Author's response to reviews

Title: Prevalence of Genetic Polymorphisms in the Promoter Region of the Alpha-1 Antitrypsin (SERPINA1) Gene in Chronic Liver Disease: a Case Control Study

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Tim Shipley, Assistant Scientific Editor

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Dear T. Shipley

Thank you for reviewing our paper, we have revised our manuscript closely following the suggestions made by the reviewers.
Please find below our point-by-point response to the reviewers’ comments.

Reviewer 1, Pascal Pineau
In their report Karin Kok and coworkers analyse a series a 297 patients with liver disease for the presence of three polymorphisms in the SERPINA1 gene responsible of a1-antitrypsin deficiency (A1AT). Patients are compared with a series of 297 controls matched for age and sex. The major aim of the study was to determine the potential role played by a recently discovered polymorphism (c.-1973T>C) suspected to be preferentially involved in the liver manifestations of A1AT. The novel c.-1973T>C polymorphism was found in the same proportions in patients and controls. It was found independent of any risk factors and did not influence the age at disease onset. The authors found, however, a significant association of S allele (p.G264V) with drug induced liver injury. Given the number of cases with adverse effects involving the liver recorded in each year, this latter aspect of the work is potentially of great medical importance although it is barely commented by the authors.

Major Compulsary revision
Discussion : Were the drugs involved in the 11 patients with DILI identified ?
Yes, we included 11 patients with DILI, 3 of them were S allele heterozygote. The 3 S heterozygotes patients had DILI due to anesthetic compounds (isoﬂurane/nestonal), celecoxib and clavulanic acid. A total of 8 wildtype patients had DILI caused by clavulanic acid (3 patients), azathioprin, sulfasalazin, pantoprazole, methotrexate and quetiapin. We have added this information to our manuscript.

The power of the study is a key issue of this work as the authors recognized it honestly. They suggest that future research should include patients homozygous for PiZZ, absent from the present study.

I consider, therefore, that the authors should somewhat attenuate their conclusion claiming that they « demonstrate that c.-1973T>C polymorphism is not associated with liver disease ».

We thank the reviewer for his comments and have adjusted our conclusion accordingly.

Finally, the reader will find a great interest in a more detailed development of the relationships between SERPINA1 and drug-induced liver disease. To my knowledge, very few reports exist on this topic. I suggest to cite at least the work of Mindikoglu and coworkers (Hepatogastroenterology. 2003 Sep-Oct;50(53):1338-40).
Thank you for your valuable comment, we added this citation to our discussion.
Minor essential revisions
Abstract:
« these divergent expressions » is a bit misleading for geneticists. Using « divergent disease expressions » will be more appropriate
c.-1973T>C polymorphism... is increased : should be replaced by « more frequent » or by « the proportion/rate of c.-1973T>C polymorphism... is increased ».
« In addition, S allele... » : This « addition » comes after a minimal explanation and without any explicative context. I think that it deserve to be more developed in the conclusions inasmuch it is the only results associated with a significant P value.
Results:
« Contrary, in healthy control ... » : « contrary » does not seem appropriate in this instance. "Similarly", "symetrically", "on the other hand" might be preferred.
Discussion:
« COPD » : should developed at least once.
« Z and S allele heterozygosity » : in the context, I suspect that « homozygosity » is more appropriate.
« pR25P is associated with advanced hepatic fibrosis...but not in patients with chronic liver disease » : this sentence is far to be clear. « other chronic liver disease » will maybe clarify this point.
We thank the reviewer for his excellent suggestions and apologize for the typos. We have adjusted our manuscript accordingly.

