World Journal of Gastroenterology

Dear Editor

Please find enclosed our revised manuscript in Word format (file name:ESPManuscript NO79977edited.doc)

Title: Relevance of α-defensins (HNP1-3) and defensin β-1 in diabetes

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The manuscript has been improved according the suggestions of Reviewers and the Editor.

All of the corrections and supplements are highlighted with bold letters in the manuscript.

The english linguistic problems of the manuscript have been corrected**.**

Author contributions, COMMENTS are provided.

Table 1. 2, 3 were merged into one table named Table1.

Revision has been made according to the suggestions of Reviewers.

I enclose our replies to the Reviewer's' comments, answered point by point.

We fully appreciate the valuable comments and questions of the Reviewers

Answers to Reviewer 1.

The author should precisely explain the clinical relevance of the results obtained.

Our main question was whether the high HNP 1-3 level in the circulation is genetically determined, i.e., is there any relationship between the copy number polymorphism of DEFA1/3 and the plasma levels of the protein. Secondly, the aim of the study was to prove the relevance of human defensin beta-1 (HBD1) in diabetes by determining the genotypes of DEFB1, which might influence the expression of HBD1. The results may contribute to a better understanding of the roles of defensins in the pathomechanism of diabetes. (See COMMENTS)

1. *Authors should explain the mechanism how HNP1-3 cause atherosclerosis or vasculopathy.*

HNP 1-3 promote the accumulation of low density lipoprotein in the vasculature, inhibit fibrinolytic activity on the surface of vascular cells, and accumulate in the intima of atherosclerotic plaques. Therefore, HNP 1-3 may have clinical implications in diabetic patients with hypercholesteremia or vascular dysfunction[7, 27].

2. Are there any studies showing that the neutrophil hyperactivity like exaggerated degranulation is linked to diabetic complications like nephropathy, neuropathy, or CV events?

3. *Are there any studies showing that the neutrophil activity like degranulation is higher in DM patients than in controls, or higher in DM patients with complications than in those without?*

There are no direct data showing that exaggerated degranulation is linked to diabetic complications, or it is higher in DM patients than in controls. However, it has recently been published that pro-inflammatory conditions during hyperglycemia favor NET – neutrophil extracellular traps – formation (Joshi M B et al. FEBS Letters 2013[30]), and HNP 1-3 are also involved in the formation of neutrophil extracellular traps[9]. It is noteworthy that diabetes is associated with low grade, sub-clinical and chronic inflammation characterized by abnormal cytokine production. Therefore, the diabetic microenvironment can induce NET formation, which may result in a basic high HNP-1 concentration in the circulations. Discussion section of the revised manuscript is supplemented with these observations, and the References section is supplemented with this citation.[30]

4. Are HNP1-3 levels correlated with blood glucose, LDL-cholesterol, or HbA1c levels?

HbA1c levels rather than glucose were compared with HNP 1-3 levels, but circulating HNP 1-3 levels were unrelated to HbA1c. The mean LDL was 2.56 ± 0.5 mmol/L, and 2.50 ± 0.5 mmol/L in patients with type 1 and type 2 diabetes,

respectively. There was an inverse relation between HNP 1-3 and LDL levels, but we did not include this result in the manuscript because this plausible fact has been interpreted earlier (Lopez-Bermejo et al. Arterioscler Thromb Vasc Biol 2007; 27:1166-1171).

5. Do the patients with CC genotypes show lower beta-defensin-1 levels compared with those with other genotypes or do they show high glucose levels?

According to literature data, the GG genotype at the -44 locus of DEFB1 is considered to be a protective genotype, whereas subjects carrying the CC genotype were at greater risk of acquiring infections. We accepted it, and we did not measure the beta-defensin-1 levels in the circulation, as it was not measured by others investigating the relevance of DEFB1 mutation. Human beta-defensin-1 is constitutively expressed in epithelial cells, but is not normally expressed in a considerable level in the circulation (LooWT et al. J.Transl Med 2012 Sep 19. Suppl 1: S9 doi: 10.1186/1479-5876) or presented at least in nanomolar concentration in human plasma (Bensch KW et al. FEBS Lett 1995, 368; 331-335). Recently, a lower DEFB1 gene expression has been observed in diabetic patients with latent tuberculosis, but no circulating levels of the peptide were measured (Gonzales-Curiel I et al. Hum. Immunol. 2011, 72; 656-662).

Patients carrying the CC genotype did not reveal higher glucose or HbA1c levels than patients with other genotypes. So we suppose that DEFA1 SNP itself is not involved in glucose metabolism. However, it has to be kept in mind that insulin treatment was able to restore decreased expression of defensin-beta-1 in animal experiments (Froy et al. [23]).

