Author's response to reviews

Title: Role of the ABCG8 19H risk allele in cholesterol absorption and gallstone disease

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Version: 2 Date: 11 January 2013

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To the Editors-in-Chief

BMC Gastroenterology

11. January 2013

Dear Sirs,

We respectfully submit our revised manuscript “Role of the ABCG8 19H risk allele in cholesterol absorption and gallstone disease” (MS:1319025332774359) for publication as an original research article in "BMC Gastroenterology". We would like to thank you and all reviewers for their helpful and constructive criticism. We feel that the reviewers’ comments helped to significantly improve the quality of the manuscript.

As suggested, we included detailed recruitment criteria of the study cohort in the section “Subjects” and added the required information for the detection methods applied. Furthermore, we explicitly explained each statistical testing and corrected some terms. We performed additional statistical analysis and verified the main points of the manuscript. Please find a detailed point by point response below (reviewer comments are shown in italics, our statements are in bold letters). For your convenience, all changes in the manuscript are underlined with grey color.
1) Amount of gallstone carriers is low, only 34 patients. Furthermore, the subgroups of normal weight and overweight gallstone carriers consist of only 11 and 23 patients, respectively. The data in the Table 2 reveal that there are actually no statistical significant findings between gallstone carriers and the control subjects within these subgroups. When the whole group of the gallstone carriers and the controls are compared with each other, the surrogate markers of cholesterol absorption indicate lower cholesterol absorption among the gallstone carriers compared to controls. However, the mean BMI of the gallstone carriers is significantly higher than that of the controls. This difference in the BMI value may explain the difference also in the absorption of cholesterol. Unfortunately, patients and controls are not matched with BMI.

We thank reviewer for this advice. As suggested above, we now matched 68 of our study subjects according to age, gender and BMI to 34 pairs. In this comparison, cholesterol absorption was significantly lower in gallstone carriers than controls, suggesting that this difference is not due to excess weight. This data is given as supplemental information Table 1A. It should also be taken into account that the 1.4 point difference in BMI is statistically significant but much less than the difference between normal weight and controls of about 5 points.
2) Absorption of cholesterol occurs in the proximal part of the human intestine, i.e., in the duodenum and the jejunum. For the purposes of the present study, biopsy samples were collected during colonoscopy from the distal ileum. Would it have been more proper to take biopsies during gastroscopy from the distal part of the duodenum, which is a more essential site in the absorption of cholesterol, and which is easy to access during gastroscopy?

Furthermore, in the abstract on line 4, the authors mention “…ILEAL cholesterol transporters…”.

ABCG5/G8 and NPC1L1 are actually INTESTINAL sterol transporters, not only ileal.

This is an interesting aspect regarding the exact localisation of intestinal cholesterol absorption. Therefore, controversial information can be found in the literature. For instance, Sane et al. 2006 in JLR [16] reported highest expression of NPC1L1 in the jejunum. However, in the recent publication of Masson et al. 2010 in PLOS ONE [53], although no significant differences were found for ABCG5 between duodenum, jejunum und ileum; ileal levels of ABCG8 and NPC1L1 were significantly higher compared to expression levels in the duodenum. We therefore think that the ileal biopsies are appropriate.

According to the reviewer’s suggestion, we now changed the term ILEAL to INTESTINAL in the abstract on line 6.

Minor Essential Revisions

1) What was the amount of biopsy samples taken from the ileum in a single patient for the purposes of the present study? This could be mentioned in the
Methods section on page 7. The amount/volume of mucosal tissue samples is an important issue for other scientists planning studies dealing with, e.g., tissue gene expression.

Thank you for this remark; we now added this information in the methods section on page 6 line 17-20.

2) It could be mentioned more clearly in the Methods section dealing with RT-PCR, Western blot analysis, etc., from what tissue/cells were the determinations made. From leukocytes or from the ileal mucosa?

According to the reviewer’s advice, we included this essential information in the methods sections on page 7 lines 2-3 and 7, page 8 line 4, page 8 line 26 and page 9 lines 1-3 and 5.

3) Were any of the subjects studied here on cholesterol lowering drugs or were they consuming phytosterol preparations at the time participating the study?

Thank you for this point. None was on lipid lowering drugs or phytosterols. We now integrated the detailed description of the recruitment guidelines for study participants on page 6 lines 7-14.

4) In the abstract, on line 4, and also in other parts of the manuscript, it is mentioned that ABCG5/G8 and NPC1L1 are CHOLESTEROL transporters. Is this the exact expression, because particularly the former one transports much more effectively plant sterols than cholesterol out of the enterocytes and hepatocytes? These are actually STEROL transporters.

The reviewer is right. Corrections were made.
1) I found that the presentation of the results made the paper difficult to follow at times. I felt that the stratification of patients by BMI was not particularly well justified. The WHO categorises a BMI of less than 25 as normal weight, 25-29.9 as overweight and >30 obese but the authors stratify their patients into <25 and >25 and also use the terms 'overweight' and 'obese' interchangeably. In the Results section values for the BMI's are wrongly reported as BMI +/- 25.2 and +/- 26.6, Table 2 has the correct values and SEM's. These are reported as being significant, although it is hard to reconcile this clinically.

The reviewer is correct with respect to the WHO criteria which are meant for large populations and not for smaller study cohorts as in this investigation. Furthermore, the changes in body compositions associated with ageing may result in BMI increase in both men and women (loss of height begins at around 30 years of age [36]), even when body weight remains constant [37, 38]. Instead, some experts propose age-related BMI criteria [39, 40]. We therefore in the manuscript avoided the term obese with the exception of the new analysis (Supporting Information, Table 1B). As suggested, we stratified the group of individuals with BMI ≤24.9 as normal weight, with BMI over 25 into overweight (BMI>25) and obese (BMI>30) subgroups. The calculation of cholesterol absorption ratios reveals the reduction in each gallstone carrier subgroup irrespective of BMI. The strongest effect was then observed for normal weight gallstone carriers compared with relevant controls. However, after adjustment
for multiple comparisons, the observed significance was lost. To avoid this
type of overstratification we suggest to maintain the definition we chose based
on our cohorts. As discussed above, the results of Table 2 were verified in a
matched cohort and confirmed. To avoid confusing interpretation of
manuscript data, we now explained the definition of the subgroups
stratification according to body weight explicitly in the results section page 11,
lines 5-11.

2) On page 12 it is inferred that the differences in HNF1alpha expression are
different but they present a p value of 0.0564 which is not significant. The
values reported for the overweight group in Table 2 also are reported as being
significant at the 0.047 level whereas the levels of 7667 +/- 1295 versus 7274
+/- 1759 suggest otherwise. Throughout the paper I would like to see the
statistical test that was used to generate the probabilities reported explicitly. A
number of statistical tests were used and it would help the readers if they
knew which test applied to what data. It would also add to the credibility of the
paper if Power calculations could be included since the possibility of a Type II
statistical error is always a risk in these types of studies.

The reviewer is right. We corrected the $P$-value of 0.0564 as not significant and
also the HNF1α levels in overweight group. According to the reviewer’s
demand we explained in the section “statistics” explicitly each statistical
testing and provided the explanation of every performed statistical data test.
As the D19H polymorphism was already reported to be associated with
gallstone disease in various populations and different ethnic groups [29, 41,
the prevalence of this variation in gallstone carriers was only confirmed in our small cohort. Therefore, the main focus of the manuscript was on the functional role of the mutated allele but not on comprehensive statistical calculations of genotype frequencies. Therefore, power calculations regarding allele frequencies were not included (and is as expected low (8%) in the actual small cohort for genetic investigations).

Minor Essential Revisions

1) I would like to see a Table showing the diagnoses / indications for colonoscopy since chronic constipation is a known risk factor for the generation of gallstones.

We have now included the indication for colonoscopy which was screening for colon cancer in the German population. Unfortunately, we do not have any information on their bowel habits. A detailed description of the recruitment guidelines for study participants was actually added on page 6 lines 7-14. The identification of gallstones by ultrasound prior to colonoscopy was an additional medical investigation required for this study.

2) The patients did not suffer from gallstone disease and so this term should not be used interchangeably with gallstone carriers. Similarly, some of the patients in the overweight group may have been obese, but not all and therefore this term should not be used interchangeably.

Thank you for this advice; we changed the descriptions in each part of the manuscript.
Please let us know if you have any further questions.

We look forward to receiving your kind response.

Sincerely,

Eduard F. Stange, MD  Dr. Simone Harsch  Dr. Olga Renner