**Author's response to reviews**

**Title:** Mechanistic biomarkers of liver safety and benign elevations in serum aminotransferases - A study in healthy volunteer treated with cholestyramine

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**Author's response to reviews:** see over
Referees’ comments:

Referee: 1

Abstract
• The abstract also does not appear to have an important conclusion – I assume they set out to find a biomarker that DOES distinguish between benign and serious rises in liver injury (according to the first line), but since they ALL go up, perhaps this concept is merely ideological?

Comment: The hypothesis as noted in the abstract was that newly proposed mechanistic biomarkers would not be elevated in response to cholestyramine treatment, which is not associated with clinical liver injury. The hypothesis was based on the fact that cholestyramine has never been reported to cause clinically significant liver injury, yet elevation in serum ALT was observed in some subjects, which returned to normal by the end of the cholestyramine treatment. Similar elevation in ALT has been observed with some other drugs like tacrine and heparin which, like cholestyramine, do not cause clinically significant liver injury. We have amended the abstract conclusion to clarify the findings in the context of the hypothesis. We found that mechanistic injury biomarkers are elevated along with ALT (contrary to the original hypothesis) and further conclude that elevations in these biomarkers must be interpreted with caution because elevations alone may not portend a serious drug-induced liver injury risk for novel drug compounds.

• Is miR-122 the most sensitive biomarker measured (22-fold change) or is HMGB1 the most useful at distinguishing between benign and important biomarker elevations (only 1.7 fold different)?

Comment: The two biomarkers provide different information and we intended them to be considered in concert. miR-122 is specifically expressed in hepatocytes and its release into the blood provides evidence of hepatocellular membrane damage, specifically. HMGB1 is not liver specific, but it is a good marker for cellular necrosis in general. Taken together, the two markers provide information that the injury is occurring within hepatocytes and that necrosis is one route of hepatocellular leakage that is occurring in the context of cholestyramine administration.

The authors should clearly state the most important finding of their study.

Comment: The conclusion of the abstract has been amended to more clearly state the findings, which is that ALT release following cholestyramine administration is associated with actual hepatocellular injury as determined by the biomarker measurements.

Introduction
• The introduction is very good and sets out clearly why the study was performed: to understand the source and/or mechanism of release of clinically benign ALT levels. This could be reflected in the title and abstract.

  **Comment:** As suggested by the reviewer, we have made changes in the title of the manuscript and conclusion in the abstract.

**Results**

• There may be an association between the young women who were taking oral contraceptives. Is there any evidence that oral contraceptives exacerbate the potential of DILI in the literature with any other drugs?

  **Comment:** Intake of oral contraceptive and estrogens has been associated with elevation in serum aminotransferases, and liver damage and carcinogenesis. While it cannot be ruled out entirely, it is unlikely that the contraceptive use affected the liver outcomes in our study. As noted in the Results, only one woman taking oral contraceptives exhibited ALT >5X ULN, despite the fact that the majority of the women enrolled in the study were also taking oral contraceptives.

CYP upregulation or RXR/PXR activation?

  **Comment:** A literature search did not reveal any studies linking the effect of cholestyramine treatment on RXR/PXR and expression or activity of CYPs and RXR/PXR was not measured in our study. Such studies would require liver biopsies be taken, which would pose some risk to the study subjects.

• What form is miR-122 (or the other biomarkers) release in? microvesicles, exosomes? Evidence exists that miR-122 is released in exosomes and may explain why they rise in blood before ALT with respect to time. Could they investigate this experimentally in human hepatocytes?

  **Comment:** This is an intriguing question and an active area of research in many labs. There exists some evidence that miRNAs are packaged into microvesicles and some evidence that miRNAs may be released in protein complexes that protect them from degradation. In the present study, we focused on miR-122 as a biomarker that indicated the presence of hepatocellular injury. It would not be productive to further investigate the sensitivity or temporal release of miR-122 in concert with other markers using the cholestyramine model, because cholestyramine administration never progresses to clinically important liver toxicity. However, these concerns are currently being researched by the IMI SAFE-T consortium in the European Union as part of an effort to validate and qualify novel liver injury biomarkers.

• Has the mechanism cholestyramine been previously explored in human cell lines, hepatotocytes etc. Could these biomarkers be applied here in a more translational approach to the problem?
Comment: Because cholestyramine does not cause clinically important liver injury, the mechanisms of ALT release have not been studied in in vitro or animal models. Our data are the first to suggest that hepatocellular injury does occur upon cholestyramine treatment (albeit at a very low level) and an interesting future direction would be to further investigate the mechanistic basis.

Conclusion
• Well written to support the data but provides more questions than answers. A more focused conclusion on the outcome of the paper and what this means for the development of these markers to predict DILI in man and in preclinical development.

Comment: We have revised the conclusion on the outcome of the paper following the discussion as requested.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.

Referee: 2

Title: Mechanistic biomarkers of liver safety and benign elevations in serum aminotransferases - A study in healthy volunteer treated with cholestyramine

Version:1 Date:6 February 2014

Reviewer: shashi K ramaiah

Reviewer's report: Minor Essential Revisions

The author can be trusted to make these. For example, missing labels on figures, the wrong use of a term, spelling mistakes.

- The authors use “mechanistic Biomarkers” in the manuscript to refer to new biomarkers. It is not clear if miR-122, CK, HMGB1, SDH are biomarkers that help explain the mechanism or liver injury; this wording should be deleted. May be the authors interpret “necrosis versus apoptosis” as mechanistic biomarkers.

Comment: The biomarkers we use have been previously termed “mechanistic” but we have dropped the term at the request of the reviewer.

- Study design: it is somewhat difficult to understand the clinical study design of which should be modified (similar comment is made on the figure 1). There is a washout phase after investigational drug with cholestyramine. The rationale for the inclusion of a positive control should be included. Also Figure 1 bidirectional arrows may not be easy for the reader to interpret and should be rewritten.
Comment: The original study was conducted to evaluate the potential for an active investigational drug to prolong the QT interval. For negative controls, subjects didn’t receive active drug treatment – Placebo; for positive control of QT prolongation, as the investigational drug was tested for its effect on QT interval, subjects received placebo followed by a single dose of Moxifloxacin on the last day of active drug treatment regimen. We have rephrased the study design and highlighted the rationale for the inclusion of a positive control.

We have also removed the bi-directional arrows.

Table 1: Peak ALT, AST, ALP and total bilirubin are post-dosing fold changes as shown in the table which should be clarified in the title of the table. It can be confusing to readers when they read both the absolute value as well as fold-change.

Comment: We have clarified in the table footnote that both absolute value and fold changes have been included for ALT.

The rationale for including only the proposed biomarkers is briefly included in the introduction section. Since there are several novel biomarkers (including PON1, MDH, purine nucleoside phosphorylase) that represent several aspects of liver injury, it is not clear why the authors hypothesized only a select biomarkers.

It is understandable to not measure a long list of biomarkers but the authors may have some previous knowledge why their proposed biomarkers were short listed which should be stated.

Comment: We intended to compare serum concentration of biomarkers in our study to our previously published study where the same biomarkers were analyzed in serum samples obtained from the healthy volunteers treated with four types of heparin. Similar to cholestyramine, heparin treatment also caused elevation in biomarkers of hepatotoxicity. However, no clinically relevant liver injury has been diagnosed in either case. In addition, we only had a limited amount of sera available to extend our analysis to other biomarkers.

The authors measured investigational biomarker samples only from samples containing the highest level. Is it possible that these biomarkers were also increased in samples at other timepoints when ALT increase was not high? This is less likely but measuring such samples would further add value to the hypothesis.

Comment: We concur with the reviewer that measuring these biomarkers at different time points during and after the drug treatment would have provided more insight on the mechanism of elevation in biomarkers. Unfortunately, only the baseline and the serum sample with the peak ALT value was archived from the subjects and made available for biomarker analysis and further characterization is not possible.
The authors claim to test the liver injury mechanism (apoptosis versus necrosis) by measuring total and cleaved CK 18. Based on the lack of differences in the ratio of cleaved to total CK18, the authors interpret that there is no shift from apoptosis to necrosis. Did the authors measure additional parameters to test apoptosis versus necrosis mechanisms? Without such data the interpretation is speculative.

**Comment:** We did not measure other parameters and we have softened out conclusion here.

It does not appear that the authors measured these new biomarkers when same subjects were assessed after the treatment was stopped after the patient follow up. Did these parameters decline or normalize? Would measuring these parameters during the drug free period provide some idea on the ongoing liver necrosis/apoptosis?

**Comment:** Only samples at the time of the baseline and peak ALT value was archived from the subjects and made available for biomarker analysis. We were not able to measure these parameters after the drug treatment was stopped.

• Discussion: The ‘new” biomarker data generated is quite interesting to support additional hepatic changes beyond benign transaminase elevations. The authors discuss the mechanistic aspects (apoptosis versus necrosis) as well as additional mechanisms to understand whether the increases are due to true hepatic injury or a benign change. There is no new data generated to explain or confirm the authors speculations other than citing literature changes that these new biomarkers change due to necrosis and/or apoptosis. It would have been compelling to test some of the mechanistic parameters of liver injury.

**Comment:** We agree that furthermore mechanistic analysis would be of potential interest and that this would represent an important future direction. We believe that the biomarkers measured here sufficiently demonstrated that low-level hepatocellular injury had occurred due to cholestyramine administration, which had not been previously shown.

• Discretionary Revisions

Figure 2: X-axis: EOS should be defined.

**Comment:** EOS has been defined as ‘end of study’ and mentioned in figure 2 legend.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that i have no competing interests.