

ARTICLE

# Case report: type 1 diabetes in monozygotic quadruplets

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Type 1 diabetes (T1D) is an autoimmune disease characterized by the lack of insulin due to an autoimmune destruction of pancreatic beta cells. Here, we report a unique case of a family with naturally conceived quadruplets in which T1D was diagnosed in two quadruplets simultaneously. At the same time, the third quadruplet was diagnosed with the pre-diabetic stage. Remarkably, all four quadruplets were positive for anti-islet cell antibodies, GAD65 and IA-A2. Monozygotic status of the quadruplets was confirmed by testing 14 different short tandem repeat polymorphisms. Serological examination confirmed that all quadruplets and their father suffered from a recent enteroviral infection of EV68-71 serotype. To assess the nature of the molecular pathological processes contributing to the development of diabetes, immunocompetent cells isolated from all family members were characterized by gene expression arrays, immune-cell enumerations and cytokine-production assays. The microarray data provided evidence that viral infection, and IL-27 and IL-9 cytokine signalling contributed to the onset of T1D in two of the quadruplets. The propensity of stimulated immunocompetent cells from non-diabetic members of the family to secrete high level of IFN- $\alpha$  further corroborates this conclusion. The number of T regulatory cells as well as plasmacytoid and/or myeloid dendritic cells was found diminished in all family members. Thus, this unique family is a prime example for the support of the so-called 'fertile-field' hypothesis proposing that genetic predisposition to anti-islet autoimmunity is 'fertilized' and precipitated by a viral infection leading to a fully blown T1D.

*European Journal of Human Genetics* (2012) 20, 457–462; doi:10.1038/ejhg.2011.212; published online 23 November 2011

**Keywords:** type 1 diabetes; monozygotic quadruplets; enteroviral infection; gene expression array; cytokine array; genetic predisposition to diabetes

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease, the complexity of which is underlined by undesirable interactions between genes and environmental factors in genetically predisposed individuals.<sup>1</sup> Although T cells are central to the mechanism of beta-cell destructions,<sup>2–5</sup> a critical involvement of other cellular and humoral components of adaptive and innate immune system have also been demonstrated.<sup>1,6–8</sup> Among environmental factors, infectious agents such as viruses primarily sensed by innate immune mechanisms are considered very potent triggers of T1D.<sup>9–12</sup> Notably, and in this context, several recently published findings suggest a strong link between T1D and enteroviral infections.<sup>13–15</sup>

To prevent the clinical onset of T1D, immuno-intervention should be undertaken in the clinically silent pre-diabetic phase. However, it is difficult to identify suitable candidates for such interventions.<sup>16</sup> The pre-diabetic phase is usually marked by the presence of autoantibodies to beta-cell antigens,<sup>17,18</sup> but the presence of these antibodies alone is not sufficient to induce destruction of beta cells.<sup>2</sup>

Here, we report a case of a family with monozygotic quadruplets where after an apparent enteroviral infection, two sisters were diagnosed with T1D while a third quadruplet was at pre-diabetic stage. To gain an insight into the molecular mechanism involved in the pathogenesis of this disease, all family members were studied for the presence of islet cell antibodies, gene expression profile of immune regulatory pathways, cellularity of T regulatory cells (Tregs) and dendritic cells (DCs), and cytokine responses.

## MATERIALS AND METHODS

### Family history

The quadruplets were born into a family with an older sibling after a physiological conception. Their development followed a typical path. At the age of 5, two of these quadruplets were simultaneously diagnosed with T1D, while a third quadruplet was in a pre-diabetic phase. Interestingly, one month before the clinical manifestation, the two diabetic girls suffered from an apparent mild infection with respiratory symptoms. Laboratory tests showed that all quadruplets and their father suffered from an enteroviral infection. All family members were subjected to the glucose tolerance test one week after the diagnosis. Upon obtaining a parental informed consent, blood samples of all

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Received 14 June 2011; revised 13 October 2011; accepted 21 October 2011; published online 23 November 2011

family members were collected and analysed. A family tree and clinical data are provided in Figure 1.

### HLA typing, confirmation of monozygosity and viral studies

All family members were HLA typed using allele-specific primers.<sup>19</sup> Serum samples from both the parents and the quadruplets were examined for signs of viral infections by ELISA, complement fixation and non-direct immunofluorescence. We focused on enteroviruses and coxsackie viruses as they are suspected to have a role in T1D development.<sup>13–15</sup> Monozygotic status of the quadruplets was confirmed by testing 14 different short tandem repeat polymorphisms using Aneufast multiplex QF-PCR kit (molGENTIX, S.L., Barcelona, Spain), recommended by CODIS (Combined DNA Index System).<sup>20</sup>

### Immune cell isolation

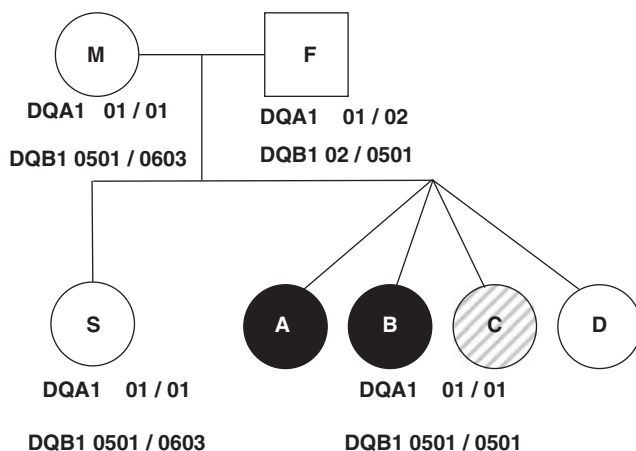
Peripheral-blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Paque density gradient centrifugation and cultured as described elsewhere.<sup>21</sup> After 72 h, cells were washed and subjected to RNA extraction and supernatants were frozen at  $-80^{\circ}\text{C}$  until used for cytokine analysis.

### Gene expression profiling

RNA isolated from PBMCs was amplified and hybridized to a high-density gene array chip (NimbleGen, Roche-NimbleGen Inc., Madison, WI, USA) where 47 633 human genome targets are presented with 8 probes per target. Chips were imaged (InnoScan 700 scanner, Innopsys, Carbonne, France), raw data were analysed using NimbleScan 2.4 software (Roche-NimbleGen Inc.) and used for further statistical analysis.

### Statistical and gene array analysis

The goal of this analysis was to identify differences in the activity of the immune signalling pathways between the diabetic quadruplets and their non-diabetic siblings. The gene array statistical analysis was performed using the network linking (GeneSpring GX10, Agilent Technologies, Inc., Santa Clara, CA, USA) and the pathway analysis softwares (MetaCore, GeneGo, Inc., St Joseph, MO, USA). Using unpaired *t*-tests, only the differences in gene expression with *P*-values  $<0.05$  were considered as statistically significant. The numbers used to generate the heat map represent normalized intensity values calculated as a  $\log_2$  ratio of gene expression to the chip median expression value.



**Figure 1** The family tree with indicated HLA DQ types. The quadruplets (A–D) are genetically monozygotic and DQ homozygous. The quadruplets A and B had simultaneous presentation of T1D just 1 month after an apparent enteroviral infection. The quadruplet C is in the pre-diabetic phase. The quadruplet D and an older sister (S) are diabetes free. All four quadruplets (A–D) produce anti-islet cell antibodies to GAD65 and IA-A2 autoantigens. HLA DQ types of mother (M) and Father (F) are also indicated.

### Cellularity of Tregs and DCs

Tregs ( $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ ) and DCs (plasmacytoid, pDC as well as myeloid, mDC) from freshly isolated PBMCs were counted and identified by flow cytometry (FACS) as described elsewhere.<sup>22</sup> Family data were compared with the control samples derived from 23 healthy volunteers (12 females/11 males), median of age 18 years, range 14–26 years.

### PBMC cytokine responses

To assess the cellular responses to Toll-like receptor 9 (TLR9) stimulation,  $2 \times 10^5$  freshly isolated PBMCs were stimulated with ODN 2216 (Invivogen, Toulouse, France) for 24 h ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ ) and cytokine productions were measured using multiplexed antibodies (Milliplex, Millipore, Billerica, MA, USA) on luminescent beads (Luminex 100 IS, Luminex Corporation, Austin, TX, USA). Similarly, a spontaneous secretion of cytokines was measured in the supernatants derived from PMBCs using a cytokine microarray (Ray Biotech, Norcross, GA, USA). Cytokine concentrations were compared with the relevant value ranges of the control population without statistical analysis.

## RESULTS

### Short tandem repeat polymorphisms test, HLA typing, anti-islet cell antibodies, glucose tolerance test and anti-viral antibodies

All quadruplets exhibited identical results in all 14 short tandem repeat polymorphisms tested. The quadruplets are homozygous for HLA DQA 01 and DQB 0501 (Figure 1). The HLA class II alleles in the quadruplets are not associated with an increased risk for T1D in the Czech population.<sup>19</sup> Remarkably, all four quadruplets were positive for anti-islet autoantibodies GAD65 and IA-2A, indicating an ongoing anti-islet autoimmunity in the non-diabetic quadruplets. The quadruplet C had a reduced first-phase insulin response and a 1-hour glucose concentration of 8.7 mmol/l, a typical characteristic of a pre-diabetic stage. Only this quadruplet had an evidence of current Enterovirus 68–71 infection with IgM, IgA and IgG antibodies. The father and the other quadruplets had anamnestic Enterovirus 68–71 IgG antibodies only.

### Functional genomics

Among the 47 633 probe-sets tested, 2136 entities (with proved difference  $P \leq 0.05$ , and fold change  $\geq 1$ ) were unique for the non-diabetic quadruplets and 2589 entities were typical of the two diabetic sisters. Out of 10 immune signalling networks with the largest differences in the gene expression profile between the diabetic and the non-diabetic quadruplets (Table 1), 4 are related to antiviral responses: (i) antiviral actions of interferons, (ii) TRIF-specific

**Table 1** Ten most differently regulated immune signalling networks between the diabetic (A and B) and the non-diabetic (C and D) quadruplets

Signalling pathways	<i>P</i> -value
Antiviral actions of interferons	0.0003
Role of TLR-3 and -4 in antiviral response:	0.0011
Trif-specific signalling pathways	
IFN alpha/beta signalling pathway	0.0019
IL-27 signalling pathway	0.0034
Antigen presentation by MHC class I	0.0056
IL-4-antiapoptotic action	0.0141
IL-12-induced IFN-gamma production	0.0260
IL-9 signalling pathway	0.0359
Human NKG2D signalling	0.0366
IL-4 signalling pathway	0.0456

TLR3 and TLR4 signalling pathways, (iii) IFN alpha/beta signalling and (iv) antigen presentation by MHC class I. Important differences were also found in four other regulatory pathways related to the interleukin signalling and T-cell differentiation pathways (IL-4, -9, -12 and -27). A gene cluster analysis of these pathways is presented in the Figure 2 where the gene expression for the diabetic and the non-diabetic quadruplets is compared.

### Cellularity of Tregs and DCs

The relative number of Tregs was decreased in all family members in comparison with the value range of the control group (Supplementary Figure 3a). Similarly, the absolute numbers of pDCs and mDCs were also decreased compared with the mean of the control subjects (Supplementary Figure 3b and 3c, respectively). The only exception for the decrease in pDCs was the older sister and that for the decrease in mDCs was the quadruplet B.

### Spontaneous and induced cytokine production by PMBCs

PMBCs from the diabetic quadruplets produced high levels of TGF-beta1 and low or undetectable levels of IL-10. A completely opposite pattern of production of these two cytokines was observed in the pre-diabetic quadruplet C (Supplementary Figure 4). Remaining healthy members of the family produced intermediate to low levels of IL-10 and low to undetectable levels of TGF-beta1. All family members produced comparable levels of IL-13 (Supplementary Figure 4). Thus, IL-10/TGF-beta-1 imbalance between the diabetic and the non-diabetic members of the family was the most striking result of this analysis. In addition, we detected higher spontaneous levels of IFN- $\gamma$  and other Th1 cytokines mainly in the non-diabetic quadruplet D. Moreover, only the father's PMBCs produced detectable amounts of IL-17 (data not shown).

The stimulation of PMBCs with TLR9-specific ligand led to the increased production of IFN- $\alpha$  in the non-diabetic quadruplets, the older sister and the mother. Concentration of IFN- $\alpha$  was slightly increased in the diabetic siblings as opposed to the controls. In contrast, PMBCs from the father were refractory to TLR9 stimulation (Supplementary Figure 5).

## DISCUSSION

A simultaneous clinical manifestation of T1D in siblings is rare. Taken into account that the probability of a natural conception of quadruplets is extremely low,<sup>23</sup> the chance for the quadruplets to also be monozygotic and DQ homozygous is truly exceptional. Here, we documented a unique case of a family with naturally conceived quadruplets where two of them have already developed T1D and the third is in a pre-diabetic state with impaired first-phase insulin response in ivGTT. Moreover, all four quadruplets are positive for anti-GAD65 and IA-2A antibodies. Thus, the probability for an early onset of T1D in the two, so far non-diabetic quadruplets is quite high.<sup>24</sup>

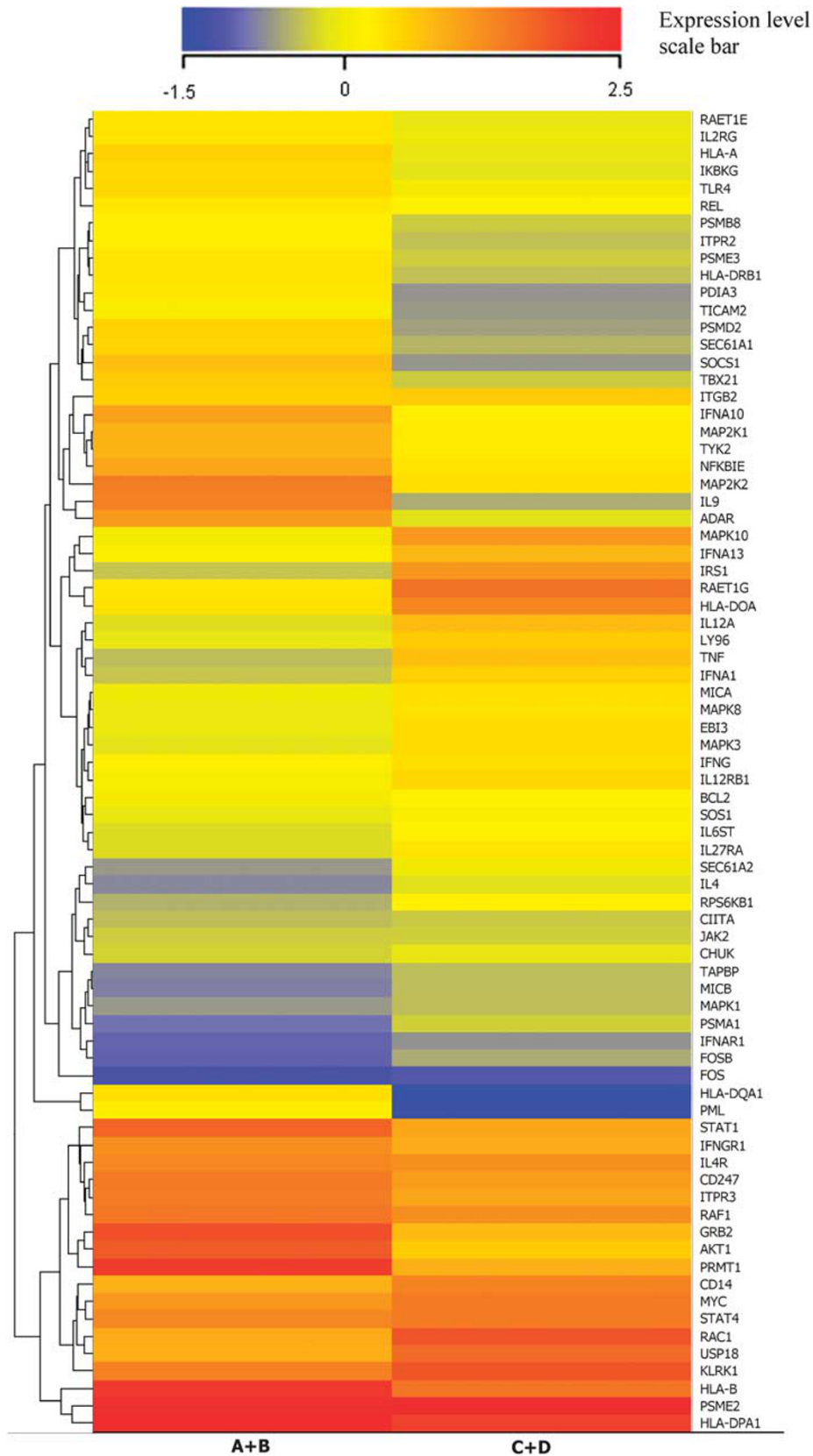
An interplay between genetic predisposition and environmental factors is implicated in T1D pathogenesis.<sup>25,26</sup> External factors such as food, infections and stress factors should be taken into account. The quadruplets had the same duration of lactation and they have similar eating habits. They all attend the same kindergarten and follow a standard national vaccination programme. According to their medical records they did not suffer from any major childhood diseases and infections. The parents reported that both diabetic quadruplets showed visible signs of a mild respiratory infection just 1 month before the onset of diabetes. No other infection or medical conditions were reported before the onset of T1D. Serological examinations

revealed that, indeed, all the quadruplets suffered from an enteroviral infection, still ongoing in the pre-diabetic quadruplet C. Viral infections are considered to have an important role in T1D pathogenesis, but the exact molecular mechanisms are still unknown.<sup>25,26</sup> In this context, our data are consistent with the recently published meta-analysis of 33 prevalence studies suggesting that enterovirus infection is common among patients with T1D.<sup>27</sup>

Our microarray data provided additional evidence to support the notion that viral infection may have contributed to the onset of T1D in the two quadruplets. Notably, cellular anti-viral responses were more prominent in the diabetic sisters where the antiviral signalling of interferons was the most significantly affected pathway. In the diabetic and the non-diabetic sisters, three other virus-sensing pathways exhibited different activation status, namely, TRIF-dependent TLR3 and TLR4 antiviral responses, IFN $\alpha/\beta$  signalling and antigen presentation by MHCI. Various evolutionarily conserved microbial structures, so-called pathogen-associated molecular patterns, are recognized by pattern recognition receptors. Toll-like receptors (TLRs) represent a prototypical class of such receptors that recognize pathogen-associated molecular patterns.<sup>28</sup> Moreover, TLR3 and TLR4 can signal the presence of viral RNAs and proteins, respectively. In addition, only TLR3 and 4 signal via adaptor protein TRIF, which triggers antiviral immune responses through the production of type I interferons (IFN $\alpha/\beta$ ) and inflammatory cytokines.<sup>29</sup> TRIF signalling also leads to MyD88-independent DC maturation.<sup>30</sup> In turn, interferons may not only significantly contribute to the development of Th1 immune responses considered as prodiabetogenic<sup>31</sup> but they can also cause the upregulation of MHC class I molecules on pancreatic beta cells, thus initiating the diabetogenic process itself.<sup>9,32</sup> An alternative pathway able to transduce the detection of enteroviral infection to interferon  $\alpha/\beta$  production involves the cytoplasmic helicase receptor MDA5 (melanoma differentiation-associated protein 5).<sup>33,34</sup> A recent genome-wide association study implicated MDA5 in the pathogenesis of T1D.<sup>35</sup> Thus, although we cannot preclude the contribution of other external factors and type of viruses, our serological and microarray data argue for a possible involvement of enteroviruses of EV68–71 serotypes in the development of T1D in our quadruplets. The propensity of stimulated PMBCs isolated from non-diabetic members of the family (with the exception of the father) to secrete high levels of IFN- $\alpha$  compared with the controls lends further support for this conclusion.

The other four pathways with the most prominent differences between the diabetic and the non-diabetic siblings are cytokine IL-27, -4, -12 and -9 signalling cascades affecting T-cell differentiation. Among these interleukins, the most promising candidates for diabetes-associated markers seem to be IL-27 and IL-9. Notably, it has been demonstrated that IL-27 functions as the key regulator of IL-10 and IL-17 production in human CD4<sup>+</sup> T cells.<sup>36</sup> Nowak and colleagues<sup>37</sup> also identified IL-9 as a mediator of Th17-driven inflammatory disease. Moreover, it has also been shown that IL-9 synergizes with TGF-beta1 to differentiate naive CD4<sup>+</sup> T cells into Th17 cells and postulated an additional role for IL-9 in mediating the suppressive function of Tregs in experimental autoimmune encephalomyelitis.<sup>38</sup> Thus, our microarray data are coherent with these findings and provide further support for the importance of IL-27 and IL-9 cytokine signalling in the development of T1D.

FACS analysis of freshly isolated PMBCs from all family members showed that the first-degree relatives of the diabetic quadruplets display a general decline in the number of pDCs and/or mDCs. Moreover, all family members displayed a lower number of naturally occurring Tregs. This is in agreement with a previously documented



**Figure 2** Microarray heat map of genes belonging to 10 most differentially regulated pathways between the diabetic and the non-diabetic quadruplets. A simplified view with averaged gene expression values of diabetic (A and B) and non-diabetic sisters (C and D) is presented. Red and blue indicate an increase and a decrease in the gene expression levels, respectively, as indicated in the scale bar. The affiliation of all listed genes with one of the ten signaling pathways listed in Table 1 is presented in the Supplementary Table.

situation where siblings of T1D patients often reveal lower number of Tregs with impaired functional capabilities.<sup>39</sup> We have also previously reported that PMBCs from young patients with T1D, predominantly produced Th3 cytokines IL-10 and TGF- $\beta$ .<sup>40,41</sup> However, a predominant secretion of TGF- $\beta$  over IL-10 seen in our diabetic quadruplets has not been reported so far. Strikingly, this production pattern exhibited a reverse relation in their pre-diabetic sister. Although the mechanism underpinning this cytokine production pattern is not known at this time, it is suggestive of a switch in the regulation of cytokine expression during the transition from pre-diabetic to clinically manifested T1D phase.

The open question is then why, if all quadruplets show signs of enteroviral infection and concomitantly are positive for anti-islet autoantibodies GAD65 and IA-2A, only two of them have developed diabetes despite their identical genetic background? In this respect, the lack of information concerning the quadruplets' autoantibody positivity before enteroviral infection precluded us to establish direct causative links between the enteroviral seropositivity, islet autoimmunity and the onset of diabetes.<sup>42</sup> Enteroviruses enter the body via ingestion and young children are their main target and reservoir. The incubation period lasts usually 3–6 days and symptoms of infection are usually subclinical, or very mild in the form of uncomplicated summer cold. Initially, enterovirus-specific antibodies of IgM class are detectable in 1–3 days after infection and are present for several months. After 7–10 days post-infection, a seroconversion to IgG class occurs and long lasting antiviral IgG antibodies, called anamnestic, are produced.<sup>43</sup> The quadruplet C is the only individual from this family that is still serologically positive for the presence of enterovirus-specific IgM antibodies. Thus, although a pre-diabetic stage of the quadruplet C correlates with a more recent occurrence of enteroviral infection, its positivity for anti-islet autoantibodies GAD65 and IA-2A indicates that this infection might not be a primary cause of the anti-islet autoimmunity, which rather results from a genetic predisposition of quadruplets to diabetes.<sup>44</sup> This line of arguments is further supported by a recent study suggesting that the progression from islet autoimmunity to fully blown T1D may increase after an enterovirus infection.<sup>15</sup> In this respect, the timing of infection and its periodic recurrence as well as a dose of the virus are certainly important parameters when considering the effect of viral infection on the same diabetes-susceptible genetic background. It is of note that a pair-wise concordance of T1D is <40% among monozygotic twins (data from monozygotic quadruplets are not available) strongly advocating for a critical role of exogenous factors in the development of T1D.<sup>45,46</sup> Taken altogether, the identical genetic background among quadruplets does not guarantee the synchronization of the onset of diabetes. In addition, an individual medical history and immunological experience reflected in differential level of gene hypermethylation could represent an important endogenous factor causing a discordance among genetically identical siblings.<sup>47</sup> Thus, the correlation between our microarray data indicating an involvement of viral infection in the development of diabetes and the appearance of clinically apparent mild respiratory, possibly viral infection in two girls 1 month before clinical diagnosis of T1D is indicative that a viral infection contributed to the autodestructive diabetic process. Although the identity of this viral agent remains uncertain, EV68–71 serotypes positivity points to enteroviruses as obvious suspects. However, as we have no direct evidence linking enteroviruses to the onset of diabetes in these quadruplets, caution has to be exercised in the interpretation of these results.

In sum, this unique family case study provides a support for the so-called 'fertile-field' hypothesis proposing that genetic predisposition to

anti-islet autoimmunity is 'fertilized' and precipitated by viral infections.<sup>48</sup> Our microarray data suggest that the viral activation of TRIF-mediated TLR signalling amplified by ensuing overproduction of IFN  $\alpha/\beta$  cytokines could lead to an imbalance between anti- and pro-inflammatory signals towards the latter, resulting in a relatively rapid progression of T1D in genetically susceptible young quadruplets.<sup>18</sup> Several lines of evidence also point to the contribution of enteroviral insult that preceded the clinically manifested onset of a fully blown and pre-diabetic stage of T1D in three out of four quadruplets. The finding that the remaining quadruplet is anti-islet autoantibody-positive, but still diabetes free with normal glucose tolerance test likely attests to her genetic predisposition to anti-islet autoimmunity. Thus, this family case is a prototypical example of the efficiency of environmental factors to cause disease in genetically identical predisposed individuals.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

Gene expression study was exclusively supported by project NPVII 2B06019 from the Czech Ministry of Education. Studies on Tregs and DCs were supported by the project VZ MSM 0021620812 from the Ministry of Education, Youth and Sports of the Czech Republic, and by the project MZOFNM2005 from the Ministry of Health and by the project GACR P302/10/1679. AN and DF as well as the cytokine analysis were supported by the project NPVII 2B08066 from the Czech Ministry of Education, Youth and Sports. This work was also partially supported by Grant AVOZ50520514 from the Academy of Sciences of the Czech Republic.

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