EMBO Molecular Medicine

Gut Microbiota Influences Pathological Angiogenesis in Obesity-driven Choroidal Neovascularization

Elisabeth Andriessen, Ariel Wilson, Miss Gaelle Mawambo, Agnieszka Dejda, Khalil Miloudi, Florian Sennlaub, and Przemyslaw Sapieha

Corresponding author: Przemyslaw Sapieha, University of Montreal

Review timeline:

Submission date:	28 April 2016
Editorial Decision:	06 July 2016
Revision received:	21 September 2016
Editorial Decision:	05 October 2016
Revision received:	06 October 2016
Accepted:	11 October 2016

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Céline Carret

1st Editorial Decision 06 July 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. Although the referees find the study to be of potential interest, they also raise a number of concerns that need to be addressed in the next final version of your article.

You will see that the referees find the study to be of interest, but regret poor citing and discussing of previous published work and ref3 suggestions should be taken into account. Additional experiments would be needed to add more data on mechanisms and thereby increase the conclusiveness of the findings. Finally, we would like to suggest changing the format from "Report" to "Article" in order to better reflect the current knowledge in the literature.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the space and time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible. Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Remarks):

Andriessen et al. have analyzed the relation of obesity, gut microbiota and the development of laser-induced choroidal neovascularization, an established model mimicking features of age-related macular degeneration. The study is principally of high interest as a direct connection of all these three aspects has not been demonstrated before despite publications of several human epidemiological data linking overweight with late AMD progression. The paper is principally well written but lacks precision in wording of introduction and conclusion. Several major issues should be addressed to support the authors conclusions and to make the data more solid regarding the molecular and cellular mechansisms.

Points of criticism:

1. Title: The title states that obesity exacerbates AMD by altering gut microbiota. The wording must be used more carefully as the authors did not perform a study in AMD patients (as implicated by the title) but analyzed the laser-CNV model. This model is indeed a relatively good model for nvAMD but can only reflect some issues related to immunity and angiogenesis.

2. Wrong and missing references in the introduction/discussion. I was surprised when I noticed that the authors used several citations that did not reflect the meaning of the written statements properly. Ref #2 is a paper on diabetic retinopathy and not related to AMD, ref #13 does not list increased systemic cytokine levels in AMD, ref #17 is on xenobiotic metabolism and does not discuss inflammation. The following important references have not been included in the introduction or in the discussion: 1. the newest genetic paper on AMD risk genes is missing (Fritsche LG et al Nat Genet 2016), 2. Zhang et al. IOVS 2016 57:1276 and 3. Maralani et al. Retina 2015 35:459 provide comprehensive meta-analyses of large prospective studies relating obesity/metabolic syndrome with AMD and should be included. 4. Horai et al. Immunity 2015 reports on microbiota-dependent immune activation in the retina, this is directly related to the topic presented here. 5. An ARVO abstract from 2007 annual meeting (Vol 48, 1768) also found that high fat diet increased leaky lesions and CNV size in the laser model.

3. In most figure with mean value bar graphs: Individual data points (either mean values per eye or individual leasons) should be plotted. This is important as the reader can directly estimate n-numbers and variation in the data set.

4. How was CNV size determined in Figs 1D, 3E, per eye ? per lesion ? The materials/methods section is not detailed enough here.

5. Vascular leakage data are usually determined to complement flat mount stainings of IB4/FITC. Sometimes inflammation-driven leakage is not identical with neovessels.

6. Fig 2A/B would significantly profit from a more sophisticated analysis of mononuclear phagocyte reactivity (microglia vs macrophages) and if possible complete retinal sections to demonstrate the location of cells in the inner vs outer retina.

7. It is left open how the changed microbiota may contribute to the different aspects of disease progression in this model. Given the recent findings from Erny et a. Nat Neuroscience 18:965 and Horai et al. 2015 Immunity one could hypothesize that resident retinal microglia have been primed by the changed gut microbes before the laser CNV model was applied. In this sense, it would be good if the authors could expand their cytokine expression analysis in serum (ELISA) and choroid (RNA) to isolated (FACS) immune cells and immune marker expression profiling of retina vs choroid/RPE. An additional time point where inflammation is maximum (e.g. at days 3 or 7) would be better suited than day14 where most immune events have already calmed down.

Referee #2 (Comments on Novelty/Model System):

While laser induced CNV is often questioned as a model for CNV in AMD it is certainly the most widely used one.

Referee #2 (Remarks):

The manuscript raises very interesting issues about multifactorial diseases like AMD. I found the paper straight forward and very well argues. I do not find any issues to be raised.

