23,1	100	>100				+			
	DH (father DN)	M		DR3 DR5 DQ2 DQ5			0,1	0,3	0,6							
	DF (mother DH)	F		DR3 DR10 DQ2 DQ5			0,1	0,4	0,6				+			
	DA (brother DM)	M		DR5 DR10 DQ5			0,1	0,5	0,7							
FA	FA (patient)	M	9	DR3 DR6 DQ1 DQ6	12,2	0,3-0,3	>100	0,4	34					+		
	FMas (father FA)	M		DR6 DR16 DQ5 DQ6			0,1	0,3	0,8				+	+		
	FMat (brother FA)	M		DR3 DR16 DQ2 DQ5			0,7	0,4	1,4					+		
	FPA (mother FA)	F		DR1 DR5 DQ2 DQ5			0,1	0,4	0,9				+	+		
GG	GG (patient)	M	5	DR4 DQ2	8	2,8-3	0,1	27	1,7		+					
	GN (patient, brother GG)	M	4	DR3 DR4 DQ2	7,6	0,3-0,5	0,5	0,2	3,7		+					
	GV (father GG/GN)	M		DR1 DRB3 DQ2 DQ5			0,1	0,2	0,6		+					
	GCMC (mother GG/GN)	F		DR4 DR 10 DQ2 DQ5			0,1	0,2	0,8		+					
HSG	HSG (patient)	M	14	DR1 DR4 DQ3 DQ5	14	0,3-0,5	1,3	48,9	10,3				+	+		
	HY (sister HSG)	F		DR1 DR4 DQ3 DQ5			ne	ne	ne				+	+		
	HF (father HSG)	M		DR4 DQ3			ne	ne	ne				+	+		
	HRG (mother HSG)	F		DR1 DR4 DQ3			ne	ne	ne				+	+		

LM	LM (patient)	F	8	DR1 DR4 DQ3 DQ5	10,7	1,39	ne	ne	ne	+						
	LN (father LM)	M					ne	ne	ne	+						
	LB (sister LM)	F					ne	ne	ne	+						
MT	MT (patient)	F	13	DR4 DR11 DQ3	16,8	0,3	ne	ne	ne				+			
	MA (father MT)	M		DR4 DR7 DQ2 DQ3			ne	ne	ne					+		
	MR (sister MT)	F		DR4 DR14 DQ3 DQ5			ne	ne	ne							
	MMP (mother MT)	F		DR11 DR14 DQ3 DQ5			ne	ne	ne					+		
MC	MC (patient)	F	6	DR2 DR5 DQ1 DQ3	14	0,3-0,4	62	6,8	13,4					+		
	ML (sister MC)	F		DR1 DR4 DQ3 DQ5			0,1	0,3	0,9					+		
	MCN (brother MC)	M		DR3 DR4			0,1	0,3	0,8					+		
	MFB (mother MC)	F		DR3 DR4 DQ2 DQ3			0,1	0,3	0,6							
PA	PA (patient)	M	16	DR3 DR4 DQ2 DQ3	14	0,09	ne	ne	ne					+		
	PM (brother PA)	M		DR3 DR7 DQ2			ne	ne	ne							
	PA (sister PA)	F		DR3 DQ2			ne	ne	ne							
	PPF (father PA)	M		DR3 DR7 DQ2 DQ7			ne	ne	ne					+		
	PSG (mother PA)	F		DR3 DR4 DQ2 DQ3			ne	ne	ne					+		
RM	RM (patient)	M	6	DR3 DQ2	14	0,4-0,5	102	nd	nd							+
	RA (twin sister RM)	F		DR3 DR11 DQ2 DQ3			0,1	0,4	0,9							
	RL (father RM)	M		DR3 DR14 DQ2 DQ5			0,1	0,3	1							
	RMR (mother RM)	F		DR3 DR11 DQ2 DQ3			0,1	0,3	0,9							
SC	SC (patient)	M	14	DR3 DR4 DQ2 DQ3	16,6	1,6-2,1	9,8	31,2	0,7		+					
	SM (father SC)	M		DR3 DR13 DQ2 DQ3			0,1	0,2	0,9							
	SA (brother SC)	M		DR3 DR4 DQ2 DQ4			0,1	0,3	1		+					
	SMT (mother SC)	F		DR4 DQ3			0,1	0,3	0,7							

SM	SM (patient)	M	3	DR3 DR4 DQ2 DQ3	9,3	<0,3-<0,3	0,1	2,3	1			+					
	SG (twin sister SM)	F		DR3 DR16 DQ2 DQ5			0,1	0,2	1			+					
	SG (father SC)	M		DR3 DR4 DQ3 DQ5			0,1	0,2	0,8								
	SSS (mother SC)	F		DR4 DR16 DQ3 DQ5			nd	nd	nd								
UG	UG (patient)	F	11	DR3 DR4 DQ2 DQ3	12,4	0,6-1,8	4,1	30,6	>100		+						
	UM (patient, brother UG)	M	9	DR3 DR4 DQ2	7,7	2,2-2,6	0,1	>100	>100		+						
	UF (father UG)	M		DR4 DR14 DQ3 DQ5			0,1	0,3	1								
	UDBC (mother UG)	F		DR3 DR11 DQ2 DQ3			0,1	0,5	0,6								

Abbreviations: ne, not executed.

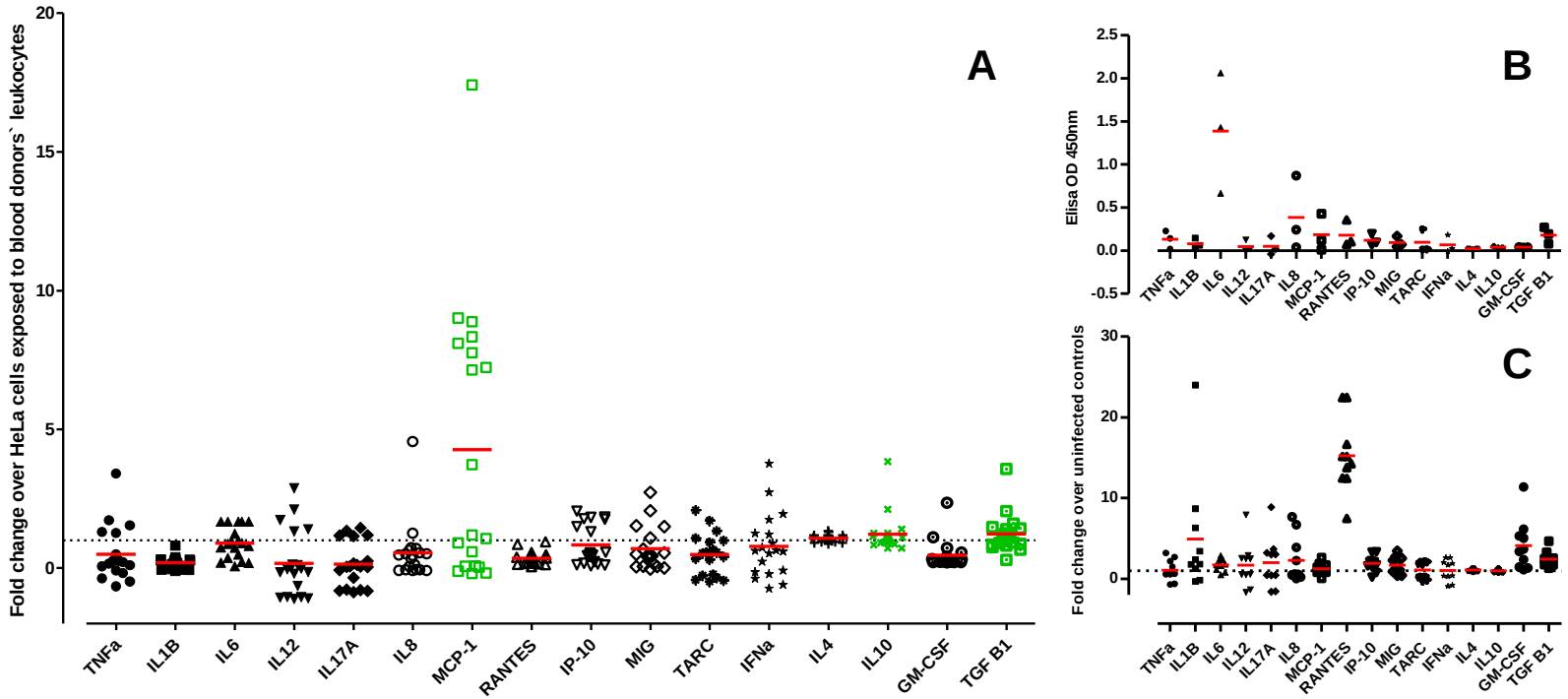
Figure 22. Temporal and geographic clustering of T1D cases. A) Distribution of family cases. B) Clusters of T1D cases in Sep-Dec 2005. C) Clusters of T1D cases in Sep-Dec 2007.



6.1.5 – Cytokine and chemokine expression

Cytokine expression was analyzed in supernatant of HeLa cells co-cultured with leukocytes from peripheral blood of T1D patients. After co-culture for one week, HeLa cells underwent 4-5 weekly passages by trypsinization and washing. At that time, it is expected that no more leukocytes were present in the cultures. The cytokine expression was tested in the supernatant of the above cultures (n = 19) in comparison to cultures treated in the same way with peripheral blood leukocytes (PBL) obtained from blood donors (n = 10; control group). Expression level is represented as fold over the control group. HeLa cells not exposed to leukocytes were used as background control for blood donors' data (n = 6). As reported for other epithelial cell types (Basolo et al., 1996), high levels of IL6 were observed in HeLa cells alone as well as in cultures exposed to blood donors` or patients` leukocytes. As a result, IL-6 levels were not changed by exposure of HeLa cells to T1D patients` leukocytes (Figure 23). High levels of the MCP-1 chemokine were observed in T1D patients (i.e., up to five fold over controls; $p<0.01$).

Figure 23. T1D patients cytokines and chemokines level. A) T1D patients citokines fold change over uninfected controls (n=19). B) HeLa cells cytokines level (n=3). C) Blood donor cytokines level fold over HeLa cells (n=10).



Abbreviations: TNFa, tumor necrosis factor alpha; IL1B-IL12-IL17A-IL8-IL4-IL10, Interleukin ; MCP-1, Monocyte chemotactic protein-1; RANTES, regulated upon activation normal T-cell expressed, and presumably secreted; IP-10, Interferon-inducible protein-10; MIG, Monokine Induced by Interferon Gamma; TARC, Thymus and activation-regulated chemokine; IFNa, Interferon alpha; GM-CSF, granulocyte macrophage colony stimulating factor; TGF B1, transforming growth factor-beta-1.

6.2 – The post-polio syndrome (PPS)

During the past years, a number of different biological samples were collected from 65 patients with diagnosed post-polio syndrome. Cerebrospinal fluid (CSF), tissue samples, whole blood, saliva and urine from Varese district were investigated for HEVs. When available, blood samples from first-degree relatives were collected.

A total of 61 samples were used as controls, including relatives (n=30), blood donors (n=20), and pathologic controls (neurologic patients with non-infectious, autoimmune, or neoplastic disease; n=11).

Table 27. Samples from PPS patients and controls.

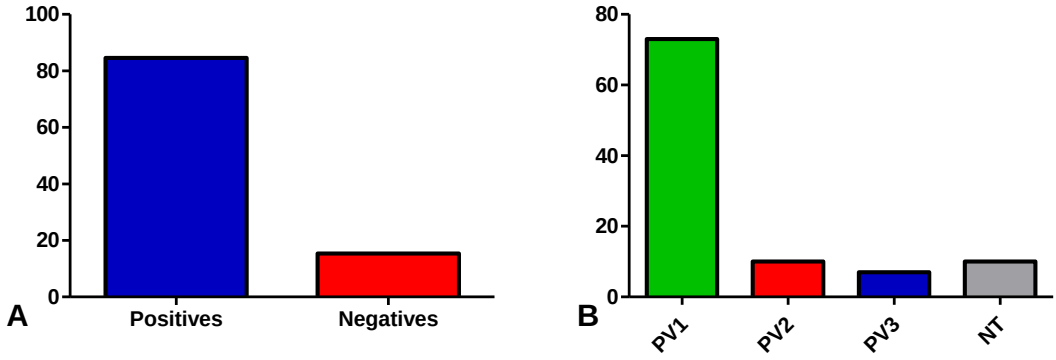
Specimen	PPS samples	Controls samples
CSF	20	11
Whole blood	60	50
Saliva	3	3
Urine	3	3
Tissue (skeletal muscle, peripheral nerve, duodenal mucosa)	3	4
Total	89 (Patients no. = 65)	71 (Controls no. = 61)

Since PVs tropism is specific for motor neurons, CSF samples were analyzed for HEV presence. Because of the difficulty to obtain this specimen, tests were made on a variety of samples. We chose to collect whole blood samples because it's simple to obtain and it appeared to have the same sensitivity of CSF for HEV detection. HeLa cells were co-cultured with peripheral blood leukocytes and after five consecutive passages, the supernatant was analyzed for enterovirus presence.

6.2.1 – PCR results

An optimized PCR method was set up in order to detect PV genome fragments in PPS samples. Initially, a pan-polio primer pair directed against the 3D region was utilized to amplify genome fragments. Subsequently, amplicons were sequenced for identifying the PV type. Afterwards, the method was modified to discriminate between the three PV types. PV genome fragments and low-level infectivity have been detected in 55/65 (84.6%) of PPS patients.

Figure 24. PV detection in PPS patients by RT-PCR. A) Distribution of PV-positive patients positives vs. PV-negative patients (n=65). B) PV types detected by RT-PCR of the 3D region in PPS patients. NT, not typed.



The majority of PPS patients was positive for serotype 1 (n=40), whereas PV2 and PV3 serotypes were less frequent (n=6 and n=4 respectively). For the rest of the cases, viral fragments were not typable (n=5). PV genome fragments could be detected in only 2 of 58 control subjects (3.4 %).

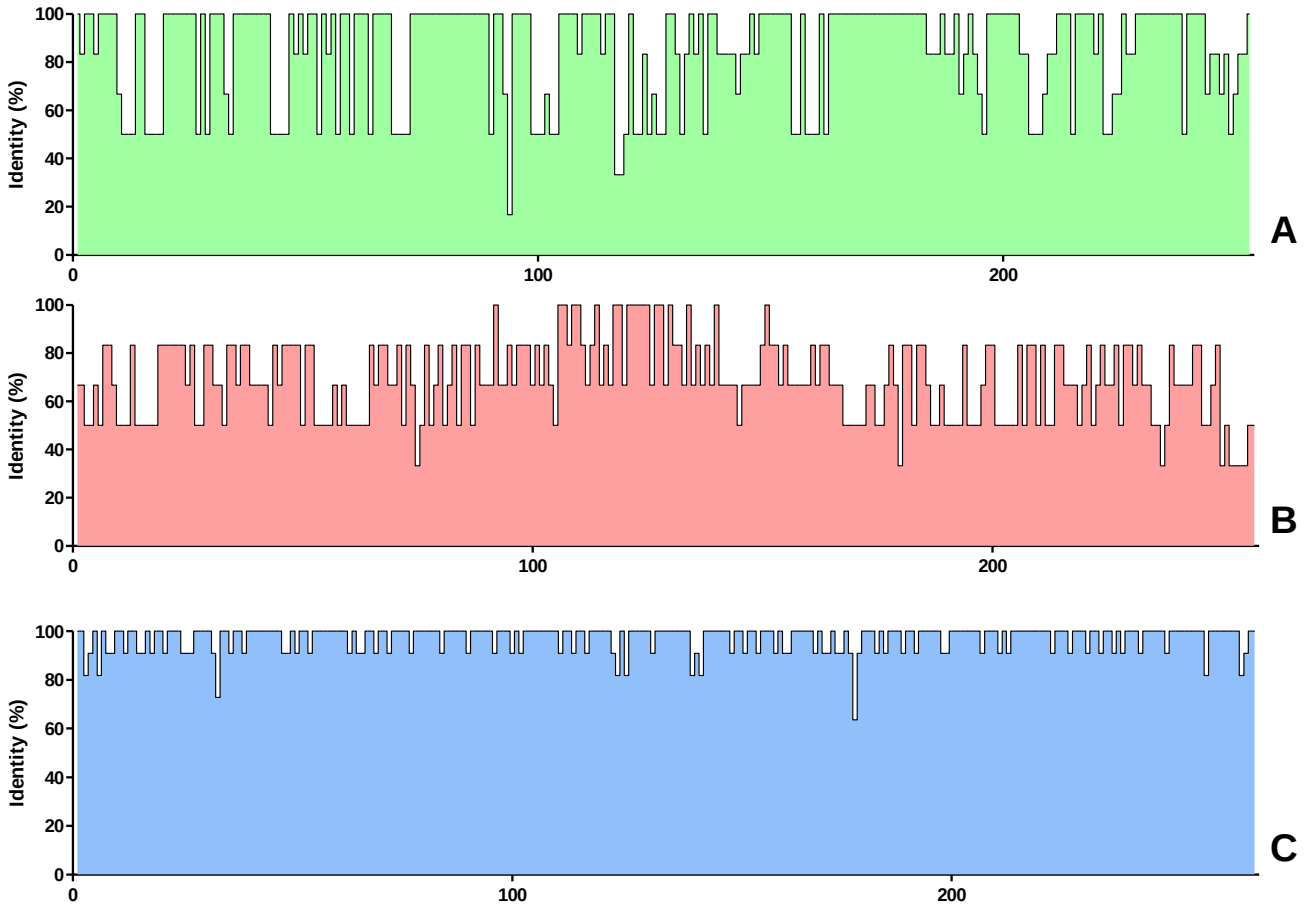
6.2.2 – Poliovirus sequencing

A study was conducted in an attempt to sequence fragments of genomic poliovirus from PPS patients. Different regions were analyzed and sequenced with primers directed against the entire poliovirus genome. Attempts to sequence three different regions were performed: the 5'UTR region, the VP1 region, the 3D region.

In particular, some patients were deeply investigated and extended fragments were sequenced. These data allowed us to identify the viral type and analyze

the genomic variability of viral RNA fragments exposed for decades to the selective pressure of defense systems.

Figure 25. Conservation of PV genome fragments in PPS patients. A) Conservation of the 5'UTR region (nt 285-533; n=5). B) Conservation of the VP1 region (2984-3240; n=4). C) Conservation of the 3D region (nt 6576-6845; n=9).

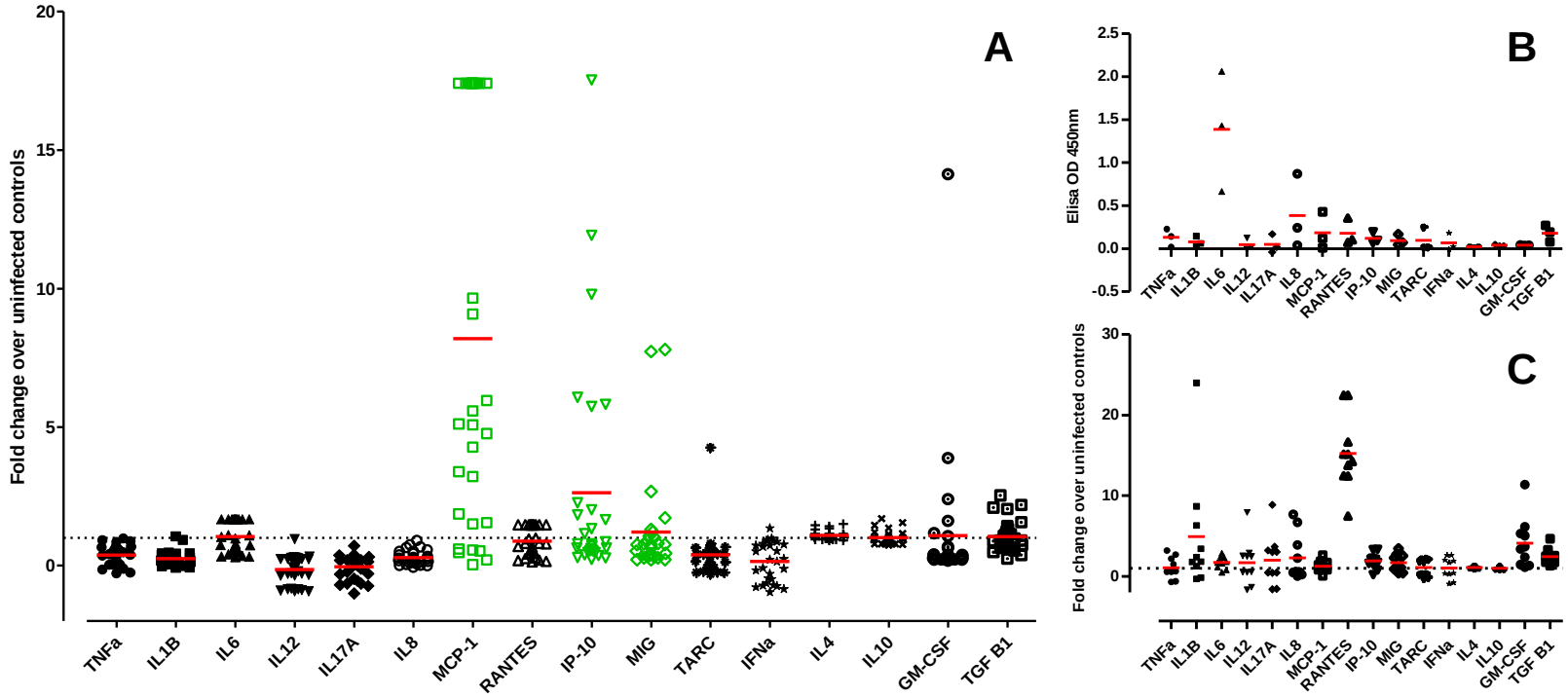


6.2.3 – Cytokine and chemokine expression

As in the case of diabetes (Figure 18), cytokine expression was analyzed in the supernatant of HeLa cells co-cultured with leukocytes from peripheral blood of 29 PPS patients. Expression levels are represented as fold over controls (cell cultures exposed to peripheral leukocytes of blood donors; n = 10). HeLa not exposed to human leukocytes were used as background control for the blood donors group (n=3).

As shown in Figure 21, high levels of MCP-1 and IP-10 were observed in cultures exposed to leukocytes from PPS patients (up to 7 and 5 times, respectively; $p<0.05$). MIG and GM-CSF levels were elevated in a minority of PPS patients.

Figure 26. PPS patients cytokines and chemokines level. A) PPS patients citokines fold change over uninfected controls (n=29). B) HeLa cells cytokines level (n=3). C) Blood donor cytokines level fold over HeLa cells (n=10).



Abbreviations: TNFa, tumor necrosis factor alpha; IL1B-IL12-IL17A-IL8-IL4-IL10, Interleukin ; MCP-1, Monocyte chemotactic protein-1; RANTES, regulated upon activation normal T-cell expressed, and presumably secreted; IP-10, Interferon-inducible protein-10; MIG, Monokine Induced by Interferon Gamma; TARC, Thymus and activation-regulated chemokine; IFNa, Interferon alpha; GM-CSF, granulocyte macrophage colony stimulating factor; TGF B1, transforming growth factor-beta-1.

6.3 – Viral cardiomyopathy

A retrospective analysis of 12 archived tissues, 3 fresh samples from lethal cases of suspected myocarditis and three blood samples from subacute clinical cases was performed. The aim of the study was to ascertain whether HEV genomes could be detected. Archived cases were fixed in formalin and fresh tissues were taken post-mortem and frozen at -70°C. Peripheral blood leukocytes were separated from peripheral blood and co-cultured with HeLa cells (using the already reported protocol). After co-culture for one week, HeLa cells underwent 4-5 weekly passages by trypsinization and washing.

Table 28. Viral cardiomyopathy samples.

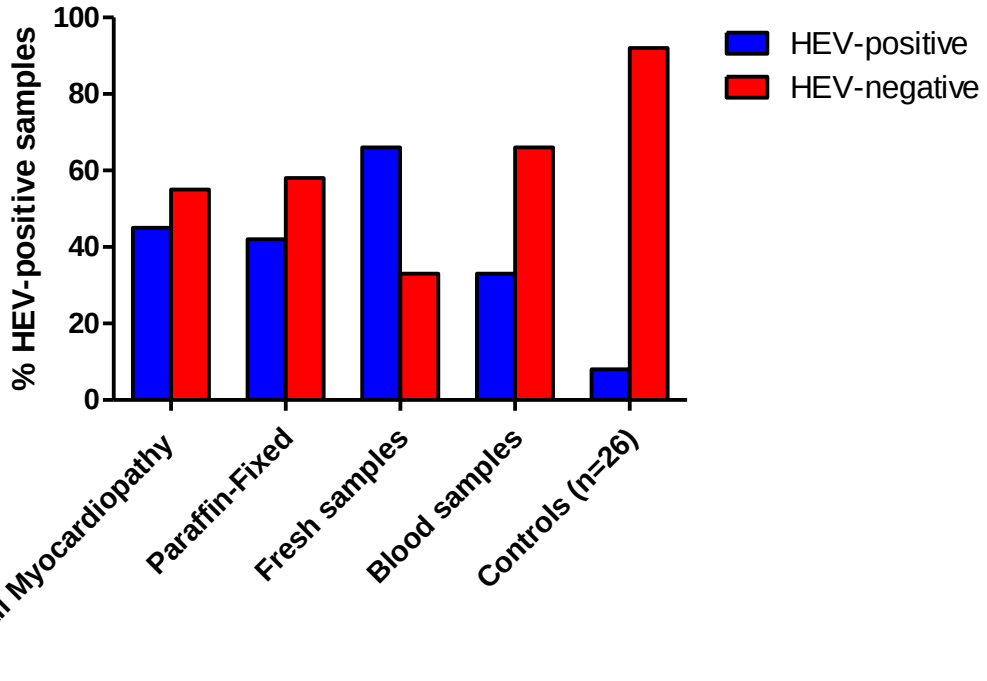
Sample	#	Age	Sex	Diagnosis
Paraffin-fixed tissue	1	3	M	Acute inflammatory cardiomyopathy (fulminant myocarditis) with associated cardiogenic shock
	2	55	F	Hypersensitivity myocarditis
	3	23	M	Lymphocytic myocarditis
	4	18	F	Inflammatory cardiomyopathy (dilated cardiomyopathy with myocarditis)
	5	11	F	Fulminant lymphocytic myocarditis
	6	49	F	Hypersensitivity myocarditis
	7	1	M	Fulminant lymphocytic myocarditis
	8	66	M	Giant cell myocarditis
	9	44	F	Giant cell myocarditis
	10	2	F	Inflammatory cardiomyopathy (dilated cardiomyopathy with lymphocytic and hystiocytic myocarditis)
	11	44	F	Giant cell myocarditis
	12	52	M	Dilated cardiomyopathy associated with hypersensitivity myocarditis
Fresh tissue	13	41	F	Mild inflammatory changes
	14	58	F	Fulminant lymphocytic myocarditis
	15	60	F	Mild inflammatory changes