6. *What about the relationship between HNP1-3 levels and infectivity in the DM patients?*

Infections with bacterial, viral and fungal etiology are frequent in diabetes, but the duration, the severity and the time period is highly variable among diabetic patients. Therefore, we did not select a special group of patients with infections. However, a further stratification with diabetic foot ulceration has been created (according the question of the Reviewer) as foot ulcerations in diabetic patients are often combined with infections. In a relatively smaller group of patients (n =28) with diabetic foot

ulcer (20 with type 1 diabetes and 8 with type 2 diabetes), the HNP1-3 plasma levels were 35. 9±1.1 ng/mL. These high HNP1-3 levels might be the consequence of the degranulation of recruited neutrophils from the skin frequently following infections. It is noteworthy that none of these patients had GG genotype of C-44G SNP of the DEFB1 gene.

We supplemented the Results, the Discussion and Figure 2 of the revised version with these data.

Answers to Reviewer 2

1. *The current manuscript addresses the role of defensins in patients with type 1 and type 2 diabetes. The authors should,in addition to the protein levels, correlate absolute mRNA expression to the diabetic patients; most likely this is also enhanced.*

Diabetic patients displayed higher plasma levels of HNP 1-3 compared to controls. In order to detect whether increased mRNA expression is responsible for elevated defensin levels, quantitative RT-PCR reactions were performed. Expression of specific mRNA in leukocytes was observed for HNP 1-3 but not parallel with HNP1-3 plasma levels. The mRNA values between patients and controls were rather equal (relative expression 1.5±0.28 vs. 1.49±0.35, respectively) suggesting that degranulation rather than increased gene expression may be responsible for increased plasma levels of HNP 1-3. Similarly, there was no correlation between HNP 1-3 mRNA levels and copy number polymorphism of the DEFA1 gene (Fig. 5). Our findings were in good correlation with the observations of Fang X M et al. (Eur J Clin Invest 2003 33; 82-87), that is, α-defensin genes were constitutively transcribed at low level in mature neutrophils, but they were not inducible. The Discussion section of the revised manuscript is supplemented with these observations, and the References section is supplemented with this citation.[31]

2. *Further, the HNP1-3 levels should be correlated to blood glucose, and HbA1c levels*.

HbA1c levels rather than glucose levels were compared with HNP 1-3 levels, but circulating HNP 1-3 levels were unrelated to HbA1c. (See answer to Reviewer 1)

3. *The authors should more explain the biological role/relevance of the defensine increase: Is this associated with the level of inflammation; as well as the severity of complications like nephropathy, neuropathy, or CV events. If possible the association between HNP1-3 levels and infective disease/inflammation should be given.*

In our study, we demonstrated that the diabetic patients (both with type 1 and with type 2 diabetes) exhibited overall higher plasma levels of HNP 1-3 than the healthy controls. The highest concentrations of HNPs were detected in patients with nephropathic or neuropathic and cardiovascular complications. The potential mechanisms are discussed in the Discussion with citations.

Furthermore, the Discussion section is now supplemented with the explanation on the connection between high HNP1-3 levels and atherosclerosis: HNP 1-3 promote the accumulation of low density lipoprotein in the vasculature, inhibit fibrinolytic activity on the surface of vascular cells, and accumulate in the intima of atherosclerotic plaques. Therefore, HNP 1-3 may have clinical implications in diabetic patients with hypercholesteremia or vascular dysfunction[7, 27] (See answers to Reviewer 1).

We did not investigate the level or severity of complications because this aspect was not the main question of the study, but it was rather the possible genetic background of the altered defensin levels in diabetes.

4. *If possible the association between HNP1-3 levels and infective disease/inflammation should be given.*

See comments to the question of Reviewer 1:

Infections with bacterial, viral and fungal etiology are frequent in diabetes, but the duration, the severity and the time period is highly variable among diabetic patients. Therefore, we did not select a special group of patients with infections. However, a further stratification with diabetic foot ulceration has been created (according to the question of the Reviewer) because foot ulcerations in diabetic patients are often combined with infections. In a relatively smaller group of patients (n =28) with diabetic foot ulcer (20 with type 1 diabetes and 8 with type 2 diabetes), the HNP1-3 plasma levels were 35.9±1.1 ng/mL. These high HNP1-3 levels might be the

consequence of the degranulation of recruited neutrophils from the skin frequently following infections. It is noteworthy that none of these patients had GG genotype of C-44G SNP of the DEFB1 gene.

We supplemented the Results and the Discussion sections, and Figure 2 of the revised version with these data.

Thank you again for publishing our manuscript in the **World Journal of Gastroenterology.**

Sincerely yours:

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