Referee #3 (Remarks):

The authors examine the role of microbiota in influencing CNV (not AMD) in a high fat diet model. There is a vast body of literature other than the body mass indices that implicates abnormalities in lipid homeostasis in AMD. Multiple GWAS have demonstrated polymorphisms in numerous genes involved in regulation of cholesterol homeostasis. The clear message from these studies is that the polymorphisms are suggestive of a complex interaction at a tissue level.
The authors demonstrate that there is increased CNV after a high fat diet in the laser CVN model and state that this is the first demonstration of this. There is a highly cited study in Cell Metabolism from 2013 that shows that in diet induced obesity there is increased CNV. This was a seminal paper in the field and as such these data are not novel. The authors should conduct a careful reading of the literature so that appropriate credit can be given to prior literature.
RD is a confusing term for regular chow as it is too similar to conditions of the retina such as retinal detachments and retinal degenerations that are also called RD.
A 35% reduction in CNV in a mouse laser induced CNV model with high variability is unlikely to be clinically relevant. The molecular mechanisms behind this effect are not elucidated in this study and given that the finding that mice on high fat diets are prone to increased CNV is not a novel finding, it would be interesting to see at a molecular level what the effects are on lipid composition and how it influences CNV.

1st Revision - authors' response 21 September 2016

Detailed response to reviewers
Reviewer #1:
We thank the reviewer for their thoughtful comments and positive assessment of the study and thank them for acknowledging that "this study is principally of high interest." Based on the recommendations and queries of the reviewer, we have performed a series of new experiments. Most notably, in the revised manuscript, we now provide a new mechanism of action for diete-induced para-inflammation by demonstrating elevated gut permeability in mice receiving high fat diets. We demonstrate that this heightened permeability can be rescued with microbiotal transplants. We also now profile infiltration of mononuclear phagocytes and microglia in different dietary paradigms by FACS.

Query 1. *Title: The title states that obesity exacerbates AMD by altering gut microbiota. The wording must be used more carefully as the authors did not perform a study in AMD patients (as implicated by the title) but analyzed the laser-CNV model. This model is indeed a relatively good model for nvAMD but can only reflect some issues related to immunity and angiogenesis.*
Response- We thank the reviewer for bringing-up this important point. We have now modified the title to: Gut Microbiota Influences Pathological Angiogenesis in Obesity-driven Choroidal Neovascularization
Query 2. *Wrong and missing references in the introduction/discussion.*
Response – We are grateful to the reviewer for bringing these studies to our attention and we have incorporated all references suggested by the reviewer and more into our revised manuscript. We also modified the incorrect ones.
Query 3. *In most figure with mean value bar graphs: Individual data points (either mean values per eye or individual leasons) should be plotted. This is important as the reader can directly estimate n-numbers and variation in the data set.*

Response- We fully agree with the reviewer and have proceed to modify all graphs to scatter plots in order better visualize the spread of data and number of data points.

Query 4. *How was CNV size determined in Figs 1D, 3E, per eye ? per lesion ? The materials/methods section is not detailed enough here.*
Response – We agree and have added additional information on the methodology in the 'Methods' section of the paper: The neovascularization was captured in a Z-Stack, and the lesion caused by the laser impact was captured in a single plane image. The Z-stacks were compressed into one image and the FITC-dextran labeled neovascular area and the area of the lesion were measured per lesion in Image-J.
Query 5. *Fig 2A/B would significantly profit from a more sophisticated analysis of mononuclear phagocyte reactivity (microglia vs macrophages).*
Response- We agree with the reviewer and we conducted an extensive characterization of infiltrating immune cells by FACS in our dietary paradigms. The new data is presented in the revised Figure 2.

Query 6. … *it would be good if the authors could expand their cytokine expression analysis in serum (ELISA) and choroid (RNA) to isolated (FACS) immune cells and immune marker expression profiling of retina vs choroid/RPE. An additional time point where inflammation is maximum (e.g. at days 3 or 7) would be better suited than day14 where most immune events have already calmed down.*
Response- We thank the reviewer for this suggestion. We addressed both points in the revised manuscript. First, we profiled by FACS infiltration of MPs and Microglia at 3, 7 and 14 days as suggested (New Figure 2). We also profiled inflammatory genes in the choroids of mice with different dietary paradigms (New Figure 2) and after microbiotal transfer (New Figure 4).

Reviewer #2
We thank the reviewer for their very positive evaluation and appraisal of the study and for acknowledging that: "The manuscript raises very interesting issues about multifactorial diseases like AMD ». The reviewer « did not find any issues to be raised.»

Reviewer #3
We thank the reviewer for their insightful, very helpful and pertinent comments. We believe that the reviewer's suggestion to further explore a mechanism was significantly beneficial for the current study. We were planning on keeping portions of this mechanism for a follow-up article but agree that it strengthens the current study. We now provide evidence that the gut microbial dysbiosis caused by high-fat diets heightens intestinal permeability leading to increased circulating pathogen associated molecular patterns PAMPs, and a inflammatory response through pattern recognition receptors.

Query 1. *There is a highly cited study in Cell Metabolism from 2013 that shows that in diet induced obesity there is increased CNV. This was a seminal paper in the field and as such these data are not novel. The authors should conduct a careful reading of the literature so that appropriate credit can be given to prior literature.*
Response – We agree with the reviewer and now cite the seminal and highly cited Cell Metabolism paper from the RJ Apte group. We also now reference additional recent studies that highlight obesity as a predisposing factor to late AMD such as Maralani et al. (Retina 2015 35:459) and Zhang et al. (IOVS 2016 57:1276).
Query 2. *RD is a confusing term for regular chow as it is too similar to conditions of the retina such as retinal detachments and retinal degenerations that are also called RD.*
Response – We thank the reviewer for bringing this point to our attention. We have now carefully included text explaining that RD is short for Regular Feed in all legends and part of the text.
Query 3. *A 35% reduction in CNV in a mouse laser induced CNV model with high variability is unlikely to be clinically relevant.*
Response – Thank you for the comment. If this study were testing a drug or treatment paradigm, then the reviewer is absolutely correct. However, obesity is condition that will affect para-inflammation in a very protracted manner. Hence even smaller effects may have compounded outcomes in a disease with long-term outcomes such as AMD.

Query 4. *The molecular mechanisms behind this effect are not elucidated in this study.*
Response: We agree with the reviewer. We now provide evidence that the gut microbial dysbiosis caused by high-fat diets results in heightened intestinal permeability which increases circulating pathogen associated molecular patterns (PAMPs), leading to low-grade endotoxemia that triggers an inflammatory response through pattern recognition receptors (PRRs). Ultimately, this exacerbates choroidal neovascularization. We provide evidence for this mechanism in figure 3 and a rescue mechanism with microbiotal transfer in figure 4.

2nd Editorial Decision 05 October 2016

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee who was asked to re-assess it. As you will see this reviewer is now fully supportive and I am pleased to inform you that we will be able to accept your manuscript pending final editorial amendments.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The techniques including the laser CNV-model and the microbiota analyses are state of the art.

Referee #1 (Remarks):

The authors have taken the comments of the reviewers very seriously and addressed all points appropriately. I think that they did a very good job by performing additional experiments to further characterize the relevant immune cell populations by stainings and FACS assays. The flow of the paper is now also much better as key references have been included and the methods are given in greater detail. Overall, this is a highly interesting study that clearly shows the direct influence of gut microbiota on angiogenesis in the posterior eye.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sapieha Przemyslaw
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2016-06531

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The effect sizes of the respective methods we experienced from pilot experiments or earlier publications. We maintained a minimum of n=3 for all experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See 1a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Mice were weighed weekly and their general health was monitored. Mice that were not in good health(weight loss or or other measurese of morbidity) were excluded. If mice developed any complications during or post laser burn such as (sub)retinal bleed or cataract, they were excluded. RNA quality was analyzed before qPCR, and meltcurves were analyzed after qPCR. If the samples did not meet criteria of standard PCR method, they were excluded. For experiments with the RAW-Blue cell line, positive and negative controls were used. I either one of the controls was abnormal, the assay was excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization was used.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Confocal pictures of the choroidal burns were saved using a randomly assigned number to each mouse unknown to the evaluator. This allowed for a blinding of the investigator for picture analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Evaluation of extent of choroidal neovascularization was performed in a blinded manner where the investigator scoring the burns was blinded to the treatement.
5. For every figure, are statistical tests justified as appropriate?	Appropriate statistical tests are included in the manuscript.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis was performed using GraphPad software.
Is there an estimate of variation within each group of data?	Variations are included in all graphs. Scatter blots showing every single data point and the mean plus/minus standard error of the mean were choosen to depict the spreading of the individual data points.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	There was normal variation in the data as every single animal reacts individually to the laser damage or the respective treatment. Variation within the experiments was reduced by using disease-free animals of similar age. Scatter blots showing every single data point and the mean plus/minus standard error of the mean were choosen to depict the spreading of the individual data points.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibody catalog numbers are provided in the manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	RAW Blue cells were obtained from Invivo Gen.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All information is included in the manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	A statement is included in the manuscript.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We consulted the ARRIVE Guidelines Checklist.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA