Author’s response to reviews

Title: Atherosclerosis, inflammation and lipoprotein glomerulopathy in kidneys of apoE-/-/LDL-/- double knockout mice

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Version: 2 Date: 19 May 2010

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

This is a re-submission of our revised manuscript entitled “Atherosclerosis, Inflammation and Lipoprotein Glomerulopathy in Kidneys of ApoE-/-/LDL-/- double knockout mice”.

The authors thank you and the reviewers for the comments and recommendations. We answered concerns on a point-by-point basis and have indicated all changes made in the manuscript.

We hope that the present version of the manuscript is now acceptable for publication in your journal.

Yours sincerely,

Alexander C. Langheinrich

Reviewer's report
Atherosclerosis, Inflammation and Lipoprotein Glomerulopathy in Kidneys of ApoE-/-/LDL-/- double knockout mice

Title: Atherosclerosis, Inflammation and Lipoprotein Glomerulopathy in Kidneys of ApoE-/-/LDL-/- double knockout mice

Version: 1 Date: 5 April 2010

Reviewer: Georgette M Buga

Reviewer's report:

Ms #4349194973581801:

The authors propose to test the potential for quantitative imaging the renal vasculature as a marker of kidney function in a mouse model of advanced atherosclerosis using high resolution micro-CT and histology to correlate plaque formation and inflammation with alterations in kidney vasculature and morphology.

Major Compulsory Revisions are listed page by page:

1. Page 6:
Quantification of vasa vasorum in the two groups of mice would make a more compelling argument for the proposed hypothesis that histopathological changes occur in the experimental group. Please include in the resubmission.

AUTHORS: The authors agree that VV neovascularization of the renal artery is of great interest and might enhance our hypothesis. Indeed, VV neovascularization has been described in detail in different vascular beds in our mouse model of atherosclerosis (Langheinrich AC et al. ATVB 2006; Atherosclerosis 2007, Invest Radiol 2008). Our findings of VV neovascularization in the adventitia of the renal artery support our previous findings concerning neovascularization of the aorta and branching neck arteries. Moreover, VV were not present in the renal artery in C57/BL mice. The present study was not designed to quantitate VV neovascularization of the renal artery.

2. To support the authors claims that inflammatory cells are present in the renal artery and in the kidney, data is required showing inflammation. It is not clear that specific cell markers were used to identify the two types of inflammatory cells mentioned in the manuscript? I strongly recommend using the methods described in the reference 12 (Ishimura et al., 2009).

AUTHORS: Inflammatory cells in the kidney were detected at light microscopy of hematoxylin and eosin stained slides. Lymphocytes and plasma cells could be clearly detected without any additional immunostaining. It was beyond the scope of the manuscript to evaluate the inflammatory cells in detail.

3. Page 7-First paragraph:
Although Atherosclerosis and Lipoprotein Glomerulopathy (part of the manuscript title) represent important factors contributing to the vascular modifications described in this manuscript, there is no experimental evidence that these mice have increased/ altered serum lipoproteins. Total cholesterol, LDL-C, HDL-C (which is markedly decreased in apoE null mice and contributes to the pro-oxidant environment in these mice) and triglycerides, are important parameters that need to be presented in the manuscript. It would give a more clear indication of the mechanisms involved in the vascular modifications described.
AUTHORS: Cholesterol, triglycerides, LDL, HDL levels have been described in detail in the apoE-LDL double knockout mouse. Examples of such publications are:


We use the same commercial available mouse model as described by several publications, therefore, re-measurements of serum lipoproteins will not contribute to the mechanism(s) involved in the vascular modifications as described in our manuscript.

4. It is surprising that serum creatinine levels were not different from control mice as indicated at the end of the first paragraph on page 7 that refers to Table 1, although Table 1 does not have any entry on creatinine. Please include it or modify the text.

AUTHORS: The serum creatinine levels have been deleted in the revised version of the manuscript as suggested by the reviewer.

5. Also, it is highly possible that these mice are diabetic. Fasting blood glucose levels should be measured as well to be able to include or exclude Type 2 diabetes mellitus as an additional risk factor contributing to the vascular and renal pathological changes in the experimental mice.

AUTHORS: Our findings of reno-vascular alterations and glomerulopathy in this mouse model of atherosclerosis might be affected by additional risk-factors such as diabetes. Up to now, there is no evidence that our mice are diabetic. Diabetes is a well known risk factor contributing to atherogenesis. The authors agree that there is a possibility that our mice are diabetic. Our well-established mouse model of atherosclerosis is based on lipoprotein disturbance. Additional investigations concerning metabolic disturbancies are the focus of ongoing studies.

6. Discussion Section Page 7:
It is not clear how the lipoproteins were identified in the renal vasculature described as "factor 8 containing lipoprotein embolys", factor 8/vWF being an endothelial not a lipid marker? Oil Red O or Sudan III for lipids need to be used. I strongly recommend using the methods described in the reference 12 (Ishimura et al., 2009).

AUTHORS: Factor 8 related antigen is certainly an antigen present in/ on endothelial cells. Beyond it is well known that immunostaining allows detection of Factor 8 in the cytoplasms of megakaryocytes and it allows the detection in platelets as well as Factor 8 protein within the blood plasma. Thus factor 8 immunopositivity of emboli within the renal vasculature was interpreted as a marker of plasma and thrombocyte aggregates. From the literature we drew the conclusion that the emboli will probably contain lipoproteins. Thus, we changed the term "factor 8 containing lipoprotein emboli" to "factor 8 containing emboli".

7. The second paragraph contains the references that were missing in the Background section on pages 4-5. Please either include them in that section or
modify the information in Discussion, to have the references listed appropriately.

AUTHORS: Please specify the references that should be listed appropriately.

8. Figure Legends:
Fig. 2:
Usually magnification results from multiplying the Ocular magnification (10x) with the Objective magnification (5, 10, 20 or 40x). Please indicate what is the total magnification (50x?) in each of the two images which appear to be of different magnification.
In addition, Fig 2 B exhibits the cortico-medular junction, where the glomerular size is usually increased, whereas Fig 2A represents the cortical area of the kidney. Please include similar areas of the kidney to be shown at the same magnification for both experimental groups.
Alternatively, sections from both cortical and medular areas could be compared between the groups.
AUTHORS: The total magnification in figure 2 has been implemented as suggested by the reviewer. We added additional images (Figure 5) showing the cortex and the cortico-medullar junction.

9. Please indicate the type of inflammatory cells shown, the staining method used and magnification.
Fig. 3B:
Fig 3 B appears to be of a different magnification than the others. Please select images from the same area and of the same magnification.
AUTHORS: Inflammatory cells in the kidney were detected at light microscopy of hematoxylin and eosin stained slides. Lymphocytes and plasma cells could be clearly detected without any additional immunostaining. We added the used staining and magnification in Figure 3 as suggested by the reviewer.

10. Figures 5-8:
Redundant. They duplicate the information presented in the Table 1. I suggest eliminating them.
AUTHORS: Figures 5-8 have been deleted in the revised version of the manuscript as suggested by the reviewer.

Minor Essential Revisions:
11. Page 4:
Please indicate the type of diet used and the age of controls.
AUTHORS: We used a standard chow diet and used age-matched controls. This has been implemented in the revised version of the manuscript (Methods, experimental design).

12. References 15-18 are missing and appear only on Page 7 in the 2nd paragraph of the Discussion Reference List and Methods section need to be coordinated.
AUTHORS: The references are now implemented in the revised version of the manuscript.

13. Page 5, paragraph 2 in Histology Section
The Immuno Histo Chemistry portion is not clear. Please explain: (a) the reason for using factor VIII/vWF antibody which is a marker for endothelial cells and for not using any markers for the inflammatory cells present in the atheroscerotic
plaques of the renal artery and in the periglomerular areas? (b) the reason that
the link antibody and APAAP were used in the absence of a mouse monoclonal
antibody or a dual staining. Were there any immune complexes that needed to
be identified? Please elaborate and include in this section.

AUTHORS: a) Please refer to question # 2 & 7. b) the link antibody and APAAP
method served as a negative control for the specificity of the immunoreactivity of
factor VIII/vWF staining. Dual staining was not performed.

14. Page 8, Paragraph 3:
Regarding the use of V V or vasa vasorum : It is also important to maintain
consistency in the use of abbreviations throughout the text.
Additional evidence that hypertension is probably present in male mice used in
this study is provided by (Trieu, V. N., and F. M. Uckun. 1998. Male-associated
Jun 18;247(2):277-9)

AUTHORS: Thank you. We changed the wording to consistently to „Vasa
vasorum”

15. Page 8 paragraph 4:

AUTHORS: The reference (12) has been implemented to clarify the relationship.

