Final publishable summary report

PEVNET Beneficiaries

1 University of Tampere
2 Uppsala Universitet
3 University of Siena
4 University of Oslo
5 University of Exeter
6 King's Collage London
7 Karolinska Institutet
8 Centre Hospitalier Regional et Universitaire de Lille
9 University of Turku
10 University of Cambridge
11 University of Helsinki
12 Vactech
13 Diamyd
14 Linköping University

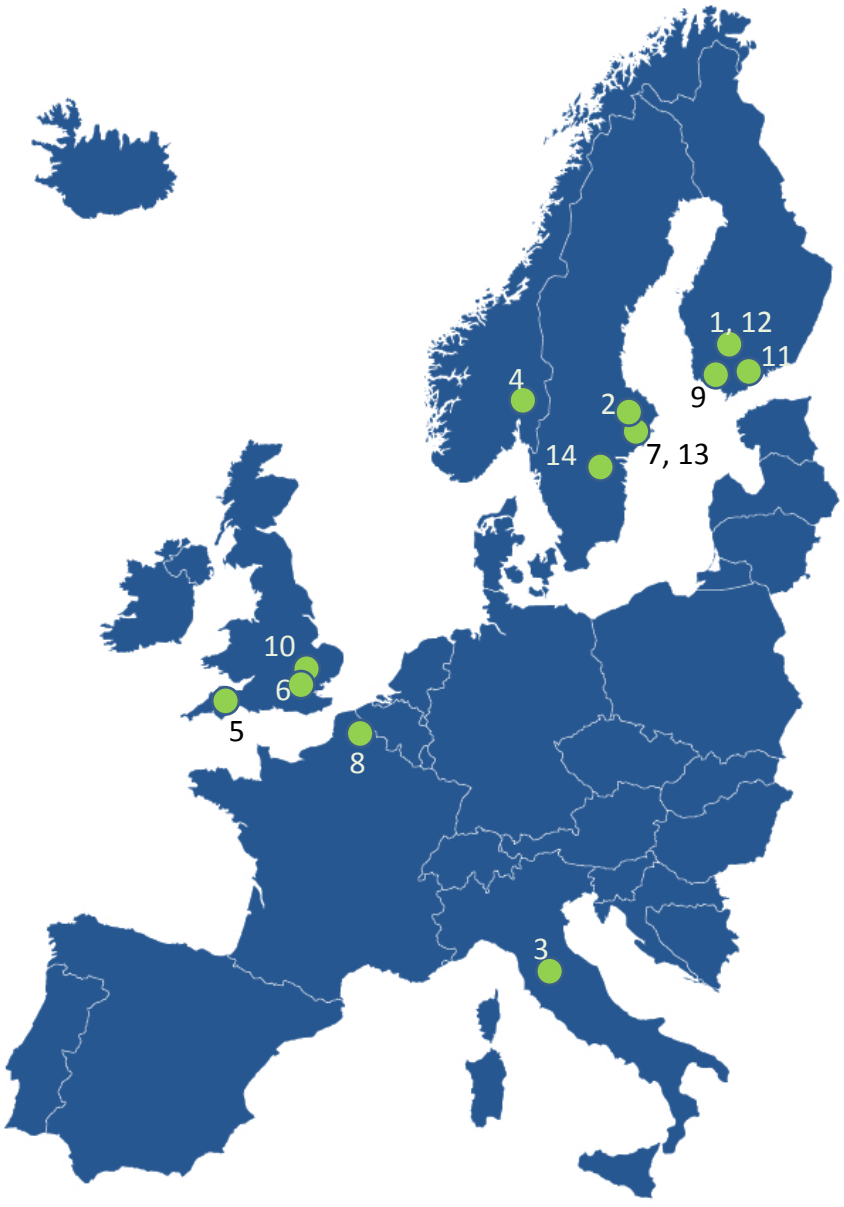

Figure 1. Map of PEVNET beneficiaries.

1. Executive summary (max 1 page)

PEVNET programme aims at providing a rational basis for the development of therapies based on one of the most promising pathogen-disease associations, namely the association between enteroviruses and type 1 diabetes. A causal association between enterovirus and type 1 diabetes has become more and more likely. The aim of PEVNET is to create a new strategy and network of unique resources that make it possible to achieve a breakthrough in this field and develop new treatments for type 1 diabetes. The main focus has been in the detection and characterization of persistent enterovirus infection in the pancreas of diabetic patients and inflammatory responses induced by the infection. In addition, PEVNET has created unique biobanks and has a strong translational component that aims at developing new therapies for the prevention and treatment of type 1 diabetes and new assays for the diagnosis of enterovirus infections.

PEVNET includes 14 partners from six countries and 12 work packages which supplement each other and have a lot of mutual interactions. The project has led to several cutting-edge discoveries and created new biobanks containing pancreas tissues collected from type 1 diabetic patients and non-diabetic subjects, blood and stool samples collected during prospective observation of children from birth until they developed type 1 diabetes and enterovirus strains isolated from prediabetic and diabetic subjects. In addition, PEVNET has actively collaborated with other studies evaluating enterovirus-diabetes association (e.g. nPOD study).

One of the most important achievements of PEVNET is the confirmation of the presence of enteroviruses in the pancreas of type 1 diabetic patients. This important milestone was reached by extensive collaboration and utilizing sensitive and validated enterovirus detection technologies developed by different partners. Another highlight is the identification of the cells which are infected in the pancreas – these cells turned out to be beta cells, the same cells that produce insulin and are selectively destroyed in type 1 diabetic patients. The fact that the majority of diabetic patients had enterovirus positive beta cells in the pancreas but the viral titers and the amount of infected cells was generally low fit with a slowly replicating persisting infection rather than an acute fulminant infection. Additional support for viral persistence was obtained by detecting dsRNA molecules in the infected tissues and by showing that the virus strain in the pancreatic islets was unique and different from the one detected simultaneously in other tissues during an acute enterovirus infection of the same individual. Experimental models for persistent enterovirus infection were also established in pancreatic cell lines in the laboratory. Host defence mechanisms were activated in enterovirus-infected tissues of type 1 diabetic patients, and this activation pattern was similar to that observed in pancreatic cells that were infected in the laboratory. One additional important discovery was made when diabetes-associated enteroviruses were characterized in detail – it was found that group B coxsackieviruses are among these viruses. This fits well with another finding showing that the receptor that Coxsackie B viruses use to enter the cell is strongly expressed on beta cells, thus making them susceptible for these viruses. PEVNET also studied possible therapeutic approaches that could be used to prevent and/or treat type 1 diabetes (GAD65-alum treatment, antiviral drugs and virus-like particles). Altogether, PEVNET has provided fundamentally new information about the role of enteroviruses in the pathogenesis of type 1 diabetes. These discoveries will help to develop new therapies and interventions

for type 1 diabetes, among which enterovirus vaccines and antiviral drugs are currently considered as the most attractive options for future development.

2. A summary description of project context and main objectives

The major hypothesis which this research program is based on states that a persistent (chronic) enterovirus infection of pancreatic islets is the main causative factor in the development of the inflammatory process leading to type 1 diabetes. We assume that the development of such persistent infections and the lack of resolution of those are likely to be regulated by genes that modify the host's immune response against these viruses. Such genes may also regulate later steps of the process, such as the nature of the inflammatory process that develops and its progression to clinical disease. Therefore, the objective of the PEVNET research programme is to provide a rational basis for preventing and treating type 1 diabetes by preventing or eradicating the causative infectious agent or interfering with virus-induced inflammatory processes. An improved understanding of the virus-host interactions is a prerequisite for the development of such treatments.

The specific research aims of PEVNET are the following:
1) to develop a new research strategy, to create a network of investigators and assemble unique facilities and biobanks for studies evaluating the role of enteroviruses in the pathogenesis of type 1 diabetes;
2) to confirm the role of enteroviruses in the pathogenesis of type 1 diabetes;
3) to confirm the role of enterovirus persistence in human gut mucosa and pancreas in the pathogenesis of type 1 diabetes;
4) to identify and characterize the mechanisms allowing the development of enterovirus persistence in human type 1 diabetes;
5) to identify mechanisms mediating the development of chronic inflammation as a consequence of viral persistence in type 1 diabetes;
6) to facilitate the ongoing efforts to develop new therapies for the prevention and treatment of human type 1 diabetes;
7) to develop improved tools for better diagnosis and prediction of type 1 diabetes in humans.

The PEVNET programme is based on a combination of complementary and synergistic expertise of top-level scientists, existing biobanks and a newly created unique tissue biobank, cutting-edge technologies in genetics, transcriptomics, immunology and virology as well as product development aimed at generating new treatments and diagnostic methods. It brings together 15 European top research groups together with two SMEs from six countries, thus creating a critical mass of researchers and clinicians having multidisciplinary experience and high-level technological expertise and providing these collaborators with access to world-leading biobank collections that are required to fulfil the goals.

This innovative and multidisciplinary research model covers the critical steps required to prove causality in diseases which are mediated by complex virus-host interactions. One of the major assets of this approach is the combination of direct analyses of target tissues and prospective epidemiological

studies as well as cell and animal models, which make it possible to study pathogen persistence and related immunological phenomena during different stages of the pathogenic process. An additional strategic strength is the large virus biobank which includes wild-type enterovirus strains which have been isolated from children during different stages of the beta cell damaging process in several studies and which contain presumably diabetogenic enterovirus variants (PicoBank). Thus, the study takes full advantage of the excellent biobank networks and a long tradition in biomedical and clinical research in Europe.

The work was organised in a way which would create maximal synergy between Partners and altogether 12 work packages (WPs) were formed (Table 1). The summary of the work plan and the interactions between different study components are illustrated in Figure 1. Table 2 shows the key resources of PEVNET.

Table 1. *Summary of PEVNET Workpackages.*

WP No	WP title	Lead Beneficiary No
1	Project management	1
2	Biobanks	4
3	Detection of Viruses	5
4	Persistent infection	8
5	Innate Immune system	7
6	Inflammation	3
7	Virus specific immune response	6
8	Virus induced changes in islet cell cultures	2
9	Host genes	10
10	Therapies and diagnostics for virus-induced inflammatory diseases	13
11	Data management and quality assurance	1
12	Dissemination	1

