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Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 23142-revised manuscript.docx).

Title: Serum Vitamin D and Colonic Vitamin D Receptor in Inflammatory Bowel Disease

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The manuscript has been improved as outlined below (changes are highlighted in manuscript):

1. To address comments from reviewer 02529831, we have rechecked all the statistics and all the p values were indeed accurate. However, during this process we noted a data entry error for one of the control patients in our file for correlations. The inflammation score for control patient C10 was entered as 0 instead of 0.5. This has been corrected, and all pertinent graphs and correlations were redrawn and recalculated, respectively. The indicated graphs were affected as follows: Figure 3A, the correlation was weakened (r=-0.3616) and is no longer significant (p=0.0714); Figure 5A, the correlation was somewhat strengthened (r=-0.5737) and more significant (p=0.0041); and Figure 5B, the correlation was somewhat strengthened (r=-0.3538) and more significant (p=0.0275). The manuscript has been edited accordingly. With regard to the comment that '*VDR IHC scores were (non-significantly) higher amongst inflamed tissue than non-inflamed tissue in patients with IBD, yet negative correlation is demonstrated with inflammation scores. How is this explained?*' we reply as follows: The difference in average VDR IHC scores per group between the three groups are neither significantly nor substantially different: normal mucosa from control patients, 2.03 ± 0.20; normal appearing mucosa from IBD patients, 1.93 ± 0.24; and disease mucosa from IBD patients, 2.30 ± 0.21 (Figure 2B). However, tissue from IBD patients with lower inflammation scores tended to have higher VDR IHC scores. Note in Figure 3B that all of the IBD patients with a VDR IHC score of 3 had an inflammation score lesser than or equal to 2, whereas as all of the IBD patients with a VDR IHC score lesser than 3 had an inflammation score greater than or equal to 2. Note in Figure 3C that all of the Crohn's disease patients with a VDR IHC score of 3 had an inflammation score lesser than or equal to 2, whereas 3 of the 4 Crohn's disease patients with a VDR IHC score lesser than 3 had an inflammation score greater than 2. These two correlations were found to have Spearman correlation coefficients of -0.4355 and -0.6872, both of which were statistically significant ($p<0.05$). These correlation coefficients

indicate a moderate negative relation between VDR IHC scores and inflammation scores in IBD patients, and a moderate-to-strong correlation between VDR IHC scores and inflammation scores in IBD patients with a diagnosis to Crohn's disease. With regard to the comment '*Why was inflammation scored amongst controls? Why is this relevant, and what does this add to our knowledge?'* we reply as follows. Histologic activity, assessed using an inflammation score, was one of the variables studied. Therefore, it was necessary to examine histologic activity in our controls to confirm that inflammation was absent or minimal in the collected tissue specimens, as shown in Figure 2A. Confirming the histologic activity, or inflammatory status, of colonic tissue from our control patients is essential for a scientifically sound study. We do not find that there is any justification for not examining inflammatory status in controls if we examine inflammatory status for IBD patients.

2. To address comment from reviewer 00070280, "*they should not conclude the results apply to IBD as there were only 3 UC patients. They should consider using only CD patients or using equal number of patients with UC"* we reply as follows. We agree that an equal number of patients with CD or UC would have been ideal for this study. We were unable to obtain an equal number of CD and UC patients and acknowledge that this is a limitation for our study. The pilot study was limited to 10 IBD patients and 10 controls. Subjects were recruited as they were scheduled for colonoscopy, and many were excluded because they were using vitamin supplements. As our Center for IBD has a larger volume of CD, the sample in the study is representative of our population and of those scheduled for colonoscopy. A larger study (as opposed to a pilot) with longer duration could correct this issue. Nevertheless, as all 10 IBD patients had endoscopic colitis (by protocol requirement), we felt that they were an adequate representation of IBD. We have made every effort to communicate the fact that our IBD group contains more CD than UC patients, as evidenced in Table 1, Supplemental Table 1, the second sentence of the results section, and Figures 3-6 (where each IBD patient is labeled as either CD# or UC#). Nevertheless, our CD and UC patients for the most part behave similarly with regards to serum vitamin D levels, colonic VDR IHC, inflammation scores, and age as shown in Figures 3-6. For this reason, we continue to refer to the cases as the IBD group and not the CD group.

3. To address comment from reviewer 02529166, "*Statement made page 2 line 2 of the introduction part need to be corrected. Effectively, ChIP-seq experiments reveal the presence of Vitamin D response element in the promoter region of VDR in intestine and bone, and thus contribute to the regulation of VDR expression (PMID: 24466413),"* we reply as follows: We have modified the problematic sentence and have added a sentence regarding the Vitamin D response elements in the promoter region of VDR mentioned in the reference by Pike et al., (2014; PMID: 24455413) as follows: "Importantly, the mechanisms by which circulating vitamin D levels regulate colonic VDR expression in homeostasis and disease states are also incompletely understood. Studies in bone cells have revealed that the VDR gene contains VDRE, suggesting that vitamin D can increase colonic VDR expression."
   To address the comment "*A decreased of Vitamin D-mediated activity in colorectal cancer is due to an increased expression of CYP24A1, the enzyme responsible of Vitamin D catabolism (PMID: 23674869). Moreover CYP24A1 overexpression increases aggressiveness and proliferative potential of colorectal tumors (PMID: 26238339). Please insert these works,"* We have added the references by Kosa et al., (2013; PMID: 23674869) and Hobaus et al., (2015; PMID: 2638339) to the introduction right after the aforementioned sentence with the following sentence: "However, treatment of Caco-2 colon cancer cells with calcitriol resulted in increased transcript levels of CYP24A1, the major 1,25(OH)2D3 inactivating enzyme (Kosa et al., 2013), and xenografts of HT29 colon cancer cells

overexpressing CYP24A1 are more proliferative and invasive (Hobaus et al., 2015)." We have also added the following sentence: "Additionally, SNAIL1 has been shown to repress VDR expression in colon cancer cells (Bhatia et al., 2015; Larriba et al., 2010; Palmer et al., 2004)," to address another source of regulation for the vitamin D-VDR system in the colon.
"Insert representative H&E staining of a normal and an inflamed mucosa." These pictures have been added to Figure 2.
"Where is located VDR in normal and inflamed mucosa? Representative IHC are required to identify subcellular localization of VDR (immune cells, epithelial cells or other). Please also discussed this point." These pictures have been added to Figure 2 and the subcellular localization of VDR has been added to the results section with the following sentence: "VDR immunostaining was mainly found within glandular epithelial cells and lamina propria cells and was observed in both the nuclear and the cytoplasmic compartments."
"Functional relevance of a decreased VDR expression needs to be address. CYP24A1 and DEFβ/HBD2 staining on adjacent section will be informative." This is a very interesting point and we thank the reviewer for the recommendation. In future studies, we would like to evaluate the expression of not only CYP24A1 and DEFβ/HBD2 but also CYP27B1 and SNAIL1 in relation to VDR expression. We feel that this would be an exciting follow up to our current study. However, this will require major revisions and work as antibodies for these four proteins will need to be acquired and optimized prior to staining valuable patient samples.
"Please state in the introduction that the main role of VDR is intestinal calcium absorption and regulation of serum calcium and phosphate levels." This has been added to the introduction.
"Scatter dot plot representation will be more informative than bar graphs." Bar graphs in Figures 1-2 have been replaced with scatter dot plot graphs.
"Introduction page 1 line 20: delete "can" and line 28: substitute "that" by "and" – this has been done.

4. With regards to comments from reviewer 000503587, we agree that a detailed understanding will require larger numbers of patients. We have edited the introduction for clarity and have ensured that any language errors have been corrected.
"It is unclear why the authors expected the age of the patients with IBD to be less than that of the control subjects. Also, why were controls not recruited to be age-matched?" IBD is more prevalent in young adults, therefore the expected age of patients with IBD undergoing colonoscopy will also be young adults. Control subjects were recruited among patients undergoing colonoscopy for other reasons, including screening for colorectal cancer. These typically will be older adults. Specifically, screening in the average population starts at age 50. The number of young adults undergoing colonoscopy in our center for reasons other than IBD is very small. Matching for age was not possible.
"What was the ethnic make-up of the 20 individuals included?" All subjects were Puerto Rican, i.e. Hispanics.
"In regards the subjects with IBD, there are many aspects not included: were these patients all newly diagnosed or did they have long-standing disease? In a related fashion, what was the length of their disease? Were these subjects in remission or did they have active disease at the time of assessment? Which medications were being prescribed for the 20 individuals - and did these correlate with the outcomes of interest?" The majority of this information was provided at the time of submission in a supplemental table (Demographic and disease information for control and IBD patients), which was cited in conjunction with Table 1 in the methods sections of the text (page 8, Patients and collection of samples). The 3 patients with UC had a disease duration of 2, 8 and 15 years. The patients with Crohn's disease had a duration of disease ranging from 2 to 16 years with a mean of 7 years. The study protocol required the presence of active (endoscopically visible) disease

for inclusion, so there were no patients in endoscopic remission. All patients had endoscopically normal areas, also a protocol requirement. Medications for IBD included aminosalicylates (3 UC and 1 CD), azathioprine (1 UC and 1 CD), adalimumab (3 CD) and infliximab (2 CD). The number of subjects and medications is too small to undergo analysis for correlation between specific medications and vitamin D serum levels or tissue receptor status.

"*The comment about the risk of colitis-associated cancer in the context of IBD needs to be qualified. The risk of cancer in the setting of colitis reflects other factors, such as the length of active disease.*" – We agree with this observation and have modified the statement to read as follows: "In fact, IBD colitis patients are six times more likely to develop CRC than the general population (Cross et al., 2011), and this risk increases with disease severity, duration, and extent (Farraye et al., 2010).

5. We thank reviewer 00227449 for their comments and agree that some of our observations are not new and that due to the small sample size we cannot be sure of a causal relationship. Determining a causal relationship would require a different approach (ie. in vitro or in vivo with animal models).

   We did not examine the expression of other genes in these IBD samples and therefore do not know if they are affected.

   "*the increased chance of false positive was not discussed*" To decrease the possibility of obtaining false positives, we limited the number of statistical analyses whenever possible. Whereas serum vitamin D levels were compared with a Mann-Whitney test (which allows for comparisons between 2 groups), the Kruskal-Wallis test (which allows for comparisons between more than 2 groups) with a post-hoc Dunn multiple comparisons test was used to compare inflammation scores and VDR IHC scores between groups. If Mann-Whitney tests were to have been used to compare inflammation scores and VDR IHC scores, a total of six additional Mann-Whitney tests would have been employed, raising the number of statistical analyses used for comparing our three variables of interest between our study groups from 3 to 7 and greatly increasing the chance of obtaining a false-positive result. Given our small sample size, we performed bivariate analyses (correlations) rather than multivariate analyses to examine the relation between different variables in our study population.

6. Decomposable figures are now included.

7. I am a native English speaker.

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

Sincerely,

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