Author's response to reviews

Title: Mitochondrial DNA haplogroup T is associated with coronary artery disease and diabetic retinopathy: a case control study

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Author's response to reviews: see over
To the editorial board,

Please find enclosed the revised version of the manuscript entitled “Mitochondrial DNA haplogroup T is associated with coronary artery disease and diabetic retinopathy: a case control study” by Kofler et al. for consideration as research article in BMC Medical Genetics.

We hope we have adequately addressed the points of the referees and appreciated their constructive suggestions for improving the quality of the paper.

REVIEWER 1

Minor concern 1

We agree with the reviewer that there might be an influence of sex and age on the statistical analysis. We have been aware that our subgroups are of different age and sex. Since the size of subgroups with similar age and percentage of sex are quite small, we did not provide such an analysis in the manuscript.

Regarding sex we found that the frequency of HG T is similar in men and women of controls and CAD patients (controls male (n=988): 8.1%, controls female (n=539): 8.7%; CAD male (n=372): 15.3%, CAD female (n=115): 13.0%).

When we performed statistical analysis of CAD patients with a similar mean of age as controls, we found that there was an even larger difference in frequencies of HG T between controls and cases (added also in manuscript at the end of the discussion, page 10). Controls [mean age 51.5±6.1; 8.3% HG T; n= 1527] compared to CAD patients [mean age 51.3±4.7; 17.8% HG T; n= 163] revealed a p-value of <0.001.
Minor concern 2
Since we did not detect a significant association of mtDNA HGs to various clinical characteristics, we did not provide such a table. We decided not to present subgroups since numbers in each group would be low.

Minor concern 3
We were not able to detect a significant association of HG T to markers of inflammation like LDL, CRP, (variables only available for controls and CAD patients, not for patients with diabetes) and homocystein (variable only available for controls). We have added a comment in the discussion to address this issue (page 9, first paragraph).

Minor concern 4
A direct comparison of HG frequencies of the present study and the Danish study is not presented because the phenotypic definition of patients in the Danish study is different. The Danish study includes cardiovascular disorders, ischemic cardiovascular disease, ischemic heart disease, myocardial infarction, ischemic cerebrovascular disease and ischemic stroke but not a group defined as CAD (angiographically documented CAD with at least one of the main coronary arteries showing ≥50% stenosis) as in the present study. We have addressed this point already in the discussion (page 10, first paragraph).

REVIEWER 2
Major comment 1
We thank the reviewer for the comment on the problem of multiple comparisons. In genetic epidemiology adjustment for multiple comparison is still a matter of debate. There is a number of studies not taking multiple comparisons into account (for example: Baudouin et al., Lancet 2005, 366:2118-2121; Rosa et al., BMC Medical Genetics 2008, 9:57; van der Walt et al., Neuroscience Letters 2004, 365:28-32; Fesahat et al., Cellular and Molecular Neurobiology 2007, 27(3):329-334) whereas others, like the Danish study (Benn et al., Circulation 2008, 117:2492-2501), did.

If we use Bonferroni correction (required significance level p= 0.05/number of comparisons) in the statistical analysis in table 3 siehe manuscript – methods [P-0.05/3x2; 3 HGs compared to HG H and 2 diseases (CAD and Valvular Heart Disease)], the significance level changes to < 0.008 and the association between HG and CAD is still significant (p=0.002) (table 3).

Statistical significance for the association between mitochondrial haplogroup and retinopathy in patients with diabetes is lost (p=0.046; required significant level after Bonferroni <0.017). This might mainly be due to the fact of the small sample size (table 4).
We have now included the adjustment of significance of the p-value in the legend of table 3 and 4 and added two sentences in the results section as well as two sentences in the methods section (statistical analysis).

Major comment 2
Since we did not observe a significant difference of the HG distribution between patients from Salzburg and those from Graz (see table below) we only mentioned this fact in the results but did not include these data as a separate table in the manuscript.

<table>
<thead>
<tr>
<th>CAD Salzburg</th>
<th>CAD Graz</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=292</td>
<td>n=195</td>
</tr>
<tr>
<td>H</td>
<td>36.7</td>
</tr>
<tr>
<td>U</td>
<td>16.1</td>
</tr>
<tr>
<td>J</td>
<td>13.0</td>
</tr>
<tr>
<td>T</td>
<td>14.0</td>
</tr>
<tr>
<td>K</td>
<td>2.4</td>
</tr>
<tr>
<td>W</td>
<td>1.7</td>
</tr>
<tr>
<td>V</td>
<td>3.1</td>
</tr>
<tr>
<td>I</td>
<td>1.0</td>
</tr>
<tr>
<td>X</td>
<td>3.8</td>
</tr>
<tr>
<td>others</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Major comment 3
We agree with the reviewer that we can not exclude that other unknown and unmeasured confounding factors are present. We address this issue as a limitation in the discussion section (page 10, last paragraph of discussion).

Minor comment 4
According to the suggestion of the reviewer we have shortened the introduction. We have omitted the following sentences, which do not change the major statements made in the introduction:

Human mtDNA is maternally inherited and consists of a 16 kb circular double-stranded DNA molecule which encodes 13 essential subunits of OXPHOS complexes.

Overproduction of reactive oxygen species (ROS) in mitochondria is either driven by β-oxidation of fatty acids or glucose oxidation. Numerous studies have linked excess ROS generation to vascular lesion formation and functional defects of endothelial tissues [1,16].

Minor comment 5
Yes, the mtDNA typing was performed in a single center.
Minor comment 6
For the information of the reviewer we have provided a table with the frequency of HG T in diabetic patients with the available data regarding vascular diseases (history of myocardial infarction, history of stroke, hypertension). Again, the frequency of HG T is higher in patients with diabetic retinopathy compared to those without. We did not include these data in the manuscript due to the small sample size.

<table>
<thead>
<tr>
<th></th>
<th>Patients with history of myocardial infarction</th>
<th>Patients with history of stroke</th>
<th>Patients with Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG T (%)</td>
<td>n=28</td>
<td>n=38</td>
<td>n=166</td>
</tr>
<tr>
<td>No Retinopathy</td>
<td>0.0%</td>
<td>0.0%</td>
<td>5.1%</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>9.1%</td>
<td>7.4%</td>
<td>15.0%</td>
</tr>
</tbody>
</table>

Minor comment 7
We have excluded only one individual who showed heteroplasmy in one of the SNPs. Heteroplasmy of less than 10% is not detectable with the method used due to baseline instability.
In general somatic differences of mtDNA is a common observation especially in tumors (Santos et al., Current Topics in Medicinal Chemistry 2008, 8:1351-1366) but the chance of a somatic mutation, leading to heteroplasmy, affecting a SNP indicative for a HG is extremely low. Therefore, we hypothesise that our patients harbour in principle all the same HG in their leucocytes and vessel walls.

Minor comment 8
According to the suggestion of the reviewer we have included the following sentence in the methods section:
From the 2387 subjects investigated, genotyping failed in 0.21% of the samples (n=5). PCR amplification failed due to degradation of DNA in 1.26% of samples (n=30).

Minor comment 9
According to the suggestion of the reviewer we have changed the description of table 3.

Minor comment 10
According to the suggestion we changed the word “diabetic(s)” to “patients with diabetes”.

Minor comment 11

No trend of HGT with severity of retinopathy was observed as can be estimated from the frequencies of HGT in non-proliferative retinopathy (13.3%) and proliferative retinopathy (10.8%).

Yours sincerely

Barbara Kofler Ph.D.