16. Page 8 paragraph 4:
Diabetes and advanced age should be considered as risk factors for LPG in
these mice.

AUTHORS: Thank you. Diabetes and age has been implemented in the revised
version of the manuscript (page 8, last para).

17. Are micro-CT features different in younger double ko mice versus the 80 day
olds?

AUTHORS: The present study was not designed to investigate the time-course
of renal alterations in this mouse model. We agree that this might be of great
interest to characterize the renal alterations over the time.

18. Page 11:
References 15-18 are not in the same order as in text. Please revise.

AUTHORS: The reference list has been changed as suggested by the reviewer.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.
Reviewer’s report
Title: Atherosclerosis, Inflammation and Lipoprotein Glomerulopathy in Kidneys of ApoE-/-/LDL-/- double knockout mice
Version: 1 Date: 7 April 2010
Reviewer: Antti Saraste

Reviewer’s report:
This study describes renal artery atherosclerosis (histology), renal vascular volume (micro-CT vascular imaging) and kidney pathology (histology) in aged atherosclerotic double knockout (ApoE-/- and LDLR-/-) mice. The results show atherosclerotic lesions in the renal artery, reduced total intrarenal vascular volume, perivascular inflammation, and glomerular changes consistent with lipoprotein disease/emboli in the atherosclerotic model. The results are interesting in providing careful description of kidney disease in this particular animal model.

- Major Compulsory Revisions
1) The manuscript introduces quantitative micro-CT imaging. I assume that the vascular volume consists mainly of venae. The manuscript would be improved by showing data on tracking/segmentation of the arteries and venae separately. Otherwise, this needs to be discussed.

AUTHORS: Thank you. In the revised version of the manuscript, segmentation of the intra-renal arterial and venous vasculature has been performed. The results have been implemented in the revised version of the manuscript (Table 1) and the figure # 4 has been changed.

2) The conclusion is “The reduced intra-renal total vascular volume is associated with renal inflammation, suggesting a direct relationship between atherosclerosis, inflammation and glomeruli alterations.” The manuscript would be strengthened by data correlating imaging data with quantitative data on inflammation, glomerular disease, and severity of atherosclerosis (renal artery or aorta). If no correlative data is shown, the discussion and conclusions should be reformulated accordingly.

AUTHORS: Thank you. Although we did not measure the severity of atherosclerosis and inflammation in the renal artery and within the kidney, we can demonstrate that those affected vessels are only present in apoE-LDL double knockout mice. Moreover, we quantitated the total amount of affected glomeruli in this mouse model of atherosclerosis and we already described atherogenesis in the aorta in the same mouse model. Up to now, we can not definitely clarify absolutely the relationship of atherosclerosis and renal alterations in this mouse model. Therefore, we re-worded the conclusion.

3) The presence/significance of superficial “plaque rupture” and superficial “intraplaque haemorrhage” in mouse vs. man remains a controversial issue. This needs to be discussed.

AUTHORS: Thank you. Indeed, plaque rupture and intraplaque hemorrhage are major complications in the life-time of an atherosclerotic plaque. Intraplaque hemorrhage has been described in this mouse model of advanced atherosclerosis in detail (Langheinrich AC et al. Invest Radiol 2007). Moreover, as demonstrated in figure 1, we found plaque rupture with concomitant
erythrocytes as shown in figure 1C. These major complications in advanced atherosclerotic lesions are similar to those found in humans and represent a critical event of vulnerable plaques. Those findings have been reported in the aorta in the apoE-LDL mouse model (Langheinrich AC et al. Invest Radiol 2007, Atherosclerosis 2007).

4) The authors found vasa vasorum in the renal arteries of hypercholesterolemic mice, but these were not quantified. Were there any vasa vasorum in control animals?

AUTHORS: Thank you. As described (Langheinrich AC et al. ATVB 2006), Vasa Vasorum are not present in C57 mice. We quantitated the total volume of VV in different vascular beds and demonstrated a relationship to different atherosclerotic lesion types (Langheinrich AC et al. Atherosclerosis 2007).

5) Methods: what kind of diet did the mouse have?

AUTHORS: We implemented the used mouse diet in the revised version of the manuscript.

Discretionary Revisions
1) Were there anatomically severe stenoses in renal arteries?

AUTHORS: ApoE-LDL double knockout mice demonstrate concentric, advanced atherosclerotic lesions in the renal artery as demonstrated in Figure 1. We did not measure pressure gradients over the renal artery. Therefore, we can not definitely clarify the severity of the renal atherosclerotic lesions.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests