ENTEROVIRUS INFECTIONS AND EXPRESSION OF IRF7 NETWORK GENES IN TYPE 1 DIABETIC CHILDREN

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Tyypin 1 diabetes (T1D) on krooninen autoimmuunisairaus, joka on seurausta haiman insuliinia tuottavien β-solujen selektiivisestä tuhoutumisesta. Nykytiedon mukaan sekä ympäristö- että perintötekijät vaikuttavat tämän kroonisen sairauden syntyyn. Enterovirusten aiheuttamien infektioiden ajatellaan olevan eräs merkittävimmistä ympäristön riskitekijöistä, jotka voisivat olla T1D:een johtavan autoimmuuniprosessin käynnistymisen taustalla. Tarkkaa diabeteksen kehittymiseen johtavaa mekanismia ei kuitenkaan vielä tunneta. Synnynnäinen immuunipuolustus toimii ensilinjassa kehon puolustamiseksi infektiota aiheuttavia patogeeneja, kuten viruksia, vastaan. IRF7-geeniverkoston geenit, kuten IRF7, OASL, OAS1 ja IFIH1, toimivat osana synnynnäistä immuunipuolustusta viruksia vastaan.

Tämän tutkimuksen tarkoituksena oli tutkia enterovirusgenomin esiintymistä sekä neljän eri IRF7-geeniverkoston geenin (IRF7, OASL, OAS1 ja IFIH1) ilmentymistä tapaus-verrokkikohortissa. Kohortissa jokaista tapauslasta kohden oli kaksi verrokkilasta, tapaukset (n = 15) ja verrokit (n = 30) olivat kaltaistettu sukupuolen, syntymäajan, näytteenottoajankohdan, paikkakunnan ja HLA-DQB1 genotyypin mukaan. Kohortin tapaukset ovat lapsia, joille on syntymästä alkaneen seurannan aikana kehittynyt kliinisesti diagnosoitu T1D. Kohortin lapsilta kerätyistä perifeerisen veren mononukleaarisista soluista eristettiin totaali-RNA. Enteroviruksen RNA ja IRF7-geeniverkoston geenien ilmentyminen todettiin RT-PCR –menetelmällä. Enterovirusanalyyseissä templaattina käytettiin eristettyä totaali-RNA:ta ja geeniekspressio-analyyseissä lähetti-RNA:ta syntetisoitua cDNA:ta. Analysoitujen geenien ilmentymistasot laskettiin suhteessa 18S rRNA -ylläpitogeeniin.

Yksi tapausnäytteistä sekä yksi kontrollinäytteistä todettiin enteroviruspositiiviseksi. Näin ollen 1,45 % kaikista tutkimuksen tapausnäytteistä sekä 0,95 % kaikista kontrollinäytteitä oli enteroviruspositiivisia. Kaikista tutkituista näytteistä viruspositiivisia oli 1,15 %. Tutkimuksessa ei todettu merkittävää eroa analysoitujen geenien ilmentymisen suhteen tapausten ja kontrollien välillä. Joissakin kohortin näytteistä todettiin IRF7-geenin ilmentymisen väliaikainen nousu. Tämä voi olla seurausta esimerkiksi akuutista infektiosta verinäytteen ottohetkellä.

Yhteenvetona voidaan todeta, että tutkimuksessa ei löytynyt merkittäviä eroja tapausten ja kontrollien välillä enteroviruspositiivisuuden tai analysoitujen IRF7-geeniverkoston geenien ilmentymisen suhteen. Tutkimuksen tilastollista voimaa rajoittaa aineiston suhteellisen pieni koko ja tulos tulisi varmistaa laajemmassa aineistossa.

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Type 1 diabetes (T1D) is a chronic autoimmune disorder that results from a selective immune - mediated destruction of the pancreatic β cells. According to current knowledge both environmental and genetic factors play a role in the initiation autoimmune process leading to the onset of T1D. Enterovirus infections are one of the major candidates for environmental risk factors for T1D. However, the exact mechanisms leading to T1D are not known. Innate immunity functions as a first line of defence against invading pathogens such as viruses. The transcripts of IRF7 network genes, such as IRF7, OASL, OAS1 and IFIH1, function as a part of innate immunity against viruses.

The aim of this study was to evaluate the presence of enterovirus genome and the expression levels of four different IRF7 network genes (IRF7, OASL, OAS1 and IFIH1) in a selected case-control cohort. Two non-diabetic control children were pairwise matched for sex, date of birth, date of sample collection, hospital district and HLA-DQB1 -conferred genetic susceptibility to children who are known to have developed clinically diagnosed T1D. Total of 15 cases and 30 controls were selected into this study. Total RNA was extracted from peripheral mononuclear cell samples. Enterovirus positivity and gene expression levels were analysed using RT-PCR. Total RNA was used as a template in enterovirus RT-PCR analyses and cDNA reverse transcribed from messenger RNA as a template in gene expression RT-PCR assays. Enterovirus analyses were done using duplicates and gene expression analyses using triplicate of each sample. Expression levels of IRF7 network genes were calculated in ratio to 18S rRNA housekeeping gene.

One case and one control were found to be enterovirus positive. Consequently 1.45 % of all cases and 0.95 % of all controls and 1.15 % all samples were analysed to be enterovirus RNA positive. There were no clear differences in the expression of the analysed IRF7 network genes between the cases and their matched controls. Gene expression levels of IRF7 peaked in some cases and controls indicating presence of factors, such as an acute infection, leading to activation of gene expression.

As a conclusion, there was no significant difference between case and control groups in enterovirus positivity or in IRF7 network gene expression levels. The reason for observed results might be limited sample number. In order to detect significant differences from a normal biological variation more samples should be analysed.

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Tuomas Mäntylä

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ABBREVIATIONS

bp	base pairs
DIPP	diabetes prediction and prevention
GADA	glutamate decarboxylase autoantibody
HLA	human leukocyte antigen
IA-2A	islet-antigen 2 autoantibody
IAA	insulin autoantibody
ICA	islet-cell antibody
IFIH1	interferon induced with helicase C domain 1
IRF7	interferon regulatory factor 7
MDA5	melanoma differentiation-associated protein 5
OAS	2'-5'-oligoadenylate synthetase
OASL	2'-5'-oligoadenylate synthetase-like
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pfu	plaque forming unit
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
T1D	type 1 diabetes
ZnT8A	zinc transporter-8 antibody

1 INTRODUCTION

1.1 Type 1 diabetes

Type 1 diabetes (T1D) is an increasing healthcare problem around the world especially in developed countries. The incidence of T1D has been increasing rapidly globally since 1950's. For the past few decades worldwide incidence has been increasing by 3 – 5 % per year (1, 2). It is estimated that the prevalence of T1D can even be doubled in the forthcoming few decades.

Incidence of diabetes is higher in Finland than in any other country in the world. T1D is usually diagnosed before age of 30, generally during childhood or adolescence. However, the onset of T1D can occur at any age: 10 – 15 % of all T1D cases are diagnosed in patients older than 30 years (3). Approximately 500 new cases of T1D are diagnosed in Finland annually.

T1D is a chronic autoimmune disorder that results from a selective immune mediated destruction of the pancreatic β cells of the islets of Langerhans. This loss of β cells leads in insulin deficiency and hyperglycemia which can manifest itself initially via symptoms such as excessive urination, thirst and weight loss. Chronic hyperglycemia can further cause or predispose to serious health complications including ketoacidosis, kidney failure, heart disease, stroke and blindness. These complications affect patients' prognosis and quality of life.

Susceptibility to T1D is modulated by a number of risk genes, with HLA genes contributing half of the genetic susceptibility (4). The other half is caused by more than 50 non-HLA genes. People with family history of T1D have approximately 10 – 15 times greater risk of developing T1D as compared to people with no family history of T1D. HLA genes HLA-DR3 or HLA-DR4 is present in 90 % – 95 % of patients with T1D compared with 45 % – 50 % in the general population (5). Although ~ 70 % of individuals with T1D carry risk-associated HLA genotypes, only 3 – 7 % of the carriers of such genetic risk are reported to develop diabetes (2).

The appearance of T1D associated autoantibodies in the peripheral blood reflects the initiation of β cell damaging process. These T1D related autoantibodies include islet-cell autoantibody (ICA), insulin autoantibody (IAA), glutamate decarboxylase autoantibody (GADA), protein tyrosine phosphatase-related islet antigen 2 autoantibody (IA-2A) and zinc transporter-8 antibody (ZnT8A). Out of the T1D patients, 85 % – 90 % have at least one, and usually multiple T1D related

autoantibodies (5). The autoimmune process and peripheral blood autoantibodies may be present for several years, accompanied by ongoing destruction of β cells, before the disease becomes clinically apparent. At the time of diagnosis, the number of remaining β cells has decreased down to 10 % – 20 % of the initial cell number. This decrease in β cell number is unable to sustain compensatory insulin secretion at a level sufficient to maintain normal blood glucose level.

Although there are several subcutaneously administered insulin analogues and oral medications available for the treatment of type 1 diabetes, none of these are either able to heal T1D or stop the progression of the chronic disease. Furthermore, according to currently existing knowledge it is not possible to stop the autoimmune process leading to onset of clinically diagnosed T1D. Regardless of extensive research, the actual trigger(s) inducing the autoimmune process leading to β-cell death are still unknown. Nevertheless, the currently available epidemiological data indicates that the rising incidence in T1D has to be attributed to environmental changes. Over the years several different factors, such as viral infections, gut's bacterial flora, cow's milk, gluten and vitamin D deficiency, have been proposed to be possible candidates (2, 6).

1.2 Enteroviruses and type 1 diabetes

Viruses, especially enteroviruses, have long been suspected as environmental agents that can initiate disease process leading to onset of type 1 diabetes in humans (7-9). Enteroviruses are small, single stranded non-enveloped positive-sense RNA viruses belonging to the picornavirus family. There are more than 100 defined human enterovirus serotypes. These include viruses such as poliovirus, coxsackie A and B viruses and Echoviruses. Enteroviruses are transmitted through faecal-oral or respiratory route and primary replication occurs in the gut mucosa. Most enterovirus infections are mild or asymptomatic, however, some serotypes are able to cause severe diseases, such as myocarditis, paralysis and meningitis in small proportion of infected individuals (8, 10, 11). Enteroviral infections are common in infants. Enterovirus is most readily detectable in faeces, where it can usually be found for up to 4 weeks after infection. In some cases enterovirus is detectable for a few months in faeces. During the time of infection enterovirus may also be detectable in blood for a few days. Viremia, i.e. presence and spread of viruses via blood, can lead to dissemination to other target organs. Increased frequency of enterovirus antibodies has been reported in patients with type 1 diabetes compared to matched control children (12). Also it has been shown that T1D is associated with enterovirus infections in gut mucosa (4). Several studies have detected enteroviral RNA in the blood of prediabetic individuals and at the onset of diabetes,

however, it is unknown whether this finding reflects persistent or acute infection. Recently Yeung et al. reported a clinical significant association between enterovirus infection and T1D based on systematic review and meta-analysis of existing data (13).

1.3 Innate immunity and viral infections

Innate immunity consists of several different mechanisms that are utilized as a first line of defence against invading pathogens such as viruses. One of these mechanism include peripheral blood mononuclear cells (PBMCs) that are capable of recognizing viruses and other invading pathogens via their pattern recognition receptors (PRR) such as toll-like receptors (TLRs). The interaction between PRRs and their ligands results in activation intracellular signalling and regulation of gene expression.

1.4 IRF7 network

Interferon regulatory factor 7 (IRF7) gene encodes four isoforms (IRF7A-D) of transcription factors that regulate the transcription of type I interferon (IFN; i.e. IFN-α and IFN-β) (14, 15). IRF7 plays a critical role in the innate immune and adaptive immune responses against DNA and RNA virus infections. Normally IRF7 exists in an inactive monomeric form in the cytoplasm of uninfected cells. Following an infection, phosphorylation of IRF7 is triggered. This leads to dimerization of phosphorylated IRF7 proteins and subsequent localization into cell nucleus where IRF7 can activate transcription of target genes (14). Aberrant production of type I IFNs has been reported to be associated autoimmune disorders. Heinig *et al.* have proposed that IRF7 network genes and their regulatory locus have role in the pathogenesis of T1D (16).

Interferon-induced helicase C domain 1 (IFIH1) gene, which is also known as MDA5 (melanoma differentiation-associated protein 5), encodes for an intracellular protein that is known to recognise double-stranded RNA during replication of certain viruses including enteroviruses and mediate innate immune response. Thus MDA5 functions as a cytoplasmic PRR (11). Polymorphisms in the IFIH1 gene have been associated with T1D. The mechanism by which the IFIH1 polymorphism modify the risk of T1D is presently unknown, but most works agree that the minor alleles

(protective from T1D) are associated with diminished transcription or reduced function of the IFIH1 (17-20).