Reviewer 2: Michele Zorzetto
In this paper Karin F. Kok and co-workers typed 297 patients with liver diseases from various aetiologies and 297 healthy controls for three different SNPs of alpha1 antitrypsin (c.1973T>C, S and Z), in order to understand the role of alpha 1 alleles in liver disease. They found no associations between the alpha 1 SNPs and liver disease.
Major
1) The principal limit of this work is represented by the fact that the patients group is composed by diseases very different one to the other. This could cause an over or underestimation of the actual contribute of the SNPs. Moreover the patients group is not in Hardy-Weinberg equilibrium for the c.-1973T>C SNP. If a population is not in Hardy-Weinberg equilibrium two different events could be happened:
· The population is stratified for a particular feature
· Some mistakes in genotyping occurred
Therefore is possible that Kok et al. stratified the patients for some features.
Indeed, the patient group is not in Hardy-Weinberg equilibrium, we hypothesise that this due to stratification for disorder; after all, we selected patients with liver disease thus we cannot exclude that we stratified for a particular feature. As our control population is in Hardy-Weinberg equilibrium, the likelihood that genotyping errors are the root cause of the finding is unlikely.
2) The only positive associations is between the heterozygous for S allele and the subgroup of DILI patients. This group is very small (11 patients) then the data is not so significant.

Indeed, only 11 patients with DILI were included. These patients developed DILI after exposure to a wide variety of different drugs (see our answer to reviewer 1). Our study was not powered adequately for investigation of the association of DILI with S allele heterozygosity. However, the differences are significant and may be a clue to clinical relevance, but needs to be confirmed in a larger population.

3) Haplotype. The table 2 is not clear. It seems not an haplotype reconstruction distribution but a multiple genotypes. Probably is better to reconstruct the haplotype with a software dedicate (e.g hplus http://cougar.fhcrc.org/hplus/).

4) It would be interesting also calculate the linkage between the different SNPs (D’ or r2)

Excellent comment, we constructed haplotypes and diplotypes; we replaced table 2 by this table.

<table>
<thead>
<tr>
<th>Diplotype</th>
<th>Controls (%) n=297</th>
<th>Patients (%) n=297</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG/TAG</td>
<td>130 (43.8)</td>
<td>112 (37.7)</td>
</tr>
<tr>
<td>TAG/TAG</td>
<td>78 (26.3)</td>
<td>93 (31.3)</td>
</tr>
<tr>
<td>CAG/CAG</td>
<td>51 (17.2)</td>
<td>63 (21.2)</td>
</tr>
<tr>
<td>CTG/TAG</td>
<td>11 (3.7)</td>
<td>12 (4.0)</td>
</tr>
<tr>
<td>CAG/CTG</td>
<td>8 (2.7)</td>
<td>7 (2.4)</td>
</tr>
<tr>
<td>TAG/TAA</td>
<td>8 (2.7)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>CAG/TAA</td>
<td>5 (1.7)</td>
<td>6 (2.0)</td>
</tr>
<tr>
<td>TAG/TTG</td>
<td>4 (1.3)</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>CTG/TAA</td>
<td>1 (0.3)</td>
<td></td>
</tr>
<tr>
<td>CTG/CTG</td>
<td>1 (0.3)</td>
<td></td>
</tr>
<tr>
<td>CAG/CAA</td>
<td></td>
<td>1 (0.3)</td>
</tr>
</tbody>
</table>

Order of alleles: c.-1973T>C- c.791A>T - c.1024G>A / c.-1973T>C- c.791A>T - c.1024G>A

Reviewer 3: Sally Chappell

The aim of this study was to investigate the distribution of the -1973 promoter polymorphism of SerpinA1 in patients with liver disease compared to healthy controls. The results show that there is no difference in the allele frequency in the patients compared to the controls for any of the aetiologies considered.

The work in this manuscript follows on from a published study which compared alpha-1 antitrypsin deficient (AATD) individuals with liver disease against AATD patients with lung disease. This previous work aimed to identify genetic variants of alpha-1-antitrypsin that would identify which AATD individuals were at risk of developing liver disease rather than COPD, and found a significant difference in the allele frequency of SNP -1973.

Major Compulsory Revisions

1. The introduction in the current manuscript sets the scene well for the consideration of AATD-induced liver disease, but the subjects included in this study were not selected on the basis of AATD. There is little justification in the current study for where alpha-1 antitrypsin may play a role in the various
aetiologies included in the liver disease group, and this is the main weakness of
the investigation. There is considerable heterogeneity in the liver disease group,
and there is little comment on how alpha-1 antitrypsin would be expected to play
a significant role in all of these causes. As a consequence of including such a
variety of conditions, the age at onset has a huge range—it is unsurprising
therefore that no significant effect was seen.

