Author's response to reviews

Title: Reduced IgM Levels and Elevated IgG Levels against Oxidized Low-Density Lipoproteins in HIV-1 Infection

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Author's response to reviews: see over
Dear Editor,

We would like thank you and the reviewers for very constructive comments. You have read the manuscript thoroughly. We have tried to comply with the raised issues. In addition to the changes specified below, we have corrected typos and improved the English language throughout the manuscript. We have particularly worked on making the Result section more fluent and on focusing the Discussion on the findings of our study. We believe that our changes have improved the manuscript substantially and hope that you now will find it suitable for publication in BMC Infectious Diseases.

Reviewer 1

1. “The study lacks significant novelty since the levels of oxLDL antibodies have previously been determined in HIV infection. In addition the findings that these antibodies are higher in HIV patients are not unexpected since HIV patients have hypergammaglobulinemia and polyclonal B cell activation and as the authors mention “there is a higher activity of the humoral immune system in these individuals” So in theory any different type of antibodies that are measured in HIV patients are higher than negative controls. They just confirm an association of HIV with inflammation and higher levels of certain antibodies and the findings are not well linked to the research question how this is pertinent to the pathogenesis of HIV atherosclerosis. Also B cell defects in HIV patients may explain low IgM levels of certain antibodies. An important analysis would be to normalize the measured oxLDL IgG levels with the total IgG levels and similarly the IgM oxLDL with the total IgM levels and redo the analysis”

Answer: We have performed calculations of ratios of specific Igs/total Igs and the analyses have been included in the Results section on page 8, 1st paragraph, and revised Figure 1. Briefly, the results showed a persisting increase in OxLDL IgG and a more pronounced decrease in MDA-LDL IgM when normalized to total IgG and IgM, respectively.

2. “It would be more interesting if they also measured actual circulating levels of oxLDL and looked at certain measurements of atherosclerosis e.g CIMT etc. The story is incomplete and the manuscript gives the impression of a constellation of measurement and associations in a small study with suboptimal design due to the limited power. There are no power analysis calculations in the methods section. The authors mention that “the size of this study is relatively small, leading to a limited power to detect some associations between biomarkers, but the study subjects are well characterized and we have a well-matched HIV control group, strengthening our findings of increased IgG levels in HIV+ individuals” This is not adequate explanation. The negative findings were most likely related to the small numbers. The study design is suboptimal to address this research question.”

Answer: The size of the study was based on a power estimation requiring 60 individuals in each group (80% power, level of significance 0.05) in order to detect a 30% change in the main variable, IgM directed against modified LDL. The major aim was not to study relationships with other variables, although these post hoc analyses were performed in order to try to explain the obtained findings. We have included the sample size as a study limitation in the Discussion, page 13, last paragraph. In regards to the requested analysis of OxLDL, we believe that performing this analysis on frozen samples that at this stage have been subject to
freeze-thaw cycles, is associated with major disadvantages such as in vitro oxidation. We have therefore decided not to perform the suggested analysis in this study.

3. “The rationale why certain measurements were performed is not explained adequately. “We measured ApoB and ApoA1 as potential markers of cardiovascular risk” is not an adequate explanation why this is important for this study since these have been measured previously in other studies? What was the hypothesis and how is this related to the main research question (levels of oxLDL antibodies)?”

Answer: The analyses of cardiovascular risk markers were assessed because anti-OxLDL antibodies have shown to be risk factors for cardiovascular disease. The relationship with cardiovascular disease has not been shown to be independent, why we considered the possibility of common measurable cardiovascular risk factors as determinants of the antibody levels. We have tried to clarify the rationale for the chosen analyses in the Results section, page 9, 2nd paragraph.

4. “Similarly “Individuals with HIV infection have been proposed to have increased microbial translocation, and we therefore analyzed sCD14 and the inflammation marker proposed as a Cardiovascular risk marker, CRP” is not an adequate explanation. How is this related to the main hypothesis and research question? This seems another irrelevant measurement that needs to be justified accordingly. IF the authors were interested in microbial translocation why did they measure only sCD14 (and not LPS)? In addition how do they explain the inverse correlation between circulating levels of sCD14 and total IgG and IgM, and the absence of relationship between sCD14 and specific IgG or IgM directed against OxLDL or MDA-LDL”?

Answer: Also the rationale for these assessments has been clarified in the Results section, page 9, last paragraph. The somewhat surprising finding of inverse correlations between sCD14 and total IgG and IgM have been included in the Discussion, page 12, end of 2nd paragraph.

5. “Both serum and plasma have been used in this study. What is the evidence that the data (oxLDL antibodies, levels of oxLDL) between the different matrices are comparable? IF this has not been previously published or studied the authors need to show pilot data that the data are comparable between plasma and sera.”

Answer: Before embarking this study we confirmed that measurements in frozen serum and frozen plasma were comparable with a strong relationship and no significant bias through parallel analyses of serum and plasma samples from 30 healthy individuals. We have included results from these analyses in the Methods section, page 7, 1st paragraph and the results are shown as graphs in the end of this document (Figure Answers). In addition, we have included details on the quality control of the analysis in the same paragraph in the Methods section. If requested, we can include the graphs in Figure Answers as a separate figure in the manuscript or as a supplementary Figure.

6. “Similarly both fresh and stored samples were used in this study. Cryopreservation can significantly affect levels of oxidized lipids including oxLDL. How are the results (oxLDL antibodies, levels of oxLDL) comparable between fresh and frozen samples?”
Answer: All analyses that were performed on both study groups were performed on frozen samples. We realize that the text in the Results section was unclear and may have resulted in misinterpretations. We have therefore moved the information on whether the analysis was performed on fresh or thawed samples from the Results section to the Methods section, pages 6 (last paragraph) and 7 (end of first paragraph, beginning and middle of 2nd paragraph).

7. “Correct typos throughout the manuscript e.g OLDL”

Answer: We apologize for text errors and have corrected the English language including typos throughout the manuscript.

8. “blood lipids were analyzed” Which lipids and how were they measured?”

Answer: Blood lipids have in the Methods section, page 6, end of 1st paragraph been changed for serum cholesterol, HDL-cholesterol and triglycerides. We have also changed the word lipids for cholesterol and triglycerides in the paragraph title in the Results section page 10, 2nd paragraph.

Reviewer 2

1. “The study reports results of IgG and IgM antibodies anti-oxidized LDL in HIV + patients with or without ARVT and in controls (HIV-). The authors found slightly lower IgM levels in HIV+, whereas higher IgG levels in this group of patients. They discuss that autoantibodies against oxidized LDL of IgM type are potentially atheroprotective, and that higher levels of IgG to different forms of oxidized LDL may have a dual role, considering immune-complex formation and the presence of immune complexes in the atherosclerotic plaque. On the other hand, it has been reported by other groups, including ours (Fonseca HA, et al, Cell Biochem Biophys. 2013 Dec;67(3):1451-60; Izar MC, et al, Diab Vasc Dis Res. 2013 Jan;10(1):32-9; da Fonseca HA, et al, Int J Cardiol. 2012 May 17;157(1):131-3; Brandão SA, et al, Am J Hypertens. 2010 Feb;23(2):208-14; and Santos AO, et al, Clin Chim Acta. 2009 Aug;406(1-2):113-8), that high titers of anti-OxLDL of IgG type may be related to less oxidized LDL being formed, due to less stimuli for lipid oxidation. They concluded that reduced IgM levels in combination with elevated IgG levels against oxidized forms of LDL in HIV+ individuals may reflect an increased risk of atherosclerotic cardiovascular disease. Subclinical or clinical atherosclerosis has not been evaluated in this study, therefore, the authors should avoid this commentary, which is an extrapolation and not a conclusion from the data.”

Answer: We thank the reviewer for these constructive comments and have modified the conclusion in the Abstract accordingly. We have also modified the text in the Discussion, page 11, last paragraph in order to better reflect the complexity of anti-OxLDL antibody levels.

2. “In addition, the authors have divided the HIV patients in four small groups, what may have reduced the power of the study, considering the variability of laboratory assays, and this should be reported as a study limitation.”

Answer: Although we see the need for dividing the very heterogeneous group of HIV+ individuals, we agree with the reviewer and have included this as a study limitation in the Discussion, page 13, last paragraph.
3. “The ART therapy, and the use of lipid-lowering agents should also impact the results, but the latter was not reported so far. LLT modify LDL oxidation and the antibody responses. As HIV treated patients develop dyslipidemia, the use of LLT should be reported.”

**Answer:** Lipid-lowering treatment in the form of pravastatin was used by three individuals in this study; two in the PI-based regimen group and one in the NNRTI group. Thus, it is less likely that lipid-lowering treatment had any major impact on the results obtained. We have included information on the use of lipid-lowering agents in the Method section, page 6, 1st paragraph. Information on lipid-lowering treatment is not available for the blood donors used as control group.

4. “Another issue is related to dosages being performed in sera or plasma. Are the results of serum samples or plasma samples equivalent, or they need a correction factor?”

**Answer:** As stated in the answer to reviewer 1, we confirmed that measurements in frozen serum and frozen plasma were comparable with a strong relationship and no significant bias through parallel analyses of serum and plasma samples from 30 healthy individuals before performing the analyses in this manuscript. We have included results from these analyses in the Methods section, page 7, 1st paragraph and the results are shown below as graphs (Figure Answers). In addition, we have included details on the quality control of the analysis in the same paragraph in the Methods section. If requested, we can include the graphs in Figure Answers as a separate figure in the manuscript or as a supplementary Figure.

Best regards
Aylin Yilmaz
**Figure Answers.** Parallel analyses of plasma and serum samples from 30 healthy individuals showed no significant bias between results obtained in serum (y) versus plasma (x): MDA-LDL IgG: y = 0.98x + 0.77, R² = 0.96, OxLDL IgG: y = 1.02x – 0.34, R² = 0.95, MDA-LDL IgM: y = 1.16x – 1.86, R² = 0.98, OxLDL IgM: y = 1.10x – 0.22, R² = 0.97. Thus, serum and plasma samples were analyzed together without factorization.