Author's response to reviews

Title: The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in C57BL/6J mice

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

Please find attached our revised manuscript entitled “The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in C57BL/6J mice” (MS: 1358856479172962).

We like to thank the reviewers for their thoughtful and helpful comments that have improved our manuscript. We carefully considered and addressed their comments and performed additional qPCR experiments, as was suggested by one of the reviewers.

All changes in the revised manuscript are underlined and a point-to-point response to the reviewer’s concerns is provided in this cover letter. We hope this manuscript is now acceptable for publication in BMC Medical Genomics.

Thank you for your consideration,

Yours sincerely,

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Response to reviewer Morihiro Matsuda:

We were pleased to learn that Dr. Matsuda finds our study well designed and the results interesting and important. We address his specific comments (italics) as follows:

1. *Based on the findings that mRNA expressions of the genes involved in lipid metabolism are modulated in small intestine by high fat diet, the authors should present the plasma lipid profiles and discuss the outcome of these mRNA changes.*

We agree with the reviewer that it is very interesting to further study the effect of the dietary fat-induced mRNA changes in the small intestine on plasma lipid profiles. So far, we have already measured total cholesterol and total triglyceride levels in plasma; however we found no significant differences between a high-fat and low-fat diet. As these plasma lipid parameters reflect whole body homeostasis it is not very surprising that they do not per se reflect the mRNA changes in the small intestine. To study the latter in more detail, we have now set up two novel studies to determine the physiological effects of the high-fat diet on the lipid metabolism-related processes, such as chylomicron synthesis and cholesterol absorption in the small intestine. The preliminary results of these studies support the mRNA changes we found in our mouse study. However, as these studies provide many interesting data, which we believe are partly also outside the scope (i.e. obesity and insulin resistance) of this manuscript, we have decided to submit the results in separate papers.

2. *In Figure 5, the authors show mRNA changes of several nuclear receptors. But as the data are determined by microarray analysis, the statistical assessment is insufficient. In order to make a statistical analysis on the findings, mRNA levels of nuclear receptors and their target genes in small intestine from 6 individual mice should be re-measured by quantitative PCR.*

In the supplementary figure S2, we already included the quantitative PCR (qPCR) results of multiple target genes of the nuclear receptors, such as Ppar target genes Slc25a20 and Hmgcs2 and Lxr target genes Abca1 and Scd1. We now additionally included qPCR data of Fxr target gene Fgf15. The qPCR data of these target genes, as well as for other genes that are not all included in supplementary figure S2, are highly in accordance with the microarray data. As the reviewer suggested, we also performed qPCR to more accurately determine basal mRNA expression of nuclear receptors along the longitudinal axis of the small intestine, in 6 individual mice. We agree that these qPCR results are indeed more informative than the microarray data that we have shown before and therefore in the revised manuscript we now included the qPCR data of nuclear receptor expression (Figure 5).

3. *Based on the findings that mRNA expressions of several secreted factors, including Il-18 and Angptl4, are modulated by high fat diet, these protein levels in plasma would be measured if possible.*

As suggested by the reviewer we measured Il-18 levels in plasma of mice fed the high-fat and low-fat diet. However, similar to what was seen for plasma lipid profiles, we found no difference between both diet groups (see figure below). Again this might
be explained by the fact that more organs might secrete IL-18 and therefore the plasma levels will probably not reflect the small intestinal gene expression pattern. The same goes for Angptl4 and moreover for this protein reliable antibodies against the murine protein are still lacking. Besides IL-18, we also measured active Glp-1 levels in plasma, which should be more in accordance with intestinal gene expression as Glp-1 secretion is restricted to the gut. As shown in table 4 of the manuscript, we found a slight up-regulation (fold change 1.4) of Glp-1 (encoded by preproglucagon mRNA) on a high-fat diet, which we also found in plasma (see figure below).

In summary, as the expression of most secreted proteins is not specifically restricted to the gut, it might be difficult to associate plasma profiles to the dietary fat-induced small intestinal gene expression changes. Plasma profiling is even further complicated by the fact that for hardly any of the differentially expressed secretion molecules mouse-specific detection assays (e.g. ELISA) are available.
Response to reviewer Leslie P Kozak:

We like to thank Dr. Kozak for his very favorable and generous comments. We address his specific comments (italics) as follows:

It would seem that if one asked, ‘How does the intestine contribute to the development of a positive energy balance in DIO?’ Two major systems come to mind, the effects of the intestine on the passage fat into the circulation and the effects of the high fat diet on the synthesis and secretion of gut peptides that regulate food intake. While the issue of nutrition metabolism was dealt with in some detail, in some respects too much detail, the effects of the high fat diet on peptides regulating food intake is almost neglected. Over 11 peptides are synthesized by the intestine (for a comprehensive review see Chaudhri et al. in Phil. Trans R. Society B (2006) 361, 1187-1209.), yet one had to search to find information on a couple. It is important sometimes to provide evidence when expression of a gene is not significantly different, as was done for the PPAR and nuclear receptors.

