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Comparative and correlative assessments of cytokine, complement and antibody patterns in paediatric type 1 diabetes

M. Abdel-Latif,* A. A. Abdel-Moneim,† M. H. El-Hefnawy‡ and R. G. Khalil*
*Division of Immunity, †Division of Physiology, Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef, and ‡National Institute of Diabetes and Endocrinology (NIDE), Cairo, Egypt

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Correspondence: M. Abdel-Latif, Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.
E-mail: mahmoud_1232000@yahoo.com

Summary

One of the most widespread and effective environmental factors is the infection with enteroviruses (EVs) which accelerate β cell destruction in type 1 diabetes (T1D). This study represented a comparison between diabetic EV^+ and EV^- children as well as correlation analysis between autoantibodies, T1D markers, cytokines, complement activation products and anti-coxsackievirus (CV) immunoglobulin (Ig)G. EV RNA was detected in Egyptian children with T1D (26·2%) and healthy controls (0%). Detection of anti-CV IgG in T1D-EV^+ resulted in 64% positivity. Within T1D-EV^+, previously diagnosed (PD) showed 74 *versus* 56% in newly diagnosed (ND) children. Comparisons between populations showed increased levels of haemoglobin A1c (HbA1c), C-reactive protein (CRP), nitric oxide (NO), glutamic acid decarboxylase and insulin and islet cell autoantibodies [glutamic acid decarboxylase autoantibodies (GADA), insulin autoantibodies (IAA) and islet cell cytoplasmic autoantibodies (ICA), respectively], interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL −10, IL −12, IL −17, C3d and sC5–9 in T1D-EV^+ *versus* T1D-EV^-. Conversely, both IL-20 and transforming growth factor (TGF-β) decreased in T1D-EV^+ *versus* EV^-, while IL-4, −6 and −13 did not show any changes. Correlation analysis showed dependency of accelerated autoimmunity and β cell destruction on increased IFN-γ, IL-12 and IL-17 *versus* decreased IL-4, −6 and −13. In conclusion, IFN-γ, IL-12 and IL-17 played an essential role in exacerbating EV^+-T1D, while C3d, sC5b −9, IL-10 and −20 displayed distinct patterns.

Keywords: complement, correlation, cytokines, enteroviruses, type 1 diabetes

Introduction

The global rapid spread of type 1 diabetes (T1D) disease cannot be explained solely by genetic predisposition but also to environmental factors, including viral infections [1]. Enteroviruses (EVs) are found to play a major role in induction of the disease based on three possible mechanisms [2]. The first is the invasion of pancreatic cells by viruses through cell surface receptors and replication followed by induction of innate immunity against the cells leading to destruction [3]. The second is altered immunity as a result of viral infection leading to autoimmunity. The third is the induction of cross-reactive immunity between EV and pancreatic β cell antigens as a result of antigen mimicry. EVs are the most likely viruses to be linked with the onset of T1D [4]. The Enterovirus genus of the Picornaviridae family consists of small, non-enveloped, positive, single-strand RNA viruses, including polio viruses, coxsackieviruses (CVs) A and B and echoviruses [5]. Transmission methods were identified to be faecal–oral or, less commonly, respiratory routes. Detection methods for EVs in serum, saliva or stool were dependent upon either reverse transcription–polymerase chain reaction (RT–PCR) or antibody assays [5].

Immunological and metabolic biomarkers were used widely in the diagnosis of T1D or β cell destruction [6]. Immunological markers included islet cell cytoplasmic autoantibodies (ICA), glutamic acid decarboxylase autoantibodies (GADA), insulin autoantibodies (IAA) and

pancreatic β cell-specific zinc transporter (ZnT-8) autoantibody. Cytokine production, including interleukin (IL)-1β, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, IL-2 and IL-17, were found to be highly characteristic for T1D patients compared to the controls, who were characterized by IL-10 [7–10]. However, IL-10, together with IL-6 and IL-13, were found to be associated with the pathogenesis caused by EV infection [11]. Haemoglobin A1c (HbA1c) is a direct indicator for glucose blood level in both of T1D and T2D, while C-peptide correlates with insulin secretion. Both of them are used as markers for pancreatic β cell destruction [12].

Inflammatory responses, including elevated C-reactive protein (CRP) and nitric oxide (NO), were found to be associated with T1D with or without EV infection, while anti-inflammatory cytokines including IL-4, IL-10 and IL-13 were found to be cytoprotective against β cell destruction [13]. Regulatory T cells (T_{reg}) by secretion of TGF-β were also found to play an essentially protective role in prevention of T1D-associated autoimmunity [14]. The inflammatory reactions also promote complement activity, which could be measured by detecting the levels of C3d and sC5b–9 in serum [15].

The current study proposed to test the prevalence of EV in paediatric patients with T1D and compare between age- and sex-matched T1D-EV^- and T1D-EV^+ patients. It also aimed to detect statistical correlations between anti-CV immunoglobulins (IgGs), complement activity and cytokines.

Materials and methods

Study population and sample collection

The study population consisted of 382 children with T1D and 100 healthy controls. These children visited the Institute of Endocrinology and Diabetes, Cairo University, Cairo, Egypt during the period from October 2013 to September 2014 for consultancies. Children with T1D ranged from 2 to 16 [9·8 ± 2·9; mean ± standard deviation (s.d.)] years, while healthy controls ranged from 3 to 14 (9·1 ± 2·7; mean ± s.d.) years. Each of the diabetic and control groups consisted of an equal number of males and females. After written informed parental consents were obtained and the study was approved by the ethical committee, blood samples were collected. Venous blood (5 ml) was collected in serum tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) through venous puncture using a 21-gauge needle. Serum samples were obtained by centrifugation at +4°C, 4500 g for 10 min and kept at −80°C until use. According to the criterion of the World Health Organization (WHO) and the American Diabetes Association, all participants were subjected to both random blood sugar and haemoglobin A1c (HbA1c) assays using commercial kits purchased from Spinreact, Girona, Spain and MyBiosource, San Diego, CA, USA, respectively. No participants (diabetic and controls) were obese or overweight. They were also free of infectious diseases, neoplastic, inflammatory and autoimmune disorders and allergies. Patients receiving immunomodulatory drugs were also excluded.

In-vitro amplification

RNA extraction from serum as well as amplification reactions has been described previously [16,17]. Primers were selected to amplify sequences within the 5′ non-coding region (5′NCR), which is a highly conserved zone among all EV serotypes. The external primers (EV/PCR1, 5′-ATT GTCACCATAAGCAGCCA- 3′; EV/PCR2, 5′-TCCTCCGG CCCCCTGAATGCG- 3′) generate a 154 base pairs (bp) fragment, whereas the internal primers (EV/PCR3, 5′-ACACGGACACCCAAAGTAGTCGGTTCC-3′; EV/PCR4, 5′ TCCGGCCCCTGAATGCGGCTAATCC-3′) generate a 114 bp PCR product.

