Author's response to reviews

Title: Comparative analysis of inflamed and non-inflamed colon biopsies reveals strong proteomic inflammation profile in patients with ulcerative colitis

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Author's response to reviews: see over
Dear Editor,

Thank you for constructive feedback on our manuscript “Comparative analysis of inflamed and non-inflamed colon biopsies reveals strong proteomic inflammation profile in patients with ulcerative colitis”. We have considered the reviewers comments carefully and have addressed these point-by-point below.

**Response to referee 1:**

- **Major Compulsory Revisions**

1. Reproducibility of 2D gels: The authors made no reference in the methods or text of the document regarding the reproducibility of the 2D gels and analysis. Were technical repeats of the gels and analysis carried out and if so what was the level of reproducibility between the reps.

   *The reproducibility of 2D gels is a relevant issue in relation to proteomic studies. In this study we choose to increase the number of biological replicates at the expense of technical replicates in the UC cohort in order to increase the statistical power of the study. This assumption was based on a technical note by Horgan (Journal of Proteome Research, 2007, 2884-2887), who reviewed statistical power in relation to the choice of sample size with emphasize on biological versus technical replicates in gel based studies.*

2. Validation of protein expressions: The authors made no reference in the methods or text of the document regarding the validation of the protein expression profiles. It is worth considering the implementation of a validation experiment such as immunoblotting for specific proteins or immunohistochemistry assays on the histological blocks. This would lend further support to the authenticity of the results and methods.

   *Clearly further validation studies using different immunoblotting techniques for specific proteins could validate and provide additional support to the results found. In the current study, the low amount of tissue in the UC biopsies meant that all the sample material was extracted at the onset of the experiment. Therefore no extra material from the examined UC cohort is available for such studies and a new experimental study including patient recruitment is needed to fulfill such findings.*

- **Minor Essential Revisions (Minor issues not for publication)**
1: Abstract, Paragraph 1: Change “wanted” to “aimed”.

*This is now changed as suggested*

1. Abstract, Paragraph 3: The authors state 44 individual protein spots were identified. Later in the document (Background, Paragraph 6, Results, Paragraph 9) the authors state 33 unique proteins were identified. The authors need to clarify if firstly these proteins are unique rather than individual, and if so change this to the correct number. If the authors are stating that in total 44 proteins were identified then perhaps more value would be added to the abstract by discussing the proteins which are unique and can act as candidate markers for UC rather than discussing proteins which were simply identified.

*The confusion in relation to identified proteins is now clarified in the sections pinpointed. 44 should be 43 (the total number of spots listed in tables 1 and 2) and this is changed throughout the manuscript. 43 is the total number of spots identified using MS. However several spots were assigned to the same protein resulting in a total of 33 individual proteins being correlated to inflammation, this is now specified. Unique is removed since the wording could be misunderstood.*

2. Abstract, Paragraph 4: The authors state that the proteins have been identified as candidate markers for disease severity. This comment needs to be changed as these markers have not been directly correlated to disease severity/activity (eg. Disease scoring indices). These proteins have been correlated to UC disease only.

*The wording is changed to “potential candidate markers for UC.”*

3. Background, Paragraph 6: See comment 2 above.

*See comment under comment 2(1) above.*

4. Methods, Paragraph 1: The authors should give information regarding the Healthy Controls and why these individuals were undergoing endoscope procedures.

*A sentence regarding the selection of healthy controls is now inserted “Four voluntary healthy controls (1:1 female-male ratio, mean age 32 (aged 18-50)) without any familial disposition for inflammatory bowel disease, daily medication, or any known diseases were recruited by announcement. They were all without inflammation at the endoscopy.”*

5. Methods, Paragraph 1: The authors state the treatments each of the 20 UC patients were receiving at the time of sampling. The number of patients described total 25. This needs to be clarified.
The number of patients described now total 20 and the text is changed to: “Of these, four patients were diagnosed with UC for the first time at the endoscopy. Among the 20 patients, ten were treated with 5-aminosalicylic acid, one with salazopyrine, one with a diuretic and renin-angiotensin inhibitor due to arterial hypertension, and eight were without daily treatment.”

6. Methods, Paragraph 5: The authors should be more specific in terms of what criteria were manually defined e.g. sensitivity, min peak values, min and max size scale etc.

In the software Progenesis SameSpots no criteria can be set regarding sensitivity, peak values, size scale etc. before the automatic procedure. As stated in the text few anchor spots were manually defined to guide the gel alignment. After this spot boarder lines are also automatically applied without any manual interference.

7. Results, Fig. 2 & 3: The size and resolution of these images are small making the referenced data points hard to see. Can the quality of these images be improved?

New figures with improved quality are included.

9. Results, Paragraph 8: See comment 3 above.

See comment under comment 2 above.

10. Results, Tables 1: insert a full stop at the end of the table legend

A full stop is now inserted

11. References Number 21: Typing error in the title of the reference “Genome-wide”.

Spelling is corrected

- Discretionary Revisions

1. The authors should consider inserting a table listing the 33 identified proteins which are unique in their expression to UC patients.

The text in the result section has been changed so it is more clear that the 33 proteins refer to the total number of individual proteins identified based after MS on the 43 selected protein spots and therefore this information should now be more easy to extract from tables 1 and 2 showing the identification details for the 43 spots.

2. It is worth commenting in the text of the discussion regarding the clinical reasons the healthy controls were undergoing endoscopes. The authors do state that the healthy controls were not patients with colorectal carcinoma, however if these individuals were undergoing endoscoping for
reasons such a polyp screening then this should be acknowledged. As stated in the introductions some inflammatory markers can be increased in polyps.

*The healthy controls were recruited by announcement from Department of Food Science, Aarhus University, Denmark and were without known diseases. We have clarified this in the Methods section.*

**Response to referee 2:**

Purpose and significance: This study retrospectively evaluates variation in mucosal & submucosal proteomic profiles of patients with ulcerative proctitis, with the intentions of identifying novel biomarkers of disease activity and elucidating new mechanisms and pathways driving inflammation in inflammatory bowel disease. There is a need for non-invasive surrogates of mucosal inflammation and disease activity in ulcerative colitis. I agree that fecal calprotectin, lactoferrin, C-reactive protein, and imaging do not necessarily reflect inflammation specific to ulcerative colitis.

Methods: The authors perform paired analysis of endoscopically inflamed and normal mucosa in those with UC, controlling for individual variation. Sample size is appropriate given bioinformatics necessary for proteomics. I do have reservations about the variation in the UC cohort, specifically that 10/20 were using 5-ASA, 4 were untreated, and two were using renin-angiotensin inhibitors. Further, the authors state (68-70) that inflammatory biomarkers and pathways of interest need to be specific to ulcerative colitis and not other causes of colitis (infectious, neoplastic, medication-induced). An additional comparison group comprised of non-IBD related inflammation (C. diff colitis mucosal biopsies) should be included to clarify pathways specific to ulcerative colitis. Ulcerative proctitis variation in severity impacts histology, cellularity, protein expression, and likely post-translational protein modification. Histology results, namely grade of inflammation is not reported and should be to characterize patient characteristics. Ideally patients would have a consistent grade of inflammation, both endoscopically and histologically, for more uniform results, especially since fold change of protein expression is reported. To demonstrate whether normal control and endoscopically normal UC mucosa are comparable, I would include a direct comparison of proteomic profiles of control left colon and non-inflamed UC left colon mucosal biopsies. Further, the variation in control group protein expression demonstrates the lack of a standard protein profile of colonic mucosal, at least in this study population. Sound statistical methods used do identify 33 unique proteins with at least a two fold expression
change between inflamed and non-inflamed mucosa in ulcerative proctitis subjects. These results do highlight metabolic pathways involved in ulcerative colitis, however nothing specific can be concluded. Characterizing the consistent elements of mucosal protein profiles serves an important role for future proteomic investigations in UC, however this study is very preliminary and few conclusions can be drawn.

This study reports unique mucosal proteins differentially expressed in inflamed mucosa compared to normal mucosal in patients with ulcerative proctitis. There is value within the field of suggesting novel metabolic pathways potentially driving inflammation in ulcerative colitis, especially as proteomics becomes more accessible. The results from this study, remain far from having clinical impact. The variance reported, even in glycerol-3-phosphate-dehydrogenase, is small. In addition, it is unclear if any of these markers can be used as a biomarker, as there were no comparisons to stool or serum detection of high-variance compounds by ELISA or other high-throughput assays. A biomarker that non-invasively better correlates to histology or endoscopic appearance of mucosa or predicts response to specific therapies, would be clinically useful.

Referee 2 specifies some very important points in relation to the relevance and the limitation of the current study. Based on the controls it is clear that the individual variation in protein expression is high. The experimental design used with non-inflamed biopsies compared with inflamed biopsies within UC patients should minimize the level variation and thus make the association with UC inflammation stronger. The significant protein spots identified in the UC patients have been evaluated in relation to the control persons in order to validate the observed differences in UC patients in relation to the control group (see fig. 3.). Furthermore the number of individuals in the two groups provides an unbalanced dataset for comparison (four controls versus twenty UC patients). Therefore, the proteomic profiles of control left colon and non-inflamed UC mucosal biopsies was not compared directly. The remaining points addressed are discussed under the specific comments below.

Major Revision
1. Clearly state in the abstract and background that the purpose of this work is to describe the proteomic profile in ulcerative colitis, not the express discovery of new biomarkers.

The wording of the abstract has now been changed accordingly.
2. Narrow the UC cohort to identical medical therapy (all 5-ASA, all treatment naïve, etc) including non-IBD medications and reanalyze the data. This should be possible given the small number of patients necessary to enroll.

We agree that it is interesting to take the difference in medical treatment into account in relation to the general interpretation of the results. We have examined this approach in relation to the multivariate statistic and did not find any grouping effect of the 5-ASA treated patients and therefore the variation in protein profile is just as broad as when looking at the patient group as a whole. This result is now included in the results section.

3. Add a non-IBD inflammation control group (i.e. C diff colitis distal colon biopsies) for comparison and clarification of proteins unique to IBD related inflammation.

This is a really good suggestion and we agree that this will be the starting point for future studies in order to validate, whether the findings in the current study are specific for UC or more general to gastrointestinal inflammation diseases. Unfortunately, we could not include an additional group in the current study as we choose to ensure a better statistical power by examining a relatively large group of UC patients instead of including a broader set-up of different patient groups.

Minor Revision

4. Please provide histology inflammation grading for UC cohort.

The biopsies were graded using a simplified method for histological assessment of inflammation in UC (Geboes et al., 2000, Gut 47: 404-409). This is now specified in methods section and included in the discussion.

We would like to thanks the reviewers for their fruitful comments, which have improved the quality of the manuscript. We have done our best to follow their recommendation in the revised version of our manuscript and with these changes in mind; we hope that the manuscript can be accepted for publication in BMC Gastroenterology.

Kind regards,

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