WP=work package

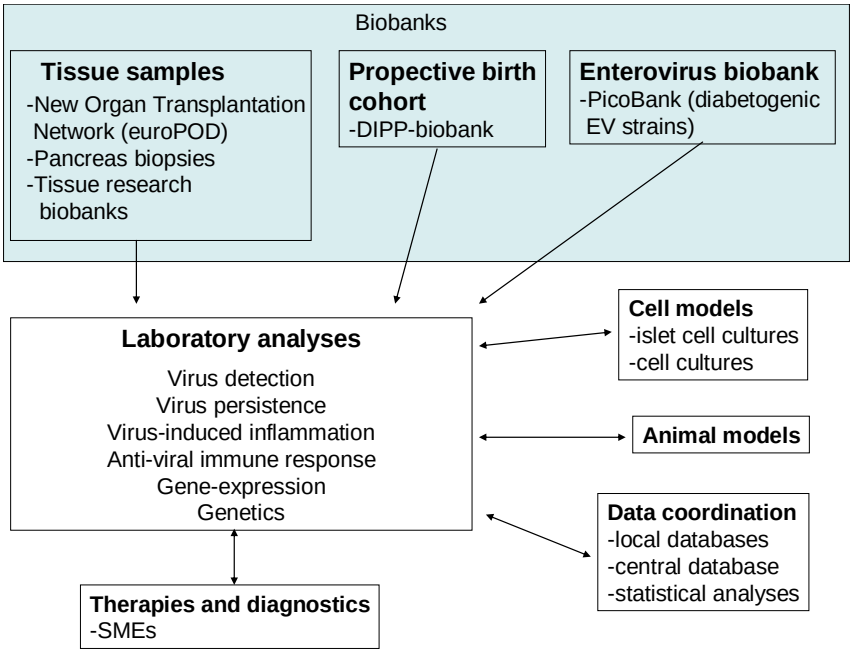

Figure 1. *Summary of the PEVNET work plan.*

Table 2. *List of key resources and research components of PEVNET program*

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1. Prospective biobanks (world's largest birth cohort study on pathogenesis of T1D)
    a. Identification of EV-T1D association
    b. Characterization of EV-induced inflammation
    c. Characterization of EV-host interactions
    d. Evaluation of EV persistence
2. Tissue biobanks
    a. Identification of pathogenic EVs
    b. Characterization of pathogen-induced inflammation
    c. Evaluation of EV persistency
3. Enterovirus biobank
    a. Provides the world's largest collection of presumptively diabetogenic EV strains for animal and *in vitro* studies
4. Animal and in vitro models
    a. Evaluation of specific EV-host interactions
    b. Identification of mechanisms leading to EV persistence
    c. Identification of mechanisms mediating EV-induced inflammation
    d. Testing the efficacy of new treatment protocols
5. Human trials
    a. Testing the efficacy of new treatment against EV-induced inflammation
    b. Obtaining pancreas and intestinal tissues from newly diagnosed T1D patients

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EV=enterovirus; T1D=type 1 diabetes

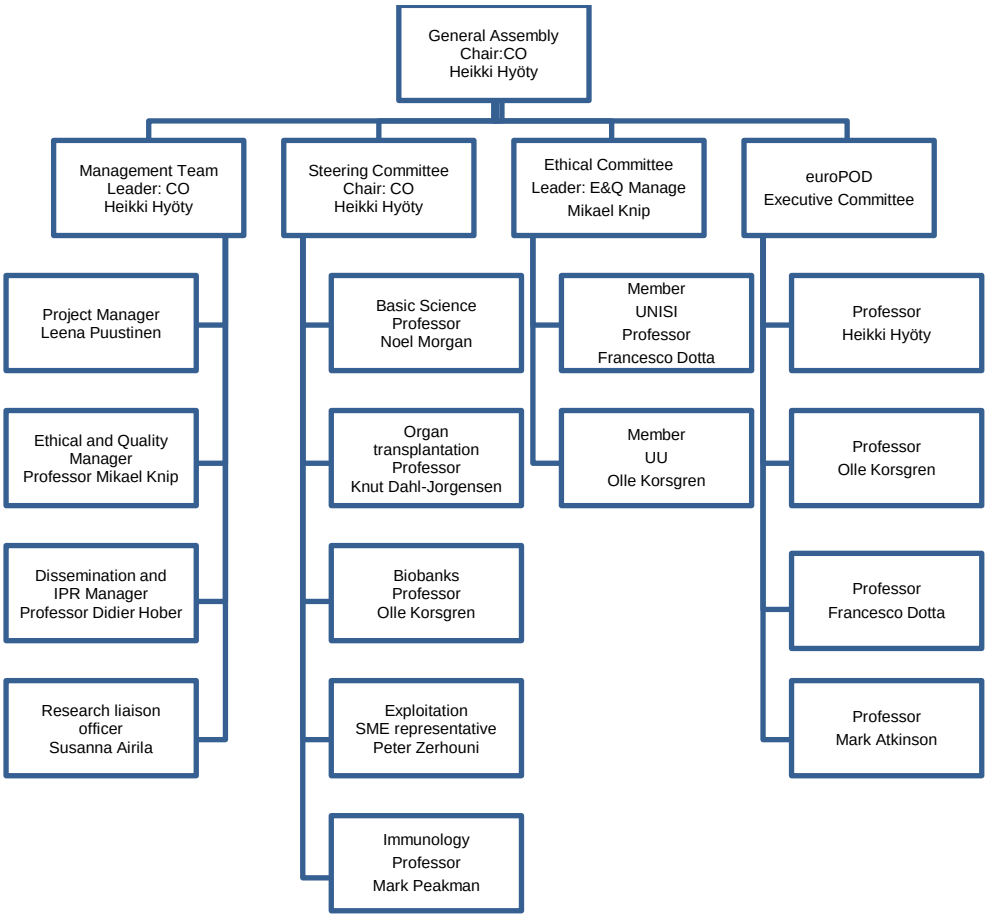
3. Description of the main S&T results/foregrounds

The main results of PEVNET program are summarized below according to the work carried out in different work packages. More details are available on the public web site of PEVNET program (http://www.uta.fi/med/pevnet/index.html).