Extracted samples (10 μl) were heated to 99°C for 5 min and placed immediately on ice. Salts, nucleotides, 0·3 μM of each primer and 100 U of reverse transcriptase (ThermoScientific, Waltham, MA, USA) were added in a 5-μl final volume. The samples were incubated for 60 min at 42°C for the RT reaction. Five μl of the RT product was added to a final volume of 50 μl of the PCR reaction mix containing 5 μl of the PCR buffer (ThermoScientific) containing 1 μm of each primer (EV/PCR1 and EV/PCR2) and 2·5 U of the Taq DNA polymerase enzyme (ThermoScientific). After a denaturation step of 94°C for 4 min, 40 cycles of amplification at 92°C for 1·5 min, 55°C for 1·5 min and 72°C for 2 min were performed with a final extension of 72°C for 10 min. The nested PCR involved adding 2 μl of first-round PCR product to a 48-μl PCR mix containing 1 μM of each primer (EV/PCR3 and EV/PCR4) and 2·5 U of Taq DNA polymerase (ThermoScientific). The same cycling conditions used for the first nested PCR were also used in the second nested PCR. PCR products (10 μl) were analysed by electrophoresis on 1·5% agarose (Applichem GmbH, Darmstadt, Germany) gels containing 0·5 mg ethidium bromide/ml. All the control non-diabetic samples were found negative to infection with EV. According to the diabetic positive and negative samples, patients were allocated into two groups of T1D-EV^+ and T1D-EV^-, respectively. For each group, newly diagnosed (ND) and previously diagnosed (PD) children were separated based on the duration (< 1 or > 1 year) of T1D diagnosis [18].

Sequencing

Ten PCR products of EV^+ samples were sequenced. Fifty to 100 μl PCR products were purified using the PCR products purification kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. Cycle sequencing was performed on 1–7 μl of the purified products with BigDye®

Terminator version 3.1 Cycle Sequencing kit (ThermoScientific) using 40 pmol of the same primers as in PCR, following the manufacturer's instructions. Cycle sequencing consisted of 25 cycles of 94°C for 10 s, 55°C for 5 s and 60°C for 4 min. Analysis of the products was carried out on an ABI prism 310 genetic analyser (Applied Biosystems, Waltham, MA, USA). Sequence data from both strands of the PCR products were aligned and compared using the Basic Local Alignment Search Tool (BLAST).

Assessment of serum C-peptide, CRP and NO

The C-peptide enzyme-linked immunosorbent assay (ELISA) kit was purchased from DRG Diagnostics (Marburg, Germany) and the CRP ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA), while NO was assayed using a colorimetric kit (BioVision, Milpitas, CA, USA). The procedures were performed according to the kit instructions provided.

Autoantibody determinations

GADA and ICA were determined using ELISA kits purchased from MyBiosource, and IAA was assessed using an ELISA kit purchased from AlphaDiagnostic International (San Antonio, CA, USA).

Determination of anti-CV IgG

The ELISA classic CV IgG kit was purchased from Serion GmbH (Würzburg, Germany). Procedure was performed according to the kit instructions. EV^+ patients who had positive (above cut-off value) and negative (below cut-off value) IgG readings were allocated into CV^+ and CV^- groups. Cut-off value was calculated by mean of control + (2 × s.d. of control).

Cytokine assays

Sandwich ELISA kits for TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL-10, IL-13, IL-20 and TGF-β were purchased from R&D Systems.

Complement activity

The complement activation product C3d was measured by ELISA, as described previously [19], where polyclonal rabbit anti-human C3d antibodies (Dako, Glostrup, Denmark) were used as the coating antibody and murine monoclonal antibody to C3d (Quidel, San Diego, CA, USA) was used as detecting antibody. Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (KPL, Gaithersburg, MD, USA) and *O*-phenylenediamine (Sigma, St Loius, MO, USA) were used for colorimetric enzyme-substrate reaction.

The soluble terminal complement complex (sC5b-9) level was assessed by ELISA using a monoclonal antibody (Quidel) against a neoepitope [20], as described previously [21].

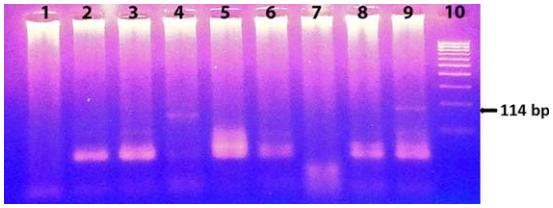


Fig. 1. Nested reverse transcription–polymerase chain reaction (RT–PCR) products of nine samples from type 1 diabetes (T1D) children. The test revealed two positive samples [lanes 4 and 9 show enterovirus (EV)-specific fragment of 114 base pairs (bp)] for EV infection out of nine samples. Lane 10 corresponds to DNA ladder. [Colour figure can be viewed at wileyonlinelibrary.com].

Statistical analysis

Data are presented as mean ± s.d. Statistical analysis was performed using SPSS version 20 for Windows (SPSS Inc., Chicago, IL, USA). The comparisons between healthy and $T1D\text{-}EV^-$ children or between $T1D\text{-}EV^-$ and $T1D\text{-}EV^+$ children were determined by one-way analysis of variance (ANOVA) least significant difference (LSD) *t*-test. A simple linear correlation analysis was processed by Pearson's method to measure the degree of dependency between variables using the MedCalc statistical program (MedCalcOstend, Belgium). Statistical significance was assumed at $P < 0{\cdot}05$.

Results

Frequency of EV RNA and anti-CV IgGs

A higher frequency of EV RNA was found in children with T1D (26·2%, 100 of 382) and none in healthy control children (0%, 0 of 100). Positive samples (two of nine samples) were identified by appearance of bands at 114 bp size (Fig. 1). Detection of anti-CV IgG revealed 2% positivity in control, 0% in the $T1D\text{-}EV^-$ (EV RNA-negative) and 64% in $T1D\text{-}EV^+$ (EV RNA-positive) groups (Table 1). The $T1D\text{-}EV^-$ group showed no change ($P > 0{\cdot}05$) in IgG levels compared to control, while $T1D\text{-}EV^+$ was significantly higher ($P < 0{\cdot}001$) than $T1D\text{-}EV^-$. Comparison between ND and PD patients for anti-CV IgG in the EV^+ group indicated 56 and 74% in ND and PD, respectively. Sequence analysis of PCR positive samples indicated 97% similarity with human CVB4 isolate CB4_Cph15 polyprotein gene (Accession no. KC558570).

Serum levels of HbA1c, C-peptide and autoantibodies

HbA1c was significantly higher ($P < 0{\cdot}001$) in $T1D\text{-}EV^-$ than controls. In comparison to the former group, HbA1c was significantly higher ($P < 0{\cdot}001$) in $T1D\text{-}EV^+$ (Fig. 2a). C-peptide was significantly lower ($P < 0{\cdot}001$) in $T1D\text{-}EV^-$

Table 1. Number and percentages of positive serum samples in reverse transcription–polymerase chain reaction (RT–PCR) [detection of enterovirus (EV)] and enzyme-linked immunosorbent assay (ELISA) immunoglobulin (IgG) [detection of coxsackievirus (CV)]

Group	No. of children	Screening RT–PCR (EV) NO. of positive samples (%)	Group	No. of children	ELISA IgG (CV) Mean ± s.d. (%)**	Group	No. of children	ELISA IgG (CV) Mean ± s.d. (%)	Duration of T1D
Control	100	0 (0%)	Control	100	0·34 ± 0·2 (2%)	Control	100	0·34 ± 0·2 (2%)	
T1D	382	100 (26·2%)	T1D-EV$^-$	100	0·33 ± 0·1 (0%)	T1D-EV$^-$ (ND)*	50	0·32 ± 0·1 (0%)	<1 year
						T1D-EV$^-$ (PD)†	50	0·34 ± 0·1 (0%)	>1 year
			T1D-EV+	100	0·96 ± 0·45 (64%)‡	T1D-EV$^+$ (ND)	50	0·83 ± 0·4 (56%)§	<1 year
						T1D-EV$^+$ (PD)	50	1·12 ± 0·4 (74%)¶	>1 year

