Format for ANSWERING REVIEWERS

São Paulo, January 24, 2015.

Dear Dr.
Yue –Li Tian
Science Editor, Editorial Office

Please find enclosed the edited manuscript in Word format (file name: **15681-review.doc**).

Title: Enterovirus and type 1 diabetes : What is the matter?

Author: Carla Sanchez Bergamin, Sergio Atala Dib

Name of Journal: *World Journal of Diabetes*

ESPS Manuscript NO: 15681

We would like to thank the reviewers for their comments and suggestions, which certainly helped to improve our manuscript. We have modified the manuscript according to the suggestions of the reviewers:

1 The format has been updated

2 The references and typesetting were corrected

3 Revisions have been made according to the suggestions of the reviewers

Reviewer 503951

(1) *On page 7, authors should specify whether diabetic children were more susceptible to CVB1 infection or vice versa.*

This is a difficult point. A recent meta- analysis (BMJ 2011;342:d35) assessing enteroviral infection (detected by both molecular and immunologic assays) showed that compared with non-diabetic control subjects, the likelihood of finding evidence of enterovirus is 10-fold higher in T1D patients and 4-fold higher in individuals with diabetes-related autoimmunity. However, we think that how the individual, guided by his genetic predisposition to autoimmunity, reacts to viral infections is also important. This issue is discussed in our revised manuscript.

(2) *On page 8, authors should discuss more about the beta-cell GAD65 autoantigen and the common amino acid sequence of the virus, especially the TCR repertoires of the T-cells in TIDM patients and healthy individuals.*

This is an important point on the relationship between virus and type 1 diabetes etiopathogenesis, and it is discussed on pages 7, 11 and 12. The relevant text is shown below for your convenience.

On page 7: Much attention has been paid to the possible immunological cross-reactivity that is induced by a homology sequence in the 2C non-structural CVB protein and a principal diabetes autoantigen, glutamic acid decarboxylase 65 (GAD65), wich share a common amino acid sequence[56-57]. GAD65 is an important target antigen in the pathogenic process of diabetes. In mice, the insulitis establishment coincides with GAD65 specific reactivity, and tolerance induction to GAD65 can prevent the disease[58-59]. Humoral and cellular responses have been detected against GAD65 before the onset of clinical diabetes[60] and auto antibodies are positive several years before diagnosis[61]. The importance of this homology in T1DM pathogenesis is supported by data showing that T cells that respond to this sequence are present both in NOD mice and T1DM patients[62-63]. This mechanism will be discussed below.

On page 11: In individuals who are genetically predisposed to T1DM, viral infections may result in the impaired activation of self-reactive T cells through a mechanism that is independent of specific T-cell receptor (TCR) stimulation[99-100]. This process, called "bystander activation", does not require specific TCR stimulation and was supported by a study of CVB4 infection in transgenic mice that resulted in the activation of circulating naive islet-specific T cells and clinical diabetes development[101].

Furthermore, the mechanism of cell destruction may be based on molecular mimicry[102]. The activation of a T-cell population against an environmental antigen results in the development of autoimmune disease if the epitope recognized shows sequence or structural similarity with a self-protein. Although virus-specific T lymphocytes are activated during an infection, antibody responses are critical in the defense against enteroviruses and are responsible for the clearance of the infection. Neutralizing antibodies are directed against the capsid surface of CVB and nonstructural proteins. These proteins are produced exclusively during the replication of the virus and are released as a consequence of the lysis of the infected cells. Directly linked to T1DM triggering was an observation of the amino acid sequence similarity between CVB4 nonstructural protein 2C and GAD65 (PEVEKEK), which suggests that the cellular anti-viral response may cross-react with the native protein, inducing an autoimmune response[103].

On page 12: All these mechanisms described may occur simultaneously. In fact, inflammatory conditions induced by virus infection will trigger autoimmunity resulting in T1DM only in susceptible individuals[38]. This hypothesis, Fertile Field, postulates that following the inflammation caused by virus infection, autoreactive T cells may be generated by bystander activation or molecular mimicry or both. The damage of beta cells and its presentation to immune system lead to antigenic epitope spreading, which explains the broad autoreactive T-cell repertoire in T1DM patients.

(3) *On page 10, authors use hygiene hypothesis to explain the association of EV and TIDM. The authors stated that "a lower prevalence of EV infection in developed countries". You need to use more scientific data to support this statement. Then, besides fecal-oral route, EV can transmit by air droplet and direct contact.*

The main scientific data to support the statement are that inverse geographical variations exist in the relationship between the frequencies of type 1 diabetes and incidence of childhood diarrheal diseases worldwide (www.cdc.gov and www.eatlas.idf.org)

The EV transmission routes were corrected in the text.

(4) *I think not all the virus infection will obey the hygiene hypothesis. For example, neonatal infection with respiratory syncytial virus will more easily to develop asthma in adult.*

We agree with you that not all virus infections will obey the hygiene hypothesis because the relationship between infections and autoimmune diseases is more complex. This issue is discussed on pages 9 and 10.

On page 9: The hygiene hypothesis was first proposed by Strachan to explain the increasing rates of asthma in highly developed countries[77], suggesting that contact with a high number of infections early in life could properly modulate the adaptive immune system, and the significant changes in human living standards and the improvement of sanitary conditions meant that people had less exposure to infection, favoring an impaired immune response to environmental triggers[35,78]. This concept may be applied to many autoimmune diseases, but it does not explain all of these diseases, as there is a complex interplay between environmental exposure, the host, and other confounding variants[78-80].
Exposure to HEV, which is typically transmitted through a fecal-oral pathway, becomes less common as individual age, and infection with HEV later in life could result in an unbalanced immune response. In other words, where enterovirus infections are frequent, children develop an efficient immune response to these viruses, and when they are exposed in the future, the effects are not exacerbated or harmful. This may explain the rising worldwide incidence of T1DM over the last decade, mainly in developed societies where enterovirus infections are less prevalent[81-83].
On page 10: In addition, the frequency of T1DM is higher in the firstborns of multiplex families than in younger children, which could be explained by a lower exposure of firstborns than siblings to infections[88].

(5) *Although EV infection may have some association with TIDM, it lack of an animal model to demonstrate. So, it is quite controversial. In addition, I suggest that the authors may discuss more before the manuscript can be published.*
The associations of EV with T1DM have been demonstrated in a animal model of type 1 diabetes (non-obese-diabetes mouse), as was discussed on page 6.
On page 6: This are convincing experimental results for the role of EV infection in T1DM development using mouse models[44-46], and some mechanisms of beta cell damage have been proposed based on experiments with non-obese diabetic (NOD) mice[47].

In addition, these controversial data and the theories about T1DM and enterovirus infection are now described in better detail in the Discussion and Conclusion sections.

Reviewer 742221

Thanks for your comment. We were very pleased.

Reviewer 1408945

(1) *These conclusions will confuse readers. Authors should mention clearly that enterovirus affects onset and/or development of type 1 diabetes mellitus or not. If it is not, the descriptions about enterovirus will be meaningles*
The conclusions were re-written to clarify the main points regarding the effects of enterovirus on the onset and/or development of type 1 diabetes. We concluded that there is a substantial amount of evidence to support the idea that enterovirus affects the onset and/or development of type 1 diabetes; however, improvements in enterovirus detection methods and randomized studies with patient follow-up are still required to confirm the importance of HEV in T1DM development and progression. The genetic predisposition to autoimmunity viral infections can, in some individuals,

be causal in the development of type 1 diabetes. (pages 16 and 17).

(2) *In the introduction section, authors mentioned the relationship between multi-virus and type 1 diabetes mellitus. However, authors jumped up to enterovirus explanation. Authors should mention why authors selected enterovirus in detail*

While several viruses have been implicated as viral factors in type 1 diabetes etiopathogenesis, the strongest evidence lies with human enteroviruses, which have been associated with an increased risk of islet autoimmunity and faster progression of this disease (Enterovirus is a member of the *Picornaviridae* family and the genus *Enterovirus*, which includes a diverse group of small RNA viruses (polioviruses, echoviruses and Coxsackieviruses) characterized by a single positive strand genomic RNA).

Therefore, we selected enterovirus to discuss in detail, this decision is justified on page 1 and by refs. 28-31.

(3) *Authors should consider the priority of articles. This may change conclusions in this study.*

We agree with you that the priority and importance of the data to discuss in a review needs to be considered and discussed carefully because the conclusions can be modified. We revised the manuscript with this point in mind and added relevant text to pages 6 to 9.

On page 6: This are convincing experimental results for the role of EV infection in T1DM development using mouse models[44-46], and some mechanisms of beta cell damage have been proposed based on experiments with non-obese diabetic (NOD) mice[47].

On page 7: Yeung conducted a useful systematic review and meta-analysis of observational molecular studies on the detection of enterovirus in T1DM patients[64]. Observational case-control studies measured enterovirus RNA or viral protein in the blood, stool or tissue of prediabetic and diabetic patients by molecular methods. The 24 selected papers and two abstracts demonstrated a clinically significant association between enterovirus infection and autoimmunity/T1DM (odds ratios ranging from 5.5 to 17.4).

On page 8 and 9: A positive association between EV infections and a rapid progression from autoimmunity to clinical T1DM was observed both in the DiMe study as well as in the DAISY follow-up study (human longitudinal studies). However, there was no agreement in the studies' conclusions between EV infection and islet autoimmunity development.

The results of these prospective studies may be controversial due to heterogeneity in the study design, the small number of patients in each study and the low sensitivity of the methods used to detect enterovirus infection. Another important confounding factor is the frequency of sampling because EV RNA can rarely be found continuously in stool samples for more than 3 months, and it is found for a shorter time in serum samples[75]. The studies that indicated a positive association between enterovirus infection and T1DM used smaller sampling intervals and a wider panel of enterovirus assays than the studies that indicated no association. Similarly, most enterovirus infections are asymptomatic, and a negative result for the virus at diagnosis does not mean that its contribution is meaningless.

The prevalence of EV infections varies in populations, and independent of this, the vast majority of people infected will not develop autoimmunity or T1DM, as illustrated by Sarmineto[76]. This study showed that in Cubans that were exposed to an echovirus epidemic, a large number of patients seroconverted to islet autoantibody positivity, but T1DM prevalence has not increased. It remains to be determined how often enteroviruses induce β cell damage, autoimmunity development and clinical diabetes.

(4) *Authors mentioned reference No. 52 as recent study. However, it is in 1993. Authors should confirm the years when articles were published.*

Reference 52 was changed, and the paragraph was reformulated on page 7.

On page 7: Much attention has been paid to the possible immunological cross-reactivity that is induced by a homology sequence in the 2C non-structural CVB protein and a principal diabetes autoantigen glutamic acid decarboxylase (GAD65), which share a common amino acid sequence[55-56]. GAD65 is an important target antigen in the pathogenic process of diabetes. In mice, the insulitis establishment coincides with GAD65 specific reactivity, and tolerance induction to GAD65 can prevent the disease[57-58]. Humoral and cellular responses have been detected against GAD65 before the onset of clinical diabetes[59], and auto antibodies are positive several years before diagnosis[60]. The importance of this homology in T1DM pathogenesis is supported by data showing that T cells that respond to this sequence are present both in NOD mice and T1DM patients[61-62]. This mechanism will be discussed below.

(5) *There are some grammatical errors in English.*

The manuscript had previously been sent to American Journal of Experts (AJE), who

provided an Editing Certificate. After this round of revision according to the reviewers' suggestions, the manuscript was resubmitted for English correction by AJE, who have provided a new Certificate with the WJD manuscript number confirming the revision.

Thank you again for considering our manuscript for publication in the *World Journal of Diabetes*.

Our best regards from São Paulo, SP, Brazil.

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