Reviewer’s report

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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Reviewer: Gregg J. Silverman

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The authors describe a potentially important clinical survey with cross sectional case-controlled investigation of the relationship between levels of serum antibodies to oxidized cardiolipin and cardiovascular events. The studies are a linear extension of the authors’ ongoing work with antibodies to OxLDL, and the immunodominant determinant, phosphorylcholine head group. It is not clear whether other anti-oxidized LDL antibodies, especially those to phosphorylcholine (PC) specificity, were also studied in these samples, but not described here. There are potential relationships with PC and oxLDL, as PC is likely to contaminate CL preps, and antibodies to CL could have polyreactivity or cross-reactivity to PC, but this is not adequately explored.

The authors should provide greater clarity as to the criteria for defining CV events. Were there uniform and consistent criteria for clinical presentation, and lab documentation, or solely by chart diagnosis?

While the authors take steps to ensure that their cardiolipin is fully oxidized, it has been argued that virtually all ELISA measure reactivity with cardiolipin, which is partially oxidized, by virtue of how it is manufactured, handled in the lab and coated and dried on to plates under lab conditions. Notably an earlier report by Witztum and colleagues have argued that PC, which is cited by the authors, did also state that PC can contaminate commercial CL preps (Hörkkö et al. J Clin Invest. 1996 Aug 1;98(3):815-25.).

The source/purification of LDL and method to make OxLDL should be described.

The organization of the results does not follow a clear rationale. The first paragraph describes prototypic OxCL binding studies, then the second the Thp-1 phagocytosis studies. Only thereafter are the clinical populations described. The results section does not explain how these are connected to explain the goals of the study.

Equally important, there are no data presented in this manuscript to support the last sentence in the abstract “Potential mechanisms include decreased uptake of OxLDL in macrophages.”

The studies are well performed and relevant and the cohort quite impressive; however there are still some questions and concerns that needs to be
addressed.

The statistical analyses in several places could be better explained. In the text some of the analysis is presenting as reaching significance at different percentiles (significant decreased risk for antibody levels above 86th percentile compared to 95th percentile etc). This is confusing and suggests that instead of an objective analysis method, the method was adapted for each parameter to present the desired results. As there already are other consistent significant patterns this analysis only weakens the manuscript.

For example, the meaning of the following paragraph is unclear. What does “trendwise” mean, especially without showing the P value?

“There were no significant associations between IgM anti-CL and CVD in our study (data not shown). However, trendwise, very high anti-CL levels were associated with increased risk of CVD among men, and above highest 98th percentile OR was 2.37 for women, (non- significant).”

2. To easier be able to follow the statistical analysis, N should be stated in all different analysis. For example in the quartiles presented in table 2a-c 3a-c the number of patients with cardiovascular events in each quartiles should be stated.

3. For P-values, presenting only 2 numbers after decimal sign would make the tables easier to read.

4. In the Table 2a-c and Table 3a-c, adding a footnote explaining that the OR and the P-values for each quartile is compared to quartile 4 would make the tables easier to follow. Also a clearer motivation in the text why this method of analysis was chosen would be beneficial. Was the analysis not significant if the lowest antibody quartile was compared to quartile 2-4?

5. The authors have recently presented similar reports regarding the protective properties of IgM anti-PC. When testing the specificity in competition studies, reactivity to PC should be included. Although oxidized cardiolipin does not have the PC epitope the cardiolipin lot could be contaminated (as it is extracted from bovine heart containing PC and only 97% pure), alternatively IgM antibodies recognizing PC may be polyreactive and also bind ox-cardiolipin. Although the later explanation is unlikely it should still be proven if possible. Similarly it would be valuable to include the results of the mass spectrometry analysis of the oxidized cardiolipin that according to the methods section was performed.

6. Even though the authors have made an effort to exclude that the detected antibody reactitivites are not influenced by beta2-GPI there is still a possibility that the antibodies recognize a protein-ox-CL complex as serum proteins are present in all assays. Hence all assays to which sera samples are added inherently include b2 gpl (and other serum factors). Therefore, the IgG and IgM must first be purified from sera and then tested for reactivity to ox-CL, which is essential to more clearly exclude the possibility of protein-ox-CL reactivity.

7. The studies of ox-CL on the uptake of Ox-LDL by macrophages are interesting but the experiment does show IgM anti-oxCL and IgG anti-oxCL.
8. The authors correctly mention in the discussion that ox-CL is generated when cells undergo apoptosis. It has also previously been shown that murine monoclonal IgM recognizing ox-CL can discriminate apoptotic cells from healthy cells (Tuominen et al Arteroscle. Thromb. Vascl. Biol. 2006). As there is a growing literature that natural IgM binding to apoptotic cells can increase apoptotic cell phagocytosis and also have direct anti-inflammatory properties (reviewed in Grönwall et al. Front. Immunol. 2012). The authors should therefore be strongly encouraged to include in the discussion how IgM binding to apoptotic cells, through PC or other apoptosis associated determinants such as oxCL, may influence the pathogenesis of atherosclerosis and cardiovascular disease.

Minor points.

1. Clarity could be improved in the last clause of this section
We have reported that antibodies to PC (anti-PC) are negatively associated with CVD and atherosclerosis, with the most striking observations at low levels, which are associated to increased risk of CVD 5-8, 21 while high levels are associated with a favourable development of atherosclerosis22.

2. The authors above describe their work that IgM anti-PC antibodies correlate with protection from CV events. Then, why do they also state the following? 
“In contrast to anti-PC, anti-OxCL was a protective factor for CVD at high levels, since high levels were associated with a 50% decreased risk.”

3. On what basis do the authors assert in the discussion that “The commercial kit used herein for determination of antibodies against CL does not detect oxCL.” The authors should also cite Bill et al. Circulation. 2000 Sep 12;102(11):1258-63.

**Quality of written English:** Needs some language corrections before being published

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

**Declaration of competing interests:**

Our lab has research support on a related but not directly competing topic. No other potential conflicts