WP1: Project management

PEVNET held seven regular consortium meetings to monitor the scientific progress and to plan the future research actions. Operational management of the project has been delegated to the Steering Committee which has met regularly. Ethical Committee has followed the project progress and monitored the ethical aspects of the research in this project in the consortium meetings. Smaller groups of PEVNET partners have had meetings to discuss and plan collaboration, distribution of the specimens, results of the studies and publications. The consortium web pages (http://www.uta.fi/med/pevnet/index.html) have been updated continuously and work as a platform where participants can share, distribute and store information related to PEVNET project (pass-word protected area). Figure 2 summarizes the organization of PEVNET programme.

Figure 2. *Management structure of PEVNET programme.*



WP2: Biobanks

The main objective was to create a comprehensive biobank resource which includes both existing biobanks
and newly collected biobanks which are supplementary in nature and which allow such research
which is needed to achieve significant progress in this research field. The detailed aims are the following:
1) To combine the pre-existing biobanks in Europe in an optimal way to form a synergistic platform for the PEVNET research programme. These biobanks include samples from prospective birth cohort studies as well as tissue samples from patients with type 1 diabetes, pre-type 1 diabetes as well as from controls.
2) To collect fresh tissue samples from living patients who have been newly diagnosed with type 1 diabetes or pre-type 1 diabetes (pancreas, lymph nodes, intestinal samples).
3) To collect new samples of fresh tissues from cadaver (brain-dead) organ donors who have type 1 diabetes, pre-type 1 diabetes and representative control donors without chronic diseases.
4) To established a close collaboration with the nPOD project (http://www.jdrfnpod.org/).

euroPOD biobank included collection of fresh tissue samples from cadaver organ donors in the Nordic Network (Uppsala) and Toscana Network (Siena). It generated snap-frozen and formalin-fixed tissue samples from the head of the pancreas of diabetic and prediabetic individuals for the analyses carried out in different work packages. In addition, isolated islets and perifusate from dynamic glucose stimulation assays are available from a majority of cases. Pancreas, duodenum, spleen, pancreatic lymph nodes, and blood from about 150 organ donors were collected annually. Organ donors were screened for islet autoantibodies (IA2A and GADA) and impaired glucose metabolism (HbA1c). In Finland (PanFin network) all donors were analysed for a complete set of diabetes-associated autoantibodies (IAA, GADA, IA2A, ZnT8A, ICA) and new rapid autoantibody assays were developed for on-call screening of organ donors. Approximately 5% of organ donors tested positive for GAD, IA-2 or ZnT8 autoantibodies.

The DiViD biobank included collection of pancreas and other tissue samples from living patients with type 1 diabetes. The tissues are of excellent quality suitable for functional analysis of beta cell insulin secretion, cell sorting and isolation, in-depth studies of pathology and immunology, and the search for viruses by transcriptome sequencing, *in situ* hybridization, immunohistochemistry, and electron microscopy.

The UK Diabetes Biobank collected by Professor Alan Foulis (Royal Infirmary, Glasgow) has been relocated to the Institute of Biomedical and Clinical Science, at the University of Exeter Medical School (UEMS). Full ethical approval for the transfer of the Research Tissue Bank was obtained. This historical collection has been of tremendous value for various components of other Work Packages, too.

The DIPP biobank includes longitudinal serum, white blood cell and stool samples collected from children who have been followed from birth until they developed islet autoimmunity. More than 10.000 children have been in the follow-up in this study since it started in 1994, and new samples and children were recruited during the PEVNET study.

The Picobank includes wild-type enterovirus strains isolated from diabetic and prediabetic subjects.

In addition, samples were available from clinical trials and other sample collections. An important additional resource and a fruitful collaboration was provided by JDRF nPOD study (PIs Professor Mark Atkinson and Professor Alberto Pugliese, University of Miami). nPOD collects pancreas and other tissues from cadaver organ donors with type 1 diabetes and pre-diabetes. Altogether, this network of biobanks created a major benefit for the study since they make it possible to obtain unique data on the role of viruses in the pathogenesis of these diseases. PEVNET biobanks are summarized in Table 3.

Table 3. *Summary of the PEVNET biobank resource*

Biobank	Samples	Created
Tissue biobanks created during PEVNET programme		
euroPOD	Several tissues from cadaver organ donors with type 1 diabetes	PEVNET research programme
DiViD	Pancreas biopsies, intestinal biopsies, pancreatic lymph nodes.	Oslo University Hospital and University of Oslo, Norway
DIABGAD-1 phase II trial	Serum and PBMC samples from T1D patients treated with GAD65 alum + Ibuprofen/Vitamin D combination	Linköping University, Sweden
Existing tissue biobanks		
PanFin	Pancreas from T1D, pre-T1D, controls	University of Tampere, Finland
Dotta	Pancreas from T1D, T2D, pre-T1D, controls	Universities of Pisa and Siena, Italy
Foulis	Pancreas from T1D, T2D, controls	University of Exeter and Royal Infirmary, Glasgow, UK
Tampere Intestinal	Intestinal biopsies from T1D and CD	University of Tampere, Finland

Biopsy bank

GAD65-Alum trials	Serum samples collected from T1D patients treated with GAD65-alum or placebo	Diamyd Medical, Sweden

Existing prospective birth cohort biobank

DIPP	Serum, PBMC, stool samples from children followed until they develop T1D	Universities of Tampere, Oulu and Turku, Finland

Existing picornavirus biobank

PicoBank	Large collection of enterovirus strains Isolated from prediabetic, diabetic and control subjects	University of Tampere, Finland

T1D=type 1 diabetes; CD=celiac disease

WP3: Detection of viruses

The objectives of this work package were to detect enteroviruses from tissue samples including endocrine pancreatic islets, pancreatic lymph nodes and duodenum, and to evaluate the risk effect of enterovirus infections in the prospective birth cohort study. In addition, the aim was to identify and type detected enteroviruses and isolate them in cell cultures.

