ANSWERING REVIEWERS

March the 2nd , 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: ESPS Manuscript NO: 8847-edited.doc).

Title: Relationship between methylation and colonic inflammation in inflammatory bowel disease

Author: Triana Lobatón, Daniel Azuara, Francisco Rodríguez-Moranta, Carolina Loayza, Xavier Sanjuan, Javier de Oca, Ana Fernández-Robles, Jordi Guardiola and Gabriel Capellá.

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 8847

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1) REVIEWER 1:

- The introduction needs to be shortened. Focus on the association between methylation and IBD-dysplasia.

This has been modified. Please see page 4 (*"In IBD, some studies already suggested that aberrant methylation might be related with the development of dysplasia and CAC. [24],25] On the other hand, inflammation has been associated with a higher methylation rate in IBD[26]"*)

- The aim of the study. "Increased risk for dysplasia/CAC" needs to be better defined. References are required.

Why was more than 8 years used as a cut-off?

In IBD patients a cut-off of 8 years from diagnosis has been proposed to identify patients at higher risk for dysplasia that need to start the colonoscopy surveillance. This has been modified in the manuscript with the appropriated references. Please see page 6.

31 Annese V, Daperno M, Rutter MD, Amiot A, Bossuyt P, East J, Ferrante M, Götz M, Katsanos KH, Kießlich R, Ordás I, Repici A, Rosa B, Sebastian S, Kucharzik T, Eliakim R, ECCO. European evidence based consensus for endoscopy in inflammatory bowel disease. *J Crohns Colitis* 2013; **7**(12): 982-1018 [PMID: 24184171 DOI: 10.1016/j.crohns.2013.09.016]

32 Lutgens MW, Vleggaar FP, Schipper ME, Stokkers PC, van der Woude CJ, Hommes DW, de Jong DJ, Dijkstra G, van Bodegraven AA, Oldenburg B, Samsom M. High frequency of early colorectal cancer in inflammatory bowel disease. *Gut* 2008; **57**(9): 1246-1251 [PMID: 18337322 DOI: 10.1136/gut.2007.143453]

- A reference is required for the histologic activity parameters.

This has been modified in the manuscript with the appropriated references. Please see page 7.

34 D'Haens G, Sandborn WJ, Feagan BG, Geboes K, Hanauer SB, Irvine EJ, Lémann M, Marteau P, Rutgeerts P, Schölmerich J, Sutherland LR. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. *Gastroenterology* 2007; **132**(2): 763-786 [PMID: 17258735 DOI: 10.1053/j.gastro.2006.12.038]

- The type of medical treatment these patients were receiving should be mentioned in the text. Plus, the authors should mention if IBD treatment modifies SLIT2/TGFB2 expression and/or methylation in any way.

The type of treatment is already specified in Table 1. We performed the analysis considering the methylation according to the different medical treatments and no statistical differences were found (data not shown). This has been added to the manuscript. Please see page 8 (*"No differences were found in the methylation status of either of the 2 genes, according to the different medical treatments (data not shown)").*

- The authors protocol for endoscopic surveillance and biopsy technique should be described.

This has been specified in the manuscript. Please see page 6 *("A total of 62 colonoscopies were performed on 38 IBD patients (29 UC and 9 CC) between December 2010 and June 2012. Fifteen of these 38 patients had a single colonoscopy. A second colonoscopy was performed in 23 patients, who were included in a longitudinal analysis. Of the 62 colonoscopies, 57 were completed with indications of dysplasia surveillance, and samples were taken from the five segments of the colon (rectum, left colon, transverse colon, right colon and cecum). Five rectosigmoidoscopies were performed, and in these cases, only samples of the rectum and sigmoid colon were taken. Rectosigmoidoscopies were performed when there was no indication for dysplasia surveillance.")*

- How many endoscopists and pathologists participated in the study, and how did the authors control for interobserver variability?

This has been specified in the manuscript. Please see page 6 *("Colonoscopies were performed by 2 experienced gastroenterologists (FRM, JGC)"…. "Histological activity was assessed by to 2 experienced pathologists (XSJ, CL").* Interobserver variability was not assessed.

- "Distal colon" should be better defined.

This point has been modified in the manuscript. See pages 8, 10 and 11.

- Were any rectal specimens taken?

This point is already explained in page 6 (…*"samples were taken from the five segments of the colon (rectum, left colon, transverse colon, right colon and caecum"…)*.

- Was there any correlation between methylation and the patients clinical status?

We did not find differences between the methylation status and the clinical activity although this should be interpreted carefully since the majority of them were in clinical remission at the moment of the endoscopy (57/62).

- Median time to the "longitudinal evaluation" should be added.

This has been added to the results. Please see page 9 ("*The median time between the 2 colonoscopies was 238 days (range 98-366).").*

- Were there any variations between UC and CC patients?

We did not find differences between UC and CD patients although this should be interpreted carefully since the majority of them were UC (29/9).

- Significant English language revision is required.

The manuscript has been reviewed by the American Journal Experts (http://www.aje.com) following the guidelines from the journal. Please find the mail from the editing service confirming their work.

- Tables need to be more focused. They are quite confusing. Try to shorten the legends. NS should be replaced with the actual P value obtained. The P values should be added as a 4th column in Table 2.

This has been corrected in the corrected manuscript. The P values are already in Table 2 in the corresponding row according to the different analysis.

- Erase supplemental tables.

These tables have been deleted from the corrected manuscript.

- The authors should expand on why no correlation was found between SLIT2 and TGFB2.

The fact that the methylation rate for *SLIT2* is higher than for *TGFB2* could be related with this lack of correlation between the 2 genes. We already observed that in our previous study (Azuara D, Rodriguez-Moranta F, de Oca J, Sanjuan X, Guardiola J, Lobaton T, Wang A, Boadas J, Piqueras M, Monfort D, Galter S, Esteller M, Moreno V, Capella G. Novel Methylation Panel for the Early Detection of Neoplasia in High-risk Ulcerative Colitis and Crohn's Colitis Patients. *Inflammatory bowel diseases* 2012; **19**(1): 165-173 [PMID: 22532293]) although we don't have a molecular based explanation for that.

- Limitations of the study should be pointed-out in the discussion: non-prospective patient accrual, low patient numbers, no comparison to other methylation markers or healthy controls, and lack of long-term follow-up.

This is carefully explained in the discussion. Please see page 11 *("Our study has several limitations. First, due to the limited sample size, the association between inflammation and the methylation of these genes should be validated, in particular those observed in the longitudinal study. Second, because the study was not designed to assess the*

reversibility of methylation when healing the microscopic inflammation, further studies are needed to confirm this hypothesis due to its potential therapeutic implications. If the reversibility of methylation after the mucosa has healed could be demonstrated, it would be reasonable to propose histological healing as an endpoint of the treatment. Finally, we chose 2 genes that had previously demonstrated distinct patterns in patients at increased risk and low risks of developing dysplasia or cancer at our center. However, many other genes have been studied in this field, and therefore, in the future, a more extensive study could include a panel with more genes.").

(2) **REVIEWER 2:**

- For active disease, it is very common to confuse histologically with dysplasia even with experienced pathologists. Therefore, it is important for authors to clarify their definitions for dysplasia and how the confusions were resolved if any.

This has been added in the analysis. Please see results at page 8 ("*Samples with dysplasia were significantly more methylated than those without dysplasia (80% vs. 27.9%; P=0.027), although this result should be carefully interpreted due to the small number of events. Furthermore, 3/5 samples with dysplasia were present in patients who had some grade of histological inflammation.").*

- ***Sample size was a limitation, and authors may need to clarify why they could not recruit more during the study period. This clearly affected the results of longitudinal analysis with non-significant p-values.***

At them moment of the analysis it was not possible to include more patients in the longitudinal study, and this was not the primary endpoint for this study. We are currently completing this cohort for further longitudinal analysis.

- ***Table 1 was confusing. Rather than number and % as headings, would UC vs Crohn's more useful? Also, legend for FC-QPOCT was not provided. There were 2 reported figures for medications which I do not understand what they represent.***

Table 1 has been modified. We did not present the results as CD vs. UC since there were only 9 CD patients.

- ***Table 5 was not provided despite being mentioned in the manuscript.***

Table 5 has been added to the manuscript.

- ***Can authors explain the differential findings between SLIT2 and TGFB2?***

The methylation rate of SLIT2 is higher and this is something that we already observed in our previous study (Azuara D, Rodriguez-Moranta F, de Oca J, Sanjuan X, Guardiola J, Lobaton T, Wang A, Boadas J, Piqueras M, Monfort D, Galter S, Esteller M, Moreno V, Capella G. Novel Methylation Panel for the Early Detection of Neoplasia in High-risk Ulcerative Colitis and Crohn's Colitis Patients. *Inflammatory bowel diseases* 2012; **19**(1): 165-173 [PMID: 22532293]) although we don't have a molecular based explanation for that.

(3) **REVIEWER 3:**

- Did their patients with methylation become colorectal cancer at their follow up?

Since most of the patients did not already have the second endoscopy at the moment of the analysis, this is something that we cannot know.

- ***There has no Table 5 in the manuscript of which the authors mentioned in longitudinal analysis of results.*** Table 5 has been added to the manuscript.

- ***Meanwhile, the manuscript needs rewriting in accordance with the format of WJG.***

The manuscript has been modified according to the format of WJG.

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

Sincerely yours,

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