Executive Summary:

The collaborative project DIAPREPP specifically addressed the call FP7 Cooperation Work Programme: Health-2007-2.4.3-1 Early processes in the pathogenesis of type 1 diabetes and strategies for early prevention. A team of 11 academic groups and 2 SMEs from 7 countries form DIAPREPP. The project ran over a period of 41 months and received around 6 Mio Euros funding. The overall objective was to determine mechanisms of islet autoantigen immunization. The expected impact was new knowledge regarding how immunization against islet autoantigens can occur; how signs of self-immunization can be exploited for prediction and monitoring of disease; and how immunization or its progression to clinical type 1 diabetes can be prevented.

In meeting its objectives, DIAPREPP amassed and used a unique set of clinical material and cohorts. These included a large case-control cohort of children followed from birth to autoimmunity to diabetes; a new high intensity followed infection cohort of children genetically at risk for type 1 diabetes; and pancreas, pancreatic islets and lymph nodes from patients with islet autoantibodies or diabetes. This material was has become a DIAPREPP legacy for future studies and networks. With respect to type 1 diabetes pathogenesis, DIAPREPP showed heightened immunization against islet beta cells between the ages 6 months and 2 years. Islet autoantibodies were rare prior to age 6 months indicating a period of protection or absence of triggering factors. Consistent with this, the first months of life were characterized by an inefficient immune system possibly explaining protection in this period. Islet autoimmunity was generally abrupt, but it did not appear to be increasing in incidence as compared to previous decades. Instead, it was noted that the rate of progression to clinical disease had increased markedly in some regions in the last decade and DIAPREPP investigators could find evidence that this was linked to genes that affect host responses to viruses. DIAPREPP investigations on viral mechanisms of beta cell autoimmunity and beta cell destruction identified virus in the pancreas of some patients, but no specific virus could be associated to the pathogenesis in DIAPREPP material. However, DIAPREPP identified crucial molecules required by enteroviruses to infect pancreatic islets, as well as response profiles of islets to virus infection. These included novel findings of virus-mediated pathogenetic mechanisms for induction and exacerbation of autoimmunity to the beta cell. A key target of DIAPREPP investigations was the metabolome. DIAPREPP showed clear differences between the metabolome of children who developed aggressive islet autoimmunity as compared to children who remained free of autoimmunity. These differences were already observed at birth and pathways potentially involved in the very early processes of susceptibility and activation of islet autoimmunity were identified. Some of these, including the role of the enzyme DHAPAT were corroborated by analysis of data generated in animal models. There were also metabolome changes that occurred upon initiation of autoimmunity. The findings pave the way for combining metabolomic markers to genes for improved early prediction and further combination with markers of autoimmunity to predict progression to disease. New technologies were developed to examine antigen receptors and response profiles of antigen responsive T and B lymphocytes and which allow tracking of cells during pre-diabetes and after immune intervention. New assays were developed, standardized, and in the case of zinc transporter 8 autoantibodies, commercialized and widely introduced for autoantibody screening. Over 80 presentations at national or international meetings, plus 40 publications of original findings and another 15 review or commentary articles have thus far resulted from the DIAPREPP project.

Project Context and Objectives:

DIAPREPP (Diabetes type 1 Prevention, Early Pathogenesis and Prediction) is a collaborative project funded by the European Union under the 7th Framework Program. The project aimed at disclosing the thus far unknown mechanisms of islet autoantigen immunization. The knowledge gained will help in the development of a new generation of diagnostic tools and approaches to hinder the development of autoimmunization and progression to disease.

An international team of 11 academic groups and 2 SMEs from 7 countries coordinated by Prof. Ezio Bonifacio from the Technische Universitaet Dresden form DIAPREPP. The project ran over a period of 41 months and was funded with around 6 Mio Euros.

The collaborative project DIAPREPP specifically addressed the call FP7 Cooperation Work Programme: Health-2007-2.4.3-1 Early processes in the pathogenesis of type 1 diabetes and strategies for early prevention.

The earliest currently identifiable process in the pathogenesis of type 1 diabetes (T1D) is the development of autoimmunity to islet beta cells in the measurable form of islet autoantibodies. The autoantibodies usually 'appear' after a period of 'negativity', signifying an 'immunization' event against islet beta cell autoantigens. The aetiology and pathogenesis of the islet auto-immunization is still poorly understood, hindering attempts at early preventive therapy.

The theme of this collaborative project is the early auto-immunization against islet autoantigens, in particular to disclose events, which precede current islet autoantibody-markers. The concept is that early events prior to auto-immunization govern the likelihood and 'signature' of immunization which in turn determines progression to disease.

The overall objective is to determine mechanisms of islet autoantigen immunization.

The expected impact is new fundamental knowledge regarding i) how immunization against islet autoantigens can occur; ii) how signs of self-immunization can be exploited for prediction and monitoring of disease; and iii) how the immunization or its progression to islet beta cell destruction and T1D development can be prevented.

DIAPREPP aims to understand how islet autoimmunization arises and to disclose events preceding current autoantibody markers.

DIAPREPP will:

1) Provide Europe with a leading unique resource of clinical material from which to discover the early autoimmune immunization processes in T1D.
2) Determine mechanisms by which infection can lead to auto-immunization against islet autoantigens.
3) Provide functional metabolomic evidence for modulation of susceptibility to autoantigen immunization by host environment.
4) Identify the early autoimmune immunization 'signatures' to islet autoantigens.
5) Translate early immunization profiles to prediction and prevention strategies.

DIAPREPP is structured into 9 work packages. These include a clinical resource work package, 3 discovery work packages, and a clinical translation work package. These work packages and their relationships are summarized graphically below. Four additional work packages are dedicated to dissemination, training, ethical issues, and management.

Specific Objectives.

1. Provide Europe with a leading unique resource of clinical material from which to discover the early autoimmune immunization processes in T1D.

Milestones during project:

1.1 Identification and first delivery of a multiple site nested case-control cohort representing children followed from birth to autoimmunity to disease.
1.2 Approval and implementation of islet autoantibody screening of organ donors and patients undergoing pancreatic surgery.
1.3 Implementation of neonate 'infection/disease' episode high intensity follow-up study.

2. Determine mechanisms by which infection can lead to auto-immunization against islet autoantigens.

DIAPREPP will focus on the enterovirus model of exposure as a test system to determine changes in islets and the immune system that infection can cause.

Milestones during project:

2.1 Established enterovirus isolation and islet infection protocols for evaluation of changes to islet function and gene expression profiles.
2.2 Validated Multiple Displacement Amplification assay for novel virus detection in human islets.
2.3Elucidation of shared PTB pathway use by enterovirus and insulin granule protein expression.

3. Provide functional metabolomic evidence for modulation of susceptibility to autoantigen immunization by host environment.

DIAPREPP will investigate the novel Finnish pre-autoimmune lipidomic findings in children who develop T1D. Findings will be validated and expanded in the DIAPREPP Biobank, and functional hypotheses tested in animal models and cell-based assays.

Milestones during project:

3.1 Validated list of pre-autoimmune metabolome changes based on longitudinal data from DIAPREPP Biobank.

3.2 Generation of model systems to address the hypotheses motivated by our clinical findings, including the DHPAT+/--NOD and UCP2-/--NOD mice, as well as DHPAT gene silencing on insulinoma cell lines and islets.

4. Identify the early autoimmune immunization 'signatures' to islet autoantigens.

DIAPREPP will draw on the DIAPREPP Biobank lymph nodes, and human HLA class I molecule expressing mouse models to establish an array of measures for defining and describing islet autoimmune response profiles in the DIAPREPP nested case-controls.

Milestones during project:

4.1 Novel IFN-g Elispot assays using proinsulin, IA-2, GAD65, and newly identified autoantigen epitope panels.
4.2 Single cell PCR assay suitable for determining gene expression profiles of islet-antigen reactive CD8+ T cells sorted using cytokine capture assay.
4.3 High throughput platform assay for 'signature' humoral autoimmunity profiling.

5. Translate early immunization profiles to prediction and prevention strategies.

DIAPREPP will exploit its findings through commercial development of assays, and determine the impact of its findings on prevention strategies by application to ongoing and completed prevention trials. A facility for prevention trial implementation will be established.

Milestones during project:

5.1 Sensitive ELISA measurement of antibodies to newly identified islet granule autoantigen, enabling commercial development.
5.2 Method for selected metabolite profiling suitable for low cost screening.
5.3 Creation of Prevention Implementation facility.

Project Results:

1. The DIAPREPP Biobank.

The general objective of Work Package 1 was to provide a unique Biobank of clinical material for the specific study of T1D early pathogenesis. WP1 functioned as a central platform to the other four S/T work packages and provided a flow of resources to these work packages. The resources include (see table 1 for summary):

1. Nested case-control cohort of children followed from birth to autoimmunity to disease.
2. New high intensity followed infection cohort of risk children.
3. Pancreatic lymph nodes obtained from patients with T1D, who have undergone surgery.
4. Pancreas, pancreatic islets and lymph nodes from patients with islet autoantibodies who undergo pancreatic surgery and controls.
5. Pancreas, pancreatic islets and lymph nodes from islet autoantibody positive organ donors.

Nested case-control pre-type 1 diabetes samples.

A major achievement in this work package is the establishment of the virtual database with the nested case-control cohort of children, providing a unique resource to the DIAPREPP partners to investigate autoimmunization to islet antigens and progression to T1D, thus completing milestone 1.1. Cases and controls have been obtained from the Finnish DIPP study, German BABYDIAB and BABYDIET studies, and the Swedish DiPiS study. Cases include children who:

- have stored samples (serum, plasma, cells or stool) and
- have developed at least one islet autoantibody and
- have material for genetic analysis.

The original target of 300 children with prediabetes (islet autoantibody positive) followed from before age 1 year and 600 controls was well exceeded. The cases are further sub-grouped into i) cases that have progressed to diabetes (94 cases), ii) cases that have islet autoantibodies to two or more islet antigens, but have not progressed to diabetes (123 cases) and iii) cases with antibodies to one islet antigen who have not progressed to diabetes (235 cases). The data is inserted into the web-based BC Gene database, which has been made accessible for relevant partners. This resource has been used to identify new biomarkers, identify periods of increased seroconversion, and examine genetic determinants of seroconversion and progression to diabetes. Some of the material will be made available to collaborators outside of DIAPREPP, and it is expected that this bioresource can be utilized in future projects.

Intensively followed infection cohort.

The nested case-control pre-type 1 diabetes samples are supplemented by samples from a new study carried out by DIAPREPP partner Simell (Turku UH) whereby children who are genetically at risk for T1D and living in the vicinity of the University of Turku clinical site are being enrolled and followed intensively at the time of infection (INDUS study). Whenever a study subject presented signs or symptoms of infection, stool and nasal swab samples were collected at home. These samples were mailed directly to Roivainen (P14, THL) for virus analysis. Samples were collected every 2 days until the child has been healthy for 2-3 days and after that additional 3 times with 5 days interval. The family kept a diary of the child's symptoms. The children visited the study physician during the time of infection as clinically needed. Medications and other therapies were accurately recorded. Blood samples were drawn when time interval from the previous sampling allows (every 3 to 6 months) and additionally when blood draw was clinically justified because of the infection. Thus far, 79 children have been recruited and over 400 samples have been collected and made available for DIAPREPP investigations. This unique study will continue with separate funding and provide invaluable material for identifying infectious agents predisposing to islet autoimmunity.

Pancreatic surgery tissues.

While much is known about the pathology within the pancreas of mouse models of autoimmune diabetes, little is known with respect to man. Indeed, pancreas, islets, and pancreatic lymph nodes from patients with recent onset type 1 diabetes and from islet autoantibody positive subjects without diabetes are a rare source of material. DIAPREPP started three studies that aimed at providing this material for study. Two of these collected material from patients undergoing pancreatic surgery and one from organ donors (see below).

Patients intended for pancreatic surgery at the HSR partner site were screened for their glucose homeostasis and for islet autoantibodies prior to surgery in order to identify patients with diabetes and/or islet autoantibodies. Pancreas tissue and where possible also pancreatic lymph nodes were obtained from positive patients and relevant negative controls at the time of surgery for this DIAPREPP Biobank resource. A total of 414 patients were screened (Table 2).

In total, 18 were positive for GAD antibodies, 3 positive for IA-2 antibodies, 42 positive for insulin antibodies, and 5 positive for ZnT8 antibodies; 6 had multiple islet autoantibodies. Of these, material was isolated and processed from 49 antibody positive patients. These resources include pancreas blocks and lymph nodes, are included in the DIAPREPP Biobank, and are available for study to DIAPREPP partners and the scientific community.

A second source of pancreatic surgery tissue was from patients with type 1 diabetes who underwent a pancreas transplant. Here, pancreatic lymph nodes were collected and processed from 25 patients. These were processed for immunological studies.

Tissue from organ donors.

DIAPREPP initiated a real-time organ donor screen for islet autoantibodies in Scandinavia. To be successful, the SME partner RSR (P12) developed a fast screen ELISA (GAD and IA-2 antibodies) and provided this to P9 for use in screening. A total of 388 organ donors were screened. In total, pancreas tissue was recovered from 11 cases who were islet autoantibody positive and from 2 cases of

undiagnosed type 1 diabetes. This material is a unique and is the subject of substantial research within DIAPREPP. Moreover, it is now included in the subsequently started nPOD (http://www.JDRFnPOD.org) international program. The organ donor screening activity of partner 9 has been exported to a new FP7 collaborative project PEVNET (Hyoty) in order to ensure its continuity.

1. Virus and type 1 diabetes - identification of viruses.

An important objective of the project was to test hypotheses related to the viral aetiology of type 1 diabetes. Viruses were sought within the Biobank material.

Enteroviruses are prime candidates for a pathogenetic role in type 1 diabetes. Partner 14 (Roivainen, THL) is the reference laboratory for enterovirus detection in Finland and therefore provided the opportunity to investigate in detail the enteroviruses found within the DIAPREPP samples. In a first study of samples from German neonates, virus was isolated and sequenced from stool samples obtained in the first year of life in 104 children, including 22 who developed islet autoantibodies. Analyses were performed in relation to the appearance of islet autoantibodies, dietary intervention, maternal type 1 diabetes and clinical symptoms (collaborative study between partners Ziegler (P5 FDeV) and Roivainen (P14 THL). Human enterovirus (HEV) was detected in 32 (9.4%) samples from 24 (23.1%) of the 104 children examined (Figure 4). Altogether 13 serotypes were identified, with HEV-A species being the most common. Children with gastrointestinal symptoms had norovirus (3/11) and sapovirus (1/11) infections in addition to HEV (1/11). Of the 104 children, 22 developed islet autoantibodies. HEV infections were detected in 18% (4/22) and 24% (20/82) of islet-autoantibody-positive and -negative children, respectively (p=0.5). The prevalence of HEV was similar in the early and late gluten-exposed groups and in children from mothers with type 1 diabetes or from affected fathers and/or siblings (p=1.0 and 0.6, respectively). According to these results no correlation was found between the presence of HEV in the first year of life and the development of islet autoantibodies. Likewise, there was no association between HEV infections and dietary intervention, maternal diabetes or clinical symptoms. Phylogenetic trees were generated for the isolated viruses in the samples. The phylogenetic clustering of these viruses indicates that they are not unique and are similar to strains circulating in the general community. These findings were published (Simonen-Tikka et al, Diabetologia).

Virus detection was also performed in stool samples and nasal swabs obtained from the 110 children within the high intensive follow-up study, INDIS (P13, TuH) in order to reveal frequencies of early childhood infections (P14, THL) and to identify the virus infections associated with early pathogenesis of T1D. In the INDIS study 260 stool and 262 nasal swap specimens were analyzed for enteric (enterovirus, norovirus, rotavirus, sapovirus, astrovirus) and respiratory viruses (enterovirus and rhinovirus), respectively, using virus-specific real-time RT-PCR methods. In addition, stool samples were studied for the presence of human parechovirus (HPeV) and the newly discovered human cardiovirus, saffold virus, which has been recently associated with gastrointestinal tract infections in humans. Altogether, HEV, HPeV, NoV (genotype GII) were detected in 9.6%, 15 %, 6.2% of the samples. Other intestinal viruses were present in 0.8-1.5 % of specimens. 19.1% and 51.5% of nasal swab samples were positive for enteroviruses and rhinoviruses, respectively. Furthermore, the enterovirus strains causing infections have been isolated and identified. These studies will be supplemented with work in which the isolated viruses are characterized for beta cell

tropism and pathogenesis, thus providing a comprehensive list of childhood enteric and respiratory viruses that could induce beta cell damage and hence trigger or exacerbate islet autoimmunity.

With respect to virus identification in islets, Roivainen (P14, THL) and Korsgren (P9, UU) each examined pancreatic islets from islet autoantibody positive individuals and/or new onset type 1 diabetes. Specifically, Korsgren performed virus analysis of isolated tissue (islets, pancreatic biopsies, exocrine tissue, spleen and PBMC) from two organ donors that died at onset of type 1 diabetes. Some islets in pancreatic sections from the two T1D organ donors were stained positive for PKR, INF and dsRNA which is consistent with virus infection. Ultrastructural studies of islet cells from the two T1D organ donors showed that insulin granules were fused and disrupted, no sign of ongoing apoptosis was seen and in a few ß-cells most intracellular organelles were missing. Morphological examination of the sections revealed that in some islets dilated endothelium and bleedings could be seen. Functional studies of isolated islets from the T1D donors revealed that a major loss of insulin secretion was detected. No virus has been isolated. Pancreatic sections from an islet autoantibody positive donor were also examined and found to stain positive for enterovirus, dsRNA and PKR and in a few islets bleeding could be seen. Enterovirus specific PCR performed in the 5´region were positive, but attempts to amplify the VP1 region has failed. Search for viral genome expression in the pancreatic islets using High throughput sequencing (P4, ULUND) is ongoing. Roivainen searched for enterovirus in islets from 8 islet autoantibody positive subjects, and found that one islet preparation was enterovirus positive in two different highly sensitive, validated RT-PCR-methods targeting highly conservative 5' non-coding region of the enterovirus genome (unpublished). The positive result was confirmed by sequencing the amplification product. So far, attempts to gain more genetic information on this specific virus strain by sequencing other genome regions have been unsuccessful.

Finally, with respect to virus identification, Piemonti (P8, HSR in collaboration with P14, THL) completed analysis of enterovirus RNA in 72 gut biopsies from islet autoantibody positive subjects, recent onset T1D patients and their controls. All subjects were found to be negative suggesting that the studied patients do not have an ongoing enterovirus infection in gut mucosa, excluding persistent enterovirus infection. Further confirmation was sought be protein staining. Since staining of tissues using a commercially available monoclonal antibody to enterovirus capsid protein VP1 was associated with false positive results, new monoclonal antibodies to capsid protein VP1 have been generated by Solimena (P1, TUD). The gut biopsies were also processed for High Throughput Sequencing by Dillner (P4, ULUND). Very few viruses were identified, again consistent with a lack of persistent viral infection via the gut mucosa in these patients.

Overall, the findings of virus identification show evidence for enterovirus infection in a minority of islet autoantibody positive and T1D cases.

2. Virus and type 1 diabetes - mechanisms of islet infection

The entry process of enterovirus to pancreatic islets may depend on the properties of the virus involved but also on tissues the virus has to invade to reach pancreatic islets. Three types of cells, pancreatic ß-cells, ductal cells and endothelial cells could be involved in the process and thus the interactions of enteroviruses with these cells are of importance. Roivainen (P14, THL) has studied the receptor usage of selected islet cell replicating echoviruses and shown that vitronectin receptors, alphaV-integrins, act as a cellular receptor for several echoviruses in primary human islets, whilst in primary human endothelial cells, these receptors work only for some of the studied serotypes

(Ylipaasto et al., J. Gen Virol 2010). Genetic diversity of enteroviruses and other RNA viruses is based on error prone replication of the genomic RNA which results in generation of multiple genetic lineages during long infection in a given individual. This means that enteroviruses exist as mixtures of microvariants in clinical isolates which may have differences in their pathogenic properties. By taking advantage of the clinical echovirus 9 isolated from a 6-week old baby at acute onset of T1D, Roivainen (P14, THL) produced parallel infectious clones of the virus and showed that some of them were more destructive in primary human islets than the others. By help of the whole virus genome sequencing and site-directed mutagenesis the viral amino acids responsible for the cell tropism were identified. The results showed that two clones differed only by two amino acids, but nevertheless had a substantial phenotypic difference in primary human pancreatic islets (lytic vs non-lytic).

In order to infect pancreatic islets, the virus has to pass through endothelial cells either by infecting them or by transcytosis. The infection of endothelial cells by enteroviruses might also induce pro-inflammatory cytokines and other toxic molecules, thereby destroying beta cells indirectly or leading to the activation of autoreactive T-cells. The capabilities of selected T1D associated enterovirus strains to infect primary human endothelial cells were studied by Roivainen (P14, THL) and Piemonti (P8, HSR). The results showed that a recently described highly prevalent enterovirus serotype enterovirus 94 (EV-94) was able to damage pancreatic islet cells, to infect, replicate, and cause necrosis in human pancreatic islets (Figure 5), and to induce proinflammatory and chemoattractive cytokine expression in endothelial cells (Smura et al., 2010).

Korsgren (P9, UU) additionally studied coxsackievirus B5 infection in exocrine and endocrine pancreatic tissue. In addition to laboratory strain they used a clinical strain isolated at onset of T1D. According to their results both virus strains replicated in endocrine cells but not in exocrine cell clusters. When two pancreatic exocrine cell lines were used a strain specific variation was seen in virus replication and cell destruction. Ultra structural studies of infected endocrine cells revealed that virus particles were often associated to insulin granule. The intracellular distribution of virus was demonstrated by gold labelled EV antibody.

In addition to endothelial cells and exocrine cells, other pancreatic cell types which might relevant to T1D are epithelial and mesenchymal cells. The results obtained in studies of Roivainen (14 THL) and Piemonti (P8 HSR) suggest that enteroviruses are able to infect, replicate, and cause necrosis in epithelial and mesenchymal cells, but with some virus strains the host cell specific factors involved in e.g. viral replication and/or translation restrict the viral growth thereby modulating the outcome of the infection (lytic, non-lytic infection, acute, chronic). Pancreatic ductal cells are also in close contact to the pancreatic islets. According to our data several different enterovirus serotypes are able to infect and produce viable progeny in human pancreatic duct epithelial cell line (HPDE). Some of the virus types were highly cytolytic whereas other closely related serotypes multiplied in these cells without apparent cytopathic effect. Furthermore, when a non-lytic strain was passaged five times in HPDE cells, its phenotype changed from non-lytic to lytic. The phenomenon was unique for pancreatic ductal cells, since both the original and ductal cell adapted virus strains replicated and induced cell lysis in green monkey kidney (GMK) cells (which is regularly used for enterovirus culture). This provided a unique model for studying viral genetic features and virus-cell interactions behind virus induced cytolysis. The virus induced lytic cell death has specific importance for the pathogenesis of T1D, since it represents a severe form of death where the cell content leaks into the surrounding tissue possibly causing damage to bystander cells (such as beta-cells) and inducing strong inflammatory response. Consistent with this, lytic virus infection was shown to induce the release of interleukin 1 alpha and beta in ductal cells.

In summary, it is demonstrated that several enterovirus strains can infect and replicate in beta cells and molecular requirements for this infection were identified. These viruses can also infect endothelial cells, mesenchymal cells and pancreatic duct cells, but not exocrine cells. Infection in these non-islet cells is variable in its outcome with respect to cell lysis. The findings demonstrate that enterovirus from several strains is capable of entering, replicating and destroying islet cells or nearby cells and therefore confirms virus multiple entry pathways for beta cell lysis.

3. Virus and type 1 diabetes - islet response profiles

As described above, effects enterovirus infection on islet cells varies according to virus strain. Response to virus infection was collaboratively examined by global gene expression and cytokine profiles (P14 THL, P9 UU and P8 HSR). The findings show that strong, sustained and temporally increasing gene expression levels of mediators of innate immunity, such as dsRNA recognition receptors, antiviral molecules, cytokines and chemokines were induced by the islet cell destructive enterovirus. The quantity and timing of the expression of genes (e.g. IL-1, TNF-alpha and TRAIL) that are considered to be key mediators in cytokine induced beta-cell dysfunction were associated with the destructive viral phenotype. Furthermore, parallel echovirus clones that induce varying degrees of necrosis, ranging from highly lytic to near benign in human pancreatic islets in vitro, were utilized for the detection of distinct gene expression patterns, representing enterovirus replication leading to necrosis or replication with no (or minor) cellular death, with minimal involvement of the genetic background of a virus. The results suggest a distinct gene expression pattern leading to pancreatic islet destruction and pro-inflammatory effects after enterovirus infection. However, neither viral replication nor cytotoxic cytokine production alone are sufficient to induce necrotic cell death.

Islet response to virus infection was also examined using islet metabolomic profiles. Decreases in ether lipids were found in the medium of infected cells as compared to mock infected cells. The findings of infected islets also found large differences in lipidomic profiles of islets obtained from islet autoantibody positive donors (Figure 6).

4. Virus and type 1 diabetes - the PTBP1 pathway

There is sound evidence that the polypyrimidine tract-binding protein 1 (PTBP1) binds to the RNAs of picornaviruses and enhances their IRES-mediated translation. Previous work by Solimena (P1b, TUD) has independently shown that PTBP1 is required for the glucose-stimulated rapid up-regulation of insulin granule biogenesis in pancreatic beta cells. Specifically, it was shown that PTBP1 binds to the 3'- and 5'-UTRs of mRNAs encoding insulin granule components, thereby enhancing their stability and translation. The list of granule components regulated by PTBP1, includes the autoantigens of type 1 diabetes insulin and ICA512/IA-2. In DIAPREPP the Solimena laboratory, in close collaboration with Merja Roivainen (P14, THL), has investigated therefore whether hijacking of the glucose-induced "PTBP1 response" by enteroviruses accounts for: a) the beta cell tropism of these viruses; and b) the changes in beta cells that could lead to loss of tolerance toward granule components.

They have discovered that infection of insulinoma cells with two distinct Coxsackievirus B5 (CVB5) strains blocks cap-dependent mRNA translation through the cleavage of eIFG4 and PABP, but does NOT prevent the glucose-stimulated, PTBP1-dependent, IRES-mediated translation of pro-insulin,

pro-ICA512, pro-Chromogranin A, pro-PC1/3 and pro-PC2 mRNAs. Remarkably, however, in CVB5-infected cells the total levels (intracellular content + media) of the corresponding mature granule proteins as well as of insulin granules are dramatically reduced. This depletion cannot be attributed to increased granule exocytosis, which instead is inhibited. Moreover, they found that CBV5 infection correlates with an increment of LC3+-structures, suggesting the induction of autophagy. Hence, we hypothesize that enteroviruses divert the traffic of newly synthesized insulin granule precursor proteins from granules to autophagosomes. Thus these proteins would be 1. obscured from normal processing pathways and 2. more available for degradation pathways and presentation via MHC. Since in enterovirus infected beta cells cap-dependent translation of most other proteins is inhibited, this mechanism could account for a sudden and dramatic increased presentation of peptides from granule antigens in MHC I in the context of an inflammatory process, and thus in the preferential loss of self-tolerance toward granule components or increased presentation of self or altered self peptides to CD8 T cells. These are novel findings from DIAPREPP.

5. Virus response gene and type 1 diabetes

The intriguing findings with respect to virus and type 1 diabetes were further examined at the level of genes involved in virus response. A gene conferring type 1 diabetes susceptibility is Interferon inducible helicase 1 (IFIH1). As part of the Biobank resources, DNA was available for genotyping of the children followed from birth. This was performed by Ziegler (P5, FDeV) and Bonifacio (P1, TUD) in the German BABYDIAB cohort to examine whether new susceptibility genes, including IFIH1, were associated with seroconversion to islet autoimmunity and/or progression to diabetes after seroconversion. The findings were striking with respect to IFIH1 which showed no association with seroconversion but which showed that islet autoantibody positive children with type 1 diabetes susceptible genotypes progress to diabetes two times faster than islet autoantibody positive children with the protective genotypes (Winkler et al, Diabetes; Bonifacio et al, Diabetes; Figure 8). The findings suggest that viral responsiveness of cells is relevant to the rate of development of type 1 diabetes and that viral infection may have the potential to influence type 1 diabetes development at multiple stages of pre-clinical disease. These genes are also are being examined in the Finnish DIPP cohort.

6. The metabolome at birth in children who develop islet autoimmunity.

It was suggested in a preliminary analysis of data from the Finnish DIPP study that there were differences in the metabolome at birth in children who developed type 1 diabetes as compared to children who did not. These data were elaborated within DIAPREPP and eventually published (Oresic et al, J Exp Med). A larger set of cord serum samples from the DIPP cohort were subsequently studied. Cord serum metabolome was investigated in progressors to T1D (n=33), newborns who developed 3 or 4 (n=32), two (n=34), or one (n=50) islet autoantibody (Aab) during follow-up, and controls (n=160) matched for gender, HLA genotype, city and period of birth. The analyses were performed using the established metabolomics platforms based on Ultra Performance Liquid ChromatographyTM coupled to mass spectrometry for lipidomics and two-dimensional gas chromatography - time-of-flight mass spectrometry for profiling small polar metabolites. The data indicate that T1D progressors and likely progressors (3 or 4 Abs) are, in comparison to nonprogressors in the control group, characterized by distinct metabolic profile which includes

diminished phospholipids, dominated by low sphingomyelins as well as several ether lipids, and diminished lactic and fumaric acids, among others. Such a metabolic profile is not found in children who developed only one islet autoantibody during the follow-up, except for lactic acid, which was also diminished in this group. In fact, none of the lipids were different when comparing this group with nonprogressors from the control group. This study confirms earlier findings as well as suggests that the observed diminishment of specific phospholipids and other metabolites in cord blood is specifically associated with islet autoimmunity that leads to T1D but not with single islet autoimmunity which is known to infrequently progress to diabetes. In children (n=23) who developed T1D before 8 years of age in the Swedish DiPiS study the cord blood metabolomics profile (determined by Oresic, P6, VTT) was different from age, gender and HLA-matched controls for a set of 6 phospholipids. The findings which confirm observations in the Finnish DIPP study are remarkable with respect to demonstrating that already at birth children destined to develop type 1 diabetes may have a signature metabolomic profile.

7. The metabolome is affected by the appearance of islet autoantibodies.

DIAPREPP provided longitudinal metabolomics analysis in the DIPP cohort performed by Oresic (P6, VTT) which indicated that that inflammation and protection against oxidative damage play crucial roles in the pre-autoimmune stages of type 1 diabetes pathogenesis. Specifically, ether phospholipids functioning as endogenous antioxidants were reduced throughout the follow-up in those who developed diabetes, while lysophosphatidylcholines, known chemoattractants of T lymphocytes, were up-regulated several months prior to seroconversion to autoantibody positivity (Oresic et al, J Exp Med). Thus, akin to what was observed at birth, the metabolome was able to show alterations prior to seroconversion. These analyses were extended to the BABYDIAB study which had postulated that children who seroconverted during early childhood had different immunization profiles to children who seroconverted late in childhood. Metabolomic analysis of the BABYDIAB samples by partner 6 (VTT) gave support to this hypothesis (Pflueger et al, Diabetes). First, lipidomic changes were observed in islet autoantibody positive children regardless of the age of seroconversion. Importantly with respect to heterogeneity, the amino acid methionine was markedly reduced in serum samples from children who seroconverted by age 2 years as compared to children who seroconverted at age 8 years and to islet autoantibody negative children (Figure 9). The findings were persistent after seroconversion, but were not observed prior to seroconversion. The metabolomic data were further extended to a second large set of samples from the Finnish DIPP study which are in the process of being analyzed. Nevertheless, thy again show differences in lipids between antibody positive and negative children at and after the time of seroconversion. Moreover, additional differences were observed with respect to the age of seroconversion. Thus, the metabolome is shown by DIAPREPP to be a rich source of biomarkers for the pre-type1 diabetes period, and a potential source to identify novel pathways involved in the preclinical process.

8. DHAPAT - a novel pathway of pre-type 1 diabetes from the metabolome.

A striking finding from the metabolomics data was the early decrease in plasmalogens in children who developed type 1 diabetes. DHAPAT (dihydroxyacetonephosphate acyltransferase) plays an important role in production of plasmalogens. Consistent with this Oresic (P6, VTT) performed a bioinformatics analysis of publicly available gene expression data from pancreas of NOD mouse and

confirmed the importance of DHAPAT in early stages of autoimmune diabetes pathogenesis (Sysi-Aho et al; PLOS Computational Biology). Convergence on DHAPAT was therefore proposed as a pathway for type 1 diabetes development. DIAPREPP experts in beta cells (Solimena, P1) and mouse models (van Endert, P2) sought to examine whether deficiencies in DHAPAT had consequences in vitro and in vivo. Knockdown of DHAPAT from beta cell lines confirmed the phenotype of decreased plasmalogens and ether lipids in DHAPAT deficient cells, but there was no alteration of insulin secretion associated with this. This suggests that if DHAPAT is involved in the pathogenesis of type 1 diabetes, it is unlikely to be directly through effects on insulin secretion. Definitive proof of DHAPAT involvement in autoimmune diabetes came from the mouse studies. Partner 2 produced hemizygous DHAPAT deficient mice on the NOD autoimmune diabetes background. These mice have an accelerated onset of diabetes as compared with litter matched control mice (Figure 10). Lipid profiles and immune function in the mice is currently under investigation.

9. Islet autoimmunity has a peak incidence around 1 year of age.

The DIAPREPP birth cohort resources from Germany and Scandinavia are unique and provided the opportunity to examine closely factors which determine seroconversion to islet autoantibody positivity. A striking observation was the timing of seroconversion in the children. Partners 5 (FDeV) and 1 (TUD) created incidence data on the German BABYDIAB and BABYDIET study children which demonstrated a clear peak incidence around age 9 months to 2 years. This was particularly enhanced in the early period when children with strong genetic risk for type 1 diabetes were examined (Figure 11).

Even more striking was the absence of seroconversion in the age range 0 to 6 months. Finnish data in children without a family history of type 1 diabetes generated by partner 13 (Turku UH) was consistent with these observations. Analysis of both the German and the Finnish data confirmed their previous reports that autoantibodies to insulin were the earliest to appear in the children. Thus, DIAPREPP shows that there is a period of high susceptibility to seroconversion which starts after age 6 months and then declines after age 2 years. This is highly relevant to triggers of islet autoimmunity.

10. Increased type 1 diabetes incidence appears to be due to faster disease progression.

Most European countries report an increased incidence of childhood diabetes over the last decades. It was unclear whether this increase is due to more seroconversion i.e. triggering of islet autoimmunity or to a faster progression to disease after the autoimmunity has initiated. Through its collaboration with the international TEDDY study, DIAPREPP investigators were able to address this question in Germany. Partner 5 (Ziegler, FDeV) examined the probability of developing islet autoimmunity, diabetes, and the rate of progression from seroconversion to diabetes in the BABYDIAB study children and compared this to matched children in the more recent data obtained in the same region within TEDDY (Ziegler et al, J Autoimmunity). A higher proportion of children developed type 1 diabetes by age 5 years in the more recent TEDDY study cohort as compared to the BABYDIAB study children confirming the overall increase in type 1 diabetes trends. However, islet autoantibody risk was the same during the two study periods. Explaining the increase in type 1 diabetes was a marked increase in the rate of progression to type 1 diabetes after seroconversion in children who developed islet autoantibodies (Figure 12). The findings point to a decrease in the ability to control autoimmunity as a cause for the rising incidence of type 1 diabetes. Together with the findings that

susceptible genotypes of the viral response gene IFIH1 are also associated with a faster progression to diabetes in the same cohort (Figure 8), it is suggestive that viral infections occurring after seroconversion may be important in determining the rate of progression to diabetes. This opens new avenues for prevention in children with pre-type 1 diabetes.

11. Autoimmunity against the insulin granule protein Zinc Transporter 8 (ZnT8)

Shortly before the start of DIAPREPP, the insulin granule protein ZnT8 was identified as an autoantigen in type 1 diabetes by investigators in the USA. DIAPREPP investigators had also gathered evidence that this was a target of autoantibodies in pre-type 1 diabetes. Partner 8 (HSR) and partner 4 (ULUND) cloned the intracellular portion of the three polymorphic variants of ZnT8 for protein expression and use in immunoassay. Partner 4 tested over 2500 patients at onset of type 1 diabetes for antibodies to the three variants and found antibodies to at least one of the variants in 64% of patients (Vaziri-Sani et al, J Imm Methods; Figure 13). ZnT8 antibodies were also examined with respect to other islet autoantibodies, age, and residual beta cell function and were found to be useful markers in addition to the established antibodies to insulin, GAD65 and IA-2, and were independent of age (Andersson et al, Autoimmunity; Nilsson et al, Autoimmunity).

Lampasona (P8, HSR) examined ZnT8 antibodies in over 1000 adult onset diabetes patients and found that their presence in patients with also GAD antibodies and/or IA-2 antibodies identified a phenotype of autoimmune diabetes with younger age of onset and characteristics of more severe insulin deficiency (Lampasona, Diabetes Care). Finally, Achenbach (P5, FDeV), Lampasona (P8, HSR) and Bonifacio (P1, TUD) extensively studied ZnT8 autoantibodies in the German BABYDIAB samples and found that they appeared at a similar time frame and frequency as IA-2 antibodies and conferred increased risk for progression to diabetes when found together with islet autoantibodies (Figure 14). They further showed that the genotype for the ZnT8 polymorphism at amino acid 325 is strongly associated with the autoantibody response to the respective polymorphic variant in a manner that is consistent with more response to the self polymorphic variant. Moreover, ZnT8 antibody positive children who were homozygous for a variant were more likely to progress to diabetes than ZnT8 antibody positive children who were heterozygous.

Overall, the DIAPREPP findings with respect to ZnT8 autoantibodies demonstrate that it is a major autoantigen in type 1 diabetes and that measurement of ZnT8 autoantibodies is highly useful for the prediction of type 1 diabetes, in particular in individuals with other islet autoantibodies. Hence, the development of a kit for the measurement of ZnT8 antibodies by the SME partner RSR as originally planned became a high priority activity. RSR used propriety knowledge to develop an ELISA for measurement of antibodies to the two more common ZnT8 variants. A prototype ELISA was made in 2009 and optimized. It underwent blinded evaluation in the Diabetes Autoantibody Standardization Program (Lampasona et al, Clin Chem) and performed extremely well in comparison to other assays. Moreover, the ZnT8 antibody workshop led by DIAPREPP investigators Lampasona and Achenbach showed high level of performance and concordance among multiple ZnT8 antibody assays. The RSR ZnT8 antibody kit has now been put on the market.

While autoantibodies are excellent markers of pre-type 1 diabetes, it is generally considered that autoreactive T cells and in particular CD8 T cells are the effectors of beta cell destruction. Hence, an ability to measure these T cells is considered an important goal in the type 1 diabetes field. Peter van Endert (P2, INSERM) provided expertise in CD8 T cell epitope identification and assays. He used this

expertise to identify a set of potential peptides that could be recognized by CD8 T cells from patients who had a HLA A2 allele (note that CD8 T cells recognize antigenic peptides bound by HLA class I alleles such as HLA A). His group were able to test blood samples from patients and controls who were HLA A2 positive and showed that ZnT8 peptides are potent stimulators of CD8 T cells from paediatric patients with type 1 diabetes, and that 5 of 8 selected peptides had responses restricted to patients (n=35; Figure 15). Overall 37 percent (13 of 35) children with diabetes had CD8+ T cell responses against ZnT8 versus 9 percent (1 of 11) controls. Thus ZnT8 is an important CD8+ T cell antigen in T1D. CD8+ T cell reactivity against ZnT8 was also observed in 50 percent of a small group of adult patients. Interestingly, T cells from adult patients recognized at least one epitope never recognized by paediatric patients (ZnT8-1), suggesting that the epitopes recognized might vary according to age of onset. Note that this study is the first to document CD8+ T cell reactivity against pancreatic autoantigenes in paediatric patients by Elispot. These data are the subject of DIAPREPP intellectual property foreground that was considered, but not processed for patent application. It is now part of a manuscript in preparation for publication.

12. Single immune cell technology

Immune responses to beta cell antigens in type 1 diabetes are almost exclusively measured at the bulk responsive level. This includes the measurement of the circulating autoantibody pool, and the circulating autoreactive pool via proliferation, tetramer binding, or bulk cytokine production. DIAPREPP considered it a major step forward if responses could be measured at the level of the single cell. In particular, there was interest in establishing technology that could capture single responsive cells and provide information with respect to their antigen specific receptors that distinguishes autoimmunity.

B lymphocytes have an immunoglobulin receptor which is highly variable between B lymphocytes and which provides the B lymphocytes their ability to recognize antigen with high affinity. The IgG form is typical of responses that are known as memory and which represent previously activated B lymphocytes. These make up a small proportion of the circulating pool of B lymphocytes. The task was to isolate these IgG positive B lymphocytes which was achieved with magnetic bead isolation of B lymphocytes followed by FACS isolation of the IgG positive B lymphocytes. The diversity of immunoglobulin genes is due to recombination of a large number of genes encoding different segments of the immunoglobulin molecule and somatic mutation to insert and/or remove sequences. The large number of possible genes that encode the IgG molecule means that methods to clone the IgG from any single B lymphocyte require the combination of a large number of primer pairs in a single PCR reaction. This is not trivial. Based on previously published work by others and using DIAPREPP foreground established by partner 1 (TUD), a set of 22 primer pairs were generated that together provided specific and efficient amplification of almost all possible gene families relevant to the cloning of IgG from the single B lymphocytes (Figure 16). This resulted in successful amplification of both the heavy and light chain genes in around 60% of single B lymphocytes which is markedly more efficient than previously published methods. This could be applied to preparations of single IgG B lymphocytes isolated from the DIAPREPP Biobank to obtain the gene sequences of memory B lymphocytes from individual patients (see below). A key application for the single B lymphocyte technology envisaged in DIAPREPP was to obtain the immunoglobulin product of B lymphocytes that were against beta cell autoantigens. This required expression of the cloned immunoglobulin gene which was achieved by cloning into a cassette that allowed full expression of the heavy and light chain after transfection into mammalian cell lines. The methodology was

established and provides an efficiency of around 40% for the cloned genes with an overall efficiency of between 10% and 30% for the successful expression of IgG from single B lymphocytes. This compares to around 1% efficiency for other methods such as EBV transformation of B lymphocytes. The methodology was used to generate human monoclonal IgG from unique sources of cells within the DIAPREPP Biobank (see below).

Although not a pre-described deliverable within DIAPREPP, the success of the technology development for human B lymphocytes led partner 1 to develop single cell PCR technology for cloning of the T cell receptors (TCR) from autoreactive T cells as a new part of the DIAPREPP project. Like IgG, the T cell receptor is highly diverse between T cells and provides specificity to antigenic peptides. Amplification of the TCR from single T cells requires multiple primer pairs. Surveying the literature and knowledge from Peter van Endert (P2, INSERM), Partner 1 was able to eventually create a set of primer pairs that could successfully amplify the TCR beta chain and the TCR alpha chain from single T cells with around 60% efficiency. This compares to around 5% using previously established methods. Thus, partner 1 was able to use material from the DIAPREPP Biobank to determine the full TCR sequences of single T cells that were responsive to autoantigens (see below). As for IgG, it was considered beneficial if the TCR products could be expressed and used to identify to target peptide of the T cell. This would eventually allow identification of peptide sets relevant for individual children regardless of their HLA and should eventually provide the scientific community with a large set of novel antigen peptides for use in T cell monitoring. Expression was also not trivial, but has now been achieved in T cell lines lacking TCR (Figure 17). Thus, DIAPREPP has developed novel technology that allows the identification of antigenic peptides from single T cells. This is now being considered for patent file application.

Finally, with respect to single cell technology, DIAPREPP partner 2 (INSERM) used proprietary knowledge to bring together primer pairs that could successfully amplify and quantify the expression of multiple markers of CD8 T cell activation state and phenotype. This has enabled partner 2 to examine single T cells that recognize specific antigen peptides in a mouse model of autoimmune diabetes and also globally in patients with type 1 diabetes and their controls (see below).

Overall, the single immune cell technology is a highlight technological achievement of the DIAPREPP project. Some of this technology could be applied within the DIAPREPP project, but it is expected to have a major application in the future.

13. Human monoclonal autoantibodies using DIAPREPP single cell technology.

Over 1000 human monoclonal IgG molecules were generated by partner 1 using the single cell technology described above (see 13) from 4 sources of Biobank material which included both patient blood and pancreatic lymph nodes from three patients who were positive for islet autoantibodies (organ donors and type 1 diabetes sources provided by partners 8 and 9). Assays were developed within DIAPREPP that would allow efficient screening of the monoclonals against known antigens. These were based on technology provided by partner 8. A dual Lucifer's assay was established that could screen for antibodies against GAD65, IA-2 and the two major variants of ZnT8 in a single assay (Figure 18).

For identification of antibodies to insulin, the Proinsulin molecule was cloned with a tag allowing capture onto solid phase and a chemiluminscence assay established. Screening of the generated monoclonal antibodies is ongoing. Thus far, 2 human monoclonal antibodies against the IA-2 antigen

and 6 monoclonals against Proinsulin have been identified. The IA-2 monoclonal antibodies have been characterized at the epitope level and show similar but distinct reactivity. This activity will continue to complete the screening of the human monoclonals and fully characterize the positives.

14. Human GAD65 autoreactive CD4+ T cells using DIAPREPP single cell technology

Assays were developed for identification and isolation of GAD65 responsive memory (CD45RO+) T cells using either whole antigen plus proliferation or GAD65 peptide plus tetramer isolation (Figure 19).

TCR alpha and beta chains from the responsive cells were sequenced and annotated using a bioinformatics pipeline developed by partner 1. The findings show that responsive cells with the same TCR can be identified in multiple wells from the same blood sample and from both peptide and whole antigen stimulated cells. However, unlike what was observed for the recall antigen tetanus toxoid these highly representative TCRs against GAD65 were rare and most of the TCRs from antigen or peptide responsive cells did not overlap. This was especially so for peptide responsive TCRs which were rarely found in the antigen responsive repertoire. It was also possible to examine peripheral blood and pancreatic lymph node T cells from the same islet autoantibody positive organ donor. The lymph node CD4 T cells were highly responsive to GAD65 and numerous TCRs from these responsive T cells were obtained. However, none of the sequences matched those found in peripheral blood responsive T cells suggesting that repertoires at the lymph node differ from those in the peripheral blood. Of particular interest, TCR from GAD65 responsive CD4 T cells from the same subject and also from different patients can share the same TCR alpha chain with several beta chains. This is interesting with respect to public TCR domains and potential diagnostic assay development.

15. CD8 T cell expression profiles using DIAPREPP single cell technology

Initially van Endert (P2, INSERM) applied the single cell expression technology in the model of the non-obese diabetic (NOD) mouse, where issues such as the relationship between CD8+ T cells in the blood and in islet infiltrates, and the value of CD8+ T cell monitoring with respect to disease prediction and to monitoring after treatment with anti-CD3 antibodies can be studied rapidly and efficiently. Methods for FACS sorting of bulk CD8+ T cells and T cells recognizing the immunodominant epitope IGRP206-14 were set up, and cells from peripheral blood and from islet infiltrates of pre-diabetic mice aged 7, 11, 17, or >30 weeks, as well as from diabetic mice, treated or not with anti-CD3 antibodies, were sorted. Using the protocol established by van Endert, mRNAs encoding 18 different genes whose products are involved in CD8+ T cell differentiation, effector functions, homing, cytokine secretion and uptake were quantified in single cells from these periods. The findings show a highly skewed expression in the IGRP pentamer positive cells as compared to pentamer negative cells sorted from islets of NOD mice (Figure 20). Additional treatment related findings were observed.

Human CD8 T cells were also examined by van Endert. Gene expression of CD8+ T cells from patients were examined without selection for antigen specific T cells. An initial study on T cells obtained from adult and pediatric patients at T1D onset has been completed and is prepared for publication. Multiplex single cell PCR analysis was performed separately on single CD45RA+ (mainly naïve) and CD45RA- (memory) CD8+ T cells. The analysis was limited to 14 genes

including granzyme A and B, perforin, IL-2, IFN-gamma, TNF-alpha, TGF-beta, FasL, KLRG1, IL10R, IL7R, CCR7, RANTES and MIP1-beta. Twelve adult and 12 paediatric patients were studied together with an equal number of controls. For each T cell subpopulation and patient or control, a minimum of 40 cells was analyzed. Since the single cell PCR technology allows for identification of T cell populations expressing defined sets of genes, a biostatistical cluster analysis was performed which identified differences with respect to the relative abundance of defined subpopulations between patients and controls. This study demonstrated that T1D onset is associated with substantial alterations in gene expression by CD8+ T cells both from paediatric and adult patients. A surprising finding is the striking discrepancy between adult and pediatric patients with respect to expression of some genes, particularly those encoding TGF-beta and the IL-10 receptor. Interestingly, expression of some markers is altered both in cells from adult and pediatric patients however in opposite directions (down-regulation in adults vs. up-regulation in children or vice versa). While the biological significance of this finding remains to be determined, it suggests significant differences in the pathophysiology of pediatric and adult T1D (Figure 21).

16. The IL-7/IL-7 receptor pathway

DIAPREPP investigators (P8, 5, 1) examined cytokine/chemokines profiles in neonates with the aim to identify pathways that were supportive of autoimmunization. Results emerged showing that the homeostatic cytokine IL-7 was increased in the first year of life. Thus, the effects of IL-7 on CD4 T cell activation were examined. The data conclusively shows that antigenic stimulation in the presence of increased IL-7 concentrations increases the number of naive antigen responsive CD4 T cells that are activated and increases the proliferation of memory antigen responsive CD4 T cells. Thus, DIAPREPP has proposed that the IL-7 pathway is involved in early immunization against beta cell antigens. These data have been submitted for publication. Further evidence was obtained by examining serum concentrations of soluble IL-7 receptor. sIL-7R is shed when cells are activated by IL-7. sIL-7 concentrations were increased in children at onset of type 1 diabetes as compared to matched control children (P=0.0001).

17. Preparing for prevention - PICCTURE

As part of the translation prospects of DIAPREPP findings and other findings in the scientific community, DIAPREPP developed a web site whereby information regarding the establishment of investigator based type 1 diabetes related clinical trials in Europe would be available. Partner 4 (Lernmark, ULUND) had extensive experience in clinical trials and performed this task.

The PICCTURE (Prevention Implementation Coordinating Centre for T1D United Research in Europe) platform has been initiated and it is available for access through direct link from http://www.diaprepp.eu to Lund University Diabetes Centre (LUDC) website http://www.ludc.med.lu.se/research-units/diabetes-and-celiac-disease (Figure 22).

The PICCTURE platform was designed to contain FIVE main disciplines:

- T1D Trial Clearinghouse

The PICCTURE clearinghouse provides an updated resource on all registered primary, secondary and tertiary prevention and intervention clinical trials on T1D. These trials have been listed according to prevention level and whether they were completed or currently ongoing. Each trail identified by specific Trial Identifier that provides a direct link to the trial page on the clinical trials website (http://www.clinicaltrials.gov/ct2/search). Tables provide summary information for nearly all preventive trials in T1D and will be updated regularly. These tables will provide a handy resource to fully follow completed and ongoing prevention and intervention clinical trials on T1D.

- DIAPREPP Trial Guide

The DIAPREPP guide provides protocol templates for prevention and intervention trials. These templates will be available as downloadable files from the Lund University Diabetes Centre website http://www.ludc.med.lu.se/research- units/diabetes- and-celiac-disease. The guide will also contain information and guidance on designing and implementing prevention trials.

- Ethics Approval Applications

This discipline provides detailed information on the requirement to submit an ethical approval proposal. It also provides guidance on the EU regulations and requirements for ethical approval of clinical trials. Direct links and ethical application forms will be accessible from member countries.

- Drug Agency Applications

This discipline will be available for drug agencies, which are willing to participate or initiate a prevention trial on T1D. Applications will be available for each member country in DIAPREPP and specific forms for the DIAPREPP PICCTURE platform trial application are also available for direct downloading. Through this service DIAPREPP will coordinate and cooperate with drug agencies based on regulation and guidance from DIAPREPP Trial Guide.

- DIAPREPP Screening and Recruitment Services

This discipline provides detailed information concerning screening programs implemented in participating centres, screening facilities in addition to recruitment criteria.

Potential Impact:

Type 1 diabetes incidence is increasing dramatically in young children within Europe. It is a lifelong disease with increased morbidity, substantially decreased life quality, and important economic burden to countries. Unlike type 2 diabetes where lifestyle interventions could reduce disease incidence, there is at the moment no known way to prevent type 1 diabetes. Thus, understanding the reasons for the increase in incidence, finding early markers of disease, and identifying pathways that could be targeted to reduce type 1 diabetes incidence are important objectives to reduce socio-economic burden of disease. DIAPREPP's potential impact to these objectives are discussed below.

DIAPREPP Contributions to Impacts listed in the original work programme.

1. Contribution to: 'Translational research for better understanding of the pathophysiological processes in the development of type 1 diabetes with emphasis on early events'.

DIAPREPP has focussed its research project almost entirely on the earliest events in the pathogenesis of T1D. Its major activities were expected to elucidate mechanisms of islet autoimmunity, and markers and signatures of the first stages of islet autoimmunity. It has taken some bold steps and innovative steps to do so. First, the three major holders of 'from birth' followed cohorts in Europe opened their doors to a collaborative effort in defining the early auto-immunization events. This was a mammoth showing of collaborative spirit amongst scientists who have dedicated decades of their lives to establishing their respective cohorts. The impact of this resource should be sufficient to make Europe's leadership in this field long lasting.