Blood samples	16	18	F	Inflammatory cardiomyopathy
	17	51	M	Subacute myocarditis
	18	31	M	Inflammatory cardiomyopathy

6.3.1 – PCR results

HEV genome fragments have been investigated with a RT-PCR directed to conserved domains in the 5'UTR region. Results were confirmed by three different primer-pairs (Table 16). Paraffin-fixed tissues from non-viral myocarditis (n=3), PBLs from blood donors (n=20) and autopsy tissues obtained form trauma-associated death (n=2) were used as controls.

Figure 27. Viral cardiomyopathy: HEV detection by RT-PCR.



Five of the 12 lethal cases of myocarditis in paraffin-fixed samples were HEV positive. Previously, serological and cell culture methods failed to identify any infectious agents associated with the disease. Following the successful set up of a protocol for recovery viral RNA from autopsy specimens, molecular investigation of this study demonstrated a close association between the

detection of viral genome and adverse cardiac events. Two of the three post-mortem samples were also positive to the PCR test. Two of the myocardiopathy patients analyzed by RT-PCR on blood samples were HEV-positive. This result was in agreement with the detection of HEV IgM and high titers of IgG in enterovirus-carrying patients.

7 – Summary of results

In this work, different persistent viral infection models have been studied. In particular, we focused our attention on T1D, PPS, and viral cardiomyopathy.

Traditionally, the diagnosis of enteroviral disease is based on the isolation of virus in cell culture. However this method is labor-intensive and requires at least 7 days for primary virus isolation and identification. For a negative report, 21 days are required in order to complete blind virus passages. A rapid diagnostic test might therefore have a strong impact on the management of patients with HEV infections, especially in light of the availability of novel antiviral drugs. Recently, PCR has been employed in rapid detection of viral sequences in a variety of tissues and bodily fluids, proving to be a sensitive and specific diagnostic tool. In particular, the newly developed PCR method, based on the conserved viral 3D region, was proven effective in revealing small quantities of viral genomes. Combined with our technique of biological amplification of virus, the RT-PCR method became more sensitive than when applied directly to non-cultured samples.

The purpose of this study was to analyze the viral agents potentially associated with slowly progressing clinical syndromes. A number of different biological samples were investigated to correlate molecular findings with pathologic features and immunohistochemistry. While immunohistochemical methods allowed the detection, identification and enumeration of inflammatory cells within the specimens, PCR allowed the detection of viral agents even if present in minute amounts.

7.1 – T1D discussion

The analysis of over 100 T1D patients from two different cohorts has led to the detection of HEVs in over 80% of patients at clinical onset. HEVs of the B species were most frequently detected. Epidemiological and geographic data showed numerous clusters of cases especially in the last quarter of the year, supporting the hypothesis of a viral infection. In particular, in the Varese cohort, T1D seasonality seems to fit with the local seasonality of HEV

infections. Cases were, in fact, concentrated in the months of September-December, whereas enterovirus infections appear to peak during summer (June-August). Based on HbA1c data, we can assume that hyperglycemia (and possibly infection) was present at least 2-3 month before clinical onset.

