RESEARCH PLAN
Strategic Research Agreement - Single-Project

Overview of the nPOD-Viral Group plans, project management, interactions, and leadership plans.

A. Background and Significance of this work to Type 1 Diabetes

A1. A role for viruses in Type 1 Diabetes.

Type 1 Diabetes (T1D) is thought to have a multifactorial pathogenesis, in which genetic factors create a predisposition to the triggering of multiple autoimmune responses against pancreatic β-cells (1). The term 'insulitis' (2) describes the inflammation and infiltration of pancreatic islets by autoreactive lymphocytes and other immune cell types. Insulitis is eventually followed by β-cell loss, which over time can become virtually complete. Many of the genetic susceptibility loci linked to T1D map to regions containing genes that modulate the immune response, its magnitude, regulation, as well as its autoantigen specificity. However, studies in identical twins report 30-50% (higher with longer follow-up) disease concordance rates (3-5), suggesting the involvement of factors beyond those of inherited genes. Indeed, a variety of such factors have been implicated, including epigenetic regulation of inherited genes, dietary components, toxins and infectious agents (6-11); just to name broad categories.

The hypothesis that viruses (and in particular enteroviruses), may play a role in T1D etiology and pathogenesis has been extensively reviewed in recent literature (12-17). This possibility was widely recognized (for its day) in 1969 (18), based on the initial observation that seasonal variations in diabetes onset correlated with the seasonality of enterovirus infection and (later) with increased antibody levels to Coxsackie virus B4. Since then, an immense number of studies have continued to address the question of seasonal variation in disease incidence and investigated the association of viral RNA and immune responses to enterovirus in relation to T1D and islet autoimmunity (19-80). The effects of enteroviral infection might interact with exposure to cow's milk, another putative environmental risk factor for T1D (81;82), as well as to dietary insulin (83). Enterovirus infections in mothers have been linked to increased T1D risk in the offspring, albeit not in all studies (84-89). As recently reviewed by Stene and Rewers (16), results of clinical studies have not always been concordant, in part due to methodological limitations as well as the timing and frequency of sampling. Taken collectively, an impressive number of studies have linked enterovirus infection to islet autoimmunity and make a case for the role of enterovirus in disease pathogenesis that cannot be ignored. However, a number of key research questions still require answers to moving this notion forward as a contributing factor to T1D development.

Beyond serological studies, which represent most of the association studies between viruses and T1D, investigators had rare opportunity to examine pancreas from T1D patients. Specifically, these studies have sought to detect both viral RNA by RT-PCR and in situ hybridization (ISH), as well as viral antigens by immunostaining approaches. We would emphasize that several of the investigators who are participants in this application are leading experts in this field of study and have successfully detected viral antigens and/or markers of viral infection in the pancreas from deceased T1D patients. From an extensive review of this literature, it is apparent that the methodological improvements that have occurred over the years have been linked to much improved detection rates (90-97). Indeed, in a recent study from the U.K., the presence of viral antigen was observed ten times more often in patients with T1D and, to a much lesser extent, in patients with T2D, compared to non-diabetic donors (98). Studies have also provided evidence that enteroviruses can infect and damage β-cells, as well as induce expression of HLA class I antigens and alpha-interferon (92-95), which could amplify inflammation and provide a link to islet autoimmunity. Enterovirus infection may induce functional changes and influence β-cell replication (15;99;100), and perhaps influence thymic selection of T cells (13).

Importantly, genetic studies also point at a potential role for viruses in T1D. This was suggested by the reported association of IFIH1 gene variants with T1D risk (101-104). Such variants influence the recognition of double-stranded RNA (dsRNA), signaling, or other functions of IFIH1 (105). This gene codes for the interferon induced with helicase C domain protein 1, also known as MDA5 (melanoma differentiation-associated protein 5). This helicase is involved in the cytoplasmic recognition of dsRNA, which is generated during the replication of enteroviruses in infected cells (viral RNA polymerase produces a negative stranded RNA copy of the positive

stranded viral RNA genome leading to the formation of dsRNA intermediate). Upon recognition of dsRNA, IFIH1 signals to promote interferon and NFκB responses followed by production of inflammatory cytokines. Functional studies associate predisposing variants with increased expression of IFIH1 mRNA in peripheral blood mononuclear cells, which include antigen presenting cells (104). In contrast, rare IFIH1 variants associated with disease protection (106) are associated with reduced function when experimentally expressed in cell lines (105). Thus, IFIH1 predisposition may be explained by stronger innate responses to viral infection. In the presence of IFIH1 predisposing alleles, viral infection of pancreatic ß-cells might be expected to lead to more sustained interferon responses and up-regulation of MHC class I expression. This would, in turn, increase the potential to expose self-antigens and trigger autoreactive responses leading to ß-cell destruction (107). The discovery of a mechanism of genetic predisposition linked to viral infections epidemiologically associated with T1D (10), together with the more recent studies demonstrating enterovirus capsid protein in patients' pancreatic islets (98), collectively make an increasingly convincing case for a role for enteroviruses in disease pathogenesis.

Recent studies have also highlighted the concept that viral infection may not only favor the development of T1D, but could, in some cases, be protective (11;108). Taken together, the identification of enterovirus serotypes associated with T1D, combined with an improved molecular characterization of the virus host interaction and relationship to pancreas pathology, could lead to the identification of novel, T1D-specific, biomarkers of infection and, potentially, novel therapeutic targets. If a virus plays a role in islet autoimmunity, a vaccine or drugs that target viral responses could, perhaps, open important therapeutic avenues for preventing the triggering of autoimmunity or its progression.   This, in turn, could reduce patient burden and potential exposure to immunosuppressive or immunomodulatory treatments, which have been associated with significant side effects and long term concerns, especially when children are concerned.

A2. The JDRF nPOD research model.
Historically, the generalized lack of access to human pancreas and other disease relevant tissues has represented a major obstacle towards a better understanding of T1D etiology and pathogenesis. Indeed, while clearly beneficial and serving a noble purpose, mouse studies may not unequivocally lead to the identification of human disease relevant molecular therapeutic targets. Recognizing this fundamental obstacle, the JDRF supported the establishment of the Network for the Pancreatic Organ Donors with Diabetes (nPOD; please see website www.JDRFnPOD.org). The mission of the JDRF nPOD is three-fold:

1) To collect tissues (i.e., pancreas, spleen, pancreatic lymph nodes, blood, etc.) from organ donors with T1D, and unaffected donors who at the time of organ donation express T1D-associated autoantibodies;

2) To distribute such tissues, worldwide, to nPOD approved investigators for diverse studies of human T1D; and

3) To promote collaboration and data sharing among nPOD investigators, to achieve an improved and more comprehensive understanding of the human disease.

Since its inception in 2007, the nPOD program has shown the feasibility of obtaining high quality tissues for research. As of April 2012, nPOD has provided specimens in support of research studies for approximately 130 investigators worldwide. The JDRF has recognized that the nPOD 'organizational' model could be extended to other settings relevant to T1D, including, but not limited to, the area of pancreas/islet cell transplantation. This is in recognition that T1D represents a clinical spectrum of conditions that evolve from the pre-clinical stage to disease onset, often leading to chronic complications and transplantation to reverse kidney failure and/or diabetes.
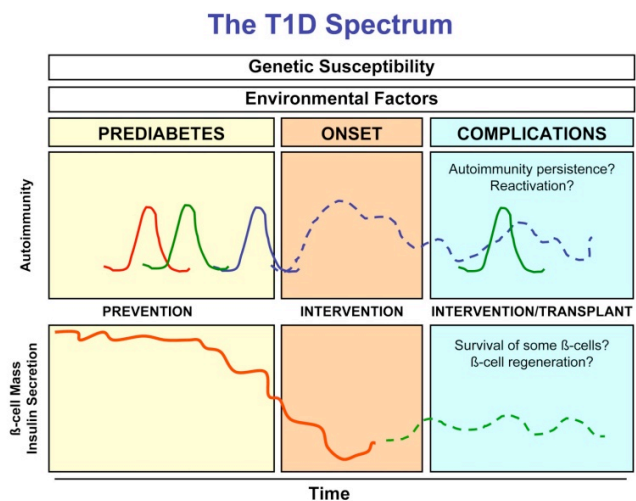

Fig. 1 – The Type 1 Diabetes Spectrum

The disease process is not necessarily exhausted after clinical onset, and we know little about what happens to autoimmunity as well as to ß-cells in the long term, as well as whether any changes are brought about by

chronic immunosuppression once patients are transplanted. The nPOD-Transplantation (nPOD-T) effort, recently launched, will contribute specimens from both deceased pancreas transplant recipients and biopsies from live recipients in the context of recurrent autoimmune diabetes, which has been recently shown in several patients in the absence of rejection (109). Such nPOD-T samples are also available to the nPOD and proposed nPOD-V group, noting that in some cases with recurrent diabetes, we found evidence of enterovirus infection in β-cells of the transplanted pancreas (109).

A2.1. nPOD project interactions. As noted above, a critical mission goal of JDRF nPOD is to promote data sharing and collaboration amongst nPOD investigators. Traditionally, the nPOD Tissue Prioritization Committee (TPC), chaired by the PI of this application, reviews all incoming applications for nPOD tissues. Whenever possible, investigators are notified of potential synergies with other nPOD projects by JDRF nPOD Administrative staff, and are requested to coordinate activities across related projects, so that we can best utilize this limited resource and gain scientific value from collaboration and integrated approaches. Thus, the JDRF nPOD functions not just as a biobank, but also as a synergistic research network with multiple but well defined goals. Taking advantage of web-based connectivity, including on line pathology, nPOD has begun regular interactions with extended groups of investigators to review data emerging from notable nPOD donors. The nPOD program has indeed built a substantial team of investigators who subscribe to the concepts of data sharing and collaboration, and ongoing efforts capitalize on the collective strength of nPOD as a coordinated, scientific powerhouse. Indeed, we envisage that this will only increase. In late April, 2012, nPOD will unveil a beta test version of a product called "_JDRF DataShare_". This innovative open source data acquisition and analysis program, based on improvements to a product known as LabKey, has the potential to revolutionize the way collaboration occurs in settings of T1D research.

A2.2. The nPOD Working Groups. Based on an intense desire to change the way research in T1D is performed, we are at the stage where we have assembled a "cloud" of investigators with diverse expertise, who are interested in collaborative studies across a broad range of topics we deem of potential interest to the JDRF. Based on the data generated so far, we believe we are in a position to take the nPOD research model to a higher level: the nPOD working groups. These groups are intended to collectively tackle key questions in T1D research, which were main topics of discussion at the JDRF nPOD Annual meeting, held in January 2012 in Miami. Addressing these questions, as well as others deemed of interest by JDRF and the T1D research community, should improve our understanding of the disease pathogenesis and lead to the identification of new, T1D-specific and human-specific therapeutic targets. Critical areas of research include, but are not limited to: 1) viral infections, which could perhaps be averted by vaccination if responsible viruses were identified and isolated; 2) autoreactive T and B cells, which could be targeted by phenotypic and functional features, by antigen specificity and by knowledge of T cell receptor sequences; 3) pathways of ß-cell regeneration, which could perhaps be stimulated with drugs; 4) potentially, additional pathogenic mechanisms that may have yet to be uncovered and could contribute to disease heterogeneity, and perhaps help explaining the limited success of clinical trials, which mostly have relied on manipulation of single pathways of the immune system (110;111). Thus, there is clear need for coordinated research through the study of human tissues from T1D patients as well as those deemed of interest to address important issues in the disease. Results could inform strategies for combinatorial therapies that target multiple disease pathways, both immune and non-immune.

A2.3. The JDRF nPOD-V working group. The nPOD-V group proposed here represents the first of the newly formed "_workgroup_" based efforts, and as such will develop much of the logistics, operational models, policies and conceptual framework that will guide the formation of future working groups (i.e., the beta test). An innovative operational concept that will be implemented here is "_real-time_" data sharing, made available through the above-mentioned "_JDRF DataShare_" to help coordinate studies as progress is being made and to inform strategy adjustments. This approach should accelerate the rate of discovery and maximize the potential for new and robust advances with the contribution of many investigators to the development of a synergistic research strategy. As samples are studied by multiple investigators, with a variety of synergistic approaches, this will help developing a comprehensive and integrated understanding of the role of enteroviruses in disease pathogenesis. Importantly, the sharing of tissues and analysis by multiple investigators affords a key unifying element in science and the rare opportunity to coordinate studies that take into account multiple approaches and design input from multiple investigators. Importantly, the nPOD-V Working Group _largely self assembled_ in response to solicitation provided in the form of webinar discussions promoted by the nPOD leadership. The nPOD-v group

began activities last summer recognizing that demonstrating a pathogenic role for one or more viruses in T1D could have very important therapeutic implications. Several investigators in this group have already made key contributions that support a role for viruses, and especially enteroviruses (66;95;98;99;112-117). At that Miami nPOD meeting, there were preliminary presentations from the nPOD-V group and since that occasion, this group has worked enthusiastically to develop plans for a comprehensive strategy to address the viral question in T1D. Indeed, this effort has culminated in this grant proposal, following the successful approval by JDRF of a letter of intent submitted by the nPOD-V group.

B. Proposed research (What?)

The **overarching hypothesis** of the nPOD-V Working Group is that enteroviruses (possibly an individual enterovirus species or a subgroup of these viruses) play an important role in the pathogenesis of human T1D. Furthermore, the group believes that such viruses can be detected and identified more frequently in tissues from organ donors with islet autoimmunity and/or T1D than in unaffected organ donors without evidence of islet autoimmunity; finally, based on heterogeneous patterns of β-cell loss identified in the nPOD cases (118), there could be correlations with different pathological patterns.

Based on the above background and hypothesis, the nPOD-V investigators have extensively discussed what methodological approaches are required to address the **key outstanding questions** that will allow for significant progress in understanding the role of enteroviruses (or other viruses) in the pathogenesis of T1D. These questions form the major **strategic objectives** of our proposal:

1. Is enteroviral infection (often or rarely) associated with T1D?
2. Can a relevant virus be isolated?
3. Can this virus be identified and sequenced?
4. Is there an acute or chronic (i.e., persistent) viral infection?
5. Is there a defective enterovirus associated with T1D?
6. Can viral proteins be identified in the tissues of T1D patients?
7. What tissue/cells are infected (e.g., pancreas, islets cells, β-cells, spleen, PBMC)?
8. What are the functional consequences of infection (e.g., replication, cell death, inflammation) in key cell types?
9. Is viral infection in peripheral blood a marker of pancreatic infection?

To address these questions, the nPOD-V group has organized itself into **6 Task groups**, with specific aims that will synergistically contribute to address the key questions. We propose a multi-faceted approach, that includes a variety of methodologies (e.g., immunohistochemistry, RNA sequencing and gene expression, protein analysis, immunology, etc.) to study viruses in T1D. To the best of our knowledge, and perhaps the most important aspect of this application to emphasize, **there has never been a study that has brought together this diversified expertise, multiplicity of approaches, and innovative techniques (e.g., RNAseq and proteomics, and more) and applied them to the examination of human tissues from patients with T1D.** Further, and to the merit of nPOD-V investigators, they recognize the need to cross-validate reagents and standardize readouts, and are contributing their own novel reagents and reference samples to apply to the study of nPOD specimens. Collectively, we believe that this will provide the best chance of generating robust results and new assays that will become available to the scientific community. Put bluntly, the goal of nPOD-V is not to provide another mere incremental advance to our understanding of the potential role for viruses in T1D, but to provide the most significant thrust forward in this area since interest first arose more than 40 years ago. We acknowledge that this represents a lofty goal. Nevertheless, it is our belief that without addressing the issue with an approach similar to that proposed here, it could be that many more years, if not decades, will pass, without a clear picture emerging with reasonable confidence about the roles (if any) for viruses in T1D. We believe, therefore, that the time is right and the correct infrastructure is in place to make a significant advance.

With this lofty goal in mind, the main aims of each task are summarized below to provide an integrated overview

of the scope of work. Importantly, several investigators serve on multiple tasks, inherently facilitating coordination and data sharing. Each task also has designated Task Leaders who will help coordinating the activities within each task and promoting cross-fertilization of ideas and data discussion across tasks. They will also assume responsibility for coordinating progress reports, publications etc. A diagram illustrating how the various tasks will perform synergistic work and how they are expected to contribute to address the key questions is shown in Fig. 2.

Task 1: Controls and cross-validation

Investigators (in alphabetical order): Campbell-Thompson, Martha; Chapman, Nora; Dotta, Francesco; Frisk, Gun; Giepmans, Ben; Hyöty, Heikki; Kusmartseva, Irina; Morgan, Noel; Oikarinen, Maarit; Richardson, Sarah; Toniolo, Antonio.

Task Leaders: Sarah Richardson and Martha Campbell-Thompson.

Circumstantial evidence has implicated enteroviruses in T1D pathogenesis. In earlier work, several viral workgroup members have shown that pancreatic ß-cells of patients with T1D display clear evidence of an enteroviral infection by immunohistochemistry (IHC). One antiserum in particular has been used extensively in these studies (VP-1, Dako). Task 1 addresses the need to demonstrate viral infection with multiple approaches and reagents, as each will also generate additional information about the biology of the virus. Task 1 investigators will develop and validate methods to identify and screen additional enteroviral antibodies as well as other methodologies for detection of viruses that are capable of reproducibly detecting the relevant enteroviral strains in human islet cells. It is also believed that enterovirus infection of pancreatic β-cells does not lead to large-scale viral replication and extensive cell lysis. Rather, the infections appear to be persistent and do not cause acute damage to islet cells. These infections appear to exist in a more persistent form with minimal virus replication. This may lead to subtle changes in islet cell physiology, which ultimately culminate in the development of autoimmunity rather than cell lysis. It is clear that there are quantitative differences between the viral protein expression in acute and persistently infected cells, therefore it is key for future studies that we identify suitable models and enteroviral antisera capable of efficiently detecting the persistent enteroviral infections, when viral protein expression is likely to be reduced. These models will also be utilized to fully characterize the serotype recognition profile of the enteroviral specific *in situ* hybridization (ISH) probes. This effort will take advantage of controls samples and reagents, which were developed and/or contributed by task investigators. The proposed cross-validation will support the work proposed in other tasks and advance the field overall by validating reagents and resources. Importantly, a key strength is the direct access to unique serotype information from large epidemiological studies (Hyöty's group) and a viral biobank, which contains viruses originating from T1D cases or from viruses that have been passaged in human pancreatic islets (Frisk and Lloyd). Systematic screening of different enterovirus serotypes in large clinical series has recently supported the role of certain CBV serotypes in the pathogenesis of T1D (ISPAD meeting, 2012). Based on this information, Task 1 will aim at validating serotype-specific reagents which can specifically detect these T1D-associated enterovirus serotypes in nPOD samples, but which do not react with other enterovirus types and confirm the presence of viral RNA or particles through in situ hybridization and electron microscopy, respectively.

TASK 2: Virus RNA Analysis

Investigators (in alphabetical order): Ferreira, Ricardo; Gerling, Ivan; Hyöty, Heikki; Lloyd, Richard; Oikarinen, Maarit; Plagnol, Vincent; Petrosino, Joseph; Thackray, Larissa; Virgin, Skip.

Task Leaders: Joseph Petrosino and Larissa Thackray.

This task plays a fundamental role towards the ultimate goal of the nPOD-V Working Group: to evaluate the association of viruses with nPOD samples. To this end, Task 2 investigators will apply an innovative and integrated approach for RNA analysis towards the identification of viruses associated with T1D. Due to the inherent genetic diversity of viruses, use of nucleic acid dependent-analyses is a key component of our efforts towards these detection goals. Collaboration across tasks will guide our efforts in Task 2 and help prioritize the work while, conversely, Task 2 work will guide other tasks. We will focus on identifying enteroviruses in nPOD samples in order to test our hypothesis that enteroviruses play a role in the generation of T1D and can consequently be found more frequently in tissues from patients with T1D than in control tissues. However, it is important to emphasize that the analysis tools used in the sequencing arm of this task includes unbiased mining for virus sequences and signatures that would permit identifying novel enteroviruses and/or additional viruses, if these existed. This effort will leverage the controls and resources in Tasks 1 and 6, and will inform work conducted in Tasks 3, 4 and 5, and vice versa. Thus, state of the art next generation deep sequencing

technologies, including HiSeq, RNA-Seq, and 454 platforms, will be applied to study nPOD tissues in an attempt to provide **a definitive test** for the presence of viruses as well as providing **specific identification** of species and variants. To our knowledge, this is the first time that this approach is applied to the study of viruses in tissue specimens, including pancreas, from T1D patients. Importantly, RNA analysis will also include in situ hybridization and RT-PCR approaches, as well as laser capture microscopy to enrich for islet cell RNA.

Task 3: Viral Protein Identification
Investigators (in alphabetical order): Campbell-Thompson, Martha; Dotta, Francesco; Gianani, Roberto; Giepmans, Ben; Frisk, Gun; Hyöty, Heikki; Morgan, Noel; Morris, Margaret; Nadler, Jerry; Nyalwdhe, Julius; ; Oikarinen, Maarit Richardson, Sarah.
Task Leaders: Noel Morgan and Heikki Hyöty.
The goal of Task 3 is to obtain robust evidence for the presence of virus in pancreas specimens and other tissues from patients with T1D. This will be achieved by using a multi-faceted and a sophisticated approach that involves the study of multiple and novel viral antigens, with increased ability to define viral serotypes, tissue distribution and the identity of viral proteins. To this end, the task will utilize a series of complementary methodologies, including immunohistochemistry, immunofluorescence, electron microscopy (EM) and proteomics. Importantly, EM and proteomics have rarely been applied to the study of the virus question in T1D; preliminary data show the power of these methodological approaches (95). The work in Task 3 interfaces closely with the activities of Task 4, where correlations will be made between islet immune responses and markers of enteroviral infection. It will make use of reagents generated in Task 1 (plus others that are available independently) and it also interfaces with parallel studies in Task 2, where methods will be employed to identify viral genomic material in the sample collection. The data will be made available and analyzed via Task 6. Together, these tasks will advance the understanding of the role of viruses in T1D and should lead to the generation of more robust evidence of viral infection and identification of serotypes, with implications for future diagnostic and therapeutic developments.

Task 4: Viral Immunity and Autoimmunity
Investigators (in alphabetical order): Campbell-Thompson, Martha; Coppieters, Ken; Dotta, Francesco; Homann, Dirk; Hyöty, Heikki; Kent, Sally; Morgan, Noel; Oikarinen, Maarit; Richardson, Sarah; Sarkar, Suparna; Schneider, Darius; Toniolo, Antonio; von Herrath, Matthias.
Task Leaders: Sally Kent and Dirk Homann.
Task 4 will investigate viral infection in other tissues besides the pancreas, the extent to which infection of other tissues may relate to pancreas infection, and what changes and responses are induced in the target cells, both within and outside the pancreas, particularly in relation to autoimmunity. We hypothesize that viral infection may affect also the spleen, gut and lymph nodes in patients with T1D, where immune cells relevant to disease pathogenesis reside. Thus, we propose to examine tissues (pancreas, spleen, lymph node, gut tissue) from subjects with T1D and from control and/or subjects with autoantibodies (preclinical) and Type 2 diabetes (control for hyperglycemia) for evidence of prior enteroviral exposure. Markers of prior viral infection of tissue will include evidence of the virus, of cellular responses to virus, and of immunological viral challenge. We will examine non-pancreatic tissues for viral proteins (cross-talk with Task 3) and cellular signaling proteins responding to virus. We will examine tissue for evidence of immunological challenge including infiltration of immune effector cells, hyper-expression of MHC and MHC-related molecules, pro-inflammatory cytokine signatures, cytokine response proteins, chemokine profiles and for evidence of enterovirus infection of autoreactive T and B cells. This effort also interfaces with independent studies by Drs. Oikarinen and Hyöty, who are examining the type of enteroviruses that are associated with T1D in large prospective studies and with ongoing studies from the Persistent Virus Infection in Diabetes Network (PEVNET), directed by Dr. Hyöty. Reagents developed by PEVNET will be applied to the study of nPOD samples, for example type-specific tetramers detect enterovirus-reactive T cell responses. These collaborative studies will provide evidence of prior viral infection of tissues contributing to the environment of pancreatic ß-cells, and for responses to virus that may provide novel insight into the viral pathogenesis of human T1D. As this task overall examines the immune response to the virus, in pancreas and other tissues, this is also an area where advances can be made towards novel therapeutic approaches.

TASK 5: Virus isolation and amplification
Investigators (in alphabetical order): Chapman, Nora; Frisk, Gun; Lloyd, Richard; Petrosino, Joseph; Toniolo, Antonio.
Task Leaders: Richard Lloyd and Gun Frisk.
Isolating virus from pancreas, spleen, blood or other tissues is essential to implicate viruses in T1D pathogenesis.

Moreover, it will aid and facilitate studies into the biology of the virus, including the eventual development of novel therapeutic strategies and assays to detect infection and help improved prediction, if an association is confirmed. This task will use a combination of approaches to ensure the most sensitive and effective virus amplification and recovery methodology is applied to nPOD and EuroPOD samples.
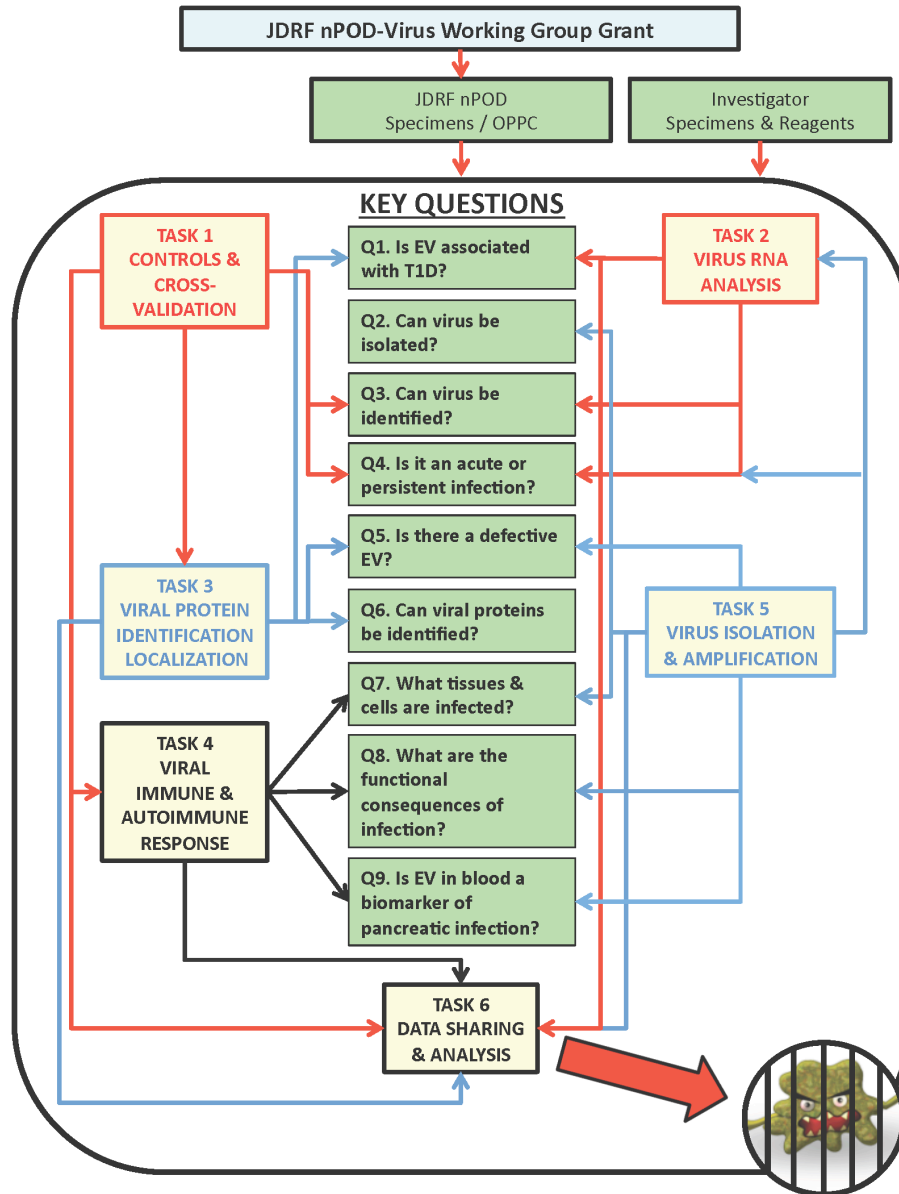

Fig. 2. Organizational flow-chart of scientific interactions of proposed tasks, and how each task contributes to address the key questions that represent the major strategic objectives of the nPOD-V group.

This will provide assurance that sufficiently exhaustive testing for rare or defective enteroviruses in samples is achieved. Thus, the goal of Task 5 is to amplify, isolate and recover virus from as many nPOD samples as possible so that definitive virus sequences can be derived to: (i) identify the specific virus (in conjunction with

Task 2), (ii) determine the degree that virus may reoccur in multiple patients, (iii) produce statistical rigor of virus isolates for the whole project, (iv) produce internal reproducibility of isolates, and (v) recover live virus that can be examined for terminal deletions or other types of mutations that may influence T1D pathogenesis. The virus isolation approaches proposed here will complement each other, and will operate in synergy with the efforts of Tasks 2, 3 and 4, providing the greatest probability that any infectious viruses present in nPOD samples will be detected and identified. Determining the virus sequence will allow an exploration to establish if these viruses are prevalent within the population during relevant periods or if they are recombinants or perhaps even new enteroviruses with properties differing from those currently recognized among enterovirus types. Ultimately, these data may guide the development of diagnostics. Of course, a primary output from Task 5 will be the isolation of live virus(es) from nPOD tissues that will provide a rich resource of new clinical virus isolates that can be characterized for "diabetogenic" potential and other detailed analysis in follow-up studies. The major advantage with virus isolation as compared to just showing the presence of virus is that it will allow construction of in vivo and in vitro models for understanding the pathogenic mechanisms of virus-induced T1D. Such experiments will ultimately lead to development of a vaccine or other therapeutic approaches.

TASK 6: Data Sharing and Joint Statistical Analysis
Investigators: All nPOD-V Investigators
Task Leaders: John Kaddis, Suparna Sarkar, and Vincent Plagnol.
Task 6 addresses the need for tools and plans for data collection, sharing and analysis that are typical of collaborative studies of this magnitude. Indeed, given the multitude of approaches and types of data to be generated, we must develop means to effectively manage the documented complexities and challenges of the biological information life cycle, including generation, storage, sharing, preservation, re-use, and analysis of data. Rapidly advancing fields, such as genomics, other high throughput approaches, and imaging must in addition contend with the burgeoning growth rate in the size of the data. The collaborative analysis of the role of viruses in T1D etiology perfectly exemplifies this trend as this multi-center project brings together scientists with different expertise. As a consequence, this project will generate heterogeneous datasets, all directly or indirectly linked to the nPOD sample collection. Task 6 will address two main goals: data management and sharing plus statistical analysis. The first aim of Task 6 is to facilitate and encourage real-time data sharing and collaboration through standardization of data definitions, formats, and provision of common platforms for data analysis and quality control so that all results and datasets are searchable, accessible and reproducible. The statistical analysis aspects build on the data sharing and management goals of the first aim. Given these aims, task 6 plays a central role in this project by providing a link across all research groups. Ultimately, it is from the data sharing and analysis supported by this task that data generated by this effort can be interpreted and used to inform robust conclusions about the role of viruses in T1D.

C. Rationale for proposed research (Why?)
The rationale for this proposal stems from several decades of studies providing evidence for viruses, and in particular enteroviruses, as having a key role in T1D pathogenesis. This notion, while subject to much investigation over the years, still remains viable; yet the concept has to be fully proven. The reasons for this are complex and multiple. We believe that the approaches proposed here offer unique strengths, which have never come together before in such a way, and will empower this group to make seminal contributions to address the viral question in T1D. These strengths are listed below:

1. The *diverse and outstanding expertise* of the nPOD-V members, individually and collectively; the group includes experts in virology and other relevant disciplines who will work together with investigators with considerable knowledge of islet autoimmunity.

2. The focus on the study of tissues from JDRF nPOD cases, which affords the opportunity to *compare data from multiple relevant tissues* in individual T1D donors, and across cases.

3. The *coordinated study of shared samples* and a scientific plan that will rely on *real-time data sharing*.

4. A *multi-disciplinary approach*, which involves *state of the art methodologies*, affording complementarity and allowing for a pre-determined level of overlap between labs to address *reproducibility*.

5. The inclusion of all the necessary *cross-validation studies*, including the validation of key reagents and the study of control specimens, several contributed by the investigators themselves, which will lead to the generation of new and validated resources.

6. The *cross-fertilization with other working groups and investigators within the nPOD cloud*, who examine other relevant aspects of T1D pathogenesis, as well as *collaboration with other viral study initiatives* that already exist, such as PEVNET and the Virus in Diabetes International Study Group (VIDIS).

7. The *focus on key questions*, with a blend of basic and clinically relevant questions, with the intention to achieve the highest clinical impact possible.

A key requirement for establishing a role for enteroviruses in T1D is to distinguish whether any virus resident within tissues results from transient seasonal acute infection, or from a long-term or persistent infection. This requires the retrieval of virus sequence in sufficient depth and completeness to assign a precise quasispecies molecular signature to any virus found in a given tissue. The availability of nPOD tissues offers for the first time the opportunity to perform this essential comparison across multiple tissues from the same patient case, and to assess rapidly evolving sequences of circulating enterovirus that have been isolated during the last 30 years. The approach applies novel, next generation deep sequence analysis to multiple tissues and amplified viruses from the same cases, affording a unique opportunity to make a significant impact towards proving or disproving the enterovirus hypothesis. Further, the nPOD-V multidisciplinary approach allows cross-validation of results across platforms and examination of various aspects of detected virus and associated changes in the host tissue. Indeed, a variety of approaches undertaken by leading investigators in the field will be applied in an integrated strategy to optimally harness information from the nPOD tissues. Approaches and sample use will be prioritized and informed by real-time data emerging from the various laboratories. Indeed, we propose that real-time data sharing is a critical concept being applied here, together with the study of samples from the same patient by multiple investigators, and the sharing of samples, including those generated through the research (e.g., virus isolates, cell lines, etc) across laboratories.

D. Research Design and Methods (How?)
The experimental aspects of this work are described in detail in the research plan of the 6 Tasks. In this overview, our goal is to cover the leadership plan and management strategies which we believe are necessary to ensure that the nPOD-V Working Group will function effectively and will reach its goals.

The PI is in ideal position to manage this effort because of the following:

1. He has over two decades of research in the T1D field but, importantly, not a major involvement in research regarding viruses; hence, he does not have an inherent intellectual stake or bias.

2. The PI serves as Executive Co-Director of the JDRF nPOD, and also as Chair of the nPOD Tissue Prioritization Committee, which requires close interaction with the nPOD Organ Procurement and Processing Core (OPPC) run by Dr. Campbell-Thompson. Thus, the PI has first-hand knowledge of the nPOD program, of all nPOD investigators and their projects, and together with the TPC and OPPC, has effectively managed prioritization of tissue distribution and scientific project, also promoting project interactions. The PI is also Director of the nPOD-Transplantation program, which may contribute additional samples to this effort, and thus he could inform about which samples could be best suited for analysis by the nPOD-V Working Group.

3. The PI will be responsible for the overall management and coordination of the project. He will constructively interact with nPOD-V Investigators, and Task Leaders, providing guidance and direction through open discussion and evaluation of progress and challenges. To the largest extent, the leadership plan is one based on collective review and decision making, where the success of the nPOD-V effort is the primary goal.

4. Given the scope of the proposed work and the innovative nature of the proposed approaches, the project will require active management and both strategic and resource prioritization. It is expected that new findings, approaches and hypotheses will emerge during the course of the study. As proposed by the JDRF, the PI will manage a pilot and feasibility/discretionary fund designed to support emerging needs. For example, we have initiated a dialogue with Drs. Imagawa and Hanafusa, to deaw comparisons with fulminant diabetes, in which a viral etiology is also suspected (119-121).

5. When an executive decision becomes necessary, every effort will be made to take that in fairness, and whenever appropriate after consultation with nPOD-V Investigators, Task Leaders, the nPOD Director (Dr. Mark Atkinson with whom, the PI, has an extremely close, i.e., literally daily, interaction), the nPOD Scientific Advisory Board Chair (Dr. Ronald Gill) and an nPOD-V Advisory Board. While this has not been finalized, we would like to propose the following individuals: Steve Tracy, PhD; George Eisenbarth, MD, PhD; Richard Insel, MD; and any others deemed fit by the JDRF or its expert reviewers. We would also rely on this board should any dispute arise, or in case of potential conflict of interest.

6. In terms of managing the project, the following types of interactions are envisioned:

   a. Monthly web-based discussions (60-120 minutes), to review progress, data, challenges and solutions. This will include update presentations on a rotational basis but also prioritize on scientific importance.

   b. Monthly calls with individual Task Leaders, as above. Moreover, these calls will be important to coordinate inter-task interactions and for the preparation of progress reports.

   c. Regular data submissions to the data management system created by Task 6, with group notifications.

   d. Together with the investigators and Task 6 Leaders, we will develop key policies to ensure an effective and fair management of data sharing and communication, that also takes into account the need for blinding samples during certain phases of the study.

   e. Face to face work meetings are also planned. The first is to occur in Berlin (September, 2012), attached to the EASD meeting. An additional opportunity will occur at the next JDRF nPOD annual meeting.

   f. E-mail and web-based communications: group has made fantastic use of this unlimited resource during the preparation of this grant application. Not only there has been an exchange of data and grant documents, but there has essentially been an open forum for discussions that have effectively contributed to bring all up to speed and 'on the same page'. While there is consideration for establishing a dedicated web-site or blog, e-mail does not require login in and affords an immediacy of communication that remains unparalleled.

   g. Project management will be assisted by the JDRF nPOD Administrative Core at the University of Florida, with whom the PI has constant interactions via telephone and e-mail means of communications.

