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ANSWERING REVIEWERS DOCUMENT

Name of journal: World Journal of Gastroenterology

Manuscript NO: 36719

Title: *Helicobacter pylori* and corpus gastric pathology are associated with lower serum ghrelin

Reviewer's code: 00073423

Answers to the reviewer:

We would like to thank the reviewer for his/her comments and hope to clarify the points of concern with the information provided below and throughout the paper.

"In general, this is quite elegantly performed study. The idea in not new as there are number of studies investigating this issue. As the authors stress the role of CagA has not been so widely investigated. The presentation is acceptable, the results and findings are not adding anything new to what is already known. Moreover, I could emphasize some major and minor drawbacks in the study and manuscript:
1. There are no data about the concomitant medications, especially PPI. This may be major confounding factors"

Patients were excluded from the study if they received antimicrobials or acid suppressants during the month before enrollment. This information is described under the subhead "Patients and Ethics" within the "Materials and Methods" section.

"2. There are no data on concomitant diseases, what could also interfere with gastric function"

Exclusion criteria were antecedents of gastric surgery, neoplastic disease, diabetes, celiac disease, thyroid, renal or hepatic pathologies, coagulopathies, drug abuse and pregnancy. This information is described under the subhead "Patients and Ethics" within the "Materials and Methods" section.

"3. There are no data on previous *Helicobacter pylori* eradication?"

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Patients were excluded from the study if they received a previous *H. pylori* treatment. This information was added within the exclusion criteria under the subhead "Patients and Ethics".

"4. The histological analysis is performed using 1 sample from antrum and 1 from corpus – it does not correspond to Sydney System"

We appreciate the reviewer's comment. Although we obtained four gastric biopsies from each subject during endoscopic evaluation, one sample of each gastric site (antrum or corpus) was used for histological assessment and the others for molecular biology evaluation. Upper gastrointestinal endoscopies are routinely performed without anesthesia in the hospitals in which the protocol was carried out. Taking this limitation into account, the ethics committees approved of the obtention of four gastric biopsies from each patient instead of six. The original Sydney system considered four biopsies to be adequate, two from the antrum and two from the corpus; however, it also stated that *"Biopsies, if taken from only one gastric compartment, represent an incomplete examination but in an individual case may still answer a particular clinical problem"* (Price AB, 1991). Moreover, it was discussed that *"Most clinical and investigative work on gastritis is biopsy-based, with its inherent sampling error. Patchy lesions can be positively identified but they cannot be excluded"* (Price AB, 1991). Nevertheless, we agree with the reviewer and acknowledge that histological evaluation of one biopsy from each gastric compartment was a limitation of our study, and added this point of discussion within the manuscript.

References:

- Price AB. The Sydney system: histological division. J Gastroenterol Hepatol 1991;6:209-22.

"5. The retrograde dietary recall must be evaluated cautiously and may have significant bias"

We agree with the reviewer that dietary intake cannot be estimated without error, as described by Beaton GH (1994). We used the 24-hour dietary recall for dietary assessment in this observational study, which might limit our results particularly due to the patients' unintentional misreport (Willett W, 1998). Although these limitations have been previously mentioned, we have described this issue in detail within the "Discussion" section.

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References:

- Beaton GH. Approaches to analysis of dietary data: relationship between planned analyses and choice of methodology. Am J Clin Nutr. 1994 Jan;59(1 Suppl):253S-261S. PubMed PMID: 8279436.
- Willett W. Issues in analysis and presentation of dietary data. In: Willet W, editor Nutritional Epidemiology. 2nd ed. New York, NY: Oxford University Press, 1998: 321- 346.

"6. Many numbers and pictures with positive and negative correlations are presented, but…. Almost all of them are Low or Moderate correlations, though statistically significant, it does not add strength to the results"

We thank the reviewer's appreciation. However, although the correlations are weak or moderate, whether they reach statistical significance or not, we consider it is important to present and discuss the results as obtained.

"7. It is difficult to understand why we need the Table 3. What does it add to the results or conclusions?"

Table 3 shows the type of gastric pathology among the *H. pylori* positive and negative patients. We consider this description is important due to its relationship with the results of Table 4, in which serum ghrelin levels are presented according to gastric pathology of the whole population, regardless of *H. pylori* status.

"8. Table 4: is it HP positive patients? Negative? Both? Not indicated."

Table 4 describes serum ghrelin levels according to gastric pathology of the whole population of patients, regardless of *H. pylori* status. We have introduced this correction in the title of Table 4.

Reviewer's code: 00227403

Answers to the reviewer:

We are most grateful to the reviewer for revising our manuscript and for his/her evaluation.

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Reviewer's code: 00503623

Answers to the reviewer:

We would like to thank the reviewer for his/her constructive criticism and hope to clarify the points of concern with the information provided below.

"The manuscript, No. 36719, reports the results of studies on the relationship between Hp infection, genotype of infecting strains and gastric pathology, and the serum levels of ghrelin and leptin. The results obtained with 163patients revealed that Hp infection and severity of gastric pathology was associated with lower serum ghrelin levels, while the level of leptin did not differ between the Hp-infected and non-infected groups. Also, the level of ghrelin in patients carrying CagA-positive strains of Hp appeared to be lower. This is an interesting presentation. However, majority of the cited literature is at least 10 to 20 years old. Moreover, the authors totally disregard the literature data on the role and mechanism of modulatory mode of ghrelin action (See Inflammopharmacology vol. 21(2013) 241; vol. 23(2015)37; and vol. 25(2017)415)."

In order to address these issues, we have included other references and introduced the following paragraph within the "Discussion" head (second paragraph):

"It has been demonstrated that ghrelin exhibits gastroprotective antioxidant and anti-inflammatory effects[36-38], modulating gastric mucosal inflammation induced by *H. pylori* lipopolysacaccharide (LPS) through several mechanisms which were recently reviewed[39]. Ghrelin circulating levels were described to rise in response to severe gastric oxidative stress induced during acute gastritis or peptic ulcer disease; however, its concentration decreases concomitantly with injury of the gastric glands[36]. Our results of lower ghrelin levels in persistently infected patients are consistent with these findings, and with the ones from other studies[35, 40]."

References:

35 Kasai C, Sugimoto K, Moritani I, Tanaka J, Oya Y, Inoue H, Tameda M, Shiraki K, Ito M, Takei Y, Takase K. Changes in plasma ghrelin and leptin levels in patients with peptic ulcer and gastritis following eradication of Helicobacter pylori infection. *BMC Gastroenterol* 2016; **16**(1): 119 [PMID: 27716077 PMCID: 5050848 DOI: 10.1186/s12876-016-0532-2]

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36 Suzuki H, Matsuzaki J, Hibi T. Ghrelin and oxidative stress in gastrointestinal tract. *J Clin Biochem Nutr* 2011; **48**(2): 122-125 [PMID: 21373264 PMCID: 3045684 DOI: 10.3164/jcbn.10-16GFR]
37 Slomiany BL, Slomiany A. Induction in gastric mucosal prostaglandin and nitric oxide by Helicobacter pylori is dependent on MAPK/ERK-mediated activation of IKK-beta and cPLA2: modulatory effect of ghrelin. *Inflammopharmacology* 2013; **21**(3): 241-251 [PMID: 23563696 DOI: 10.1007/s10787-013-0169-5]
38 Slomiany BL, Slomiany A. Role of amplification in phospholipase Cgamma2 activation in modulation of gastric mucosal inflammatory responses to Helicobacter pylori: effect of ghrelin. *Inflammopharmacology* 2015; **23**(1): 37-45 [PMID: 25362585 DOI: 10.1007/s10787-014-0220-1]
39 Slomiany BL, Slomiany A. Role of LPS-elicited signaling in triggering gastric mucosal inflammatory responses to H. pylori: modulatory effect of ghrelin. *Inflammopharmacology* 2017; **25**(4): 415-429 [PMID: 28516374 DOI: 10.1007/s10787-017-0360-1]
40 Isomoto H, Ueno H, Nishi Y, Yasutake T, Tanaka K, Kawano N, Ohnita K, Mizuta Y, Inoue K, Nakazato M, Kohno S. Circulating ghrelin levels in patients with various upper gastrointestinal diseases. *Dig Dis Sci* 2005; **50**(5): 833-838 [PMID: 15906753]

Reviewer's code: 01436308

Answers to the reviewer:

We would like to thank the reviewer for his/her comments and hope to clarify the points of concern.

"This study evaluated the association of H. pylori, cagA genotype, and type of gastric pathology with ghrelin, leptin and nutritional status. The authors found that H. pylori infection and severity of gastric corpus pathology are associated with lower serum ghrelin levels. The study is interesting, and the manuscript is well-written. My comments are listed below: 1. Serum ghrelin levels should be analyzed after eradication of H. pylori."

We appreciate the reviewer's suggestion; however, as we aimed to evaluate the association of *H. pylori*, *cagA* genotype, and the type of gastric pathology with ghrelin, leptin and nutritional status, we conducted this cross-sectional study. As

a consequence of the obtained results, we are now conducting a quasi-experimental, before-after interventional study to evaluate ghrelin levels of the patients before and after *H. pylori* eradication.

"2. The authors should explain why the sample size is enough for analysis."

The following paragraph under the "Statistical analysis" subhead of the "Materials and methods" section, explained the sample size calculation for our study. We have then included 163 patients.

"A sample size of 160 individuals was calculated to be included in the study using the Statcalc program (Epi Info Version 3.2, Georgia, USA), setting an α error of 0.05, a β error of 0.20, an estimated 50% *H. pylori* infection prevalence in adult patients and a 25% expected frequency of ghrelin hormonal variation between the *H. pylori* positive and negative groups."

"3. The mechanisms by which H. pylori infection is associated with serum ghrelin levels should be analyzed, or at least be discussed in the Discussion section."

We thank the reviewer for his/her suggestion. This was a point of concern shared with another reviewer. We introduced the following paragraph within the "Discussion" head (second paragraph):

"It has been demonstrated that ghrelin exhibits gastroprotective antioxidant and anti-inflammatory effects[36-38], modulating gastric mucosal inflammation induced by *H. pylori* lipopolysacaccharide (LPS) through several mechanisms which were recently reviewed[39]. Ghrelin circulating levels were described to rise in response to severe gastric oxidative stress induced during acute gastritis or peptic ulcer disease; however, its concentration decreases concomitantly with injury of the gastric glands[36]. Our results of lower ghrelin levels in persistently infected patients are consistent with these findings, and with the ones from other studies[35, 40]."

Reviewer's code: 02536349

Answers to the reviewer:

We are grateful to the reviewer for his/her comments, and hope to clarify the points of concern, which were also shared by another reviewer.

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"The study is well designed and despite to caveats related with highly fluctuating and multifactorial hormone ghrelin it is worth publishing. It would be more expressive if the caveats and weak points of the subject written in conclusion section as a paragraph, for example the effect of patchy involvement (Thus it would be more convincing to take four quadrant biopsy from sites to minimize this). The manuscript is well written by means of grammar and scientific terminology."

Although we obtained four gastric biopsies from each subject during endoscopic evaluation, one sample of each gastric site (antrum or corpus) was used for histological assessment and the others for molecular biology evaluation. Upper gastrointestinal endoscopies are routinely performed without anesthesia in the hospitals in which the protocol was carried out. Taking this limitation into account, the ethics committees approved of the obtention of four gastric biopsies from each patient instead of six. The original Sydney system considered four biopsies to be adequate, two from the antrum and two from the corpus; however, it also stated that *"Biopsies, if taken from only one gastric compartment, represent an incomplete examination but in an individual case may still answer a particular clinical problem"* (Price AB, 1991). Moreover, it was discussed that *"Most clinical and investigative work on gastritis is biopsy-based, with its inherent sampling error. Patchy lesions can be positively identified but they cannot be excluded"* (Price AB, 1991). Nevertheless, we agree with the reviewer and acknowledge that histological evaluation of one biopsy from each gastric compartment was a limitation of our study, and added this point of discussion within the manuscript.

