Author's response to reviews

Title: A mouse model of sitosterolemia; absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol

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PDF covering letter
Response to concerns of Dr. Joyce Repa

We would like to thank Dr. Repa for her careful and thoughtful review of our manuscript. Her diligent review has uncovered errors and now that they are corrected we believe that these revisions now make this manuscript much stronger.

Minor Essential Revisions-

1. The authors need to fully describe the “standard rodent diet” used in these studies, identifying the manufacturer and providing the diet number or name.

Text in Methods section under animals and diet has been edited to include a description of the “standard rodent diet.” New text is “All mice were maintained on a standard rodent chow (Harlan Teklad mouse/rat diet LM-485) which contained 61 mg/kg cholesterol, 31 mg/kg campesterol and 105 mg/kg sitosterol, given free access to water and maintained at 25°C with a 12-h light, 12-h dark cycle.”

2. Table 1 has an extra line following Srebp-2 that causes formatting problems. This is not apparent on our electronic or print version. However, we will make corrections during proof reading, if it reoccurs.

3. In Table 2, N/A should be defined. In addition, statistical analysis should be performed and significance indicated where appropriate.

Table 2 has been edited in that N/A has been changed to ND (not detected). Additionally asterisks have been added to the table to indicate data with P values of <0.05.

4. The Y-axis of Fig. 3a should be relabeled as “sterol” rather than cholesterol.

The figure and legend have been changed to correctly indicate ‘sterols’ as opposed to ‘cholesterol.’

5. There appears to be a discrepancy between the text (CYP7A1 enzyme activity was reduced by 59%) and the results shown in Fig. 4b (the reduction is likely to be only ~30%).

The actual data from the assay revealed a 37% reduction in CYP7A1 enzyme activity. The reported 59% reduction was an error and the text has been altered to reflect this change. The numerical data are as follows:

<table>
<thead>
<tr>
<th></th>
<th>Abcg8+/+</th>
<th>Abcg8+/-</th>
<th>Abcg8-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act</td>
<td>27.9±2.4</td>
<td>22.8±2.4</td>
<td>17.6±2.2</td>
</tr>
</tbody>
</table>

P values of <0.05

6. The photograph in Fig. 6i is cropped in a way that makes it difficult to identify the orientation of the intestinal villi and location of crypts. This should be readjusted or labeled.

Arrows have been added to the figure to indicate location of intestinal villi and crypt. Additional text has been added to the figure legend to denote these locations.

7. Label or describe the panels in more detail for Fig. 7. A slide showing immunolocalization of an apical marker (such as bsep in Fig. 6) should also be included in this figure.

Figure 7 has been edited to make the histology more clear. Arrows have been added to indicate bile canaliculi and an asterisk has been added to indicate bile ducts. The figure legend has been changed to include this description. Additionally, figure legends have the same wording now to indicate ‘bile canaliculi’ as opposed to ‘canalicular membranes’ previously, which is the correct term at this level of microscopy. We do not have readily available anti-Bsep antibody against mouse and all the commercial reagents are against the human proteins. The Bsep antibody used in this report was available to our colleagues in Europe.
8. The legend to Fig. 8 indicates that statistics were performed...phospholipid secretion was not statistically different—this discrepancy should be resolved.

The data for the bile salt and phospholipid secretion during depletion and TUDC infusion are not statistically significant and this is stated in the text. Additionally, for the sterol secretion in the Abcg8-/- mice it is essentially a flat line and shows a definite biological significance. Statistical analysis is not needed in this case, as the difference is clear-cut. However, the +/- mice although they show an intermediate phenotype, this ‘trend’ is not statistically significant.

9. As Fig. 9 describes secretion RATES, these should be expressed ... Figure 9 has been changed to reflect the secretion rates as expressed in nmol/min/100 g body weight. Additionally, statistically significant findings have been indicated and described in the figure legend.

**Major Essential Revisions**

1. The targeting strategy to eliminate ABCG8 is complicated...Northern blot...The authors should definitively establish that no “exon 1-3” product is generated by their “null” allele.

   The disrupted allele contains the deletion of exons 4-13 and the partial loss of exon 3 resulting in the loss of the last 2 amino acids in exon 3 (S, S). By RT-PCR there are PCR products for exons 1-2 (data not shown). However, by quantitative RT-PCR (with exons located in exons 2 and 3), there is a very low level of product detected as shown in figure 4a. Since our targeting disrupts the important Walker A, B and C motifs we feel confident that if any peptide were produced, it would not act as a chaperone for sterolin-1 since the current evidence suggests that heterodimerization may take place through these ATP-binding domains as shown by the crystal structures of other ABC proteins.

   The description of the Northern blot probes has been clarified and edited in the text and figure legend. The initial description was an error in that the restriction sites were the sites of our vectors not the restriction sites of the cDNA.

   The reverse primer listed in the table for real time RT-PCR did have a mistake at the 3’–end. The last ‘A’ should have been a ‘T’. Additionally, we identified that the wrong cyclophilin primer set was placed in the table. These have been corrected.

2. Tissue lipid concentrations.... tissue weights... in Table 2.

   The text in the results section under the heading “Sterol levels in Abcg8/sterolin-2 deficient animals” has been edited to indicate that there were no differences in tissue weights by genotype.

3. Concerning results in Table 2...brain campesterol levels appear to show a genotype-dependent change—is this significant?....

   The table in the initial draft was in error. Inadvertently, a line was mistakenly omitted. The correct table now appears in the revision. Additionally, the plant sterol levels found in the brain are small and we did not quantitate separately individual sterol species. As for the differences in hepatic sterol levels in table 2 vs. figure 2 these animals were different sets of animals that were used for each of the experiments. The animals for the table 2 data were mixed sex, 12 weeks of age and feed a regular chow diet. The animals for figure 2 were all female, 12 weeks of age and fed a regular chow diet. The figure legend has been changed to reflect this information. The sex difference of these animals is ongoing and not reported in this manuscript.

4. There is a discrepancy between ...northern analysis...quantitative real-time PCR...

   We have addressed some of this in response to issue 1. The northern analysis of hepatic Abcg5 expression differs from the real-time RT-PCR because the northern blot was not a quantitative blot. Text has been edited to reflect the difference. Additionally, in the recently reported Abcg5 KO mouse (ref. 46), the level of Abcg8 expression is roughly half compared to wild type. Our data are consistent with these findings.
5. The use of three different anti-ABCG5 antisera... a more complete assessment of this ...the preimmune and/or “blocked” serum should be used as a negative control. In addition,...colocalization of the apical marker (bsep) and ABCG5...

Despite that fact that the SC anti-Abcg5/sterolin-1 was raised against the rat peptide there is still immunoreactivity for the mouse as shown by western blotting and transient transfection experiments using mouse Abcg5 and Abcg8 constructs. A preimmune serum western blot shows no banding pattern at all and does not show the 150 or 75 kDa bands as in the immune serum.

Responses to concerns from Dr. Gerd Schmitz
We would thanks Dr. Schmitz for his review of our manuscript.
The reviewers’ comments are in italics and the response to each concern is addressed below it.

Discretionary Revisions (which the author can choose to ignore)
1 Figure 4a) Although incorporated in the text, the authors should include in the figure, whether the measured mRNA expression levels of the 10 genes analyzed differ with statistical significance in the three mouse groups.

Statistical analysis of the mRNA expression of the 10 genes analyzed could not be performed on the three mouse groups using the Student’s t-test since there was no treatment or different diets used for these studies.