*Newly diagnosed. †Previously diagnosed. ‡Significant ($P < 0·001$) compared to type 1 diabetes (T1D)-EV$^-$. §Significant ($P < 0·001$) compared to T1D-enterovirus (EV)$^-$ (newly diagnosed). ¶Significant ($P < 0·001$) compared to T1D-EV$^-$ (previously diagnosed). **Cut-off value = 0·74.

than controls, while the T1D-EV$^+$ group was non-significantly ($P > 0·05$) lower than T1D-EV$^-$ (Fig. 2b). Similar to the pattern of HbA1c serum levels in three different groups, all the autoantibodies (GADA, IAA and ICA) were significantly higher ($P < 0·001$) in T1D-EV$^-$ than controls, but showed a further increase ($P < 0·001$) in T1D-EV$^+$ compared to T1D-EV$^-$ (Fig. 3a–c). Comparison of autoantibody levels between EV$^+$-CV$^-$ and EV$^+$-CV$^+$ revealed higher frequency of autoantibodies in CV$^+$ than CV$^-$, while comparison between ND and PD patients who were CV$^-$ or CV$^+$ showed generally higher significance in ND than PD (Fig. 3d–f). This was more prominent in GADA and ICA than IAA. All autoantibodies were significantly higher in CV$^+$ ND than CV$^-$ ND. Only ICA levels were significantly ($P < 0·001$) higher in PD (CV$^+$) than PD (CV$^-$). EV$^+$ patients were separated into IgG$^-$ (< 0·74) and IgG$^+$ who were, in turn, allocated according to the strength of IgG into + (0·74–1·0), ++ (1·0–1·4) and +++ (> 1·4). Comparison of autoantibody levels between the latter groups clearly revealed the dependence of IAA on IgG but less prominently in ICA compared to GADA (Fig. 3g–i).

Serum levels of cytokines, TGF-β and NO

Inflammatory cytokines (IFN-γ, TNF-α and IL-1β) showed a similar pattern to that of autoantibodies. NO showed also the same pattern as an inflammatory mediator (Fig. 4). Levels of T helper type 2 (Th2) cytokines were investigated where only IL-4, IL-6 and IL-13 were significantly higher in T1D-EV$^-$ ($P < 0·001$) than control and no change ($P > 0·05$) in T1-EV$^+$ compared to T1D-EV$^-$. Again, IL-10 showed the same pattern to that of autoantibodies and inflammatory cytokines with a clearly higher increase ($P < 0·001$) in T1D-EV$^+$ *versus* T1D-EV$^-$ (Fig. 5). IL-12 and IL-17 showed the same pattern as inflammatory cytokines and NO, while IL-20 and TGF-β showed different patterns between groups (Fig. 6). The TGF-β level was significantly higher in T1D-EV$^-$ than controls and in T1D-EV$^+$ compared to T1D-EV$^-$ ($P < 0·001$). IL-20 was significantly higher in T1D-EV$^-$ than controls and lower in T1D-EV$^+$ than T1D-EV$^-$ ($P < 0·001$).

Serum levels of CRP and complement activity

Similar to inflammatory cytokines and NO, CRP showed the same pattern between the groups as a marker for

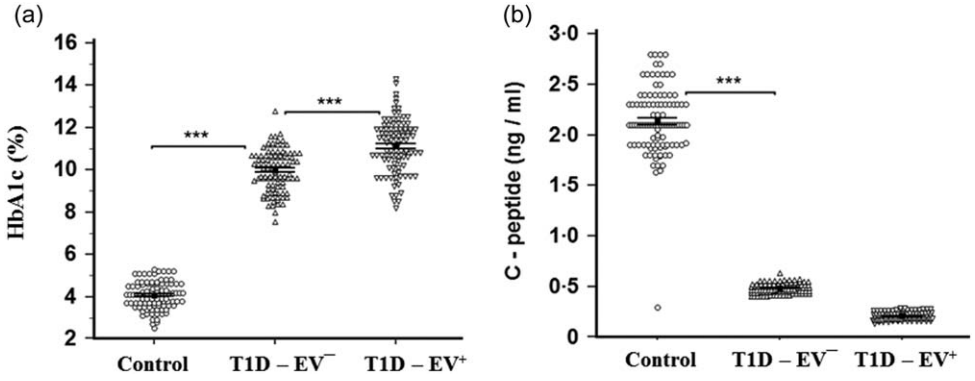


Fig. 2. Determination of both haemoglobin A1c (HbA1c) (a) and C-peptide (b) levels in human serum samples of control, enterovirus (EV)$^-$ and EV$^+$ groups. HbA1c was significantly higher ($P < 0·001$) in EV$^-$ than controls and in EV$^+$ than EV$^-$ ($P < 0·001$). C-peptide was significantly lower ($P < 0·001$) in EV$^-$ than controls, while EV$^+$ did not show any changes ($P > 0·05$) compared to EV$^-$. Means ± standard deviations are shown.

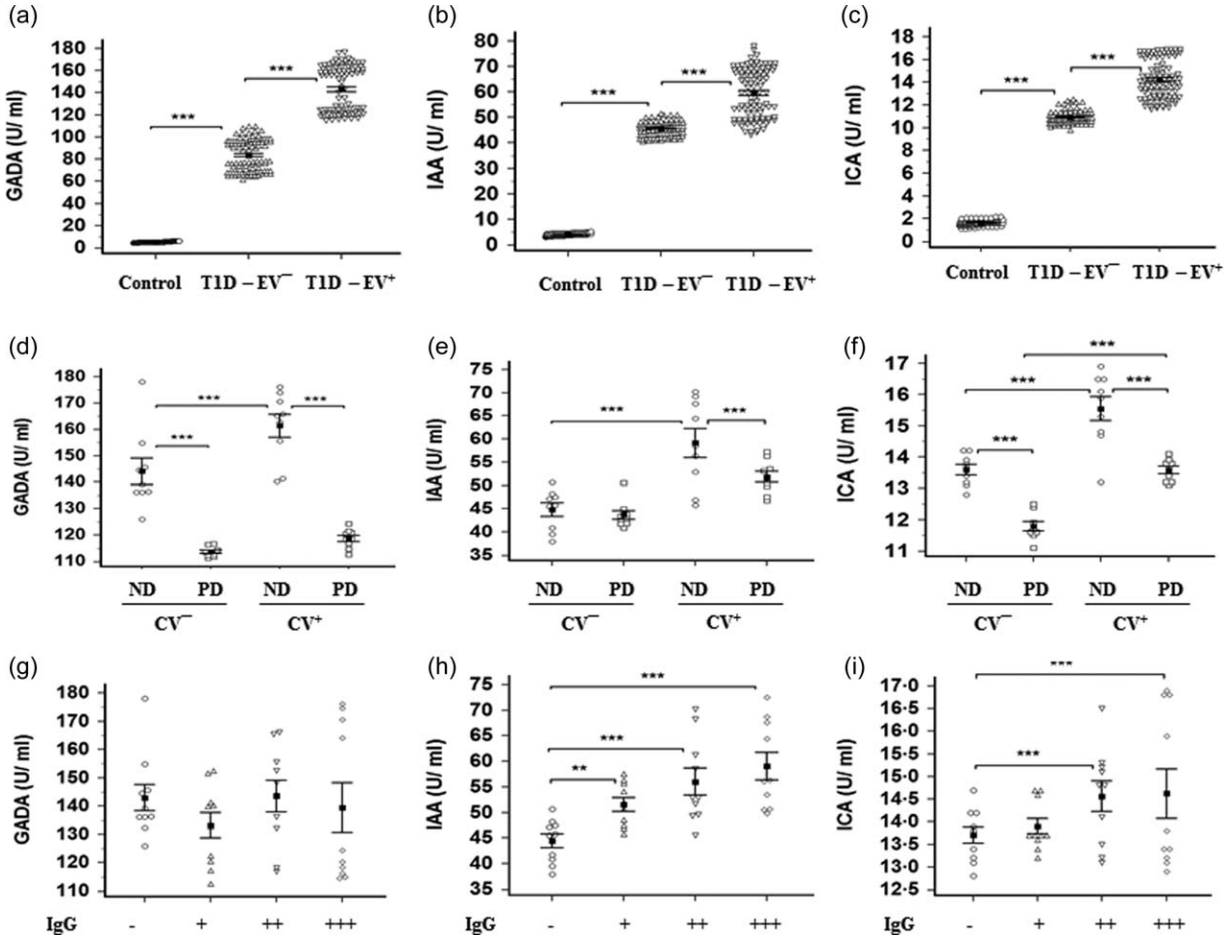


Fig. 3. Levels of glutamic acid decarboxylase autoantibodies (GADA) (a), insulin autoantibodies (IAA) (b) and islet cell cytoplasmic autoantibodies (ICA) (c) in human serum samples of control, enterovirus (EV)$^-$ and EV$^+$ groups. Autoantibodies were significantly higher in EV$^-$ than controls ($P < 0{\cdot}001$) and in EV$^+$ than EV$^-$ ($P < 0{\cdot}001$). Levels of GADA (d), IAA (e) and ICA (f) were also compared between newly diagnosed (ND) and previously diagnosed (PD) patients who were EV$^+$-CV$^-$ or EV$^+$-CV$^+$ showed generally higher significance ($P < 0{\cdot}001$) in ND than PD. The same levels were compared between EV$^+$-immunoglobulin (Ig)G$^-$, IgG$^+$ (+), IgG$^+$ (++) and IgG$^+$ (+++) showed dependence of IAA and ICA on IgG strength. Means ± standard deviations are shown.

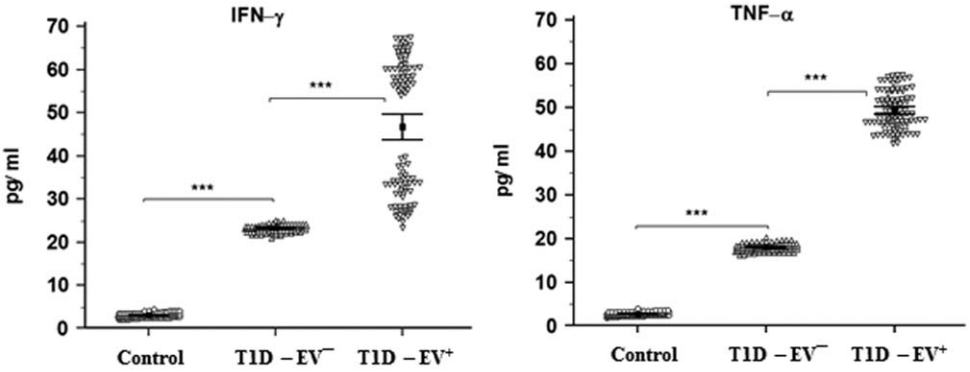
inflammation. While no changes ($P > 0{\cdot}05$) were observed between control and T1D-EV$^-$, complement activity was higher in T1D-EV$^+$ than T1D-EV$^-$ (Fig. 7). C3d was significantly higher compared to sC5b–9 ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively).

Correlations of autoantibodies, cytokines and TGF-β with HbA1c, C- peptide, NO, CRP, C3d and sC5b–9 in diabetic groups

For T1D-EV$^-$, positive correlation was found between ICA and sC5b–9 (0·45; $P < 0{\cdot}05$), while negative correlations appeared between C3d and IL-13 (–0·6; $P < 0{\cdot}05$) or sC5b–9 with IL-17 (–0·5; $P < 0{\cdot}05$; Table 2). In addition, HbA1c correlated negatively with IL-20 (–0.3; $P < 0{\cdot}01$). In T1D-EV$^+$, HbA1c, NO and CRP showed highly significant and positive correlations with autoantibodies, IFN-γ and IL-12 levels but negative correlations with IL-4, IL-6 and IL-13 (Table 3). IL-10 showed both positive and negative correlations with C-peptide (–0·2; $P < 0{\cdot}05$) and NO (–0·24; $P < 0{\cdot}05$), respectively. Negative correlations also appeared between IL-20 and CRP (–0.2; $P < 0{\cdot}05$) or IL-17 and NO (–0.23; $P < 0{\cdot}05$), while positive correlations appeared between NO and CRP with anti-CV IgG (0·25; $P < 0{\cdot}05$). Levels of C3d and sC5b–9 did not show any significant correlations in T1D-EV$^+$.

Correlations of HbA1c and C-peptide with NO, CRP, C3d and sC5b–9 in diabetic groups

While T1D-EV$^-$ did not show any significant correlations (Table 4), T1D-EV$^+$ showed positive correlations between HbA1c and each of NO (0·36; $P < 0{\cdot}001$) and CRP (0·2; $P < 0{\cdot}05$) or C-peptide with C3d (0·45; $P < 0{\cdot}05$).



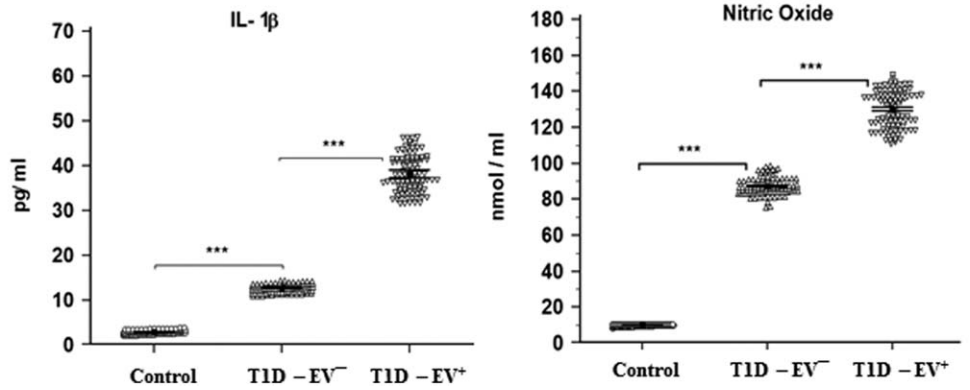
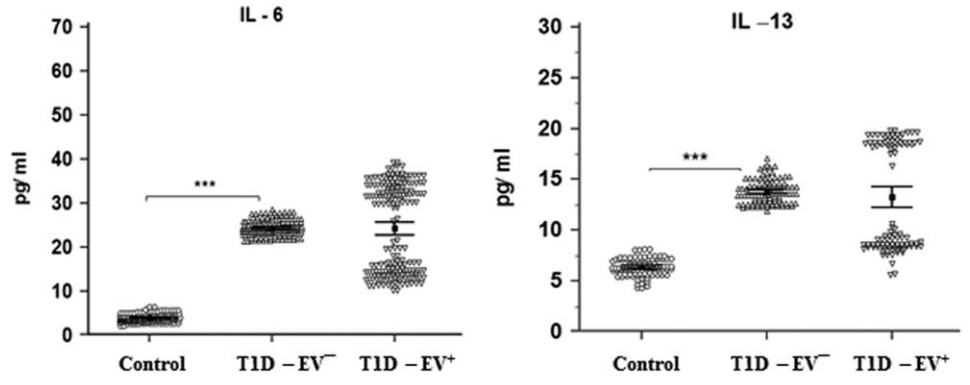


Fig. 4. Detection of interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β and nitric oxide (NO) in human serum samples of control, enterovirus $(EV)^-$ and EV^+ groups. Inflammatory cytokines and NO were significantly higher in EV^- than control ($P < 0{\cdot}001$) and in EV^+ than EV^- ($P < 0{\cdot}001$). Means ± standard deviations are shown.



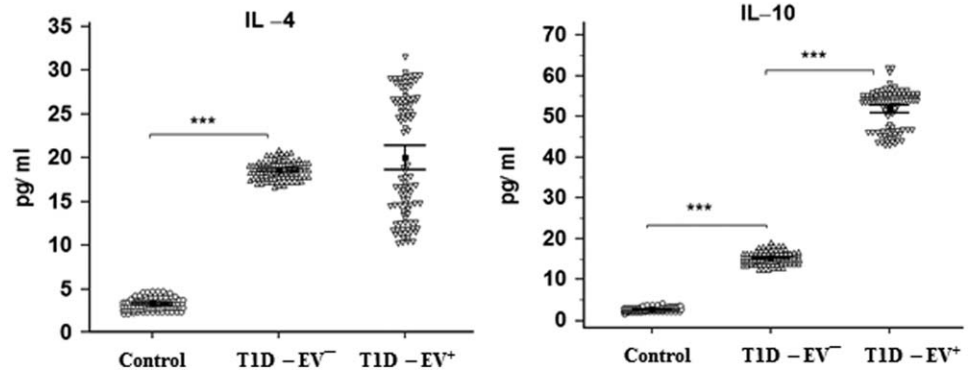


Fig. 5. Detection of interleukin (IL)-4, IL-6, IL-10 and IL-13 in human serum samples of control, enterovirus $(EV)^-$ and EV^+ groups. IL-4, IL-6 and IL-13 were significantly higher in EV^- than controls ($P < 0{\cdot}001$) and no changes in EV^+ *versus* EV^- ($P > 0{\cdot}05$). IL-10 was significantly higher in EV^- than controls ($P < 0{\cdot}001$) and in EV^+ than EV^- ($P < 0{\cdot}001$). Means ± standard deviations are shown.

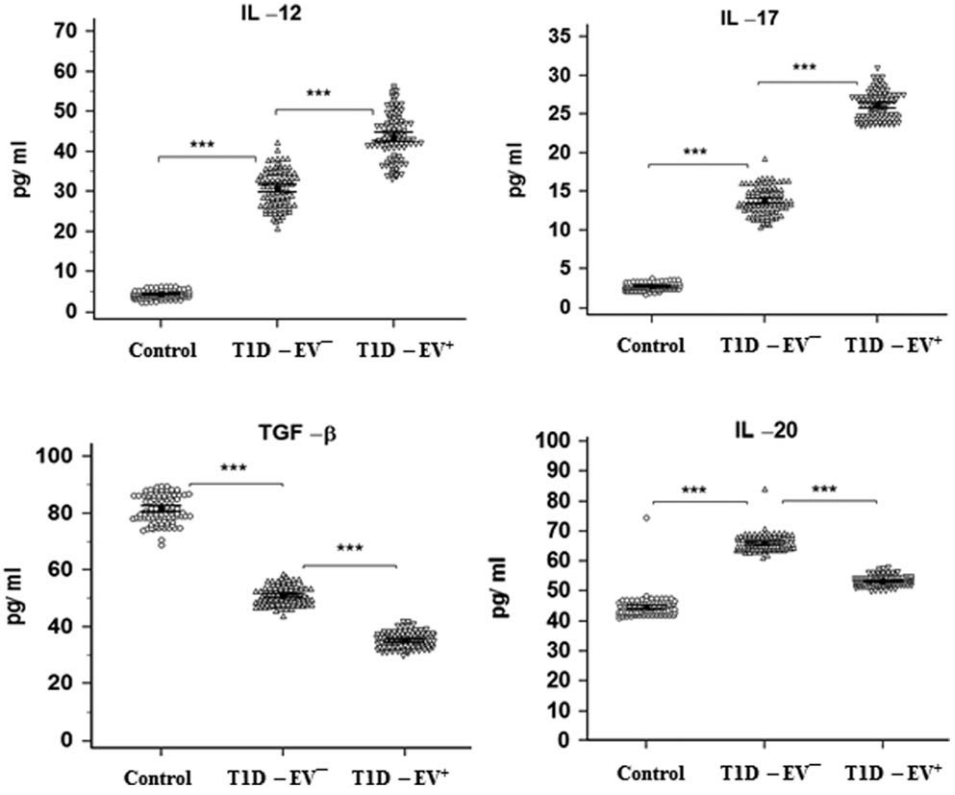


Fig. 6. Detection of interleukin (IL)-12, IL-17, IL-20 and transforming growth factor (TGF)-β in human serum samples of control, enterovirus $(EV)^-$ and EV^+ groups. IL-12 and IL-17 were significantly higher in EV^- than controls ($P < 0{\cdot}001$) and in EV^+ than EV^- ($P < 0{\cdot}001$). Conversely, TGF-β was significantly higher ($P < 0{\cdot}001$) in EV^- than controls and in EV^+ than EV^- ($P < 0{\cdot}001$). IL-20 was significantly higher ($P < 0{\cdot}001$) in EV^- than controls but lower ($P < 0{\cdot}001$) in EV^+ than EV^-. Means ± standard deviations are shown.

Correlations of autoantibodies with cytokines in diabetic groups

In T1D-EV^-, GADA correlated positively with IL-6 (0.4; $P < 0.001$), TNF-α, IL-13 and IL-17 (0·2; $P < 0{\cdot}05$) and negatively with IL-10 (–0·3; $P < 0{\cdot}01$; Table 5). IAA correlated negatively with both IL-4 and IL-12 (–0·2; $P < 0{\cdot}05$) but ICA correlated positively with IL-4 (0·4; $P < 0{\cdot}001$) and IL-13 (0·2; $P < 0{\cdot}05$). In T1D-EV^+, all autoantibodies correlated positively with both IFN-γ and IL-12 and negatively with IL-4, IL-6, IL-13 and IL-17 ($P < 0{\cdot}001$). GADA and IAA but not ICA correlated negatively with IL-20 ($P < 0{\cdot}05$), while all correlated negatively with IL-10 ($P < 0{\cdot}05$ for GADA and ICA but $P < 0{\cdot}01$ for IAA).

Correlations between autoantibodies and cytokines with anti-CV IgGs in T1D-EV^+

Anti-CV IgGs correlated positively with GADA (0·34; $P < 0{\cdot}01$), IAA and ICA (0·37; $P < 0{\cdot}001$) and both IFN-γ and IL-12 (0·3; $P < 0{\cdot}01$) but negatively with IL-4, IL-6 and IL-13 (–0·3; $P < 0{\cdot}01$; Table 6).

Discussion

The prevalence of EV infection among T1D children revealed 26·2% positive cases based on EV RNA detection in serum. This finding indicated a probability of infection with other environmental viruses which could be a cause of

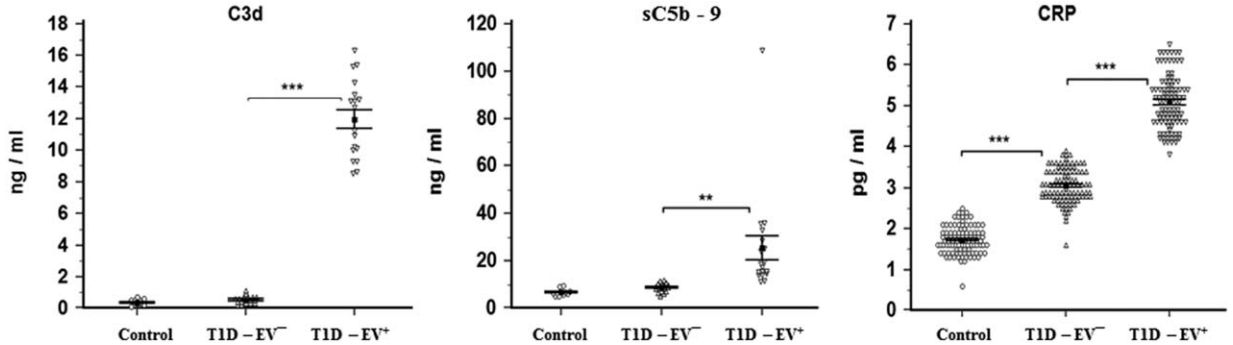


Fig. 7. Detection of complement activity and C-reactive protein (CRP) serum levels in controls, EV^- and EV^+ groups. Both C3d and sC5b–9 were significantly higher in EV^+ than EV^- ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively). CRP was significantly higher in EV^- than controls ($P < 0{\cdot}001$) and in EV^+ than EV^- ($P < 0{\cdot}001$). Means ± standard deviations are shown.

Table 2. Correlations with haemoglobin A1c (HbA1c), C-peptide, nitric oxide (NO), C-reactive protein (CRP), C3d and sC5b–9 in the type 1 diabetes (T1D)-enterovirus $(EV)^-$ groups

	HbA1c	C- peptide	NO	CRP	C3d	sC5b–9
			r (*P*-value)			
GADA	0·02 (0·849)	0·04 (0·695)	0·2 (0·05)	−0·2 (0·09)	−0·2 (0·4)	−0·07 (0·783)
IAA	−0·14 (0·175)	0·16 (0·114)	0·1 (0·336)	−0·07 (0·05)	−0·37 (0·121)	0·115 (0·65)
ICA	0·10 (0·318)	0·13 (0·181)	−0·08 (0·382)	0·007 (0·93)	−0·23 (0·359)	0·453 (0·04)*
IFN-γ	−0·12 (0·254)	−0·05 (0·6)	−0·07 (0·485)	−0·06 (0·54)	0·06 (0·81)	−0·2 (0·422)
TNF-α	0·11 (0·269)	−0·11 (0·27)	0·12 (0·233)	0·077 (0·44)	−0·006 (0·98)	−0·15 (0·546)
IL-1β	−0·02 (0·881)	−0·08 (0·43)	−0·13 (0·2)	0·122 (0·225)	0·11 (0·657)	0·3 (0·2)
IL-12	0·11 (0·287)	0·04 (0·7)	−0·03 (0·76)	0·154 (0·126)	0·017 (0·94)	−0·173 (0·5)
IL-4	0·06 (0·535)	−0·16 (0·11)	−0·04 (0·68)	−0·026 (0·8)	−0·48 (0·05)	0·27 (0·27)
IL-6	0·02 (0·808)	0·02 (0·876)	−0·04 (0·6)	0·05 (0·6)	0·063 (0·8)	−0·13 (0·6)
IL-13	−0·14 (0·160)	−0·17 (0·1)	−0·09 (0·366)	−0·12 (0·22)	−0·6 (0·01)*	0·1 (0·7)
IL-20	−0·33 (0·001)**	−0·10 (0·326)	−0·04 (0·7)	−0·06 (0·55)	−0·04 (0·86)	0·23 (0·35)
IL-10	−0·20 (0·05)	−0·08 (0·408)	−0·05 (0·6)	0·06 (0·55)	0·28 (0·25)	0·28 (0·25)
IL-17	0·14 (0·150)	0·10 (0·303)	0·1 (0·24)	0·035 (0·72)	0·136 (0·58)	−0·5 (0·03)*
TGF-β	−0·04 (0·7)	−0·015 (0·87)	−0·07 (0·46)	0·07 (0·44)	−0·3 (0·21)	0·08 (0·7)

GADA = glutamic acid decarboxylase; IAA = insulin autoantibodies; ICA = islet cell cytoplasmic autoantibodies; IFN = interferon; TNF = tumour necrosis factor; IL = interleukin; TGF = transforming growth factor. *$P < 0·05$; **$P < 0·01$.

Table 3. Correlations with haemoglobin A1c (HbA1c), C-reactive peptide (CRP), nitric oxide (NO), CRP, C3d and sC5b–9 in type 1 diabetes (T1D)-enterovirus $(EV)^+$

	HbA1c	C- peptide	NO	CRP	C3d	sC5b–9
			r (*P*-value)			
GADA	0·37 (0·0001)***	−0·14 (0·170)	0·8 (<0·0001)***	0·6 (<0·0001)***	−0·006 (0·98)	0·18 (0·47)
IAA	0.34 (0.0005)***	−0·03 (0.736)	0.8 (<0.0001)***	0.6 (<0.0001)***	0.17 (0.48)	0.35 (0.15)
ICA	0.36 (0.0002)***	−0·16 (0.122)	0.75 (<0.0001)***	0.45 (<0.0001)***	−0·14 (0.57)	0.15 (0.54)
IFN-γ	0.34 (0.0005)***	−0·15 (0.146)	0.8 (<0.0001)***	0.6 (<0.0001)***	0.27 (0.26)	0.17 (0.48)
TNF-α	0.08 (0.423)	0.04 (0.681)	−0·04 (0.7)	−0·01 (0.9)	0.002 (0.9)	0.27 (0.27)
IL-1β	−0·07 (0.501)	0.14 (0.153)	−0·15 (0.13)	−0·09 (0.35)	−0·06 (0.8)	0.09 (0.7)
IL-12	0.29 (0.003)**	−0·15 (0.126)	0.66 (<0.0001)***	0.53 (<0.0001)***	0.4 (0.1)	0.1 (0.64)
IL-4	−0·33 (0·001)**	0·09 (0·388)	−0·77 (<0·0001)***	−0·6 (<0·0001)***	0·1 (0·7)	−0·14 (0·57)
IL-6	−0·33 (0·0009)***	0·09 (0·382)	−0·84 (<0·0001)***	−0·6 (<0·0001)***	−0·27 (0·26)	−0·02 (0·9)
IL-13	−0·36 (0·0002)***	0·11 (0·283)	−0·84 (<0·0001)***	−0·6 (<0·0001)***	−0·33 (0·17)	−0·0009 (1)
IL-20	−0·11 (0·272)	0·05 (0·627)	−0·2 (0·07)	−0·2 (0·02)*	−0·06 (0·8)	0·07 (0·7)
IL-10	−0·03 (0·769)	0·22 (0·032)*	−0·24 (0·02)*	−0·14 (0·15)	0·06 (0·8)	−0·34 (0·15)
IL-17	−0·21 (0·038)	0·02 (0·824)	−0·23 (0·02)*	−0·16 (0·09)	−0·08 (0·7)	−0·05 (0·8)
TGF-β	0·37 (0·12)	−0·05 (0·6)	0·13 (0·2)	0·1 (0·26)	0·2 (0·36)	0·37 (0·12)
Anti-CV IgGs	−0·14 (0·245)	0·10 (0·389)	0·25 (0·03)*	0·25 (0·04)*	−0·05 (0·8)	0·21 (0·38)

GADA = glutamic acid decarboxylase; IAA = insulin autoantibodies; ICA = islet cell cytoplasmic autoantibodies; IFN = interferon; TNF = tumour necrosis factor; IL = interleukin; TGF = transforming growth factor. *$P < 0·05$; ** $P < 0·01$; ***$P < 0·01$.

Table 4. Correlations between haemoglobin A1c (HbA1c) & C-peptide with nitric oxide (NO), C-reactive protein (CRP), C3d and sC5b–9 in type 1 diabetes (T1D)-enterovirus $(EV)^-$ and EV^+ groups

		NO	CRP	C3d	sC5b–9
				r (*P*-value)	
HbA1c	T1D-EV^-	0·13 (0·18)	−0·07 (0·47)	0·03 (0·9)	−0·18 (0·46)
	T1D-EV^+	0·36 (0·0002)***	0·2 (0·036)*	0·02 (0·9)	−0·4 (0·12)
C- peptide	T1D-EV^-	−0·045 (0·65)	0·07 (0·5)	0·3 (0·18)	0·015 (0·95)
	T1D-EV^+	−0·2 (0·1)	0·07 (0·4)	0·45 (0·04)*	0·1 (0·64)

*$P < 0·05$; ***$P < 0·01$.

Table 5. Correlations between autoantibodies and cytokines in type 1 diabetes (T1D)-enterovirus (EV)$^{-}$ and EV^{+} groups

	T1D-EV^{-}			T1D-EV^{+}		
	GADA	IAA	ICA	GADA	IAA	ICA
	r (*P*-value)			*r* (*P*-value)		
IFN-γ	0·1 (0·2)	0·01 (0·89)	0·1 (0·44)	0·9 (<0·0001)***	0·9 (<0·0001)***	0·8 (<0·0001)***
TNF-α	0·2 (0·02)*	0·01 (0·78)	0·01 (0·69)	0·0 (0·65)	0·0 (0·83)	0·0 (0·62)
IL-1β	0·01 (0·83)	0·01 (0·72)	0·01 (0·92)	−0·1 (0·44)	−0·1(0·38)	−0·1 (0·25)
IL-12	−0·1 (0·24)	−0·2 (0·02)*	0·1 (0·45)	0·7 (<0·0001)***	0·7 (<0·0001)***	0·6 (<0·0001)***
IL-4	0·1 (0·21)	−0·2 (0·03)*	0·4 (<0·0001)***	−0·8 (<0·0001)***	−0·8 (<0·0001)***	−0·7 (<0·0001)***
IL-6	0·4 (<0·0001)***	0·2 (0·07)	0·01 (0·82)	−0·9 (<0·0001)***	−0·8 (<0·0001)***	−0·7 (<0·0001)***
IL-13	0·2 (0·02)*	0·01 (0·73)	0·2 (0·04)*	−0·9 (<0·0001)***	−0·9 (<0·0001)***	−0·8 (<0·0001)***
IL-20	−0·1 (0·15)	0·01 (0·76)	0·01 (0·84)	−0·2 (0·04)*	−0·3 (0·012)*	−0·2 (0·06)
IL-10	−0·3 (0·004)**	−0·2 (0·12)	0·1 (0·47)	−0·2 (0·048)*	−0·3 (0·007)**	−0·2 (0·04)*
IL-17	0·2 (0·01)*	0·01 (0·1)	−0·1 (0·32)	−0·3 (0·0004)***	−0·3 (0·006)**	−0·3 (0·009)**
TGF-β	−0·15 (0·12)	−0·18 (0·06)	−0·17 (0·08)	0·05 (0·63)	−0·0005 (0·1)	0·05 (0·65)

GADA = glutamic acid decarboxylase; IAA = insulin autoantibodies; ICA = islet cell cytoplasmic autoantibodies; IFN = interferon; TNF = tumour necrosis factor; IL = interleukin; TGF = transforming growth factor. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

T1D. Another possibility is the involvement of genetic risk, which contributed to autoimmunity to pancreatic β cell antigens. The study highlighted the necessity of investigation of other viral infections among T1D patients [22]. Indeed, Rubella virus, rotavirus, cytomegalovirus and mumps virus were found to be associated previously with children with T1D [23–25]. The study tested only 10 PCR products (EV^{+}) for sequencing, where all showed high homology with CVB4. The sequencing results could not be direct evidence or an indicator for the involvement of CVB4 in T1D, especially considering that the ELISA test for anti-CV IgGs gave 64% positivity. This, in turn, might also imply the involvement of other EVs such as echoviruses in the disease [26]. In this study, the T1D-EV^{+} population was selected based on the results of nested PCR for detection of viral RNA in T1D patients. The viral RNA detection in serum represented a diagnostic method for active infection, which might be sufficient for induction of autoimmunity before development of anti-viral IgG [27]. The other possibility is viral infection after ongoing autoimmunity, which could lead to T1D acceleration [28]. Indeed, the sequence of events, whether the viral infections occurred earlier and autoimmunity occurred later or vice versa, cannot be predicted because all the RNA-negative children were IgG-negative.

Increased IgG levels could be an indicator for recurrent infections with same virus in children as a result of drinking water contaminated with EVs [29]. In addition, IgG levels among EV^{+}-PD (more than 1 year) who might have been exposed to recurrent infections were significantly higher than EV^{+}-ND (less than 1 year). If we speculate that EV^{+}-CV^{-} and EV^{+}-CV^{+} represent new and recurrent infections, respectively, the recurrent infections down-regulate the production of autoantibodies. This is because the autoantibodies in ND children were significantly higher than PD. In the case that the development of anti-viral IgG occurs after a long time of first infection, this is often associated with down-regulated production of autoantibodies. However, separation of EV^{+} according to IgG strength into positive, +, ++ and +++ revealed a dependence of IAA and ICA on IgG strength. If we speculate the dependence of IgG strength on acute and recurrent infections, the latter would increase IAA and ICA, which supports the hypothesis of accelerated ongoing autoimmunity and overt diabetes after viral infection. The correlation analysis between IgG and autoantibodies supports this hypothesis further.

As most cases were anti-CV IgG-positive, it could be considered that CV invaded β cells through specific receptors, replicated and led to autoimmunity and cell destruction [30]. This cell destruction was widespread as a result of highly exposed autoantigens and activation of bystander T cells [5]. The increased expression of major histocompatibility complex (MHC-I) proteins on the cell surface with viral antigens could also activate CD8^{+} T cells [31]. Innate immunity also played an important role through activation of Toll-like receptors (TLRs) on macrophages and neutrophils which, in turn, secreted inflammatory cytokines [32]. These cytokines could recruit and activate natural killer cells (NKs), which played an important role in killing and destroying β cells [33]. Recurrent infections could invade more β cells and increase the activated CD8^{+} T cells and levels of autoantibodies.

HbA1c showed an observable increase in diabetic groups, either infected or not. However, diabetic EV^{+} showed a further increase over EV^{-}. Thus, EV infection exacerbated β cell destruction and decreased secretion of insulin. This was also confirmed by measured C-peptide which, conversely, decreased in diabetic groups. The effective role of EV in inhibition of insulin secretion was assigned to depletion of its granule stores and hence

Table 6. Correlations between autoantibodies & cytokines with anti-coxsackievirus (CV) immunoglobulins (IgGs) in the type 1 diabetes (T1D)-EV$^+$ group

	Anti-CV IgGs *r* (*P*-value)
GADA	0·34 (0·003)**
IAA	0·37 (0·0009)***
ICA	0·37 (0·0009)***
IFN- γ	0·3 (0·001)**
TNF-α	−0·07 (0·56)
IL-1β	0·13 (0·25)
IL-12	0·3 (0·006)**
IL-4	−0·3 (0·008)**
IL-6	−0·3 (0·007)**
IL-13	−0·3 (0·005)**
IL-20	−0·02 (0·86)
IL-10	−0·05 (0·7)
IL-17	−0·02 (0·86)
TGF-β	0·05 (0·68)

GADA = glutamic acid decarboxylase; IAA = insulin autoantibodies; ICA = islet cell cytoplasmic autoantibodies; IFN = interferon; TNF = tumour necrosis factor; IL = interleukin; TGF = transforming growth factor. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}01$.

inhibition of insulin secretion [34]. In addition, increased production of autoantibodies to multiple cell antigens, including GAD, IA and IC in diabetic groups, with observable exacerbation in EV$^+$ compared to EV$^-$, implicated the potential role of EV in autoimmunity induction [35]. Increased levels of proinflammatory cytokines (IFN-γ, TNF-α and IL-1β) and NO coincided with and interpreted further the observed levels of HbA1c, C-peptide and autoantibodies. These cytokines had been known to be associated with viral infections, while NO was also known to induce damage to the cell [36,37]. The direction of immune response to Th1 phenotype by the production of IFN-γ was observable in T1D-associated autoimmunity. However, IL-10, which is a Th2 cytokine and known to be secreted by T_{regs}, was also elevated in diabetic groups, with an observable increase in EV$^+$. Indeed, this finding was also observed in autoimmune T1D patients in comparison to non-diabetic controls and even in diabetic EV$^+$ *versus* diabetic EV$^-$ children [7,11,38]. IL-4, IL-6 and IL-13 appeared higher in diabetic groups, but no difference between EV$^+$ and EV$^-$ was observed. Thus, EV, rather than IL-4, IL-6 and IL-13, motivated production of IFN-γ. Increased levels of IL-4 and IL-6 in hyperglycaemic T1D children were found sustained even after 2 h of euglycaemia/correction [39]. It could be considered that the increased levels of IFN-γ due to viral infection dampened the levels of Th2 cytokines (IL-4, IL-6 and IL-13) but not IL-10, showing the latter as a distinct cytokine. IL-12 and IL-17 behaved similarly to inflammatory cytokines. Thus, increased levels of IFN-γ, IL-12 and IL-17 in viral infection revealed a predominance of Th1 and Th17 *versus* Th2 immune responses. Both IFN-γ and IL-12 were reported to be responsible for activation of natural killer (NK) cells which are, in turn, responsible for pancreatic β cell apoptosis [40]. Thus, NKs with help from IFN-γ and IL-12 initiated β cell lysis before activation of CD8$^+$ T cells and production of anti-viral antibodies. Generally, IFN-γ, IL-10, IL-12 and IL-17 were found to play an important role in induction of autoimmunity and disease progression with EV infection [38]. In addition, elevated levels of IL-12 and IL-17 were found to be associated with low frequencies of T_{regs} [9]. TGF-β decreased in diabetic groups with observably exacerbated decrease in EV$^+$ *versus* EV$^-$ children. Human serum TGF-β levels were also decreased in diabetic children with either types 1 or 2 [14]. Indeed, the levels of this growth factor in serum were indicators for the infiltration and activity of T_{regs} which decreased during autoimmunity [41]. IL-20 levels were found to be up-regulated in diabetic EV$^-$ children in comparison to healthy controls, but down-regulated in EV$^+$ *versus* EV$^-$ children. This indicated an association of this cytokine with T1D-EV$^-$ but down-regulated in T1D-EV$^+$. IL-20 belongs to the IL-10 family of cytokines and is linked to other autoimmune diseases, such as rheumatoid arthritis [42].

The levels of C3d and sC5b–9 indicated increased complement activity in diabetic EV$^+$ *versus* diabetic EV$^-$ children. Moreover, their levels in diabetic EV$^-$ children did not show any significant differences compared to healthy controls. Thus, the profile of these complement activation products (especially C3d) was distinct for diabetic EV$^+$. However, complement activation was described preciously to play a role in the disease pathogenesis, irrespective of viral infections [43]. The involvement of activated complement components in inflammatory reactions after EV infection was also found [44]. Furthermore, capsid proteins of CVB3 were found to interact with C3 and activate the alternative pathway [45]. The levels of CRP showed a similar profile to inflammatory cytokines (IFN-γ, TNF-α and IL-1β, IL-12 and IL-17), as well as IL-10 and NO. CRP was found to polarize human monocytes towards the proinflammatory M1 phenotype and was involved in complement activation [46,47].

Statistical correlations in diabetic EV$^-$ showed fewer significantly positive and negative associations. Statistical analysis showed the dependence of complement activity on ICA in diabetic EV$^-$ [48]. Conversely, complement activation was independent of IL-13 and IL-17. Furthermore, IL-20 showed an inhibitory effect on pancreatic β cell destruction. However, this was not apparent in EV infection.

A strong dependence was found of inflammation and β cell destruction in EV infection on the levels of anti-viral antibodies, autoantibodies and Th1, but not Th2, cytokines [13,49]. In addition, a positive correlation between the levels of C-peptide and C3d revealed the dependence of β cell destruction on complement activity. This can be considered as evidence for a complementary role in T1D-EV$^+$ disease.

Statistical correlation revealed also that the increased levels of both IL-4 and IL-6 were responsible for increased levels of ICA and GADA, respectively, in diabetic EV$^-$. Conversely, the decreased levels of these cytokines together with IL-13 and less potently IL-17 were responsible for increased GADA, IAA and ICA in diabetic EV$^+$. Moreover, IFN-γ and IL-12 were responsible for exposure of more β cell antigens to the immune system as a result of EV entry to the cell (breaking tolerance) leading to autoimmunity [50].

In conclusion, T1D-EV$^+$ showed increased levels of inflammatory cytokines and NO when compared to T1D-EV$^-$. Viral infection increased Th1 and decreased Th2 cytokines with remarkable down-regulation for T_{regs}. IL-10, IL-20 and complement activation products showed distinct profiles for viral infection, highlighting their roles in diagnosis of T1D-EV$^+$. The recurrent viral infections accelerate the ongoing autoimmunity and overt T1D.

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None.

Author contributions

A. A. M. designed the experimental study, M. H. H. diagnosed the disease, R. G. K. performed the experiments, M. A. L. wrote the paper.

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