References:

- Price AB. The Sydney system: histological division. J Gastroenterol Hepatol 1991;6:209-22.

Reviewer's code: 03474116

Answers to the reviewer:

We would like to thank the reviewer for his/her constructive criticism and hope to clarify the points of concern with the information provided below.

"General: In this study, the authors investigated to evaluate the association of H. pylori infection, cagA status, and type of gastric pathology in relation with ghrelin, leptin and nutritional status. Authors showed that serum ghrelin was significantly lower in infected patients than in uninfected ones, even after

adjusting for BMI and gender. In addition, the type and severity of gastric pathology in the corpus was associated with lower serum ghrelin, independently of H. pylori status.
Major comments: 1. In general, most of cased infected in H. pylori infection have chronic gastritis. Authors showed that the type and severity of gastric pathology in the corpus was associated with lower serum ghrelin. What is chronic inactive gastritis and chronic active gastritis?"

We classified chronic gastritis activity according to the The Sydney system and The updated Sydney system definitions (Price AB, 1991; Stolte and Meining, 2001).
"Chronic gastritis: There is an increase in lymphocytes and plasma cells within the lamina propria unaccompanied by the organized patterns recognizable as a special form. Activity: An infiltrate of neutrophil granulocytes may accompany chronic gastritis or, indeed, any of the special forms." (Price AB, 1991).
"Chronic active gastritis means that both lymphocytes and neutrophils infiltrate the mucosa in a characteristic manner" (Stolte and Meining, 2001).
According to what has been exposed above, we classified chronic active gastritis with the presence of neutrophils and lymphocytes in the pit region and/or the lamina propria, and chronic inactive gastritis in the presence of lymphocytes but without the presence of neutrophils in that regions.

References

- Price AB. The Sydney system: histological division. J Gastroenterol Hepatol 1991;6:209-22.
- Stolte M, Meining A. The updated Sydney system: classification and grading of gastritis as the basis of diagnosis and treatment. Can J Gastroenterol. 2001;15(9):591-8.

"2. Why did authors show ghrelin level as median? It will be better to show those as mean. In addition, author is required to reanalyze significances."

The distribution of the variable values (ghrelin levels) was not normal. For that reason, we considered it more appropriate to show median values and to apply the non-parametric Mann-Whitney U test for the analysis. In addition, variances non-homogeneity supports the use of the aforementioned statistical test. Nevertheless, answering reviewer's question, mean serum ghrelin levels were (333.64 ± 164.96) pg/mL for the *H. pylori* positive and (448.51 ± 233.00) pg/mL

for the *H. pylori* negative patients, which differed significantly after the unequal variances *t*-test was applied ($P = 0.0091$). However, we still consider that median ghrelin values represent the variable characteristics in the evaluated *H. pylori* positive and negative groups much better because, as explained above, values distribution was not normal and their variances were not homogenous.

"3. It is unclear what is P value in Table 4."

Table 4 summarizes the results obtained from the analysis of serum ghrelin concentrations according to the type of gastric pathology of the antrum and the corpus in all the patients, independently of their *H. pylori* status. The type and severity of gastric pathology in the corpus were associated with lower serum ghrelin levels ($P = 0.04$). The Kruskal Wallis post-hoc analysis revealed that ghrelin levels differed significantly among all the types of gastric pathology in the corpus except in the chronic inactive and active gastritis groups.

The last sentence was added within the "Gastric pathology, *H. pylori* infection and appetite hormones concentrations" subhead of the "Results" section.

"4. Table 3: Total in H. pylori-negative is not 100%. Please check.

We appreciate the reviewer's correction and introduced the corrected values in Table 3.

"5. In general, if patients infected in cagA-positive strain, severity of gastric mucosal atrophy is severe. Authors demonstrated that the type and severity of gastric pathology in the corpus was associated with lower serum ghrelin, independently of H. pylori status. Authors should discuss this discrepancy. In addition, patients infected in vacA s1m1 have severe of gastric mucosal atrophy."