Indeed, we included patients with a broad variety of liver disease. Likewise the age at
onset was highly variable as was to be expected given the inclusion criteria. We did not
focus on liver disease in patients with A1AT deficiency, but tried to identify SNP’s in the
SERPINA1 gene which could be (partly) responsible for the development of liver disease of
different etiologies.

2. The power calculations were also carried out assuming one group of 297
patients, whereas in fact this is really a collection of much smaller sub-groups.
Indeed, we tried to detect a SNP responsible the pathogenesis of liver disease, independent
of etiology. The power calculations were based on the complete liver disease population.

3. Inclusion criteria for the controls is lacking—how were these individuals
checked for the presence/absence of liver disease? Were they examined
clinically or was this on the basis of self-reporting?
The absence of liver disease was established on basis of self-reporting and none of the
patients used any medication. We have changed this in our manuscript.

4. What quality control measures were in place to check the genotyping results?
Were any samples done in duplicate? Were any sequenced, or analysed using
an alternative technique, to check the accuracy of the PCR method?
We compared the results of routinely performed iso-electrofocussing with the results of the
study method in patients who had phenotyping prior to our study. All checked samples led
to identical results. Our laboratory has implemented a quality assessment that consists of
in-duplo testing of quality control samples on a quarterly basis to assure the quality of the
testing system. Results of the quality control testing are regularly reviewed as part of our
ongoing DNA quality assessment. Thus we are convinced that the number of genotyping
errors are limited if present at all.

5. The authors state that the distribution of Z allele heterozygosity is similar
across all aetiologies, but do not give any further detail. Were statistical tests
done for all of these comparisons?
We calculated Odds Ratio’s for the entire group of liver patients and compared this to the
control population. In addition we calculated Odds Ratio’s for each subgroup. We have
explained this in more detail in our methods section.

6. The distribution of -1973 alleles are likely to be different in MZ individuals
compared to wildtype MM individuals – this is unsurprising, and the altered allele
frequencies of -1973 in PiZ patients has already been documented (Ref 9 in the
current manuscript). It is possible that similar results would be seen for the S
allele, although why this should be different between the patients and control
groups is unclear. Potentially these results, and the conflict with the results in
References 13-15 as mentioned by the authors in the discussion (which are
much larger studies), are likely to be caused by the small sample numbers in the
current study.
We agree with this comment, we constructed haplotypes and diplotypes. We did not
detect haplotypes and/or diplotypes which were associated with liver disease of various
etologies. We found an association of S allele heterozygosity with DILI, and thus the CTG haplotype was associated with DILI. This is changed in table 2 and throughout the manuscript.

Reviewer 4: Ahmad Settin
This study concerning the prevalence of c.-1973T>C, Z allele and S allele in a cohort of patients with liver disease of various aetiologies compared with healthy controls sounds an interesting study. Generally it is well written, well presented and well organized with statistically validated data. The main weakness, however, is the relatively non homogeneous sample of cases with liver disease, if authors would have focused on one disorder e.g HCV and enriched their sample of same affected cases, I think will be far more better, In spite of this we still can speculate that at minimum these genetic polymorphisms did not appear to be a risk factor for chronicity of liver affection by these disorder.

Indeed, the complete set of patients suffered from various etiologies of their liver diseases. This can be regarded as a weakness of our study. Originally we aimed to detect a relation between the polymorphism and the general outcome of a liver affliction i.e. liver fibrosis/cirrhosis. This is the reason for the design of this study. Unfortunately, we did not have enough HCV patients to achieve adequate power for the detection of associations with these polymorphisms.

These include more informative tables about the distribution of all genotypes and alleles pertaining to A1AT gene significance of difference related to Allele frequencies in both studied groups and also about the analysis of frequencies related to the haplotypes analysis of the 3 alleles together e.g C/S/Z, C/S/M, C/Z/M, T/S/Z, T/S/M, T/Z/M etc. I think figures can be omitted as well.

We thank the reviewer for his comments and accordingly, we replaced table 2 by a diploptotype table.

We hope that you will reconsider our manuscript for publication, and are looking forward to your answer.

Yours sincerely,

Karin F Kok, René H te Morsche, Martijn GH van Oijen and Joost PH Drenth