UNIVERSITY OF VERONA
SCHOOL OF MEDICINE

DEPARTMENT OF NEUROSCIENCE, BIOMEDICINE AND MOVEMENT

HUMAN ANATOMY AND HISTOLOGY SECTION

Strada le Grazie, 8 – Borgo Roma
Tel. 045.584564 – 045.8027155 - Fax 045.8027163
e-mail: anatvr@borgoroma.univr.it
37134 VERONA

(2) 37443-Answering reviewers Verona, December 12, 2017

Name of journal: *World Journal of Gastroenterology*

Manuscript NO: **37443**

Title: **Glucose transporter expression in the human colon**

Authors: **Flavia Merigo, Alessandro Brandolese, Sonia Facchin, Silvia Missaggia, Paolo Bernardi, Federico Boschi, Renata D'Incà, Edoardo Vincenzo Savarino, Andrea Sbarbati, Giacomo Carlo Sturniolo**

Dear Editor,

We have the pleasure to re-submit a revised version of our manuscript NO 37443. We have carefully studied the reviewers' comments and those of the WJG editorial staff. The changes in the main text have been highlighted in red.

Below we list our response to the comments and criticisms raised by the reviewers.

We wish to thank the reviewers for their positive and helpful comments that have helped to improve the quality of our manuscript.

The manuscript has been checked for grammar and style by a native English speaker and professional translator who revised the English style to ameliorate the B quality classification achieved with the first submission. A certificate attesting that he is a qualified member of the Italian Association of Translators and Interpreters is attached.

We have uploaded the figures in the main text also in a separate file as ppt images
Yours sincerely,

Flavia Merigo

Department of Neuroscience, Biomedicine and Movement,
Human Anatomy and Histology Section,
University of Verona,
37134 Verona, Italy
flavia.merigo@univr.it

A point-by-point response is given below.

The original comments of the reviewers are reported in italics

Reviewer's code: 00034437

This manuscript showed the expression of GLUT2, SGLT1, and GLUT5 in large intestinal mucosa in patients with IBD, colorectal cancer and healthy control only by immunohistochemistry study. Overall, data shown in this manuscript is all phenomenon. This reviewer strongly suggests that the authors should do more additional experiments regarding what mechanisms regulate expression of GLUT2, SGLT1, and GLUT5 in colonic mucosa in the inflamed and non-inflamed mucosa.

Our study represents the first step of future investigations on this topic.

Reviewer's code: 02520845

ESPS Manuscript NO: 35838 Title: Glucose transporter expression in the human colon: the missing ring in pathogenesis of colonic diseases? The authors investigated the expression of the members of the GLUT family of membrane proteins in the human colorecetal mucosa in inflammatory bowel disease. The manuscript is readable and presentable, but there are a few minor comments. Results & Discussion: The data is clearly presented and the discussion is well organized. The immunohistochemical data which is not presented, it would be specified as "data was not shown" (page 14).

Done (page 14).

Also, I suggest decreasing the number of Figures on the way to select the representative figures for the most important results and throw out the figures which did not show difference between the investigated groups;

We have deleted old Figure 6 of the past version. There are now 10 figures.

in the Fig.7 summarize the most important findings in the figure legend.

Accordingly, we have summarized the main data in the legend (see new Figure 6 in the revised version of the manuscript) .

In literature review, recent researches are listed to this topic. In conclusion, this is an interesting study which highlights the role of GLUT5 expression in inflammatory bowel disease associated with atypical aggregation of lymphatic vessels.

Thank you for your interest in our study.

Reviewer's code: 01047616

The authors investigated the expression patterns of GLUT2, SGLT1, and GLUT5 in the mucosal biopsies of control subjects and IBD patients by using immunohistochemistry and immunofluorescent staining. The study aim is straightforward and the results showed novel findings of glucose transporters expression in the human colonic mucosa of IBD patients. While the technical difficulties of tissue collection and rareness of the human biopsies are well recognized, a number of concerns regarding the inclusion of patients must be corrected. Major concerns are listed below. Major points:

1. The control subjects included those who had a history of colorectal surgery (n=4 out of 20). These post-surgical patients are not proper controls and should be excluded from analysis. It is known that intestinal adaptation such as upregulation of glucose transporter occurred in brush border of remnant intestine in animal models of short bowel syndrome (Martin GR et al., Am J Physiol 2014).

We have excluded from the study the patients (n=4) with a history of colorectal surgery. Data, tables, and figures have been modified accordingly.

Moreover, both inflamed and non-inflamed areas obtained from the control subjects were included in the analysis (Table 2). The inflamed tissues should not be considered as controls.

The Control group consisted of patients undergoing colonoscopy to investigate clinical symptoms, but none was diagnosed with IBD. Hence, they are not to be considered healthy controls, but rather controls not suffering from IBD. For this reason, we believe that the data on biopsies with inflammation may be relevant for this patient group . The Control group is now designated throughout the text as "controls" and not as "healthy controls". We thank the reviewer the suggestion to correct this inaccuracy.

2. *The immunostaining pattern of glucose transporters in each intestinal segment appears to be in cluster forms on the epithelial surface and only 5-33% of biopsies are positively stained. Since no difference in pattern or percentage could be definitely identified for the glucose transporters and the n number of patients are relatively low, the title is far-reaching by suggesting " a missing ring in the pathogenesis of colonic diseases". This should be deleted.*

As suggested, we have deleted " *a missing ring in the pathogenesis of colonic diseases* "from the title.

3. Has the author tried peptide competition assay to ensure the specificity of antibodies used for staining of glucose transporters? The authors have to show the absence of non-specific staining by antibody since the conclusion is mainly based on immunohistochemical staining.

We agree with the reviewer that this type of control is the best, but the corresponding peptides for GLUT5 and GLUT2 antibodies used in this study are not available. We prepared control sections by preabsorbing the primary antibody with the corresponding peptide for SGLT1. However, the specificity of glucose transporter labelling has been carefully checked. First, we tested different antibodies in addition to those used in this study: rabbit anti-GLUT2 (LifeSpan Biosciences Inc., Seattle, WA, USA, cat #LS-C15390), rabbit anti-GLUT5 (LifeSpan Biosciences Inc., cat #LS-C15414). All the antibodies were tested on human small intestine used as positive controls. The antibodies that gave the best results were the antibodies that we used in this study.
The Glut5 antibody has been used in various human studies, including in small intestine and colon sections:
-Medina Villaamil V et al. Fructose transporter GLUT5 expression in clear renal cell carcinoma. Oncol Rep 25: 315-23 (2011).
-Engelson EJ et al. An Essential Farnesylated Kinesin in Trypanosoma Bruce. PLoS One 6: e26508 (2011).
-Gowrishankar G et al. GLUT 5 is not over-expressed in breast cancer cells and breast cancer patients. PLoS One 6: e26902 (2011).
-Polito A et al. Hyperglycaemia and apoptosis of microglial cells in human septic shock. Crit Care 15: R131 (2011).
In addition, the fact that we observed a different expression pattern for GLUT5 in our study is a good indication that the GLUT5 labelling we observed is specific.
A few sentences were added in "Materials and Methods "in the new version of the manuscript. We have added additional figures (Figure 3i, j) showing GLUT5 staining (Figure 3i) and the negative control (Figure 3j) in adjacent sections.

Fig 9: Gold-labeled TEM is recommended to show if GLUT5 is localized to lymphatic vessels.

Please, see point 4

Fig 10 and 11: Stainings of GLUT5 and LYVE-1 (a lymphatic vessel endothelium marker) were not co-localized. GLUT5 seems to be present on certain cell types, lymphocytes?

They may be lympho-monocytes, but we are not sure.

4. *The stainings are mostly based on immunohistochemistry or immunofluorescence. The use of real-time PCR would be beneficial for quantification of overall expression levels.*

The small size of biopsies allowed us to use a single method. We chose IHC analysis on paraffin-coated specimens because it permits to evaluate not only the expression of glucose transporters but also their localization and distribution, which was the aim of our study. We agree with the reviewer that immunogold or PCR analysis would have provided more detailed data on the expression of glucose transporters, but they require fixation and treatment of samples completely different from the ones we used. They can, however, be used to carry out future investigations on this topic.

Reviewer's code: 02441737

Comments to the manuscript 35838 entitled: Glucose transporter expression in the human colon: the missing ring in pathogenesis of colonic diseases? From the authors Merigo F., et al. It is a very interesting descriptive study to be innovative in daily clinical practice, although it is advisable that the authors respond to the following comments. Methodology: It is recommended that the authors describe in detail the statistical procedure for determining sample sizes for the groups studied (sample size and precision for estimating a population proportion). Because this is a descriptive study, the following statistical parameters should be considered: a) confidence level, b) expected proportion of patients expressing glucose transporters in colorectal mucosa in healthy subjects and subjects with inflammatory bowel disease, and c) the accuracy of the test.

The question raised by the referee on the procedure for determining the sample size cannot be applied in the present study which remains a pilot study describing GLUT5 expression in biopsies collected from patients which underwent clinical practice in the Hospital. The sample size of the patients groups was not defined *a priori* but describes the number and the nature of the collected samples.

The question about the statistical approach is interesting but far beyond the goal of this paper. A mere control experimental group constitute by healthy volunteers is not described here due to the nature of the recruited patients. Moreover, the number of biopsies with GLUT5 expression compared to the total amount of biopsies (and not the number of patients), and the samples size of the groups itself make it difficult any statistical inference, which is a focal point and, hopefully, the goal of future studies. The main result of the present study is GLUT5 expression in lymphatic vessels, and the first description of its expression in two intestinal pathologies.

It would be of interest to describe clearly the clinical characteristics and the diagnosis of the patients who formed the control group. This, because this group was formed by patients with very different symptomatology and / or diagnosis. Patients who underwent colonoscopy for prevention of colorectal cancer or were followed-up after polypectomy or had history of colorectal surgery or lower gastrointestinal symptoms were designated as the control group. The BMI of the patients has a very wide range, could present the results of the expression of glucose tracers stratifying patients according to their BMI (obese and non-obese). The above, because the age and BMI of patients and controls vary significantly between groups, which could influence the presented results.

As suggested, we grouped the biopsies (inflamed and non-inflamed) based on patient BMI to form 3 subgroups (normoweight, overweight, and obese) in which we calculated the percentage of GLUT5-immunoreactive specimens for clusters of vessels. We have added a new table (Table 6), showing these results which are described in the various sections of

the text in the new version of the manuscript. Thank you for this important suggestion that provides new information.

It would be of interest if the researchers compared the results among the groups of subjects: 1) who underwent complete colonoscopy, biopsies were obtained from all 6 portions of the colon-rectum (cecum, ascending colon, transverse, descending, sigmoid colon, rectum) and those which the biopsies were obtained only from the endoscopically.

We felt that we could not carry out the reviewer 's suggestion because the number of biopsies with only endoscopic evaluation is very different between patient groups (2 biopsies in UC, 2 in CD, 16 in CTRL). This discrepancy would make it difficult to compare the data of the different patient groups. Idem for patients with biopsies of all intestinal tracts (7 patients in UC, 1 in CD, 6 in CTRL).

Results In Figure 1, it is recommended that the authors highlight the microscopically findings of using arrows.

Thin and thick arrows have been inserted in Figure 1 to indicate staining on the apical pole and the basolateral membrane of epithelial intestinal cells.

Make the correction at the foot of Figure 7, since these are bar graphs, they are not histograms.

We thank the reviewer for catching this error. The correct name is now indicated at the foot of Figure 6 in the revised version of the manuscript.