Investigations of family cases supported the viral infection hypothesis, since intra-familial infections have been detected, but diabetes developed only in family members carrying the high-risk HLA haplotypes. In families where more than one case developed, the presence of virus in blood was detected in even in the patient's brother/sister before clinically overt diabetes developed.

7.2 – PPS discussion

In the PV persistence study, we found that 86% of PPS patients were positive for the presence of the viral genome, while CSF, PBLs and bioptic tissues from control patients were free from demonstrable PV sequences. It therefore appears that the PVs are capable of persisting in the body for years after the first contact with this pathogen (i.e., the acute attack of paralytic polio that occurred many decades before). It is still unknown whether the virus is present in patients who, despite having contracted acute paralytic poliomyelitis, failed to progress to PPS. So far, in fact, we were unable to study sufficient numbers of patients with "stable polio" (i.e., non-progressing poliomyelitis). In the absence of predictive biomarkers, it is not possible to predict if and when a polio survivor will develop PPS. Moreover, some PPS cases can be attributed to aging or to concomitant pathologies.

7.3 – Viral cardiomyopathy discussion

The initial phase of this study is promising, since about two thirds of the three investigated patient groups were positive by PCR assays directed to the conserved 5`UTR region of HEVs.

Since the true prevalence of mild myocarditis is clinically elusive (frequently asymptomatic and self-limited), improvements in histopathological and

molecular diagnostics will likely lead to clarify the etiology of this serious pathology that can be unpredictably progressing.

8 – Conclusions

Taken together, the above data indicate that different HEV types can cause persistent infections in apparently normal subjects. These low-level infections are associated with different clinical syndromes affecting the endocrine, nervous, or cardiovascular system.

Even if these data do not prove a causal relationship between HEV infections and diabetes, the high prevalence of HEV genome sequences, the observed clustering of some T1D cases, as well as familial HEV infections (in a few cases with diabetes developing in two siblings within a few months after infection), indicate that different HEV types represent a significant biomarker of juvenile diabetes. Positivity for HEV genomes was also associated with the expression of pancreatic autoantibodies. If comparable results could be obtained in patient cohorts from different geographic areas, HEV detection methods would contribute recognizing some of the environmental agents linked to diabetes.

If a causal relationship between enterovirus infections and T1D were to be finally established, it would be interesting to evaluate the feasibility of an enterovirus vaccine for primary prevention of the disease. The efficiency of vaccination against poliovirus is an encouraging example. However, it will be necessary to have more information concerning the distribution of diabetogenic HEV types in different geographic areas.

The study of PPS patients clearly showed that PVs can persist in the body for decades after the acute infection. Sequencing results showed that the 3D region (viral RNA polymerase) remains highly conserved. These results thus suggest that the 3D region may be used as a target both for diagnosis and for antiviral drugs. Further studies will allow to sequence the entire viral genome in a larger number of patients and to assess whether the changes observed so far are common to multiple persisting strains. The complete characterization of the genome may clarify the pathogenic mechanism by which the PVs can persist in vivo for decades. The consistent finding of PV genomes in PPS patients suggests that viral persistence may play a role in

the progressive degeneration of motor units. This view appears to open new avenues both for prevention and treatment of PPS.

Sequencing data from our PPS study strongly suggest that the persisting HEVs detected in PPS and diabetes are highly mutated. In fact, most PV isolates obtained from PPS patients showed genetic alterations in the explored regions (5`UTR and VP1) that probably are affecting the ability of virus to replicate and its antigenic properties. Even these strains, however, show some residual infectivity in the sense that they remain present in the body for long periods of time (decades in PPS) and are still able – when propagated at low levels in cell cultures – to express structural proteins and to induce the production of specific cytokines. From the genetic standpoint, these viruses are known to undergo two main types of genetic variations, namely mutations and recombination events (Agol, 2006; Yang et al., 2005; Vignuzzi et al., 2005).

Finally, our results on viral myocardiopathy emphasize the relevance of newer molecular tools for elucidating the etiopathogenesis of this clinical condition. Further myocardiopathy cases will be undoubtedly investigated using the highly sensitive PCR assay targeting the 3D region of human EVs.

9 – References

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