Development of enterovirus detection assays. A detailed Concordance Pancreas Study was carried out to validate enterovirus detection methods in collaboration with the nPOD study. This has confirmed the presence of enterovirus VP1 protein in the pancreas by both immunocytochemistry and proteomic approaches. In addition, proteomics has suggested the presence of peptides derived from other enterovirus proteins. Antisera directed against the various enterovirus capsid proteins were developed and evaluated for immunodetection of enterovirus in infected cells as well as in tissue samples as well as by ELISA. Overall, the Dako clone (5D8/1) gave greatest sensitivity and specificity for enterovirus detection. The sensitivities of different methods to detect enterovirus RNA were also compared (*in situ* hybridization, RT-PCR) and these optimized assays were used to detect enteroviral RNA in tissue and other samples. Concordance with the proteins involved in the induction of viral response in host was also found to be high (HLA class I, PKR).

Studies in pancreatic tissue have led to several important new discoveries. First, the present of enteroviruses in pancreas tissue of type 1 diabetic patients was confirmed by multiple methods. The proportion of patients in whom enterovirus antigens are detected in the pancreatic islets is typically in the range of 60-70% across multiple cohorts. The work has also revealed that enterovirus antigens are located in insulin-producing beta cells. Virus antigen persisted during prolonged periods of disease since they are detected readily in pancreases recovered from individuals who died many years after initial diagnosis of type 1 diabetes. This is consistent with the concept that the profile of enterovirus infection is atypical in beta cells and, rather than causing large scale lysis, a more persistent infection

develops in which sustained (but very low) levels of virus are retained in small numbers of beta cells over an extended period of time. This was also supported by studies which searched for enteroviral RNA genome in the same pancreas tissues using sensitive RT-PCR technologies: extremely low levels of viral RNA were found in the majority of newly diagnosed type 1 patients. This also fits with a low-grade infection which may be present in this kind of atypical, persistent form. Further studies revealed also that double stranded RNA is clearly formed in the infected tissues, another indicator of the presence of the virus and persisting type of infection. The mechanisms which underlie the development of such persistent infections are not well understood but could involve a modification to the viral genome such as selective deletion of nucleotides located in the 5' terminal region.

Studies in the prospective birth cohort study revealed that enteroviruses are detected in stools several months before islet autoimmunity starts. In addition, the type of diabetes-associated enteroviruses was evaluated by screening neutralizing antibodies against group B Coxsackieviruses which were found to be associated with diabetes in our previous studies. These analyses were carried out in two clinical cohorts including children who have been prospectively followed from birth and who developed type 1 diabetes (DIPP cohort) as well as children recruited at the diagnosis of clinical type 1 diabetes in a previous EU project which Partner 1 was coordinating (VirDiab project; contract number QLK2-CT-2001-01910). The results obtained from these cohorts have further supported the risk association between group B coxsackieviruses and type 1 diabetes. Moreover, a new discovery was made suggesting that these viruses could initiate the process by facilitating autoimmunity against insulin.

In conclusion, the increasingly robust and wide-ranging evidence generated in this work package confirmed the presence of enterovirus in pancreas samples off type 1 diabetic patients among the UK, nPOD and DiViD cohorts. In addition, the prospective DIPP cohort confirmed the association between enterovirus infections and the initiation of the beta-cell damaging process leading to type 1 diabetes.

WP4: Viral persistence

The major hypothesis on which the PEVNET research program is based states that persistent (chronic) enterovirus infection is the main causative factor in the development of the inflammatory process leading to type 1 diabetes. The objectives of this work package were to identify such persistent enterovirus infection in tissues collected from different patient groups and in children who were prospectively followed and develop diabetes during the follow-up. In addition, the aim was to identify viral and host factors regulating the development of enterovirus persistence

As described in WP3 very few cells express enterovirus protein in the pancreatic islets of type 1 diabetes patients, but they are present more frequently than in non-diabetic controls. Increased expression of dsRNA molecules was found in the pancreatic islets of type 1 diabetic patients as possible sign of viral persistence. In addition, PCR-based methods were able to find small amounts of enterovirus RNA in the pancreatic islets of the majority of newly diagnosed type 1 diabetic patients. This also supports the idea of slowly replicating persisting enterovirus infection in the pancreas.

Moreover, we observed enterovirus protein and enterovirus specific genome in diabetic patients with extended duration of disease (up to 10y) and there is no evidence of lysis within the islets of the type 1 diabetes cases. These findings have been replicated in different PEVNET biobanks and in the nPOD cohort. Together this provides robust evidence of the presence of enterovirus in the pancreas of type 1 diabetes patients. In keeping with these findings we are also able to detect evidence of a response to infection (detailed in WP5/ WP6) in the type 1 diabetes patients both within the VP1+ cells themselves and in the surrounding endocrine cells within an affected islet.

Cell models were created to study the mechanisms of enterovirus persistence and methods for detecting persisting enterovirus infection in tissues and blood have been optimized. A new enterovirus antibody assay was developed for the detection of possible viral persistence and new methods have been developed to detect enterovirus RNA in longitudinal blood samples collected from prediabetic children. However, analyses of consecutive stool and blood samples gave no clear sign of enterovirus persistence since prolonged virus positivity was not seen in prospectively followed prediabetic children.

These studies have shown that enteroviruses can establish persistent infections in pancreatic cells, including beta cells that have been infected in the laboratory, and replicate in such cells for several months or even years. In addition, the markers expressed by these cells resemble the markers found in the pancreatic islets of type 1 diabetic patients. Persisting infection produces also dsRNA, a finding which resembles those made in human islet beta-cells of type 1 diabetic patients. In cell models, persistent enterovirus infection could be completely cured by a treatment with an antiviral drug, which opens possibilities to test if such drugs could eradicate possible persisting enterovirus from the pancreas of diabetic patients.

WP5: Innate Immune System

The objectives of this work package were to determine if the innate immune response to enteroviruses is abnormal in patients with type 1 diabetes, to evaluate if the innate immune response to enteroviruses regulates the development of viral persistence and to assess the ability of different polymorphic version of IFIH1 to recognize enteroviruses. Further objectives were to evaluate whether the genotype of IFIH1 determines the human pancreatic islet response to an enterovirus infection and whether the genotype of IFIH1 determines the diabetogenic effect of enteroviruses in a prospective birth cohort study

The expression of different proteins involved in virus recognition and antiviral defence was detected both in pancreatic cells infected by enteroviruses in the laboratory and in pancreatic tissues of diabetes patients. The islets in type 1 diabetic patients showed signs of increased innate immune activation and lowered capacity to counter-regulate this response. Studies with cultured human pancreatic islets revealed that the antiviral defence may be deficient in beta cells, possibly making them susceptible to enterovirus infection. In addition, different enterovirus strains differed in their ability to induce such

responses suggesting that the virus strains may be an important determinant in virus-induced inflammation in the pancreatic islets. Two distinct profiles of immune cell subtypes were found within the pancreatic islets of patients with recent-onset diabetes.

Over recent years it has become increasingly clear that the strength of the innate immune response is determined by the genetics of the host. Therefore, we studied further how polymorphisms in genes important for the host immune response affect the response to enterovirus infection. We have specifically focused on non-synonymous single nucleotide polymorphisms (nsSNPs) identified in genome wide association (GWAs) studies as being associated with altered risk for diabetes development, including for example IFIH1, PTPN22 and TYK2. Extensive studies with different diabetes-associated genetic variants of one major immune defence molecule IFIH1 revealed that diabetes-associated alleles are associated with altered response to the virus. Genotyping studies have revealed an interesting correlation between a certain type 1 diabetes -associated polymorphisms in innate immune system response genes and the risk for enterovirus viremia.

In conclusion, these studies have contributed to the characterization of the innate immune responses elicited by numerous different human cells and tissues (e.g. human islets, PBMCs, etc) in response to clinically relevant enterovirus strains. We have also determined the ability of different polymorphic versions of IFIH1 to recognize these viruses as well as examining the innate immune response in relation to IFIH1 genotype. We have also been able to study the relationship between permissiveness to enterovirus infection and beta cell damage. Using data from the DIPP study we have been able to link certain gene polymorphisms to an altered risk for enterovirus positivity. Finally, via studies in pancreas and intestinal tissue of type 1 diabetic patients we have been able to demonstrate an activated innate immune response in such tissues. Collectively, this information has allowed us to draw numerous conclusions of importance for understanding the role of host genetics, innate immune responses and enterovirus infections in the etiopathogenesis of type 1 diabetes.

WP6: Islet inflammation

The aims of this work package were to identify the nature of inflammatory infiltrates and chemokines involved in immune cell recruitment to enterovirus-infected pancreatic islets and study the secretory products released by infiltrating immune cells. In addition, the aim was to monitor the expression of inflammatory markers in the islets to characterize the gene-expression profiles and activated immunological networks in enterovirus-infected pancreas.

The results indicate islet-specific expression of proinflammatory molecules and presence of autoreactive T cells suggesting ongoing islet inflammation and active recruitment of leukocytes. In addition, insulin-producing beta cells were found to strongly express CAR, which is the major receptor for group B coxsackieviruses. On the other hand, beta cells expressed relatively weakly MDA-5 (innate immune receptor for enteroviruses) suggesting that the anti-viral innate immune response of beta cells may be weaker than that of other islet cell types potentially making them susceptible to enterovirus infection. Global gene expression profiles of cultured human pancreatic islets change dramatically

after infection with different strains of coxsackie B viruses. Certain virus strains induced changes in gene expression of much larger number of genes than other strains and diabetes mellitus type 1 pathway was enriched in differentially regulated genes. IFIH1 genotype influenced gene expression responses to the virus. IgA deposits in the intestinal mucosa of type 1 diabetic patients correlate with the expression of enterovirus protein in intestinal mucosa, and in some cases these deposits were found also in the pancreas.