DIAPREPP used the cohort to focus on the first signs of autoimmunity and the events and changes that occur prior to and around autoimmunity (seroconversion). It brought in a sophisticated state-of-the-art high-end metabolomics platform to ensure that data of sufficient quality and quantity to match the quality of the cohort was obtained during the project. This platform has generated exciting data. Especially important have been the metabolomics data generated from the samples obtained at or prior to seroconversion. They have shown clear differences between children who developed 'aggressive' islet autoimmunity (multiple islet autoantibodies and progression to diabetes) as compared to children who develop single islet autoantibodies or who do not seroconvert. These differences are already observed at birth (cord blood) and therefore have important implications for the pathogenesis of islet autoimmunity. Pathways potentially involved in the very early processes of susceptibility and activation of islet autoimmunity were identified. Some of these were corroborated by analysis of data generated in animal models. This includes the role of DHAPAT, an enzyme that is involved in the synthesis of plasmalogens (found by DIAPREPP investigators to be altered in pre-type 1 diabetes). DHAPAT deficiency led to more aggressive disease in NOD mice, highly consistent with the human data. Thus, this is an area that will be avidly pursued in the future with respect to better understanding its role in pathogenesis and as a target of prevention. The marked decrease in methionine observed at seroconversion in children who seroconvert in the first two years of life is also interesting with respect to novel pathogenetic mechanisms.

The timing of seroconversion identified in the German and Finnish cohorts is also highly informative with respect to when environmental agents come into play. Both cohorts found that the age from after 6 months to age 2 years is a high risk period for seroconversion. Both also found that insulin is the

earliest autoantibody target. The findings will focus the search for aetiological factors to this age period. Moreover, the fact that seroconversion was rare prior to age 6 months has led to new hypotheses that there is a period of protection in the neonates where autoimmunity is unlikely. This will lead to new investigations to understand these potential protective mechanisms which could be extended or copied for further protection during the risk period.

In view of the findings with respect to the 6 to 2 year high risk period, the potential role of viruses in the early pathogenesis was an important topic and indeed was heavily investigated in DIAPREPP. With respect to a viral aetiology of type 1 diabetes, DIAPREPP had hoped to identify novel viruses that could trigger islet autoimmunity. Instead, it showed that relevant samples prior to islet autoimmunity were relatively devoid of virus and that the viruses were usually common viruses found in the general population. This is nevertheless relevant since it should focus future attention on studying such common viruses and examine the role of viruses after seroconversion ie in the progression to diabetes. The latter aspect is important since DIAPREPP did find clear evidence of virus infection of beta cells in its unique islet resources suggesting that the ability of certain enteroviruses to infect beta cells is pertinent to at least some islet autoantibody positive cases. It also indirectly became highly relevant from other DIAPREPP data which showed that 1. There is a large increase in the rate of progression to diabetes after seroconversion in the last 10 years as compared to previously, potentially explaining the increasing incidence of childhood diabetes, and 2. Genotypes of the virus response gene IFIH1 strongly associate with the rate of progression. These findings further point to an important role of virus infection in propagating existing autoimmunity rather than/in addition to triggering autoimmunity and therefore opens new avenues for understanding pathogenesis and implementing prevention strategies.

Perhaps the most novel and interesting finding with respect to virus was the effect of enterovirus infection on the translation and processing of PTBP1 influenced secretory granule proteins. While the original hypothesis that infection may highjack transcription/translation of the proteins was not fully verified, a marked effect on processing and the quantity of mature protein was. The implications are extremely interesting and support a concept whereby virus infection leads to marked redirection of granule proteins to the autophagosome which would dramatically increase their immunogenicity, especially within the environment of the other changes observed in infected cells. The latter was also a subject of intense investigation within DIAPREPP. Full profiles of islet cell changes upon enterovirus infection were produced, identifying potential novel markers of virus infection and response pathways that are likely to have a role in pathogenesis. Moreover, key molecules involved in the infectivity of beta cells by enterovirus were identified.

New findings with respect to progression came from some mathematical modelling of the islet autoantibody changes over time and the relationship to type 1 diabetes susceptibility genes. Most striking were observations that children who progress rapidly are rich in type 1 diabetes susceptibility alleles whereas children who do not progress are devoid of these alleles. It appeared to be a cumulative effect of susceptible allele number. The findings are important because they dissociate the risk for seroconversion (mainly conferred by HLA and by INS genotypes) and the rate of progression after seroconversion (conferred by mostly the other susceptibility genes). Thus the findings should focus mechanisms involving these genes to the relevant phase of preclinical disease. Related to these were mechanistic studies that showed the potential for the IL-7/IL-7 receptor pathway in the activation and expansion of autoreactive T cells.

Finally, there are numerous new technologies developed that will help track autoimmunity in future studies and will help our understanding of the early pathogenesis in the future. New methods for

looking at autoreactive T cells at the single cell level were developed and although not used extensively in the pre-type 1 diabetes cohorts within the DIAPREPP project period, will now be used on these cohorts to understand when autoreactive T cells are activated and their phenotype when activated with the help of other third party funding. Preliminary data suggest it can be prior to seroconversion. The human monoclonal autoantibody resource from the single B cell studies will likely identify new potential autoantigens and from the pathogenetic mechanism side, have provided reagents that are already used to study the effect of antibodies on autoreactive T cells.

2. Contribution to: 'Research should improve early diagnosis'.

The foreground that DIAPREPP produced with respect to the early auto-immunization events provide a relevant impact on the number of tools and assays that will become available for the measurement of phenomena associated with early pre-type 1 diabetes. Already implemented is a commercial kit for the measurement of ZnT8 autoantibodies by one of the SME partners of DIAPREPP. This was a major aim of the project realized within the project period. ZnT8 autoantibodies were demonstrated within DIAPREPP to be highly relevant markers of islet autoimmunity complementing the existing GAD, insulin and IA-2 autoantibodies. Measurements of these autoantibodies (including the kit developed in DIAPREPP) were also put to the test by DIAPREPP investigators in the international Diabetes Autoantibodies Standardization Program and shown to be ready for clinical application. As a result, ZnT8 autoantibodies are now incorporated into large international studies (TEDDY, TrialNet). ZnT8 was also shown to be a target of CD8+ T cells and disease relevant epitopes (peptides) identified. This will increase the ability to identify pathogenetic T cells in the pre-diabetic phase of the disease.

There is clear potential for improved early diagnosis in the metabolomic findings of DIAPREPP. In particular, the profiles observed at birth have the potential to be developed into selection criteria for stratifying highest risk genetically susceptible children to include into research studies or intervention trials. Work will be needed to determine the actual ability to stratify risk. An excellent opportunity arises via the TEDDY study in which a number of DIAPREPP investigators take part and where metabolomics measurements will be performed. It has been proposed by the DIAPREPP investigators that the birth profiles found in DIAPREPP be tested in early samples from TEDDY for reproducibility in multiple regions and as a means of risk stratification. The metabolomic profiling analysis of the longitudinal data from DIAPREPP is still ongoing, but promises to provide early markers associated with disease progression after islet autoimmunity has occurred and should therefore increase the likelihood of early diagnosis. The data on genetic profiles are also relevant to early identification since they were very effective in identifying islet autoantibody positive children with fast versus slow progression to diabetes. Further work will be done in order to define a smaller panel of genes and genotypes. Moreover, data from both Germany and Finland is being analysed together to provide stratification for both relatives and general population islet autoantibody positive children.

Markers will be derived from the unique single cell B and T cell studies. With around 1000 monoclonal human IgG antibodies from pancreatic lymph node B ymphocytes of patients with type 1 diabetes, DIAPREPP has produced a new resource for the identification of islet autoimmunity biomarkers. It is expected that from this resource which has only been partially analyzed, and hardly at all for novel autoantibodies, a number of antibodies against pancreas proteins will be recovered. These become candidates for new markers and will be validated in the future. The monoclonal islet autoantibodies become tools for epitope recognition and for developing assays that can be used to

identify spreading of the autoimmune response, a characteristic associated with disease progression. The T cell receptor technology on single autoreactive T cells will eventually identify new antigenic peptides of autoantigens (in addition to those already found for ZnT8 within DIAPREPP) which can be used to measure autoreactive T cell responses in at risk individuals. The technology has also shown the potential for identifying autoreactive T cells at the molecular level and is expected to lead to molecular quantitative PCR based assays which will be evaluated for autoreactive T cell tracking within individuals and which may provide future prognostic tests in pre-type 1 diabetes.

DIAPREPP has also produced reagents such as purified Proinsulin and ZnT8-luciferase fusion proteins which are available for autoantibody assay development. Indeed, the Proinsulin has already been sent to other centers who are developing new (Denver) or commercial (RSR) assays for insulin autoantibodies. There are also new assays for measurement of autoantibody affinity eg IA-2 affinity, and DIAPREPP was involved in the international harmonization of GAD and IA-2 autoantibodies which included preparing world reference standards for these measurements. This level of standardization will improve early diagnosis and recruitment into prevention trials.

