Early signs of disease in type 1 diabetes

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Word count for entire manuscript: 4752

Abstract

During the past twenty years since its initiation, the Finnish Diabetes Prediction and Prevention Project (DIPP) has collected longitudinal biological samples from children with a human leukocyte antigen gene – conferred risk for type 1 diabetes. This biobank has provided detailed sample series that map the progression from health to disease, as well as the healthy maturation of risk-matched children, and has thus been the focus of a large body of research into the etiology of type 1 diabetes. In this review we summarize recent findings that have been reported in the context of the analyses of these samples, focusing on observations derived from the use of proteomics, metabolomics and transcriptomics.

Key words:

Longitudinal studies

Biological markers

Proteomics

Metabolome

Transcriptome analysis

Although type 1 diabetes (T1D) has been primarily associated with genetic susceptibility the variable outcomes of genetically alike individuals, identical twins in particular, has supported the influence of the surrounding environment, including early exposure to different dietary components, infections and hygiene (1). To determine the influence of such subtle factors and identify the triggering events that promote this autoimmune disease, wide scale screening and periodic monitoring of the study population is necessitated. During the past twenty years since the establishment of the Finnish Diabetes Prediction and Prevention Project (DIPP) (2), new born children from three major Finnish cities have been screened for T1D risk conferring variants of the human leukocyte antigen gene (HLA-DQB1). Children recruited into the study participate by visiting the clinic every three to six months and provide biological samples and/ specimens. Blood, serum and stool samples have been collected and there are now in excess of one million serum samples stored. Within this extensive collection there are numerous series that exemplify maturation and a healthy outcome, as well as reflecting the progression towards T1D. With carefully controlled sample collection, division and storage, this resource has been the seed of a vast body of high quality, ground breaking research into the aetiology of T1D. Notably, in the past two decades new and improved technologies have emerged to facilitate the determination of biological components in this prospective sample series.

Currently the situation remains such that only by the measurement of a panel of serum borne autoantibodies that the indications of an autoimmune attack can be detected. These include islet cell autoantibodies (ICA), antibodies to insulin (IAA), glutamic acid decarboxylase (GAD), protein tyrosine phosphatase (IA2) and zinc transporter 8 (ZnT8) (3, 4). A body of research from the DIPP study has considered the nature of these autoantibodies and their underlying risks and order of presentation (5-10). For instance, in the evaluation of the data from 520 children who progressed to T1D, IAA was the most common primary autoantibody (320 children), appearing at around the age of 2 years (11). Overall, including the data from several other international research centers, it has been observed that the majority of children at risk of type 1 diabetes who displayed multiple autoantibody seroconversions will progress to diabetes within 15 years (12). The timing between seroconversion and diagnosis is highly variable, ranging from months to many years and in the order of 2-5% of patients clinically

diagnosed with T1D are negative for known antibodies (13-15). Clearly there is a need for better biomarkers (16). In this review we consider he use of metabolomics and proteomics for blood serum and the application of transcriptomics analysis to whole blood samples.

Proteomics

Previous T1D orientated proteomics studies have targeted the serum proteome of newly diagnosed patients and healthy controls (17-19). However, the emergence of prospective sample collections, such as that created by the DIPP project, has created the opportunity to characterize the temporal changes in the serum proteome of children *en route* to T1D (20). Using samples from DIPP we have applied quantitative mass spectrometry based proteomics approaches to profile the time course from early infancy to seroconversion and diagnosis, comparing these with measurements from healthy controls. As albumin alone contributes over 50% of the protein content by weight, and the 14 most abundant serum proteins contribute in the order of 95% of the protein content, depletion of the high abundance proteins was used to enable the qualitative and quantitative analysis of lower abundance components (21). We used isotopic labelling of the proteolytic digests, which facilitated parallel processing of sample mixtures and compensated for quantitative differences in fractionation of the proteolytic digests prior to MS characterization, thus providing further detail of the lower abundance serum proteome. For this the 8-plex variant of isotope tagged relative and absolute quantification (iTRAQ) reagents was used in the comparison of ~180 samples from 13 case control pairs (22, 23). This approach enabled the identifications and comparisons of up to 500 proteins, spanning a concentration range of five orders of magnitude. We also used a label free approach for quantitative proteomics (LFQ) and determined complementary data from six case-control pairs (84 samples). Notably there was excellent overlap amongst the proteins most frequently quantified in all samples within subjects and between methodologies. Together the two methods provided quantitative profiles of in the order of 250 proteins across multiple samples and subjects.

In the analysis of these data we considered time based longitudinal trends, in which we observed a greater similarity in the cases alone, rather than the mixed or control only groups. Amongst the children progressing towards T1D increasing abundance of proteins related to acute inflammatory response, complement activation, humoral and adaptive immune response was observed. Notably, from hierarchical clustering of the data, differences in abundance of the complement proteins were frequently observed. To gain an overview of how the relative abundance of the proteins behave relative to the complement system, correlations were calculated for the proteins detected in the cases and controls relative to the one of the central complement components, complement 5. This analysis indicated that for the children progressing to T1D there were more pronounced changes in the complement proteins, in particular with proteins of the membrane attack complex and antigen-antibody complex.

A rank product approach was used to detect proteins that were differentially abundant at specific time windows relative to seroconversion and diagnosis. A panel of differential abundant proteins were identified including markers detected prior to seroconversion.
Using the top scoring pair method two proteins (APOC4 and AFAM) were observed to classify these data with a high success rate. Subsequent determination of receiver operator characteristics of the data indicated and area under the curve of 0.85. Overall these data demonstrated the possibility to distinguish at risk children on the basis of protein profiles measured in their serum.

Continued work from our group includes extended discovery measurements from a separate cohort and validation of the current markers using a targeted mass spectrometry platform. For the latter a selected reaction monitoring (SRM) approach is in use. In future measurements we foresee that such methodology could be used to reliably screen a wide panel of known targets to assist in risk characterisation and diagnostics.

Metabolomics

In their seminal publication, Orešič *et al*. (24) observed characteristic metabolite patterns in children who later developed T1D. Notably, with the rapid sample analysis times achievable for polar metabolites with two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS) and the use of ultra-high performance liquid chromatography (UHPLC) together with electrospray mass spectrometry (for lipids), significant sample numbers were characterized. Using UHPLC-MS, lipid profiles were determined for 56 children who progressed to type 1 diabetes and 73 controls, from a set of 1,196 samples. Their observations included reduced serum levels of succinic acid and phosphatidylcholine (PC) at birth and reduced and decreasing levels of serum triglycerides and multiple phospholipids throughout follow up. They proposed that on the basis of the reduced levels of PCs that the children could be choline deficient from birth. Moreover these differences could reflect differences in the microbiome of these children, which in turn may result from the maternal diet or intestinal flora during pregnancy. The overall model interpreted from these data suggested that these differences may affect the immune system in the offspring and that dysregulation of metabolism precedes β cell autoimmunity.

In later work from the same group, the influence of *in utero* effects was considered in samples analyzed from a Swedish study population (DiPiS) (25). Here they observed that the cord-blood lipidome was affected in children diagnosed with type 1 diabetes before the age of 8 years (76 children diagnosed with type 1 diabetes before 8 years vs. 76 healthy control subjects matched for HLA risk, sex, and date of birth, as well as the mother's age and gestational age). In keeping with the earlier observations, a significant decrease in cord-blood phosphatidylcholines and phosphatidylethanolamines was seen in children diagnosed with type 1 diabetes before 4 years of age. In summary they emphasized the potential role of phospholipids as mediators of the immune system that might contribute to early induction of islet autoimmunity, and proposed that metabolomics of umbilical cord blood may be used identify children at increased risk for type 1 diabetes. With further measurements of samples from Finnish subjects in the DIPP study (26), Orešič *et al.* considered the cord serum lipidome in infants who developed T1D relative to matched healthy controls and those

who developed several antibodies. More specifically these were as follows: infants that developed T1D (N = 33); infants who developed three or four (N = 31), two (N = 31), or one (N = 48) islet autoantibody during the follow-up; and controls (N = 143). The controls were matched for sex, HLA-DQB1 genotype, city of birth, and period of birth. These measurements revealed distinct cord blood lipidomic profiles in T1D progressors that were characterized by reduced levels of the major choline-containing phospholipids in agreement with earlier findings from the DIPP (24) and DiPiS (26) studies. On the basis of these observations they concluded that the reduction in choline-containing phospholipids in cord blood is specifically associated with early progression to T1D but not with development of β-cell autoimmunity in general.

In follow up studies from this group similar analyses were made from sample series from Germany and Sweden (27). Samples from the German BABYDIAB study (27) were considered in the context of autoantibody status and the age of seroconversion. Here the metabolite profiles from 35 children who progressed to islet autoimmunity and 35 controls were determined. In these groups there were children who developed T1D either up until the age of 2 years (n = 13) or at age 8 years or older (n = 22). In addition to the differences in the profiles of triglycerides and polyunsaturated fatty acid-containing phospholipids, they observed that children who developed autoantibodies by age 2 years had twofold lower concentration of methionine as compared to those who developed autoantibodies in late childhood or remained autoantibody negative. This study suggests that age is an important factor to consider when studying the interplay of metabolic and immune-system factors in progression to T1D.

Transcriptomics

Transcriptomics has been used to search for changes in the peripheral blood of children with various stages of the T1D disease process. Early studies mainly compared individuals with T1D to controls, and more recently samples from autoantibody positive children has been analysed (28). The time from the appearance of first T1D associated autoantibodies to the clinical disease can vary from a few weeks to two decades and hence it would be helpful if there were markers that could be used to

distinguish those who may progress rapidly to T1D from the slow progressors. Jin *et al.* performed microarray analysis of peripheral blood mononuclear cells from antibody positive children who developed T1D (n = 21) and compared this with similarly antibody positive children that had not yet developed T1D within the time span of several additional years (N = 15) (28). The children compared were matched by age, gender, genetic risk and incidence of disease in first degree relatives. The differentially abundant genes that were detected were validated by RT-PCR of peripheral blood total RNA from antibody positive children that progressed to T1D (n = 18) and their antibody positive matched controls (n = 50). From these profiling and validation measurements, a panel of five genes (*BACH2*, *IGLL3*, *TXNRD5*, *CDC20*, and *EIF3A*) were identified that might serve as potential biomarkers for risk stratification of antibody positive subjects and may be involved in T1D pathogenesis.

Our first study based on DIPP sample collection was a proof-of-concept and showed the value of analysing longitudinal whole blood samples from children who develop autoantibodies or T1D and their carefully matched controls (29). While the number of patients and controls was limited, the study demonstrated differential expression of 520 probe sets between the cases and controls, several of which belonged to pathways involved in the immune system. The longitudinal samples also revealed that the responses were dynamic in nature. Encouraged by these results we increased the number of samples to carry out a transcriptome analysis (30). Altogether 368 blood samples (191 from seroconverted and TID children, and 177 from healthy controls) from the DIPP study were analysed. The results revealed that gene expression signatures that reflect immune system activation were observed already before T1D associated autoantibodies could be detected.

A closer examination of these data revealed that some 300 probes were detected as differentially expressed between the seroconverted children and their matched controls. The retinoic acid inducible gene 1 (RIG-I)-like receptor signalling and cytosolic DNA-sensing pathways were enriched among the upregulated genes before the onset of clinical T1D. During this period the differentially expressed genes were also enriched of genes localised within T1D-associated susceptibility loci and included

major histocompatibility complex class I C gene (*HLA-C*), the protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), and IKAROS family zinc finger 1 (*IKZF1*). We discovered that a distinct interconnected IRF7-centered transcriptional network was activated in T1D progressors. This network of 32 genes also included STAT1, STAT2, and TRIM22 – all involved in interferon and innate immunity responses. Besides these, altogether, 95 unique transcription factors were differentially expressed in children who progressed to T1D.

To identify transcriptional signatures characteristic for distinct phases of the autoimmune process we analysed the data according to several time windows (i.e. before seroconversion, at seroconversion, 6-18 months after seroconversion, 1−2 years before clinical T1D diagnosis and at clinical T1D diagnosis). In the time-windows before, at, or 6−18 months after seroconversion, 604, 263, and 1,578 probes were identified as differentially expressed, respectively; 103 probes were identified that were common to all three time windows. During the intervals of 1−2 years before clinical diagnosis and at clinical diagnosis, 1,609 and 876 probes were differentially expressed between the cases and controls. Interestingly, the cytokine receptor-cytokine interaction pathway was one of the upregulated functions at T1D diagnosis. In summary, the changes were highly dynamic relative to different stages of T1D pathogenesis, suggesting that different transcriptional signatures are measured depending on the stage of progression of the disease. However, a module consisting of interferon-induced transcripts was constantly upregulated in every time-window.

To study if nucleotide polymorphisms (SNP) had an influence on differential gene expression, we carried our genotyping using the Immunochip (Illumina). Among the 118 differentially expressed genes where a cis eQTL (cis acting expressed quantitative trait loci) effect was found, were genes that have been associated with autoimmune diseases including T1D. Interestingly, such cis eQTL effects were detected in several genes that were in the centre of the interferon activated network found in children with T1D associated autoantibodies. Our findings showed that innate immune response to type one interferons can be detected throughout the progression of the disease – even before the appearance of diabetes associated autoantibodies. Since α and β interferons themselves were not

differentially regulated by the blood cells suggest that the response seen is caused by cytokines secreted elsewhere. In the paper by Ferraira *et al.* (31), which was published back to back with our study, an interferon inducible signature was identified *in vitro* with PBMCs from healthy controls, and then evaluated in terms of the onset of autoimmunity and progression to a clinical diagnosis of T1D, using samples from the German, BABYDIET, prospective birth cohort of children at high risk of developing T1D. In keeping with the findings from our study, an interferon signature was already detected before seroconversion in children developing T1D. It was hypothesized that upregulation of IFN could be associated with observations supporting the increased risk of islet autoimmunity with viral infection, and in particular with the pathogenic role of enterovirus coxsackievirus B1in the induction of β-cell autoimmunity (32).

Another recent prospective study, DIABIMMUNE, has been aimed at testing the hygiene hypothesis in T1D by comparing a range of factors in longitudinal samples collected from children born in Estonia, Finland and Russian Karelia. Children from Finland have the highest incidence of T1D, whereas Russian children have the lowest incidence of T1D among these three countries. Also Estonian children have a lower incidence of T1D than Finnish children. As these differences cannot be explained by the HLA genotype, it has been proposed that perhaps the standard of living plays an important role. We were interested in analysing umbilical cord blood collected from these infants to find out if there are differences in activation of immune system already in born in Finland, Estonia find out if there were differences in activation of immune system already *in utero*. Genome wide transcriptomic analysis revealed that gene expression profiles were very different in cord blood from children in Russian Karelia as compared to cord blood from children in Finland (33). Transcriptomes of children born in Estonia were more similar to those of children born in Finland, than to transcriptomes of children born in Russian Karelia. The genes found to be upregulated in infants from Russian Karelia, as compared to Finnish and Estonian infants, were enriched with genes involved in the activation of the innate immune system. Moreover, comparison of the data with previous studies that had investigated gene expression profiles in newborns and in one year old children revealed that cord blood of infants born in Russian Karelia had gene expression profiles characteristic for more

mature immune system that those of children born in Finland and Estonia. Our study was the first to discover differences in genes of the immune system in the umbilical cord blood samples from infants born in distinct standards of living. These findings support the hygiene hypothesis and suggest importance of immunomodulation by environmental factors already during pregnancy.

Future Directions and Perspectives

The detection of autoantibodies has to date provided the best indication of the threat and onset of T1D in genetically disposed individuals. With the complexities of the disease aetiology, there are several pathways towards T1D to be discovered. It appears that *in utero* effects may already influence the outcome. Viral and bacterial infections, microbiota, diet and vaccination programs are among the environmental factors likely to play a role. Besides T1D associated autoantibodies, additional informative and precise markers are needed to further divide subjects at risk from this heterogeneous disease into subgroups, such as those who progress rapidly to T1D and those who progress slowly, as well as according to putative causative agents and molecular mechanisms involved. Already from the recent transcriptomics, proteomics and metabolomics studies reviewed here, it is apparent that markers preceding the appearance of T1D associated autoantibodies can be detected. Identification and validation of multi-modal molecular markers will provide means to predict, diagnose and monitor T1D, and will be useful in the design and testing of prevention and therapies for T1D.

Acknowledgments

We would like to thank Dr. Matej Orešič for his valuable comments on our manuscript.
This work was supported by the Academy of Finland Centre of Excellence in Molecular Systems Immunology and Physiology Research, 2012–2017, Decision No. 250114, JDRF, The Sigrid Juselius Foundation and the National Technology Agency of Finland.

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