An extensive effort was undertaken to confirm the class I HLA hyperexpression of insulin-containing islets in the pancreas of type 1 diabetes patients. This phenomenon was now confirmed using multiple antibodies, multiple different techniques and tissue types. We can now demonstrate that HLA-A, B, C, B2M and HLA-F are all upregulated at both the protein and RNA level in type 1 diabetes patients when compared to non-diabetic controls. This increase in expression of these components is also closely associated with elevated expression of STAT1, linking interferon pathways to the altered expression of Class I HLAs. In addition, a small number of beta cells express class II HLA in type 1 diabetes patients suggesting that under certain circumstances beta cells may have the ability to present antigen to infiltrating CD4+ T cells. Together these alterations are likely to result in a situation where the beta cells are more visible to infiltrating beta cell specific immune cells.

WP7: Virus-specific immune response

The objectives of this work package were to identify the molecular targets of the adaptive cellular immune response to enteroviruses and characterise the adaptive immune response to enterovirus in terms of target cell cytotoxicity and inflammatory mediators. In addition, the aim was to examine the role of host defense in virus-induced inflammation and enterovirus persistence and determine the pattern of antibodies able to enhance enterovirus-induced inflammation.

One main strategy was to identify enterovirus peptide epitopes presented by the major HLA class I molecules, HLA-A2 (A*0201) and use these as tools to study the cytotoxic T-cell response. As a result, novel CTL epitopes were identified in enteroviruses. Enterovirus-specific CD8 IFNγ memory responses were screened in a case-control cohort utilising these epitopes. The responses were usually of low affinity and did not clearly differ between cases and controls. In addition, it turned out that none of the CD8 peptides that reacted in ELISpot assay worked in tetramer assay.

Interestingly, different enterovirus strains were able to infect peripheral blood mononuclear cells and these viruses could be detected in peripheral blood mononuclear cells in diabetic patients and controls. We also got evidence suggesting that non-neutralizing anti-enterovirus antibodies can modulate virus-specific immune response and pathogenetic effects of the virus. A correlation was found between these “enhancing antibodies” and beta cell autoimmunity in type 1 diabetic patients suggesting that an imbalance between anti-enterovirus enhancing and neutralizing activities could play a role in the pathogenesis of the disease. A peptide in viral VP4 protein was found to be the target for enhancing

antibodies. The role of enhancing antibodies in enterovirus infection and enterovirus-induced inflammation was also suggested in mice experiments.

WP8: Enterovirus-induced changes in pancreatic islets

The aims of this work package were to discover if there is a connection between previously observed epidemiological risk effect of certain enteroviruses and their tropism to pancreatic islets and insulin-producing beta cells. This kind of tropism could link to their ability to cause beta-cell damage and disturb beta-cell function.

The tropism of enteroviruses to pancreatic islets and beta cells was studied in human pancreatic islets which were infected in the laboratory by different enterovirus strains. It was found that species B enteroviruses, particularly group B coxsackieviruses, replicated only in insulin containing cells. This was in striking contrast to exocrine cells that were resistant to most of these viruses. The main receptor used by these viruses is the Coxsackie-Adenovirus Receptor (CAR). CAR was found to be expressed by beta cells but not by other islet cells. Its expression was also increased in the pancreatic islets of diabetic patients. Virus-induced cytokines and chemokines increased the expression of CAR in islet cell cultures, suggesting that an inflammatory milieu could make beta cells more susceptible for enteroviruses.

Different coxsackie B virus strains caused different types of infection in cultures pancreatic islets - some cause acute lytic infection with rapid cell-destruction while others caused a non-lytic persistent-type infection with only subtle cell damage even if there were no differences in viral titres between the strains. All strains also reduced the expression of insulin gene but none of them affected glucagon gene expression suggesting that they infected only beta cells. Strong induction of the genes encoding chemokines, cytokines and interferon response genes was seen in infected islets including genes encoding OAS-2, MX1 and PKR. The lytic coxsackie B virus strains reduced the expression of genes encoding two of the known type 1 diabetes-associated autoantigens (GAD65 and ZnT8) while the infection with the non-lytic strain had no or perhaps even a somewhat inducing effect on these genes. RNA-sequencing revealed also clear changes in gene expression in the infected islets and major differences were found between the lytic strains and the non-lytic virus strain. Infection also changed the methylation of islet DNA.

In conclusion, these studies suggest that Coxsackievirus B replicates only in human beta cells and that the replication is associated with loss of function. Infection induces genes that are involved in attracting immune cells, antiviral defence and genes coding transcription factors. The pattern obtained in infected islets resembles that seen in the pancreatic islets of diabetic patients. The genetics of the donor and the genetics of the virus strains both regulated these responses.

WP9: Host genes

The aims of this work package were to analyse associations between disease variants and the presence or absence of enterovirus infection in the type 1 diabetic patients and to analyse associations between disease variants and mRNA expression in tissue samples.

Systematic collection, processing and genotyping of samples from type 1 diabetic patients, prediabetic subjects and control subjects were carried out. An extensive effort was carried out to genotype children who participated in the Finnish Diabetes Prediction and Prevention (DIPP) study and were prospectively followed from birth. Genotyping was carried out for HLA and other established diabetes susceptibility genes. Genotype information was correlated with the presence of enterovirus RNA in blood and stools, as well as with serologically verified enterovirus infections. Multiple other variants previously associated with type 1 diabetes have been now tested for association with enterovirus infection. Although most of them were not associated, several polymorphisms have been identified that show evidence for association. Some of these polymorphisms are located in genes with known important functions in the immune system, e.g. STAT4 and GIMAP5, which suggests their potential role in controlling enterovirus infection.

The results suggest that genetic variants located in different loci affect the course of enterovirus infection. Given that these variants have previously been associated with type 1 diabetes, they may indicate a potential pathogenic mechanism leading to type 1 diabetes in genetically susceptible subjects infected with these viruses.

WP10: Therapies and diagnostics for virus-induced inflammatory diseases

The objective of this work package was to evaluate if antigen specific immunotherapy, particularly GAD65 immunotherapy, is effective in enterovirus-induced inflammation. An additional aim was to study the efficacy of selected antiviral drugs against enteroviruses and their ability to cure persisting infection in cell models were tested. A further aim was to develop a high throughput platform for the synthesis of enterovirus-like particles using baculovirus expression system and synthesize such particles of type 1 diabetes-associated enterovirus types. This task aimed at developing new enterovirus-like particle based antibody assay for the analyses of antibodies against enteroviruses.

Objectives concerning the safety and efficacy of GAD65 immunotherapy in enterovirus-induced inflammation was investigated in clinical GAD-alum trials. Participants of these trials were screened for signs of enterovirus infections and possible effect of past enterovirus exposures on the preservation of beta-cell function in the GAD65 treatment and control arms was evaluated. Additionally, a novel GAD65 immunotherapy vector was tested in mouse model. Antiviral drugs were also tested in cell models and found to be effective against diabetes-associated enteroviruses and able to eradicate persisting enterovirus infection in a cell model. This finding opens possibilities to test antiviral drugs in clinical trials to see if they can prevent the progression of beta-cell damage.

Virus-like particles of Coxsackievirus B3 were successfully produced and purified. Their immunogenicity was confirmed in mice experiments and they were used as antigens in EIA assays to measure IgG class antibodies against enteroviruses. These particles worked well as antigens in IgG analyses, and the results correlated well with results using purified intact virus as antigen. This suggests that they can be used as antigens in EIA based antibody assays and other immunological assays. They could also be useful as vaccines against enteroviruses.

WP11: Data management and quality assurance

The basic database structure has been created for PEVNET. Extensive QA programs were established for the method used for the detection of enteroviruses in clinical samples.

WP12: Dissemination activities

PEVNET has generated multiple publications in scientific journals, thesis books and presentations at scientific congresses. Altogether 92 original articles have been published in peer-reviewed scientific journals. Information about the project and its goals has been disseminated to the public audience by press releases, interviews in newspapers, TV and radio as well as by the Consortium web pages. PEVNET has disseminated research findings and enterovirus detection methods by posting them to the Consortium web pages. PEVNET collaborates closely with the nPOD project, which collects tissue samples from cadaver organ donors with type 1 diabetes in USA and in Europe. PEVNET has also close connections with the Viruses in Diabetes International Society (VIDIS). Complete list of publications is available on PEVNET webpages.

4. The potential impact and the main dissemination activities and exploitation of results

The PEVNET programme has emerged from the need to mount an integrated, Europe-wide approach to the complex question of the role of infection in a multi-factorial disease that is of huge economic and social importance. Type 1 diabetes affects more than 3 million Europeans and the majority develop the disease in early childhood or during teenage. Therefore the disease has prolonged impact, reducing life-expectancy by an average of 10 years and inducing a range of associated diseases known as "diabetic complications" that are severe and debilitating. In this sense, type 1 diabetes has a major socio-economic impact. Most critically, this is a disease with a rapidly rising trajectory of incidence and prevalence. Worse still, this rapid rise will affect the under-5 year-olds most severely, who will then be forced to live with diabetes for the longest time. This rapid rise in diabetes cannot be adjudged to be due to changes in the genetic susceptibility of the European population, as the gene pool has been stable over this period. Rather, the increasing incidence reflects the influence of crucial environmental factors, including pathogens such as enteroviruses. Thus, it is critical that a greater understanding of

the impact of infectious disease in this complex pathological process is reached, and this is the major objective of the PEVNET programme.

It has been estimated that the presentation of type 1 diabetes in childhood results in Finland in additional lifetime costs amounting to about 1 M€. A lifetime spent living with diabetes after a diagnosis in childhood results accordingly in a substantial economic burden. It is hard to think of any other disease with a potential infectious aetiology that has such a high socio-economic impact in the EU.

The driving force behind the PEVNET programme is the potential for prevention of type 1 diabetes. It is clear that the achievement of that goal would represent a seismic step in the efforts to improve the health of the population of the European Union and beyond. Since the disease has emerged as a rapidly increasing entity in the last 50 years, it follows logically that there must be environmental exposures that have induced and accelerated this – and that therefore can be identified.

PEVNET was designed to identify such agents. As infectious diseases, these should then be amenable to standard approaches – the development of vaccine regimes and the development of anti-viral agents. A vaccine approach could be deployed in early childhood, when the critical disease-susceptibility window arises and exposures to wild type agents are clearly most risky. Such an approach can be expected to be delivered at relatively low costs but with huge economic and social consequences. In this sense, the programme was implemented in an optimal time, since the first attempts to develop vaccines against enteroviruses that are associated with type 1 diabetes have been started. In fact, PEVNET generated several new research findings that can facilitate the development of such vaccines and antiviral treatments. These findings include discoveries that can be considered as scientific breakthroughs in this field.

PEVNET created a highly collaborative network of experienced scientists whose collaboration will continue also after the EU funding has ended. This has an important facilitating effect on scientific research in this field.

The address of the project public website: http://www.uta.fi/med/pevnet/index.html

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Photos

PEVNET meeting in Tampere in 2013

PEVNET meeting in Siena in 2012