Fig. 3. Organizational structure of the nPOD-V Working Group.
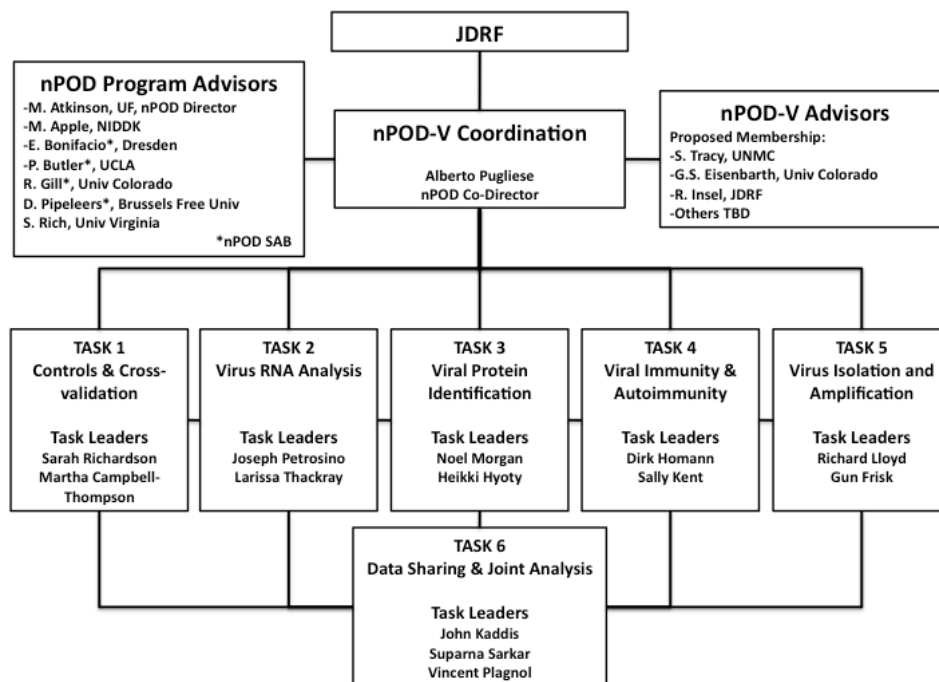
E. Milestones and timelines (Deliverables) (When?)

From the broad perspective of what this project may achieve, we believe that nPOD-V represents the first formally coordinated effort to study nPOD tissues for addressing a key question regarding the pathogenesis of T1D. The proposed plan is comprehensive in design, international in its membership, collaborative amongst its investigators, utilizes the most modern and novel of techniques, and utilizes the highly valuable nPOD tissues.

The potential for obtaining solid evidence associating a virus with T1D as well as molecular information regarding any such virus, together with information regarding changes induced by the virus, will help to define novel therapeutic strategies and targets for this disease. Indeed, we believe viral infections are potentially preventable and/or that the response to virus could be modifiable.

As the molecular features of a virus are identified and its tissue distribution determined, this could allow for the development of biomarkers for this infection. This would have *clear translational implications*. For example, such information could synergize with other biomarker validation efforts organized by the JDRF to assess whether populations (including relatives of T1D patients as well as otherwise 'control' populations) have disease-relevant viral infections. This, we believe, could lead to improved prediction and prevention.

Scientific milestones for each task are reported in the individual task research plan.

From the point of view of project management, we expect to reach the following milestones:

1. Year 1: Implementation and refinement of effective management and policies that regulate the activity of the group.

2. Years 2 and 3: as more nPOD working groups are expected to be launched in the future, contribute the experience gained towards the formation of those groups, and promote inter-group interactions.

3. Throughout the 3 years, effective management of the pilot and feasibility fund to support emerging needs and novel ideas.

4. Throughout the 3 years, further management of collaborations and ensure the commitment and participation of all nPOD-V investigators, and maximize their intellectual contribution to the project.

5. Throughout the 3 years, effective management of the project and scientific direction that will lead to addressing of the key questions identified by the nPOD-V group, with the goal of enriching the nPOD dataset with novel and useful data, and to disseminate the information in form of publications and presentations at meetings.

6. Throughout the 3 years, provide effective sample prioritization and utilization among approved investigators and workgroups. As demonstrated to date, sample distribution has been achieved with a high degree of transparency in the overall nPOD program without conflict of interest. The Tissue Prioritization Committee is committed to finding to new ways to achieve continued fair and equitable allocation to this resource. A recent discussion from an internal nPOD meeting revolved around expanding by inviting new members into the TPC committee. Current members will be given an option to retire and replaced and at least two new members will also be installed. The first new member will be a representative from PevNet; many of whom are involved directly in the viral workgroup missions. The second new member may be selected from the broader research community, through requests for volunteers.

7. Throughout the 3 years, this viral workgroup will be to create an internal sample utilization committee comprised of 1 representative from each of the 5 Tasks and a representative of each of the 3 "investigator collections" that are also contributing biosamples to this initiative (Dotta, Hyoty/Frisk, Morgan/Richardson). Members of this nPOD-V utilization committee will have over-sight on sample allocations within tasks and assist the director with equitable distribution and prioritization.

F. Advantages over alternative approaches that would address goal.

Decades of research, largely from individual laboratories, have led to important data that make a persuasive case for a role of enterovirus in the pathogenesis of T1D. Yet, individual approaches and limitations with samples and

reagents have not allowed a complete analysis of the role for viruses in T1D. This collaborative effort recognizes those limitations and addresses them with an integrated and multidisciplinary approach proposed here. Furthermore, the proposed approach will now include many innovative and powerful technologies that have not been traditionally used for studying viruses in T1D. The availability of shared tissues from the same patients and their coordinated analysis provide an unprecedented opportunity that investigations can be exhaustive and fully informative. The premise underlying this collaboration, based on the nPOD research model and the concept of data sharing and collaboration promoted by the nPOD leadership, has been fully embraced by the nPOD-V investigators; we believe that this in itself represent a major step-forward and innovation in the study of complex questions in human disease. Further, the nPOD-V working group expands the potential of nPOD to fully exploit its potential, and paves the way for more working groups to follow.

G. Future plans if research is successful.

We anticipate that the nPOD-V group will interface and collaborate with additional nPOD working groups, which will be formed over the next several months. Thus, the group will become part of the nPOD Investigator Cloud. Again, we believe that this is the ultimate goal of nPOD, to create a collaborative framework and a scientific powerhouse that through analysis of shared samples and collaboration, can and will advance our knowledge of human T1D. Findings from this effort, we believe, will lead to improvements in other research questions/areas of JDRF research interest including additional mechanistic studies, biomarker identification, understanding β-cell biology, and the development of druggable targets capable of effectively intervening in this disease. Thus, we predict that this translational effort will facilitate progress towards clinically applicable diagnostics and therapeutics, which could involve partnerships with the biomedical industry and other clinical organizations.

H. Please list any Intellectual Property (IP) filed or any commercial efforts pursued for the approach described in the current application.

N/A

I. Literature Cited (no page limit):

1. Eisenbarth,GS: Update in type 1 diabetes. *J.Clin.Endocrinol.Metab.* 92:2403-2407, 2007

2. in't,VP: Insulitis in human type 1 diabetes: The quest for an elusive lesion. *Islets.* 3:131-138, 2011

3. Redondo,MJ, Jeffrey,J, Fain,PR, Eisenbarth,GS, Orban,T: Concordance for islet autoimmunity among monozygotic twins. *N.Engl.J.Med.* 359:2849-2850, 2008

4. Redondo,MJ, Yu,L, Hawa,M, Mackenzie,T, Pyke,DA, Eisenbarth,GS, Leslie,RD: Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. *Diabetologia* 44:354-362, 2001

5. Redondo,MJ, Rewers,M, Yu,L, Garg,S, Pilcher,CC, Elliott,RB, Eisenbarth,GS: Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *BMJ.* 318:698-702, 1999

6. Forbes,JM, Soderlund,J, Yap,FY, Knip,M, Andrikopoulos,S, Ilonen,J, Simell,O, Veijola,R, Sourris,KC, Coughlan,MT, Forsblom,C, Slattery,R, Grey,ST, Wessman,M, Yamamoto,H, Bierhaus,A, Cooper,ME, Groop,PH: Receptor for advanced glycation end-products (RAGE) provides a link between genetic susceptibility and environmental factors in type 1 diabetes. *Diabetologia.* 54:1032-1042, 2011

7.   Macfarlane,AJ, Strom,A, Scott,FW: Epigenetics: deciphering how environmental factors may modify autoimmune type 1 diabetes. *Mamm.Genome.* 2009

8.   Peng,H, Hagopian,W: Environmental factors in the development of Type 1 diabetes. *Rev.Endocr.Metab Disord.* 7:149-162, 2006

9.   Akerblom,HK, Vaarala,O, Hyoty,H, Ilonen,J, Knip,M: Environmental factors in the etiology of type 1 diabetes. *Am.J.Med.Genet.* 115:18-29, 2002

10. Knip,M, Veijola,R, Virtanen,SM, Hyoty,H, Vaarala,O, Akerblom,HK: Environmental triggers and determinants of type 1 diabetes. *Diabetes.* 54 Suppl 2:S125-36.:S125-S136, 2005

11. Boettler,T, von,HM: Protection against or triggering of Type 1 diabetes? Different roles for viral infections. *Expert Rev.Clin.Immunol.* 7:45-53, 2011

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

13. Jaidane,H, Sane,F, Hiar,R, Goffard,A, Gharbi,J, Geenen,V, Hober,D: Immunology in the clinic review series; focus on type 1 diabetes and viruses: enterovirus, thymus and type 1 diabetes pathogenesis. *Clin.exp.Immunol.* 168:39-46, 2012

14. Lind,K, Huhn,MH, Flodstrom-Tullberg,M: Immunology in the clinic review series; focus on type 1 diabetes and viruses: the innate immune response to enteroviruses and its possible role in regulating type 1 diabetes. *Clin.exp.Immunol.* 168:30-38, 2012

15. Grieco,FA, Sebastiani,G, Spagnuolo,I, Patti,A, Dotta,F: Immunology in the clinic review series; focus on type 1 diabetes and viruses: how viral infections modulate beta cell function. *Clin.exp.Immunol.* 168:24-29, 2012

16. Stene,LC, Rewers,M: Immunology in the clinic review series; focus on type 1 diabetes and viruses: the enterovirus link to type 1 diabetes: critical review of human studies. *Clin.exp.Immunol.* 168:12-23, 2012

17. Coppieters,KT, Wiberg,A, Tracy,SM, von Herrath,MG: Immunology in the clinic review series: focus on type 1 diabetes and viruses: the role of viruses in type 1 diabetes: a difficult dilemma. *Clin.exp.Immunol.* 168:5-11, 2012

18. Gamble,DR, Taylor,KW: Seasonal incidence of diabetes mellitus. *Br.Med.J.* 3:631-633, 1969

19. Gamble,DR, Taylor,KW, Cumming,H: Coxsackie viruses and diabetes mellitus. *Br.Med.J.* 4:260-262, 1973

20. Nelson,PG, Pyke,DA, Gamble,DR: Viruses and the aetiology of diabetes: a study in identical twins. *Br.Med.J.* 4:249-251, 1975

21. Gladisch,R, Hofmann,W, Waldherr,R: [Myocarditis and insulitis following coxsackie virus infection]. *Z.Kardiol.* 65:837-849, 1976

22. Di,PC, Del Guercio,MJ, Paolino,GP, Barbi,M, Ferrante,P, Chiumello,G: Type 1 diabetes and Coxsackie virus infection. *Helv.Paediatr.Acta.* 34:557-561, 1979

23. Ray,CG, Palmer,JP, Crossley,JR, Williams,RH: Coxsackie B virus antibody responses in juvenile-onset diabetes mellitus. *Clin.Endocrinol.(Oxf).* 12:375-378, 1980

24. Palmer,JP, Cooney,MK, Ward,RH, Hansen,JA, Brodsky,JB, Ray,CG, Crossley,JR, Asplin,CM, Williams,RH: Reduced Coxsackie antibody titres in type 1 (insulin-dependent) diabetic patients presenting during an outbreak of Coxsackie B3 and B4 infection. *Diabetologia.* 22:426-429, 1982

25. Asplin,CM, Cooney,MK, Crossley,JR, Dornan,TL, Raghu,P, Palmer,JP: Coxsackie B4 infection and islet cell antibodies three years before overt diabetes. *J.Pediatr.* 101:398-400, 1982

26. King,ML, Shaikh,A, Bidwell,D, Voller,A, Banatvala,JE: Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. *Lancet.* 1:1397-1399, 1983

27. Mertens,T, Gruneklee,D, Eggers,HJ: Neutralizing antibodies against Coxsackie B viruses in patients with recent onset of type I diabetes. *Eur.J.Pediatr.* 140:293-294, 1983

28. Mirkovic,RR, Varma,KS, Yoon,JW: Incidence of coxsackievirus B type 4 (CB4) infections concomitant with onset of insulin-dependent diabetes mellitus. *J.Med.Virol.* 14:9-16, 1984

29. Buschard,K, Madsbad,S: A longitudinal study of virus antibodies in patients with newly diagnosed type 1 (insulin-dependent) diabetes mellitus. *J.Clin.Lab Immunol.* 13:65-70, 1984

30. Alberti,AM, Amato,C, Candela,A, Costantino,F, Grandolfo,ME, Lombardi,F, Novello,F, Orsini,M, Santoro,R: Serum antibodies against Coxsackie B1-6 viruses in type 1 diabetics. *Acta Diabetol.Lat.* 22:33-38, 1985

31. Frisk,G, Fohlman,J, Kobbah,M, Ewald,U, Tuvemo,T, Diderholm,H, Friman,G: High frequency of Coxsackie-B-virus-specific IgM in children developing type I diabetes during a period of high diabetes morbidity. *J.Med.Virol.* 17:219-227, 1985

32. Friman,G, Fohlman,J, Frisk,G, Diderholm,H, Ewald,U, Kobbah,M, Tuvemo,T: An incidence peak of juvenile diabetes. Relation to Coxsackie B virus immune response. *Acta Paediatr.Scand.Suppl.* 320:14-9.:14-19, 1985

33. Banatvala,JE, Bryant,J, Schernthaner,G, Borkenstein,M, Schober,E, Brown,D, De Silva,LM, Menser,MA, Silink,M: Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. *Lancet.* 1:1409-1412, 1985

34. Bruserud,O, Jervell,J, Thorsby,E: HLA-DR3 and -DR4 control T-lymphocyte responses to mumps and Coxsackie B4 virus: studies on patients with type 1 (insulin-dependent) diabetes and healthy subjects. *Diabetologia.* 28:420-426, 1985

35. Schernthaner,G, Banatvala,JE, Scherbaum,W, Bryant,J, Borkenstein,M, Schober,E, Mayr,WR: Coxsackie-B-virus-specific IgM responses, complement-fixing islet-cell antibodies, HLA DR antigens, and C-peptide secretion in insulin-dependent diabetes mellitus. *Lancet.* 2:630-632, 1985

36.   Hyoty,H, Huupponen,T, Kotola,L, Leinikki,P: Humoral immunity against viral antigens in type 1 diabetes: altered IgA-class immune response against Coxsackie B4 virus. *Acta Pathol.Microbiol.Immunol.Scand.C.* 94:83-88, 1986

37.   Hehmke,B, Michaelis,D, Hildmann,W, Richter,KV, Kohnert,KD: Cytotoxic islet cell autoantibodies in newly diagnosed insulin-dependent diabetes mellitus: lack of correlation to age, residual beta cell function, HLA antigens and Coxsackie B virus antibodies. *Diabetes Res.* 4:73-77, 1987

38.   Field,LL, McArthur,RG, Shin,SY, Yoon,JW: The relationship between Coxsackie-B-virus-specific IgG responses and genetic factors (HLA-DR, GM, KM) in insulin-dependent diabetes mellitus. *Diabetes Res.* 6:169-173, 1987

39.   Tuvemo,T, Frisk,G, Friman,G, Ludvigsson,J, Diderholm,H: IgM against Coxsackie B viruses in children developing type I diabetes mellitus--a seven-year retrospective study. *Diabetes Res.* 9:125-129, 1988

40.   Tuvemo,T, Dahlquist,G, Frisk,G, Blom,L, Friman,G, Landin-Olsson,M, Diderholm,H: The Swedish childhood diabetes study III: IgM against coxsackie B viruses in newly diagnosed type 1 (insulin-dependent) diabetic children--no evidence of increased antibody frequency. *Diabetologia.* 32:745-747, 1989

41.   Muir,P, Singh,NB, Banatvala,JE: Enterovirus-specific serum IgA antibody responses in patients with acute infections, chronic cardiac disease, and recently diagnosed insulin-dependent diabetes mellitus. *J.Med.Virol.* 32:236-242, 1990

42.   Frisk,G, Friman,G, Tuvemo,T, Fohlman,J, Diderholm,H: Coxsackie B virus IgM in children at onset of type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection. *Diabetologia.* 35:249-253, 1992

43.   D'Alessio,DJ: A case-control study of group B Coxsackievirus immunoglobulin M antibody prevalence and HLA-DR antigens in newly diagnosed cases of insulin-dependent diabetes mellitus. *Am.J.Epidemiol.* 135:1331-1338, 1992

44.   Hyoty,H, Hiltunen,M, Knip,M, Laakkonen,M, Vahasalo,P, Karjalainen,J, Koskela,P, Roivainen,M, Leinikki,P, Hovi,T, .: A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes.* 44:652-657, 1995

45.   Clements,GB, Galbraith,DN, Taylor,KW: Coxsackie B virus infection and onset of childhood diabetes. *Lancet.* 346:221-223, 1995

46.   Helfand,RF, Gary,HE, Jr., Freeman,CY, Anderson,LJ, Pallansch,MA: Serologic evidence of an association between enteroviruses and the onset of type 1 diabetes mellitus. Pittsburgh Diabetes Research Group. *J.Infect.Dis.* 172:1206-1211, 1995

47.   Foy,CA, Quirke,P, Lewis,FA, Futers,TS, Bodansky,HJ: Detection of common viruses using the polymerase chain reaction to assess levels of viral presence in type 1 (insulin-dependent) diabetic patients. *Diabet.Med.* 12:1002-1008, 1995

48.   Hiltunen,M, Hyoty,H, Knip,M, Ilonen,J, Reijonen,H, Vahasalo,P, Roivainen,M, Lonnrot,M, Leinikki,P, Hovi,T, Akerblom,HK: Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. *J.Infect.Dis.* 175:554-560, 1997

49. Frisk,G, Diderholm,H: Antibody responses to different strains of coxsackie B4 virus in patients with newly diagnosed type I diabetes mellitus or aseptic meningitis. *J.Infect.* 34:205-210, 1997

50. Andreoletti,L, Hober,D, Hober-Vandenberghe,C, Belaich,S, Vantyghem,MC, Lefebvre,J, Wattre,P: Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus. *J.Med.Virol.* 52:121-127, 1997

51. Roivainen,M, Knip,M, Hyoty,H, Kulmala,P, Hiltunen,M, Vahasalo,P, Hovi,T, Akerblom,HK: Several different enterovirus serotypes can be associated with prediabetic autoimmune episodes and onset of overt IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *J.Med.Virol.* 56:74-78, 1998

52. Juhela,S, Hyoty,H, Hinkkanen,A, Elliott,JF, Roivainen,M, Kulmala,P, Rahko,J, Knip,M, Ilonen,J: T cell responses to enterovirus antigens and to beta-cell autoantigens in unaffected children positive for IDDM-associated autoantibodies. *J.Autoimmun.* 12:269-278, 1999

53. Nairn,C, Galbraith,DN, Taylor,KW, Clements,GB: Enterovirus variants in the serum of children at the onset of Type 1 diabetes mellitus. *Diabet.Med.* 16:509-513, 1999

54. Luppi,P, Alexander,A, Bertera,S, Noonan,K, Trucco,M: The same HLA-DQ alleles determine either susceptibility or resistance to different coxsackievirus-mediated autoimmune diseases. *J.Biol.Regul.Homeost.Agents.* 13:14-26, 1999

55. Lonnrot,M, Salminen,K, Knip,M, Savola,K, Kulmala,P, Leinikki,P, Hyypia,T, Akerblom,HK, Hyoty,H: Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: a prospective study. Childhood Diabetes in Finland (DiMe) Study Group. *J.Med.Virol.* 61:214-220, 2000

56. Chehadeh,W, Weill,J, Vantyghem,MC, Alm,G, Lefebvre,J, Wattre,P, Hober,D: Increased level of interferon-alpha in blood of patients with insulin-dependent diabetes mellitus: relationship with coxsackievirus B infection. *J.Infect.Dis.* 181:1929-1939, 2000

57. Juhela,S, Hyoty,H, Roivainen,M, Harkonen,T, Putto-Laurila,A, Simell,O, Ilonen,J: T-cell responses to enterovirus antigens in children with type 1 diabetes. *Diabetes.* 49:1308-1313, 2000

58. Varela-Calvino,R, Ellis,R, Sgarbi,G, Dayan,CM, Peakman,M: Characterization of the T-cell response to coxsackievirus B4: evidence that effector memory cells predominate in patients with type 1 diabetes. *Diabetes.* 51:1745-1753, 2002

59. Yin,H, Berg,AK, Tuvemo,T, Frisk,G: Enterovirus RNA is found in peripheral blood mononuclear cells in a majority of type 1 diabetic children at onset. *Diabetes.* 51:1964-1971, 2002

60. Harkonen,T, Paananen,A, Lankinen,H, Hovi,T, Vaarala,O, Roivainen,M: Enterovirus infection may induce humoral immune response reacting with islet cell autoantigens in humans. *J.Med.Virol.* 69:426-440, 2003

61. Sadeharju,K, Knip,M, Hiltunen,M, Akerblom,HK, Hyoty,H: The HLA-DR phenotype modulates the humoral immune response to enterovirus antigens. *Diabetologia.* 46:1100-1105, 2003

62. Gupta,M, Nikitina-Zake,L, Landin-Olsson,M, Kockum,I, Sanjeevi,CB: Coxsackie virus B antibodies are increased in HLA DR3-MICA5.1 positive type 1 diabetes patients in the Linkoping region of Sweden. *Hum.Immunol.* 64:874-879, 2003

63. Dahlquist,GG, Forsberg,J, Hagenfeldt,L, Boman,J, Juto,P: Increased prevalence of enteroviral RNA in blood spots from newborn children who later developed type 1 diabetes: a population-based case-control study. *Diabetes Care.* 27:285-286, 2004

64. Marttila,J, Hyoty,H, Nanto-Salonen,K, Simell,O, Ilonen,J: Epitopes recognized by CBV4 responding T cells: effect of type 1 diabetes and associated HLA-DR-DQ haplotypes. *Virology.* 319:27-35, 2004

65. Viskari,H, Ludvigsson,J, Uibo,R, Salur,L, Marciulionyte,D, Hermann,R, Soltesz,G, Fuchtenbusch,M, Ziegler,AG, Kondrashova,A, Romanov,A, Knip,M, Hyoty,H: Relationship between the incidence of type 1 diabetes and enterovirus infections in different European populations: results from the EPIVIR project. *J.Med.Virol.* 72:610-617, 2004

66. Salminen,KK, Vuorinen,T, Oikarinen,S, Helminen,M, Simell,S, Knip,M, Ilonen,J, Simell,O, Hyoty,H: Isolation of enterovirus strains from children with preclinical Type 1 diabetes. *Diabet.Med.* 21:156-164, 2004

67. Frisk,G, Tuvemo,T: Enterovirus infections with beta-cell tropic strains are frequent in siblings of children diagnosed with type 1 diabetes children and in association with elevated levels of GAD65 antibodies. *J.Med.Virol.* 73:450-459, 2004

68. Viskari,H, Ludvigsson,J, Uibo,R, Salur,L, Marciulionyte,D, Hermann,R, Soltesz,G, Fuchtenbusch,M, Ziegler,AG, Kondrashova,A, Romanov,A, Kaplan,B, Laron,Z, Koskela,P, Vesikari,T, Huhtala,H, Knip,M, Hyoty,H: Relationship between the incidence of type 1 diabetes and maternal enterovirus antibodies: time trends and geographical variation. *Diabetologia.* 48:1280-1287, 2005

69. Hindersson,M, Elshebani,A, Orn,A, Tuvemo,T, Frisk,G: Simultaneous type 1 diabetes onset in mother and son coincident with an enteroviral infection. *J.Clin.Virol.* 33:158-167, 2005

70. Cinek,O, Witso,E, Jeansson,S, Rasmussen,T, Drevinek,P, Wetlesen,T, Vavrinec,J, Grinde,B, Ronningen,KS: Longitudinal observation of enterovirus and adenovirus in stool samples from Norwegian infants with the highest genetic risk of type 1 diabetes. *J.Clin.Virol.* 35:33-40, 2006

71. Moya-Suri,V, Schlosser,M, Zimmermann,K, Rjasanowski,I, Gurtler,L, Mentel,R: Enterovirus RNA sequences in sera of schoolchildren in the general population and their association with type 1-diabetes-associated autoantibodies. *J.Med.Microbiol.* 54:879-883, 2005

72. Skarsvik,S, Puranen,J, Honkanen,J, Roivainen,M, Ilonen,J, Holmberg,H, Ludvigsson,J, Vaarala,O: Decreased in vitro type 1 immune response against coxsackie virus B4 in children with type 1 diabetes. *Diabetes.* 55:996-1003, 2006

73. Elfaitouri,A, Berg,AK, Frisk,G, Yin,H, Tuvemo,T, Blomberg,J: Recent enterovirus infection in type 1 diabetes: evidence with a novel IgM method. *J.Med.Virol.* 79:1861-1867, 2007

74. Sarmiento,L, Cabrera-Rode,E, Lekuleni,L, Cuba,I, Molina,G, Fonseca,M, Heng-Hung,L, Borroto,AD, Gonzalez,P, Mas-Lago,P, az-Horta,O: Occurrence of enterovirus RNA in

serum of children with newly diagnosed type 1 diabetes and islet cell autoantibody-positive subjects in a population with a low incidence of type 1 diabetes. *Autoimmunity.* 40:540-545, 2007

75. Oikarinen,M, Tauriainen,S, Honkanen,T, Oikarinen,S, Vuori,K, Kaukinen,K, Rantala,I, Maki,M, Hyoty,H: Detection of enteroviruses in the intestine of type 1 diabetic patients. *Clin.exp.Immunol.* 151:71-75, 2008

76. Sauter,P, Chehadeh,W, Lobert,PE, Lazrek,M, Goffard,A, Soumillon,M, Caloone,D, Vantyghem,MC, Weill,J, Fajardy,I, Alm,G, Lucas,B, Hober,D: A part of the VP4 capsid protein exhibited by coxsackievirus B4 E2 is the target of antibodies contained in plasma from patients with type 1 diabetes. *J.Med.Virol.* 80:866-878, 2008

77. Al-Hello,H, Paananen,A, Eskelinen,M, Ylipaasto,P, Hovi,T, Salmela,K, Lukashev,AN, Bobegamage,S, Roivainen,M: An enterovirus strain isolated from diabetic child belongs to a genetic subcluster of echovirus 11, but is also neutralised with monotypic antisera to coxsackievirus A9. *J.Gen.Virol.* 89:1949-1959, 2008

78. Schulte,BM, Bakkers,J, Lanke,KH, Melchers,WJ, Westerlaken,C, Allebes,W, Aanstoot,HJ, Bruining,GJ, Adema,GJ, Van Kuppeveld,FJ, Galama,JM: Detection of enterovirus RNA in peripheral blood mononuclear cells of type 1 diabetic patients beyond the stage of acute infection. *Viral Immunol.* 23:99-104, 2010

79. Berg,AK, Tuvemo,T, Frisk,G: Enterovirus markers and serum CXCL10 in children with type 1 diabetes. *J.Med.Virol.* 82:1594-1599, 2010

80. Tapia,G, Cinek,O, Rasmussen,T, Witso,E, Grinde,B, Stene,LC, Ronningen,KS: Human enterovirus RNA in monthly fecal samples and islet autoimmunity in Norwegian children with high genetic risk for type 1 diabetes: the MIDIA study. *Diabetes Care.* 34:151-155, 2011

81. Sadeharju,K, Hamalainen,AM, Knip,M, Lonnrot,M, Koskela,P, Virtanen,SM, Ilonen,J, Akerblom,HK, Hyoty,H: Enterovirus infections as a risk factor for type I diabetes: virus analyses in a dietary intervention trial. *Clin.exp.Immunol.* 132:271-277, 2003

82. Lempainen,J, Tauriainen,S, Vaarala,O, Makela,M, Honkanen,H, Marttila,J, Veijola,R, Simell,O, Hyoty,H, Knip,M, Ilonen,J: Interaction of enterovirus infection and cow's milk-based formula nutrition in type 1 diabetes-associated autoimmunity. *Diabetes Metab Res.Rev.* 28:177-185, 2012

83. Makela,M, Vaarala,O, Hermann,R, Salminen,K, Vahlberg,T, Veijola,R, Hyoty,H, Knip,M, Simell,O, Ilonen,J: Enteral virus infections in early childhood and an enhanced type 1 diabetes-associated antibody response to dietary insulin. *J.Autoimmun.* 27:54-61, 2006

84. Dahlquist,GG, Ivarsson,S, Lindberg,B, Forsgren,M: Maternal enteroviral infection during pregnancy as a risk factor for childhood IDDM. A population-based case-control study. *Diabetes.* 44:408-413, 1995

85. Viskari,H, Knip,M, Tauriainen,S, Huhtala,H, Veijola,R, Ilonen,J, Simell,O, Surcel,HM, Hyoty,H: Maternal Enterovirus Infection as a Risk Factor for Type 1 Diabetes in the Exposed Offspring. *Diabetes Care.* 2012

86. Lonnrot,M, Knip,M, Roivainen,M, Koskela,P, Akerblom,HK, Hyoty,H: Onset of type 1 diabetes mellitus in infancy after enterovirus infections. *Diabet.Med.* 15:431-434, 1998

87. Smith,CP, Clements,GB, Riding,MH, Collins,P, Bottazzo,GF, Taylor,KW: Simultaneous onset of type 1 diabetes mellitus in identical infant twins with enterovirus infection. *Diabet.Med.* 15:515-517, 1998

88. Elfving,M, Svensson,J, Oikarinen,S, Jonsson,B, Olofsson,P, Sundkvist,G, Lindberg,B, Lernmark,A, Hyoty,H, Ivarsson,SA: Maternal enterovirus infection during pregnancy as a risk factor in offspring diagnosed with type 1 diabetes between 15 and 30 years of age. *Exp.Diabetes Res.* 2008:271958.:271958, 2008

89. Simonen-Tikka,ML, Pflueger,M, Klemola,P, Savolainen-Kopra,C, Smura,T, Hummel,S, Kaijalainen,S, Nuutila,K, Natri,O, Roivainen,M, Ziegler,AG: Human enterovirus infections in children at increased risk for type 1 diabetes: the Babydiet study. *Diabetologia.* 54:2995-3002, 2011

90. Yoon,JW, Austin,M, Onodera,T, Notkins,AL: Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N.Engl.J.Med.* 300:1173-1179, 1979

91. Jenson,AB, Rosenberg,HS, Notkins,AL: Pancreatic islet-cell damage in children with fatal viral infections. *Lancet.* 2:354-358, 1980

92. Foulis,AK, Farquharson,MA, Meager,A: Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus. *Lancet.* 2:1423-1427, 1987

93. Foulis,AK, Farquharson,MA, Cameron,SO, McGill,M, Schonke,H, Kandolf,R: A search for the presence of the enteroviral capsid protein VP1 in pancreases of patients with type 1 (insulin-dependent) diabetes and pancreases and hearts of infants who died of coxsackieviral myocarditis. *Diabetologia.* 33:290-298, 1990

94. Fuchtenbusch,M, Irnstetter,A, Jager,G, Ziegler,AG: No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes. *J.Autoimmun.* 17:333-340, 2001

95. Dotta,F, Censini,S, van Halteren,AG, Marselli,L, Masini,M, Dionisi,S, Mosca,F, Boggi,U, Muda,AO, Prato,SD, Elliott,JF, Covacci,A, Rappuoli,R, Roep,BO, Marchetti,P: Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. *Proc.Natl.Acad.Sci.U.S.A.* 104:5115-5120, 2007

96. Oikarinen,M, Tauriainen,S, Honkanen,T, Vuori,K, Karhunen,P, Vasama-Nolvi,C, Oikarinen,S, Verbeke,C, Blair,GE, Rantala,I, Ilonen,J, Simell,O, Knip,M, Hyoty,H: Analysis of pancreas tissue in a child positive for islet cell antibodies. *Diabetologia.* 51:1796-1802, 2008

97. Richardson,SJ, Willcox,A, Bone,AJ, Foulis,AK, Morgan,NG: The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia.* 52:1143-1151, 2009

98. Richardson,SJ, Willcox,A, Bone,AJ, Foulis,AK, Morgan,NG: The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia.* 52:1143-1151, 2009

99. Willcox,A, Richardson,SJ, Bone,AJ, Foulis,AK, Morgan,NG: Immunohistochemical analysis of the relationship between islet cell proliferation and the production of the enteroviral

capsid protein, VP1, in the islets of patients with recent-onset type 1 diabetes. *Diabetologia.* 54:2417-2420, 2011

100. in't,VP: Insulitis in the human endocrine pancreas: does a viral infection lead to inflammation and beta cell replication? *Diabetologia.* 54:2220-2222, 2011

101. Smyth,DJ, Cooper,JD, Bailey,R, Field,S, Burren,O, Smink,LJ, Guja,C, Ionescu-Tirgoviste,C, Widmer,B, Dunger,DB, Savage,DA, Walker,NM, Clayton,DG, Todd,JA: A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. *Nat.Genet.* 38:617-619, 2006

102. Concannon,P, Onengut-Gumuscu,S, Todd,JA, Smyth,DJ, Pociot,F, Bergholdt,R, Akolkar,B, Erlich,HA, Hilner,JE, Julier,C, Morahan,G, Nerup,J, Nierras,CR, Chen,WM, Rich,SS: A human type 1 diabetes susceptibility locus maps to chromosome 21q22.3. *Diabetes* 57:2858-2861, 2008

103. Qu,HQ, Marchand,L, Grabs,R, Polychronakos,C: The association between the IFIH1 locus and type 1 diabetes. *Diabetologia.* 51:473-475, 2008

104. Liu,S, Wang,H, Jin,Y, Podolsky,R, Reddy,MV, Pedersen,J, Bode,B, Reed,J, Steed,D, Anderson,S, Yang,P, Muir,A, Steed,L, Hopkins,D, Huang,Y, Purohit,S, Wang,CY, Steck,AK, Montemari,A, Eisenbarth,G, Rewers,M, She,JX: IFIH1 polymorphisms are significantly associated with type 1 diabetes and IFIH1 gene expression in peripheral blood mononuclear cells. *Hum.Mol.Genet.* 18:358-365, 2009

105. Shigemoto,T, Kageyama,M, Hirai,R, Zheng,J, Yoneyama,M, Fujita,T: Identification of loss of function mutations in human genes encoding RIG-I and MDA5: implications for resistance to type I diabetes. *J.Biol.Chem.* 284:13348-13354, 2009

106. Nejentsev,S, Walker,N, Riches,D, Egholm,M, Todd,JA: Rare Variants of IFIH1, a Gene Implicated in Antiviral Responses, Protect Against Type 1 Diabetes. *Science.* 2009

107. von Herrath,M: Diabetes: A virus-gene collaboration. *Nature.* 459:518-519, 2009

108. Coppieters,KT, Boettler,T, von,HM: Virus infections in type 1 diabetes. *Cold Spring Harb.Perspect.Med.* 2:a007682, 2012

109. Vendrame,F, Pileggi,A, Laughlin,E, Allende,G, Martin-Pagola,A, Molano,RD, Diamantopoulos,S, Standifer,N, Geubtner,K, Falk,BA, Ichii,H, Takahashi,H, Snowhite,I, Chen,Z, Mendez,A, Chen,L, Sageshima,J, Ruiz,P, Ciancio,G, Ricordi,C, Reijonen,H, Nepom,GT, Burke,GW, III, Pugliese,A: Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells. *Diabetes* 59:947-957, 2010

110. Matthews,JB, Staeva,TP, Bernstein,PL, Peakman,M, von,HM: Developing combination immunotherapies for type 1 diabetes: recommendations from the ITN-JDRF Type 1 Diabetes Combination Therapy Assessment Group. *Clin.exp.Immunol.* 160:176-184, 2010

111. Staeva-Vieira,T, Peakman,M, von Herrath,M: Translational mini-review series on type 1 diabetes: Immune-based therapeutic approaches for type 1 diabetes. *Clin.exp.Immunol.* 148:17-31, 2007

112. Stene,LC, Oikarinen,S, Hyoty,H, Barriga,KJ, Norris,JM, Klingensmith,G, Hutton,JC, Erlich,HA, Eisenbarth,GS, Rewers,M: Enterovirus infection and progression from islet

autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). *Diabetes.* 59:3174-3180, 2010

113. Lempainen,J, Tauriainen,S, Vaarala,O, Makela,M, Honkanen,H, Marttila,J, Veijola,R, Simell,O, Hyoty,H, Knip,M, Ilonen,J: Interaction of enterovirus infection and Cow's milk-based formula nutrition in type 1 diabetes-associated autoimmunity. *Diabetes Metab Res.Rev.*10, 2011

114. Oikarinen,S, Martiskainen,M, Tauriainen,S, Huhtala,H, Ilonen,J, Veijola,R, Simell,O, Knip,M, Hyoty,H: Enterovirus RNA in blood is linked to the development of type 1 diabetes. *Diabetes.* 60:276-279, 2011

115. Tauriainen,S, Oikarinen,S, Oikarinen,M, Hyoty,H: Enteroviruses in the pathogenesis of type 1 diabetes. *Semin.Immunopathol.* 33:45-55, 2011

116. Oikarinen,M, Tauriainen,S, Honkanen,T, Oikarinen,S, Vuori,K, Kaukinen,K, Rantala,I, Maki,M, Hyoty,H: Detection of enteroviruses in the intestine of type 1 diabetic patients. *Clin.exp.Immunol.* 151:71-75, 2008

117. Tracy,S, Drescher,KM, Chapman,NM: Enteroviruses and type 1 diabetes. *Diabetes Metab Res.Rev.* 27:820-823, 2011

118. Gianani,R, Campbell-Thompson,M, Sarkar,SA, Wasserfall,C, Pugliese,A, Solis,JM, Kent,SC, Hering,BJ, West,E, Steck,A, Bonner-Weir,S, Atkinson,MA, Coppieters,K, von,HM, Eisenbarth,GS: Dimorphic histopathology of long-standing childhood-onset diabetes. *Diabetologia.* 53:690-698, 2010

119. Imagawa,A, Hanafusa,T: Fulminant type 1 diabetes--an important subtype in East Asia. *Diabetes Metab Res.Rev.* 27:959-964, 2011

120. Imagawa,A, Hanafusa,T: [Fulminant type 1 diabetes mellitus]. *Rinsho Byori.* 58:216-224, 2010

121. Shibasaki,S, Imagawa,A, Tauriainen,S, Iino,M, Oikarinen,M, Abiru,H, Tamaki,K, Seino,H, Nishi,K, Takase,I, Okada,Y, Uno,S, Murase-Mishiba,Y, Terasaki,J, Makino,H, Shimomura,I, Hyoty,H, Hanafusa,T: Expression of toll-like receptors in the pancreas of recent-onset fulminant type 1 diabetes. *Endocr.J.* 57:211-219, 2010

J. Principal investigator assurance: The principal investigator agrees to accept responsibility for the scientific and technical conduct of the research project and agrees to all terms and conditions of the award.

The principal investigator agrees to abide to the responsibilities stated above.

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