Author's response to reviews

**Title:** Low levels of IgM antibodies to oxidized cardiolipin increase and high levels decrease risk of cardiovascular disease among 60-year olds: a prospective study.

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Response to reviews

We are grateful to the reviewers for their thoughtful comments, and we hope we have responded adequately to them.

Reviewer 1
General comments
PC is not present in CL, but still there may be some contaminations. However, we did not detect a strong cross reactivity between aOxCL and anti-PC, and we have now included this finding in the Result section, p 7 and also included a figure 2d where experiments are demonstrated. If there is a contamination of PC in the CL-preparations, this does not seem to play a role in the findings herein. Antibodies against oxLDL have not been determined in this cohort. We think such antibodies are very heterogeneous since the antigen is not well defined. As reported in ref 7, anti-PC was determined in this cohort.

For definition of CV, we have followed strictly the ICD10 criteria, which are uniform and consistent. We have now clarified this in the definition of CV, p 4.

As cited (ref 17), Witztum and colleagues reported in an important paper that CL coated overnight is exposed to air and undergoes oxidation. This is discussed in our manuscript, and we also find differences in the antigenicity since the antibodies in focus here are not beta2-GPI-dependent in contrast to the ones studied in ref 17. Further, aCL determinations by commercial kits nowadays seek to avoid oxidation, further emphasizing that at least the strong oxidation used here is not relevant for such assays. Whether there still can be some oxidation in these measurements by different commercial kits is beyond the scope of the present investigation, where focus was not on antibodies against CL or mildly oxidized CL but on strongly oxidized CL.

The source of oxLDL (oxidized by exposure to cupper ions) is described (p 6), and we chose a commercially available product to improve standardization. We have now moved the description of macrophage uptake to the end, to make the relations more clear and logical. We have now changed the wording in the last sentence in the abstract to more clearly reflect what was shown and the possible implications. We do not as yet have anti-OxCL antibodies which are not beta2-GPI-dependent, to make further experimental studies, but we are working on this.

In the statistical analyses, we focused on quartiles as previously in this cohort. However since aCL are known to be dangerous only at very high levels, often above 2-3 SD, we thought it was very important to also study these antibodies at high levels, which is why these data are reported. Of note, aOxCL were not dangerous (statistically) at high levels. We think with the background history of aCL, this information is important.

2. We think the tables would not benefit from including also N-values, instead they would seem confusing. We therefore suggest that the presentation is kept as it is now.

3. The tables have now been changed so only 2 decimals are showed.
4. We think the presentation with quartiles as presented is the most relevant. Comparisons between lowest quartiles and the rest is not the model chosen here, though it in general generates comparable data.

5. anti-PC in competition with aOxCL is now presented as described above, and included in the result section p 7 and in Fig 2d. The results indicated that there is no important cross reactivity; further, PC is not present in CL. We have now included the mass spectrophotometry results in a novel Figure 1.

6. We cannot exclude the possibility of oxCL-protein complexes as antigens, in fact this is an interesting possibility and also a very complex issue which should be further explored! However, the main goal was to investigate if the major aCL antigen, beta2-GPI was implicated which according to our results does not seem to be the case. If oxCL binds to other proteins and an antigen is presented to which protective aOxCL antibodies bind, this could be of major interest but is in our view clearly beyond the scope of this study. To isolate IgG and IgM and test antibodies from this against oxCL could also be interesting but is also complicated and of note, our main goal is to study the totality of binding to oxCL (IgM or IgG) and not subfractions of it. Our data clearly demonstrate that this approach yields novel not previously presented information.

7. The last sentence in the abstract has now been changed as described above, to better indicate the findings.

8. We agree that the cited findings and reports are of major importance, and have now discussed this further in the Discussion section, p 11.

Minor points
1. We have now clarified this, last section page 9
2. In contrast to anti-PC being only significant as protection marker for CVD at low levels, aOxCL was also protective at high levels. I think our statement is thus clear.
3. We have now cited Bill et al, and clarified the statement that the kit we used for aCL was developed not to detect oxCL but native CL ("Specificity: The microplate is coated with highly purified Cardiolipin and human β2-Glycoprotein I. Special coating processes, developed by the manufacturer guarantee for the native immunogenic structure of Cardiolipin after immobilisation on the solid phase. The Anti-Cardiolipin test kits are specific only for autoantibodies against Cardiolipin or to the complex of Cardiolipin and β2-Glycoprotein I").
Reviewer 2

1. We have now more thoroughly described the method also for IgG-related measures, p 5. We have also clarified that competition and co-factor experiments were related to IgM. It is currently not clear which is the antigen, e.g., its detailed structure. Even though it is not in our hands, related to the important co-factor beta2GPI, other protein – lipid complexes could be implicated, if not the lipid itself is enough for antigenicity.

2. We have now changed the last sentence in the conclusion so it is made clear that we have not been able to study effects of anti-OxCL in an experimental setting. We are working to produce such antibodies, but this is beyond the scope of the present study in our opinion.

3. The issue of the exact antigenicity of OxCL is not clear, and we have now described this more clear in the Discussion section, p 10, adding the information that it is possible that protein-OxCL complexes could play a role. However, we think it is beyond the scope of this study to define the exact antigenicity of OxCL.