2'-5'-oligoadenylate synthetase (OAS) family consists of three genes encoding active OAS enzymes (OAS1-3) and an OAS-Like (OASL) gene encoding an inactive protein (21). The encoded OAS1 protein is an interferon-induced, dsRNA-activated antiviral enzyme which plays a role in innate antiviral response. Activation of latent OAS1 by dsRNA leads to activation of latent RNase L, which in turn results in viral RNA degradation and inhibition of viral replication. 2'-5'-oligoadenylate synthetase-like (OASL) gene encodes a protein that is able to bind to double stranded RNA. OASL-protein has been shown to have antiviral activity against picornaviruses (22).

2 AIM OF THE STUDY

Enterovirus infections are one of the major candidates for environmental risk factors for T1D. Several different risk genes for T1D have also been identified. However, there are only a few reports concerning the expression of innate immune genes in genetically susceptible persons. The purpose of this study were to evaluate the presence of enterovirus genome and basal expression of four different IRF network genes (IRF7, OASL, OAS1 and IFIH1) in the peripheral blood mononuclear cells of children who developed T1D and in non-diabetic control children.

3 MATERIALS AND METHODS

3.1 Sample collection and selection

All study subjects were participants of the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) project that has been ongoing since year 1994. The protocol of DIPP study has been approved by the Ethical committee of the University of Tampere. Written consent has been obtained from all study subjects and/or their parents. In the DIPP study the families of all newborn infants at the University Hospitals of Oulu, Tampere and Turku are offered a possibility for screening of newborn infants for HLA risk genes for T1D. Screening is done from a blood sample taken from umbilical cord at the time of the birth. Families with a child carrying an increased genetic susceptibility for T1D are invited to participate in prospective follow-up starting from birth. Blood samples are then collected from study participants every three months until two year of age and every six to 12 months intervals thereafter until age of 15. Collected blood samples are analysed for T1D-associated autoantibodies (12) (www: http://research.utu.fi/dipp/index.php?mid=8&language=fi). Children who seroconvert to autoantibody positive are followed and blood samples drawn subsequently every three months until age of 15.

Peripheral mononuclear cells (PBMCs) have been separated from the whole blood samples collected during the follow-up of DIPP children. Blood samples were drawn into commercially available blood collection tubes (BD Vacutainer® CPT™ Cell Preparation Tubes with Sodium Heparin, Becton Dickinson, Franklin Lakes, New Jersey, USA). CPT-tubes were centrifuged for 20 min at 2700 rpm at RT (CR412 –centrifuge, Jouan S.A., Saint-Herblain, France). PBMC pellet was resuspensed into plasma, transferred into new centrifugation tube and centrifuged for 10 min at 1700 rpm at RT. Plasma was then removed and PBMC cell pellets were snap frozen by dipping samples into liquid N_2 and stored at – 70 °C until time of use.

Total of 15 cases and 30 controls were selected into this study: Two persistently autoantibody negative control children were matched with each case, based on the date of birth, gender, hospital district and HLA-DQB1 genotype. All available PBMC sampling time points from 4 cases and their 8 respective controls were analysed (see **Table I** for sample information). From the remaining 11 cases and 22 controls one PBMC sample collected before and one sample taken after seroconversion into autoantibody positive were analysed.

Table I: Sample information

Extraction kit	AllPrep Mini	RNeasy 96
Number of cases	4	11
Number of controls	8	22
Number of samples	108 (47 case time-points, 61 control time-points), mean 9 per each child (min=4 samples/children, max=16 samples/children)	66 (22 case time-points, 44 control time-points), 2 time points from all children (one before and one after seroconversion to autoantibody positive)

3.2 Preparation of RNA extraction controls

Control PBMCs were obtained from a healthy adult volunteer. Cells were separated from whole blood as described in section 3.1. After the second centrifugation step plasma was removed and the cells were resuspensed into RPMI cell culture medium (Gibco, Life Technologies Ltd, Paisley, UK) supplemented with 10 % foetal calf serum (Gibco). An aliquot cell suspension was mixed with trypan blue and cells were then counted with a haemocytometer under a light microscope. Next approximately 40 000 PBMCs were plated in 150 µl of cell culture medium per well on a 96 well plate. This was followed by infection of the cells with either 1 plaque forming unit (pfu) of coxsackievirus serotype B1 (CBV1) or with 1 pfu of ECHO-11 virus per cell. Cells were then incubated at + 37 °C and 5 % CO_2 for 48 h. Cells were harvested from the 96 well plate and centrifuged for 5 min at 1500 rpm at RT. Cell culture medium was removed and the cell were washed twice with warm phosphate buffered saline (PBS). After both of the washing steps cells were centrifuged 5 min at 1500 rpm at RT and PBS was removed by careful suction. Finally cell pellets were stored at – 70 °C until time of use.

3.3 RNA extraction

Two different commercially available extraction kits, AllPrep DNA/RNA/Protein Mini (Qiagen, Hilden, Germany) and RNeasy 96 (Qiagen) -kits, were used during this study to extract total RNA from PBMC samples (see **Table I**). H_2O was used as negative extraction control and coxsackieviruses of serotypes B1 and B3 (CBV1 and CBV3 respectively), ECHO-11 virus and PBMCs infected with CBV1 were used as positive extraction controls in RNA extractions. Both kits were used according to manufacturer's protocols. The extractions were carried out as follows:

Cell lysis was done by adding 600 µl of Qiagen RLT buffer on frozen PBMC pellet. Prior to this 10 µl of β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) was mixed per 1 ml of Qiagen RLT buffer. Cell pellets were then thawed at RT followed by mixing with 20 G needle attached to a 5 ml syringe. Cell lysates were then transferred to QiaShredder spin columns (Qiagen) followed by centrifugation at 12000 × g at RT for 2 minutes.

With Qiagen's AllPrep DNA/RNA/Protein Mini –kit the extraction of total RNA from cell lysates was continued by transferring lysates into DNA spin columns that were then centrifuged for 30 s at 8000 × g at RT. To obtain final ethanol concentration of approximately of 40 %, 540 µl of absolute ethanol was added into each flow through. Samples were mixed by pipetting, transferred to Qiagen RNeasy spin columns and centrifuged for 15 s at 8000 × g. Next 700 µl of Qiagen RPE buffer was added into each Qiagen RNeasy spin column followed by centrifugation for 15 s at 8000 × g at RT. The columns were then washed twice with 500 µl of Qiagen RPE buffer per column. After the first wash step the columns were centrifuged for 15 s at 8000 × g at RT whereas the second centrifugation step lasted for 2 min at 8000 × g at RT. Column bound RNA was eluted by adding 60 µl of RNase-free H_2O into each spin column followed by centrifugation for 1 min at 8000 × g at RT. **Figure 1** outlines AllPrep extraction procedure.