We agree with the reviewer that the small intestine might contribute to development of dietary fat-induced obesity is by regulating energy intake. However, in our study we carefully measured energy intake (see p11 of the revised manuscript) and found that there was no significant difference between mice that were fed the high-fat and low-fat diets. Therefore, we believe that within our study design the capacity of the small intestine to regulate food intake does not contribute to the observed development of dietary-fat induced obesity. This is also reflected in our microarray data, as we found that the expression of most gut peptides that regulate food intake is hardly affected by the high-fat diet. Except for Cck and Pyy, the fold changes were all outside the selection criteria (< -1.5 and > 1.5) that we have chosen to provide a comprehensive representation of the most interesting secretome analysis results. Moreover, the changes in gut peptide expression that we found even imply that food intake is slightly reduced on a high-fat diet. A food intake-regulating role for the small intestine in development of obesity is therefore not very likely in our study. For individual gene expression interests we refer to the Gene Expression Omnibus database (accession number GSE8582).

While it is understandable that a lab interested in the PPARs might spend a lot of effort on examining their levels of expression, to this viewer the conclusion would be the changes in these receptors is really insignificant (Table 2), but they hand on the to belief that there are important changes. If there are important changes it is in the question, why do gene targets of PPARs such as Acyl-CoA thioesterases, Cytochrome P-450 a10 show such robust changes in expression? Might there be other transcription factors more important than PPARs. Similarly, too many words are spent on genes associated with chronic inflammation, yet the short periods of time on a high fat diet would not be expected to induced high levels of expression of genes of inflammation.

We carefully considered the points that are made by the reviewer, but an (unbiased) overrepresentation analysis (ORA) of our microarray data clearly revealed that especially biological processes related to lipid metabolism and inflammation/immune response are highly affected by dietary fat. Moreover, as the reviewer also suggests the passage of fat into the circulation might be one of the major small intestinal
contributors to development of the metabolic syndrome. Therefore, we believe lipid metabolism is an important topic to focus on in our manuscript. As microarray analysis is based on gene expression regulation and it is known that nuclear receptors such as Ppars, Lxr and Fxrs (but there might of course also be other (unknown) transcription factors involved) can regulate lipid metabolism-related gene expression, we still believe it is essential to discuss the potential role of these nuclear receptors in our manuscript. Moreover, we clearly show that, despite minimal gene expression changes of nuclear receptors itself (as is noted by the reviewer), a high-fat diet does substantially effect the ligand-specific activation status of these nuclear receptors as is reflected by the differential expression of their target genes. We rephrased and shortened this part of the manuscript now to make it more clear and concise (see p14-16 of the revised manuscript).

For other peripheral organs, such as liver and white adipose tissue, it is reported that inflammatory processes can play a role in development of insulin resistance. As our ORA also showed that dietary fat affects inflammation-related processes, we believe it is important to discuss the possible small intestinal contribution of these processes to development of metabolic syndrome. However, we agree with the reviewer that at this point it is hard to draw definitive conclusions about the role of inflammation/immune response-related processes in the small intestine in development of obesity and insulin resistance.

Another essential recommendation: A small, but important request concerns the absence of important data that should be added. In the methods a protocol was described for the analysis of feces, but no data was presented in the manuscript. Was there a difference in the energy content of the feces and was there a difference in energy intake between mice on high and low fat?

To determine whether the dietary effects that we found on obesity and insulin resistance could be due to a higher energy intake on the high-fat diet we analyzed non-absorbable chromic oxide levels in feces, which are indicative for the food intake of mice fed a high-fat and low-fat diet. We found that energy intake on the high-fat diet (10.8 ± 0.9 kcal/day) was even slightly lower, but not significantly different from that on the low-fat diet (11.9 ± 0.9 kcal/day). Thus, we conclude that not the amount, but solely the composition of the Western-style humanized high-fat diet induces obesity and insulin resistance in C57BL/6J mice. As this was apparently not clearly formulated in our manuscript, we rephrased the ‘Method’ part for the fecal analysis and the ‘Results’ part regarding energy intake (see p 7 and 11 of the revised manuscript).

In our study we did not further investigate the fecal energy content, but from previous studies we know that the energy content derived from fecal fat can slightly increase from 0.1 to 0.5 kcal/day on a high-fat diet. This implies that the energy absorption on a high-fat diet would even be slightly lower than the energy intake calculated (see p 11 of the revised manuscript), due to an elevated fecal excretion of fat. However, as we believe that these fecal differences are negligible and do not contribute to a potential explanation of the observed dietary-fat induced obesity and insulin resistance, we decided not to include these data in our manuscript.