Our results demonstrated that the type and severity of gastric pathology in the corpus were associated with lower ghrelin serum levels, independently of *H. pylori* status, as shown in Table 4. However, the small number of patients with gastric atrophy or intestinal metaplasia in the *H. pylori* positive and negative groups did not allow us to seek an association between ghrelin levels and gastric atrophy according to *H. pylori* status. In addition, although *H. pylori* positivity was associated with lower ghrelin concentrations, we were not able to demonstrate a statistically significant difference in hormonal levels according to

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the *cagA* genotype despite finding a tendency towards lower ghrelin levels in *cagA* positive patients.
All these issues have been previously discussed. We hope we have clarified the reviewer's points of concern.

"6. In this study, of cagA-positive strain, 14.5% of strain was cagA-positive and vacA s1 type. Is not this prevalence is too high?"

Overall prevalence of *H. pylori cagA* positive genotype in the antrum and the corpus was 74.7% (CI95%; 64.4 - 82.8%). From the *H. pylori vacA*S1 gastric biopsies, 85.5% were *cagA* positive and 14.5% were *cagA* negative both in the antrum and the corpus. These prevalences are consistent with previous results from our group (Armitano et al., 2013), and from other authors (Mattar et al., 2005; Erzin et al., 2006).

References:

- Armitano RI, Matteo MJ, Goldman C, Wonaga A, Viola LA, De Palma GZ, Catalano M. Helicobacter pylori heterogeneity in patients with gastritis and peptic ulcer disease. Infect Genet Evol. 2013;16:377-85.
- Erzin Y, Koksal V, Altun S, Dobrucali A, Aslan M, Erdamar S, Dirican A, Kocazeybek B. Prevalence of Helicobacter pylori vacA, cagA, cagE, iceA, babA2 genotypes and correlation with clinical outcome in Turkish patients with dyspepsia. Helicobacter. 2006;11(6):574-80.
- Mattar R, dos Santos AF, Eisig JN, Rodrigues TN, Silva FM, Lupinacci RM, Iriya K, Carrilho FJ. No correlation of *babA2* with *vacA* and *cagA* genotypes of *Helicobacter pylori* and grading of gastritis from peptic ulcer disease patients in Brazil. Helicobacter. 2005;10(6):601-8.

"7. How about association with ghrelin level and severity of gastric mucosal atrophy?"

Although we found an association between lower ghrelin levels and the presence of gastric atrophy in all the patients, as has been previously mentioned and discussed in the manuscript, we were not able to seek an association between ghrelin levels and gastric atrophy according to *H. pylori* status because of the

small number of patients with that pathological condition in the *H. pylori* positive and negative groups.

"8. As observed in Ref 18, authors should check achyl-ghrelin and desachyl-ghrelin."

We appreciate the reviewer's suggestion. Although it would have been interesting to have the results of acyl- and desacyl-ghrelin levels, we have decided to measure total ghrelin serum levels due to technical and financial issues.
According to Hosoda et al. (2004), *"because the ester bond is both chemically and enzymatically unstable, elimination of the octanoyl modification of ghrelin can occur during storage, handling, and/or dissolution in culture medium"*. The authors added that *"Because of increased interest in ghrelin measurements, a standardized method of sample collection is required*". In addition, Jeffery et al. (2011) described that *"acylated ghrelin is highly unstable and degrades rapidly to unacylated ghrelin"* (reference was made to Hosoda et al., 2004). *"The optimum method of plasma ghrelin measurement is a contentious issue - a number of researchers maintain that measurement of total ghrelin is reflective of active ghrelin levels and is an adequate approach"*.

References:

- Hosoda H, Doi K, Nagaya N, Okumura H, Nakagawa E, Enomoto M, Ono F, Kangawa K. Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. Clin Chem. 2004; 50(6):1077-80.
- Jeffery PL, McGuckin MA, Linden SK. Endocrine impact of *Helicobacter pylori*: Focus on ghrelin and ghrelin o-acyltransferase. World J Gastroenterol 2011; 17(10): 1249-1260.

"9. Please add information of clinical trial registration."

Our study design was observational, not a clinical trial.