3. Contribution to: 'Research should lead to approaches for tackling prevention of type 1 diabetes'.

The foreground produced by DIAPREPP in understanding mechanisms of islet autoimmunity and in developing assays to identify and track autoimmunity during pre-T1D will provide both new possibilities in preventing T1D, particularly at an early stage of autoimmunity, and new ways to monitor progression during intervention. DIAPREPP did not test new therapies, but identified potential pathways that can be targeted with therapy for prevention. The pathways include IL-7 whereby down-regulation is expected to reduce type 1 diabetes risk. Indeed, partner 1 (TUD) is developing antagonist IL-7 mutants that may have therapeutic value in this context. DHAPAT is also an obvious candidate pathway for therapeutics in view of its repeated identification in metabolomic screens and in the NOD mouse DHAPAT deficiency studies. Therapeutics that increase DHAPAT activity are expected to reduce type 1 diabetes risk. Anti-viral or anti-inflammatory therapies appear as an interesting possibility for prevention in islet autoantibody positive children in view of the data emerging with respect to IFIH1 genotypes and progression to type 1 diabetes. If the proposed mechanism regarding protection via dampening cellular responses to viral infection are true then our data would support the use of mimicking this after seroconversion, especially in children with IFIH1 susceptible genotypes.

The impact of DIAPREPP will also be on mechanistic assays for drug discovery and for use as an early indicator of therapy efficacy. For example, the metabolomic 'abnormalities' observed might be used to screen potential drugs using cell based assays where the defect is expressed, and measurement of metabolome after treatment may be used to determine whether the treatment has corrected the defect. The novel assays for T cells will become valuable tools for measuring efficacy of prevention trials. We see that an ability to monitor at the clonal cell level circulating autoreactive CD4+ and CD8+ autoreactive T cells will greatly improve our immediate measures of intervention effectiveness. These measurements will also provide an ability to determine immune vs disease effect and can further be applied to mechanistic studies aimed at identifying responders versus non-responders to intervention.

Finally, DIAPREPP has provided a T1D early prevention platform for Europe. This was considered an important undertaking in view of the difficulty and getting through the very many regulatory issues of clinical trials in Europe. It was not specifically meant to aid translation of DIAPREPP findings into trials, but designed to aid European investigators and pharmaceutical companies in trial planning and processing of all the material required as well as provide contacts and information on existing trials. It is hoped that this web-based information site will be utilized by EU investigators in the future.

Impact beyond diabetes

Type 1 diabetes is an autoimmune disease and therefore DIAPREPP findings with respect to pathogenesis and novel technologies that are relevant for type 1 diabetes become relevant for other autoimmune diseases. Moreover, some are also relevant to immune response per se which include infectious immunity and vaccination. For example, assays and technologies that were developed to obtain antigen specific human monoclonal autoantibodies or the T cell receptor of antigen specific T cells can have broad applications to these other settings by enabling better understanding of antigenic targets, monitoring responses to antigen and vaccine, examining responses to new variants, and developing and testing new vaccines. DIAPREPP has also provided new standards with respect to metabolomic analyses, assessing reproducibility, the effects of sample storage, and developing new analytical tools; and autoantibody measurements with new levels of international standardization.

Dissemination

Dissemination of new knowledge within the scientific community is an intrinsic interest of research, and aims at strengthening and reinforcing the European research activities by multiplication and initiation of networking and collaborations beyond the consortium. Dissemination of results and new knowledge obtained in DIAPREPP is of high priority, emphasized by the dedication of a workpackage to dissemination issues. The areas focussed on by DIAPREPP were publication and public information, management of intellectual property, and commercial products.

Publication remains the main measure of productivity at the scientific level and the widest reaching means of scientific communication. At the time of completing the report, DIAPREPP includes 40 published or in press original articles and a further 15 reviews, commentaries or chapters. Of these, 34 are directly related to DIAPREPP deliverables/objectives, and the remainder have used DIAPREPP findings or resources to obtain their findings which indirectly contribute DIAPREPP objectives. A number of other manuscripts have been submitted for publication. Highlight publications include Oresic et al, Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes, J Exp Med, 2008, and Pflueger et al, Age- and Islet Autoimmunity-Associated Differences in Amino Acid and Lipid Metabolites in Children at Risk for Type 1 Diabetes, Diabetes 2011 (together with an accompanying commentary) in the area of metabolomics; Winkler et al, An interferon-induced helicase (IFIH1) gene polymorphism associates with different rates of progression from autoimmunity to type 1 diabetes, Diabetes 2011; and Ziegler et al, Accelerated progression from islet autoimmunity to diabetes is causing the escalating incidence of type 1 diabetes in young children, J Autoimmunity, 2011 in the area of pathogenesis; and Lampasona et al, Diabetes Antibody Standardization Program: First Proficiency Evaluation of Assays for Autoantibodies to Zinc Transporter 8, Clin Chem 2011 in the area of biomarkers. As expected in a

relatively short project period (41 months), major DIAPREPP findings are still unpublished. Some have been submitted to high impact journals, and many are in preparation.

Presentations at scientific meetings. Data generated by DIAPREPP have been presented at numerous international and national scientific meetings as oral presentations or posters. Moreover, the senior investigators from the partner institutes have had numerous opportunities through invited lectures to present DIAPREPP data and show EU FP7 support for the work. In total, DIAPREPP has been represented by DIAPREPP members in 87 presentations (excluding the restricted DIAPREPP meetings) during the project period. A highly relevant aspect of what has changed in the presentations is that while previously the individual DIAPREPP investigators usually spoke about their own studies alone, the DIAPREPP interaction has made the these investigators distinctly more aware of their competitors as providing comparative and complementary data, allowing them to hughlight also the findings or their DIAPREPP collaborators. DIAPREPP also organized one open scientific symposium at the occasion of the 2010 European Association for the Study of Diabetes meeting and co-sponsored another symposium at this meeting. Since a number of DIAPREPP scientists are members of prominent international bodies, networks, and studies, relevant DIAPREPP findings have also been discussed at strategic planning meetings for future studies and policies. For example, partners 4, 5, and 13 are the European members of the Steering committee of the international TEDDY study which examines environmental triggers of type 1 diabetes, and together have enrolled over 60% of the study cohort. They have major input into the studies and analyses performed in TEDDY and have used DIAPREPP findings to guide this. Similarly, partner 1 co-chairs the islet autoantibody harmonization committee and has been able to introduce methods and the ZnT8 autoantibodies to international studies.

Considered important with respect to dissemination has been the training of younger scientists for the future. DIAPREPP has trained numerous PhD students and young post-doctoral´scientists, sometimes giving them the opportunity to give their firrt scientific presentation. DIAPREPP has included a number of scientific meetings amongst the investigators always encouraging participation of the younger scientists, and has had 5 training workshops for scientific methodology and application. Thus, DIAPREPP has hopefully made an important contribution to the training of future leaders in type 1 diabetes research.

The website has also been an important medium for communication. All published material which is from the DIAPREPP project is listed on the website. Currently there are 55 publications listed. In addition, the website links to all the websites of the partners allowing the public access to additional diabetes research that is ongoing in the partner institutes, as well as to organizations relevant to type 1 diabetes. Meetings relevant to DIAPREPP, including internal or DIAPREPP sponsored meetings or events are also posted. The website will be kept open and updated by the coordinator institute until the scientific publications of DIAPREPP findings are completed.

Non-scientific publications have been an important aspect of communication to the public. There have been official lay and business journal articles and television presentations which are dedicated to DIAPREPP or in which DIAPREPP has been discussed. Moreover, DIAPREPP investigators frequently speak to patients with type 1 diabetes and to representatives of patient groups, and even government officials about type 1 diabetes and therefore continuously update the public at these levels regarding relevant DIAPREPP findings. A prime example was the recent presentation by partner 5 (Ziegler) at the 2011 World Health Summit of the impact of global change on diabetes in which she was able to include relevant DIAPREPP findings.

With respect to commercialization, the intellectual property related to measuring ZnT8 antibodies in diabetes mellitus is covered by the following patents:-

1. European patent no EP1563071 B1 "Proteine specifique des cellules pancreatiques beta des Ilots de Langerhans et ses applications" and US Patent no 7,851,164 B2 "Protein specific to pancreatic beta cells in Islets of Langerhans and applications thereof" and related patents in other countries (Mellitech).

2. Patent application PCT/US2007/089125 "Diagnostic and therapeutic target for autoimmune diseases and uses thereof" (University of Colorado).

RSR Ltd (DIAPREPP Partner) has exclusive License Agreements to thesepatents. The ZnT8Ab ELISA kit was been developed by RSR Ltd and assessed in the Diabetes Autoantibody Standardization Program 2010 where it scored 99% specificity with 68% sensitivity, comparing well with in-house 35S-based assays. ZnT8Ab ELISA kits manufactured by RSR Ltd are now available with sales in the USA, Japan, Europe and other countries proceeding. The market for ZnT8Ab ELISA kits is estimated to be similar to that of the other markers of autoimmune diabetes GADAb and IA-2Ab. The kit description can be found at: http://www.rsrltd.com/pdf%20ifu/ZnT8%20Ab%20ELISA.pdf.

In addition, a patent application is pending for the technology developed by Partner 1 in regards to the identification of antigen-specific T cell receptors and their peptide epitopes.

List of Websites:

Updates of DIAPREPP can be found at http://www.diaprepp.eu.