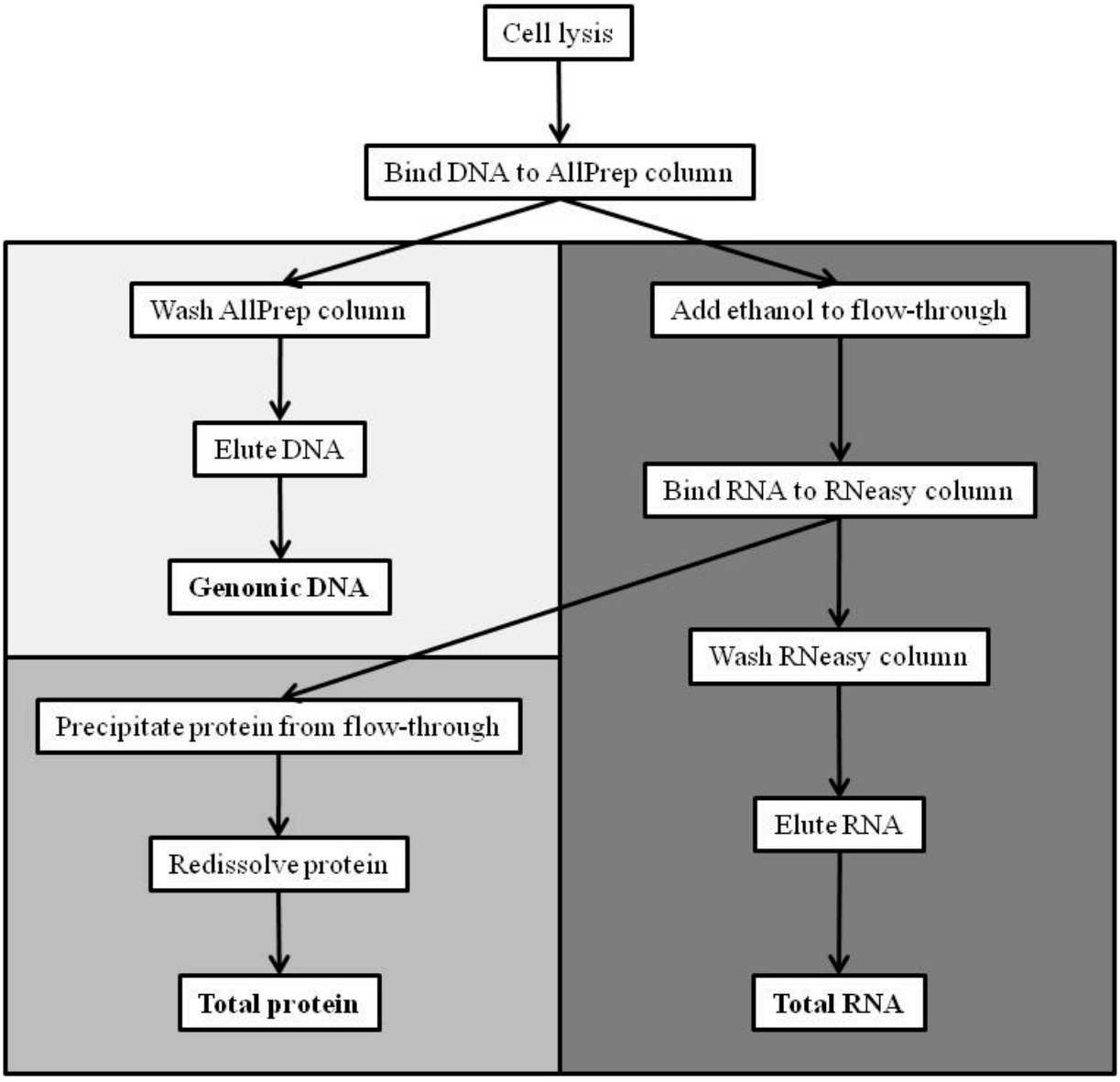

Figure 1: Schematic outline of sample processing protocol with AllPrep –kit, figure modified from AllPrep DNA/RNA/Protein Mini Handbook (23) **.**

With Qiagen's RNeasy 96 –kit, 540 µl of absolute ethanol was added into each cell lysate to obtain approximate final ethanol concentration of 40 % into each sample. After mixing the lysates by pipetting, the lysates were applied to wells on RNeasy 96 plate and centrifuged at 5600 × g for 4 min at RT. Next each sample on RNeasy 96 plate was washed by pipetting 800 µl of Qiagen RW1 buffer per well followed by centrifugation of the RNeasy 96 plate at 5600 × g for 4 min at RT. This was followed by pipetting 800 µl of Qiagen RPE buffer into each well and centrifugation at 5600 × g for 4 min at RT. Wash step with Qiagen RPE buffer was repeated with exception to the centrifugation step that lasted for 10 minutes in order to dry the membranes on RNeasy 96 plate. Finally the RNA was eluted by pipetting 60 µl of RNase-free H_2O per well and centrifuging the

plate at 5600 × g for 4 min at RT. For the duration of each centrifugation step the RNeasy plate was sealed with a sheet of AirPore tape. RNeasy 96 extraction protocol is shown in **Figure 2**.

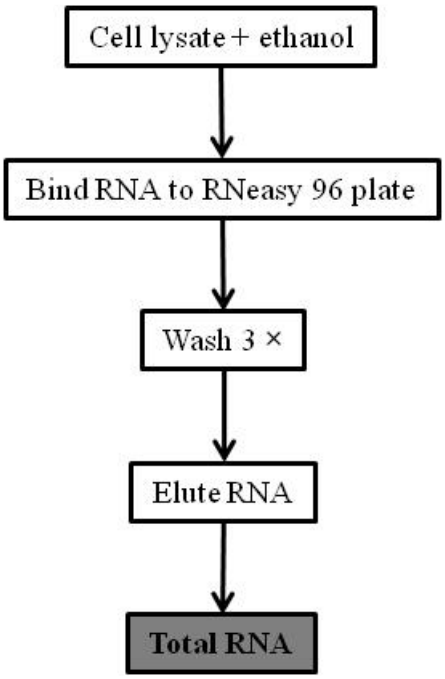

Figure 2: Outline of extraction protocol of RNA with RNeasy 96 –kit, figure modified from RNeasy 96 Handbook (24) **.**

All RNA containing samples and controls obtained from AllPrep and RNeasy 96 extractions were stored at – 70 °C until further use.

3.4 Reverse transcriptase reaction

Extracted RNA samples were reverse transcribed into cDNA by polymerase chain reaction with random hexamer primers. This was done as follows: First, for one reaction, 10 µl of extracted RNA and 1.4 µl of random hexamers (TAG Copenhagen A/S, Copenhagen, Denmark) were mixed and incubated at +70 °C for 5 min on a 96 well plate. Next the sample plate was placed on ice for cooling followed by a short centrifugation step to spin down the samples. RT master mixture was prepared by mixing 5.75 µl of dH_2O, 5 µl of RT enzyme buffer (Promega, Madison, WI, USA), 1.25 µl of 10 mM dNTP (Qiagen), 0.6 µl (24 U) of RNase inhibitor (Promega) and 1 µl (200 U) of Maloney murine leukemia virus reverse transcriptase (M-MLV RT) enzyme (Promega). RT master mixture was mixed with RNA/random hexamer –solution on a 96 well plate (total volume of each RT reaction adding up to 25 µl). This was followed by incubation of the plate at + 37 °C for 1 h.

The plate was stored frozen at – 70 °C until RT-PCR. The wells were covered with adhesive cover for the duration of each incubation step and storage at – 70 °C. Each 96 well plate contained negative control reactions where template was substituted for equal volume of dH_2O.

3.5 Real time-PCR

3.5.1 Enterovirus RT-PCR

The presence of enterovirus genome was analysed by RT-PCR as follows: RT-PCR master mixture was prepared by mixing multiplicity of following volumes: 5 µl of 2 × QuantiTect Probe master mixture (Qiagen), 0.86 µl of dH_2O, 0.9 µl of 10 µM 4- primer, 0.9 µl of 10 µM 636+ primer, 0.12 µl of 25 µM enterovirus probe 1, 0.12 µl of 25 µM enterovirus probe 2 and 0.1 µl of QuantiTect RT mix (see **Table II** for primer and probe sequences).

Table II: Enterovirus RT-PCR primers and probes and their sequences

Primer / Probe	**Primer sequence (5'-3')**
fwd 636	CGG CCC CTG AAT GCG GCT AA
rev 4-	GAA ACA CGG ACA CCC AAA GTA
Enterovirus probe 1	FAM-TCT GTG GCG GAA CCG ACT A-TAMRA
Enterovirus probe 2	FAM-TCT GCA GCG GAA CCG ACT A-TAMRA

Next 8 µl of master mixture was pipette per reaction (well) on an optical 96 well plate on ice. Finally 2 µl of extracted RNA was applied per well. The plate was covered with an optical adhesive cover and centrifuged for 1 min at 300 × g at + 4 °C. The reaction plates were stored frozen at – 20 °C until time of RT-PCR assay. Prior to start of RT-PCR run, plate was thawed on ice and centrifuged for 1 min at 300 × g at °C.

Each sample was analysed as duplicates using ABI Prism 7900 HT (Applied Biosystems, Life Technologies Ltd, Paisley, UK) RT-PCR equipment. The RT-PCR run consisted of the following steps: First a reverse transcription reaction was carried out at + 50 °C for 30 min. Next the plate was held at + 95 °C for 15 min to activate the HotStarTaq DNA polymerase. Then the samples were cycled through 50 cycles of programmed amplification (denaturation, + 94 °C, 15 sec; combined annealing and extension, + 60 °C, 1 min). Every assay included samples of known enterovirus serotypes as positive controls as well as several negative control samples. A sample was considered

enterovirus positive if RT-PCR analysis gave a positive signal in at least one of the duplicate assays.

3.5.2 IRF7 network RT-PCR

The expression of IFIH1, OASL, OAS1 and IRF7 were analysed using RT-PCR, 18S rRNA was used as a housekeeping gene. RT-PCR was carried out as follows: Master mixture was prepared by mixing 6.75 µl of dH_2O, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer and 10 µl of SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) per one reaction (see **Table III** for primer sequences and amplicon sizes). Next 18.8 µl of master mixture was applied per well on an optical 96 well plate followed by pipetting of 1.3 µl of template cDNA per well. The plate was then covered with an optical adhesive cover, centrifuged for 1 min at 300 × g at + 4 °C and stored frozen at – 20 °C until RT-PCR analysis.

Table III: IRF7 network primers and their sequences

Primer name	Primer sequence (5'-3')	Amplicon size (bp)
18S RNA forward	ATC CCT GAA AAG TTC CAG CA	154
18S RNA reverse	CCC TCT TGG TGA GGT CAA TG	
IFIH1 forward	GCT CAG AAA GCA ATG CAG AG	95
IFIH1 reverse	CAG ATT TGG GTG AAC TGT GG	
OASL forward	CGG GTG CTG AAG GTA GTC A	111
OASL reverse	CTC CTG GAA GCT GTG GAA AC	
OAS1 forward	CTG GCT GAA TTA CCC ATG CT	116
OAS1 reverse	TCT GAT ACC TCC TGG GAT CG	
IRF7 forward	AAG CTG GAA CCC TGG CTG T	122
IRF7 reverse	TCG TCA TAG AGG CTG TTG GCG	

Each sample was analysed as triplicate with each of the primer sets using CFX96 real-time PCR equipment (Bio-Rad). The RT-PCR program consisted the following steps: first the samples were denaturated for 30 sec at + 96 °C, then through 40 cycles of programmed amplification (denaturation, + 96 °C, 2 sec; annealing and extension, + 60 °C, 5 sec) and finally for melt curve analysis from + 65 °C to + 95 °C at 0.5 °C increments during 10 sec. Each assay included several negative control samples where H_2O was pipetted into the well instead of cDNA. End products were stored frozen at- 20 °C until time of agarose gel electrophoresis.

3.6 Calculation of gene expression levels of IRF7 network genes

Gene expression levels of IRF7, IFIH1, OASL and OAS1 were calculated using equation $2^{(-(\text{target gene-18S rRNA}))}$, where target gene and 18S rRNA values are averages of Ct-values obtained from triplicate RT-PCR analyses. Individual Ct-values were acquired from RT-PCR run data using the control software (Bio-Rad CFX manager, Bio-Rad) of CFX96 real-time PCR equipment. 18S rRNA is a ribosomal RNA with a size of 18 Svedbergs. It is present and expressed in eukaryotic cells at fairly constant level, this ribosomal RNA was used as a house-keeping gene in this study.

3.7 Agarose gel electrophoresis

The amplification of correct size cDNA sequence during RT-PCR run of each of the analysed IRF7 network gene was confirmed by agarose gel electrophoresis. Calculated amplicon sizes of each of the genes can be seen in **Table III**. The RT-PCR products were separated by running samples on 2 % agarose, 0.01 % ethidium bromide (EtBr) -gel prepared in 1× Tris-Acetate-EDTA (TAE) -buffer. Prior to loading 10 µl of sample was mixed with 2 µl of 6× DNA loading dye (Thermo Fischer Scientific, Waltham, MA, USA). Samples and 3 µl of GeneRuler 100 bp DNA ladder (Thermo Fischer Scientific) were applied to the wells of agarose gel and electrophoresis was carried out for 60 to 70 min at 120 V. Gels were photographed on an UV light to visualize product size.

4 RESULTS AND DISCUSSION

All case children from the cohort of this study have proceeded to clinically diagnosed diabetes. In addition, three control children have been analysed later on to gone through seroconversion to pancreatic islet-cell antibody (ICA) positive.

4.1 Detection of enteroviruses

The presence of enterovirus RNA was analysed by RT-PCR. Out of 15 cases and 30 controls analysed during this study, one case and one control was analysed to be enterovirus positive at one sampling time point (**Table IV**). Consequently 1.45 % of all cases and 0.95 % of all controls and 1.15 % of all samples were enterovirus RNA positive. Both of the enterovirus positive samples were from longitudinal cohort group. RNA of these samples was extracted using AllPrep extraction kit.

Table IV: RT-PCR analysis of enterovirus genome in selected DIPP samples

	Number of time points (samples)				**Enterovirus positive samples**		
	Extraction method						
	AllPrep	**RNeasy**	**Total No.**		**Total No.**	**% of cases / controls**	**% of all samples**
Cases	47	22	69	174	1	1.45	1.15
Controls	61	44	105		1	0.95	

Previously Oikarinen *et al.* reported that the age of the child had an influence on the frequency of enterovirus RNA in serum (12): In children who were younger than 6 months of age, only 1.0 % of the samples were enterovirus RNA positive compared with 3.5% of samples in 6- to 18-month-old children and 5.0% of samples in children aged 18-24 months. There after the frequency started to decrease and in children older than 2 year the frequency of enterovirus positive samples was 2.0%. Schulte *et al*. have reported detection of enterovirus RNA in four out of ten PBMC samples collected within month of T1D diagnosis from study patients (25). They did not detect enterovirus RNA from any of the control subjects. Plasma samples of two out of these four enterovirus positive patients were also found to be positive for enterovirus. The number of enterovirus positive children in the cohort of current study is somewhat lower than values reported by Oikarinen *et al.* and Schulte *et el.* (12, 25). Reason for the observed difference might be explained at least partly by differences in study designs: Number of analysed samples in the current study was almost five

times lower as compared to the study of Oikarinen *et al.*. Also Oikarinen *et al*. used serum as a starting material for RNA extraction whereas frozen cell pellets were used in current study. Schulte *et al.* collected blood samples within one month of diagnosis of T1D. In the current study analysed PBMC samples were collected before and after seroconversion into autoantibody positive. Time interval between the analysed samples was approximately one year. Thus the timing of blood sampling from study participants might have an effect on the presence of enterovirus genome in the blood. The shortest interval between blood samples in DIPP study is 3 months. The incidence of enterovirus infection shows a seasonal variation. Schulte *et al.* did not provide information of seroconversion. Therefore it cannot be excluded that initiation of autoimmune process leading to T1D manifestation might have started earlier and enterovirus positivity is a coincidence since these infections are still quite common.

There was no amplification of enteroviral RNA detected from the negative extraction controls (H_2O) or from the negative RT-PCR controls (H_2O) confirming that there was no contamination present during sample preparation or RT-PCR analysis. However, RT-PCR analyses of control samples containing RNA extracted either from CBV1, CBV3, ECHO-11 or from PBMCs infected with CBV1 were found to be enterovirus positive. This confirmed successful RNA extraction and proper functioning of the RT-PCR reagents and assay.

4.2 Expression of IRF7 network genes

No clear difference was detected in the expression of any of the IRF7 network genes between cases and controls (**Figures 3-5**). The expression of IRF7 mRNA peaked in some individual samples in both case and control groups (**Fig 3-5**). These increases in gene expression levels may have been caused by possible infections at the time of blood sample collection. The expression of IRF7 was higher throughout the analysed cases and controls than the expression of other analysed IRF7 network genes. In samples that had increased expression of IRF7 mRNA the expression of IFIH1, OASL and OAS1 was also increased.

From the longitudinal set of samples one case and control was analysed to be enterovirus positive as already mentioned previously. In these two enterovirus positive samples peaking of gene expression of analysed IRF7 network genes coincided with enterovirus positivity (**Figures 4 and 5**). In the case child 1, the seroconversion to autoantibody positivity took place before enterovirus positivity.

However, it cannot be ruled out that this child could have been infected previously by another enterovirus.

No amplification of IFIH1, OASL, OAS1 or IRF7 was detected in the negative extraction controls (H_2O and CBV3 supernatant) or from the negative RT-PCR control (H_2O) indicating that there was no contamination present in the RNA extraction or RT-PCR assay. However, a clear gene expression was detected from the positive assay control samples prepared from PBMCs infected either with 1 pfu of CBV1 or ECHO11 per cell. The expression of IRF7 was highest in the CBV1 infected cells (**Fig. 3**). The expression levels of IFIH1, OASL, OAS1 and IRF7 in analysed case-control cohort was lower than the expression levels detected from positive assay control samples. This might be explained by the fact PBMCs infected *in vitro* were used as an assay control. The infection of control cells had lasted for 48 h before cell were harvested and stored frozen. This set up can be considered to mimic an acute phase of an infection and therefore the expression of IRF7 network genes is upregulated. However, appearance of similar number of enterovirus infected cells and high expression of IRF7 network genes *in vivo* might not be possible even during acute viremia.

Results, i.e. gene expression levels, were calculated by using equation $2^{(-(\text{target gene-18S rRNA}))}$. 18s rRNA is a housekeeping gene that is expressed in a fairly constant level in all nucleated eukaryotic cells. Thus the obtained results should be in ratio to the cell number used for RNA extraction. According to the manufacturer RNeasy 96 extraction kit is optimised for processing up to 0.5×10^6 cells/well whereas in case of AllPrep RNeasy spin column it is suggested that no more than 10×10^6 cells/spin column should not used. There is no data available of the absolute cell number in any of the case-control PBMC samples. Therefore the overloading of spin columns cannot be ruled out. This might affect the obtained results.

Agarose gel electrophoresis of the RT-PCR end products confirmed the amplification of correct size IRF7 network gene sequences.

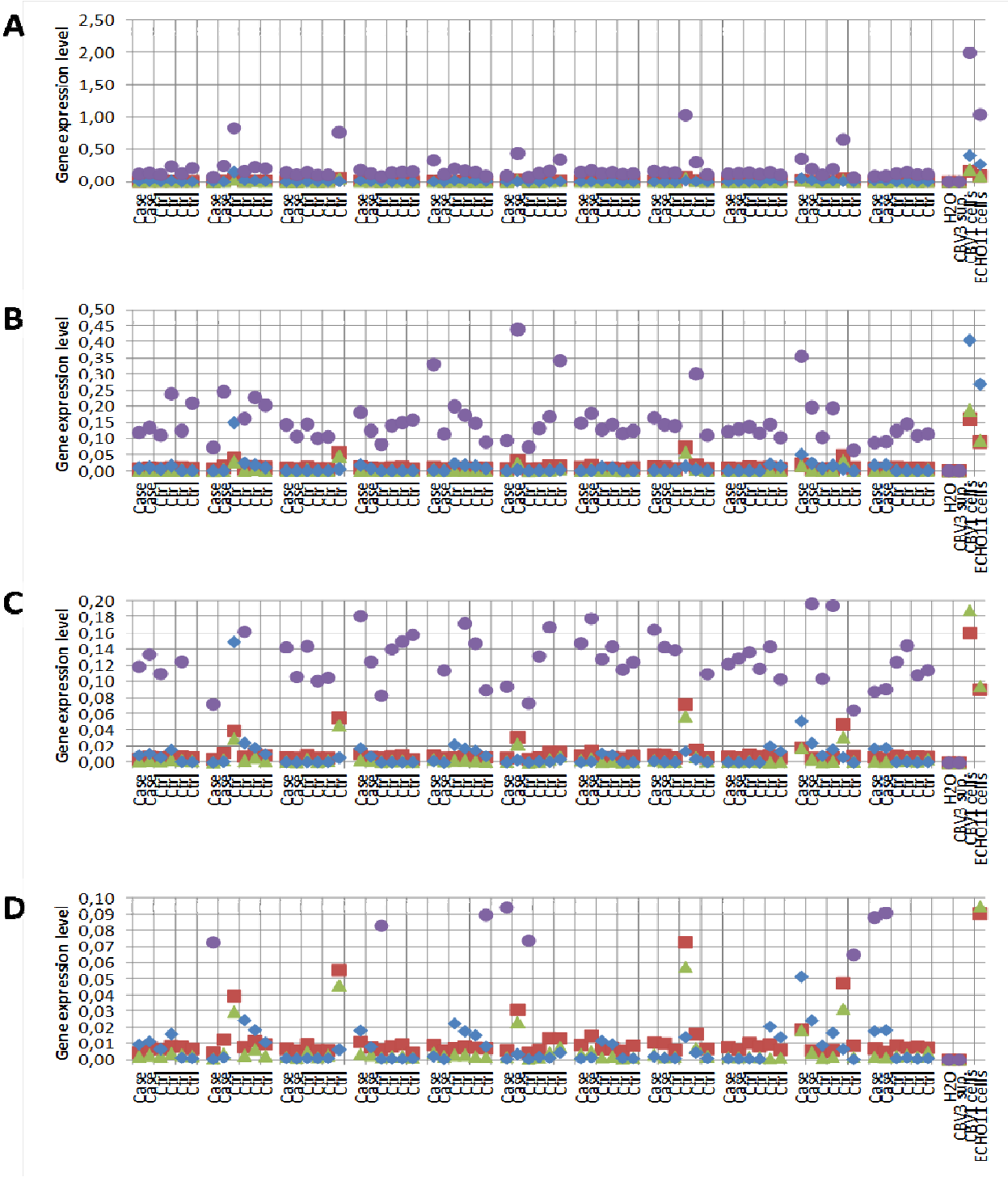

Figure 3: Gene expression levels of IFIH1 (■), OASL (▲), OAS1 (◆) and IRF7 (●) in pair wise case-control cohort. Each figure is from the same results, the only difference being scale down of y-axis from A to D (same results are shown using different y-axis). Cases (case) and controls (ctrl) have been divided into sets of six samples corresponding selected case subjects before and after seroconversion and two corresponding control subjects at same time points. Gene expression levels of IRF7, IFIH1, OASL and OAS1 were calculated using equation $2^{(-(\text{target gene-18S rRNA}))}$, where target gene and 18S rRNA are averages of Ct-values obtained from triplicate RT-PCR analyses. Controls: H_2O; CBV3 sup., cell culture medium containing CBV3; CBV1 cells and ECHO11 cells, PBMCs infected *in vitro* with 1 pfu of CBV1 or ECHO11 per cell.

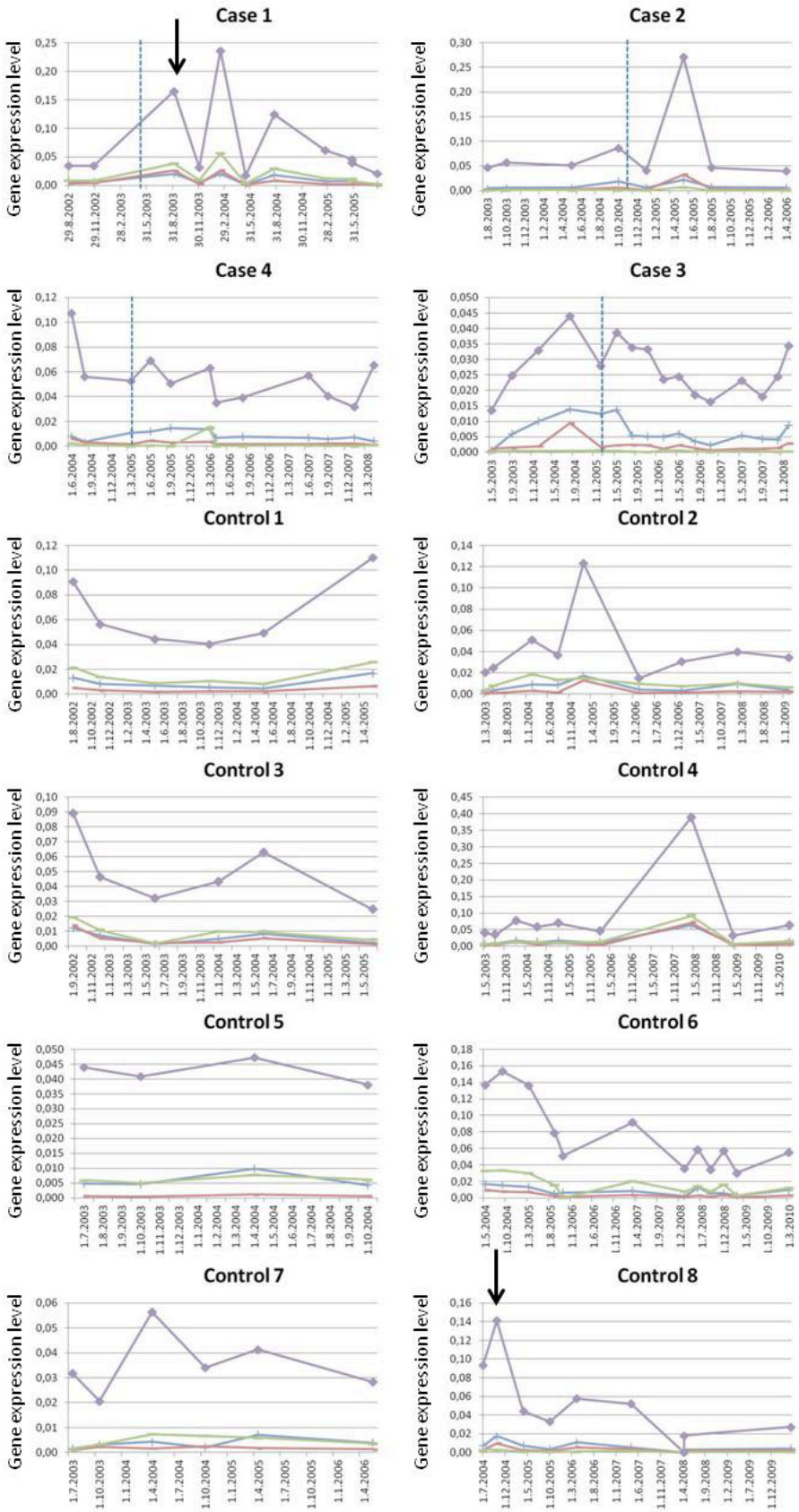

Figure 4: Gene expression levels of IFIH1 (–), OASL (–), OAS1 (–) and IRF7 (–) in longitudinal case-control cohort. Dashed vertical lines in cases 1 – 4 indicate the time point of seroconversion. An arrow in case 1 and control 8 shows the samples analysed to be enterovirus positive by RT-PCR.

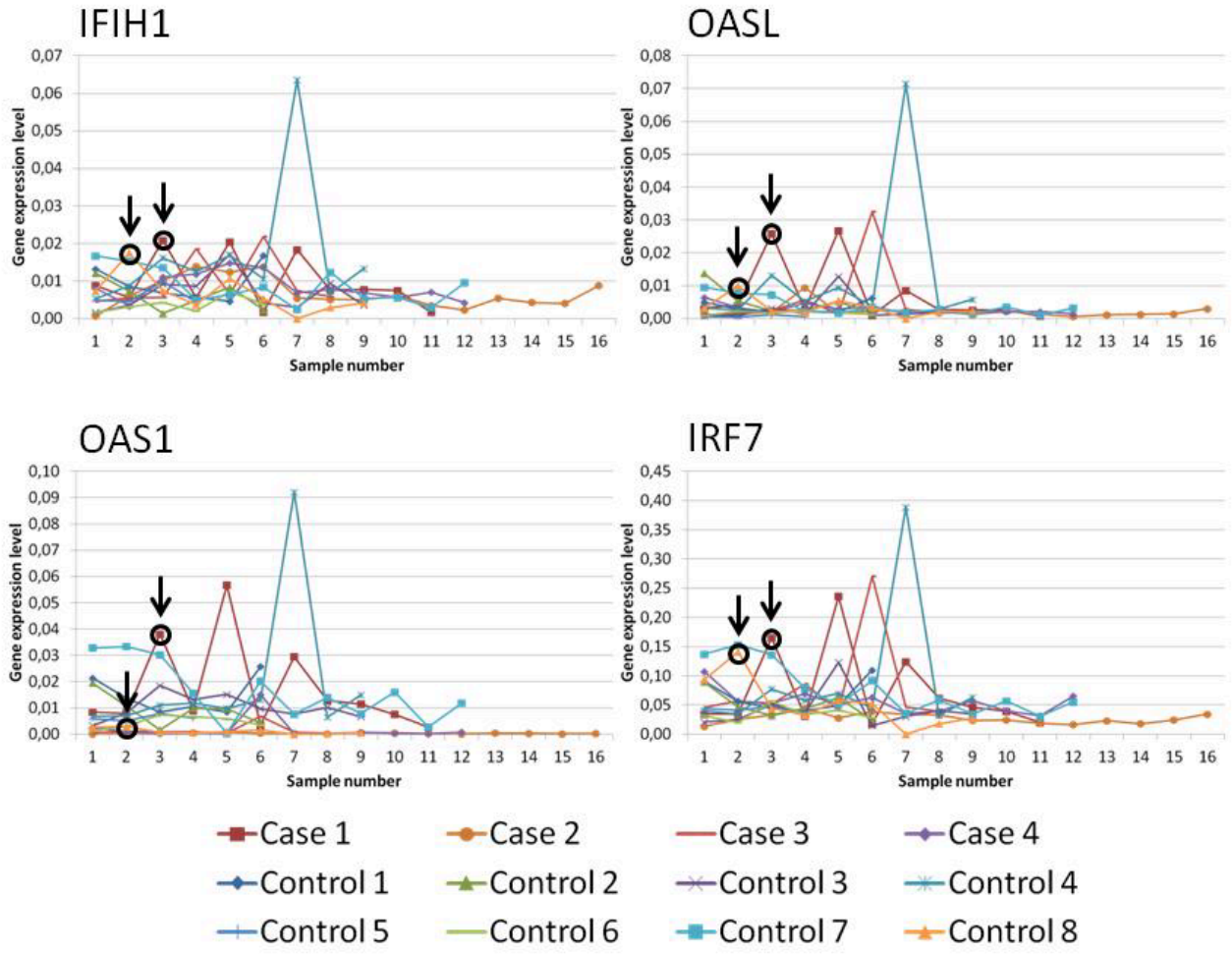

Figure 5: Gene expression levels of IFIH1, OASL, OAS1 and IRF7 in longitudinal case-control cohort matched by sample number. Gene expression levels were analysed by RT-PCR and calculated using equation $2^{(-(\text{target gene-18S rRNA}))}$, where target gene and 18S rRNA are averages of Ct-values obtained from triplicate RT-PCR analyses. One case (case 3) and one control (control 8) were detected to be enterovirus positive at time points indicated by an open circle and arrow.

5 CONLUSIONS AND FUTURE PROSPECTS

No clear differences were found in the expression of IFIH1, OASL, OAS1 or IRF7 or in the presence of enterovirus genome between the selected cases and their control subject. Gene expression levels varied considerably between individuals and between samples, and therefore larger case-control series should be analysed to detect possible diabetes-associated changes in gene expression.

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