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Peer Review File

Lipid Droplets Sequester Palmitic Acid to Disrupt Endothelial Ciliation and Exacerbate Atherosclerosis in Male Mice

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

-The authors have responded to the majority of the comments made by reviewer 1; however, there are several points that still need to be addressed in this work:
The authors demonstrate an unexpected role for palmitic acid on endothelial protection. While the majority of studies show a proatherogenic, proinflammatory role for palmitic acid, the authors demonstrate a protective role compared with a diet rich in unsaturated fatty acids. Moreover, the authors claim that this process could be due to endothelial ciliation that is maintained in palmitate enriched diet whereas oleic diet induced a decreased in ciliated endothelial cells.
The study of the endothelial function as a whole is based only on the secretion of proinflammatory cytokine (that is increased by oleic acid and not by palmitic acid correlated with the absence of cilia).
It would have been interesting to determine the level of autophagy and senescence in response to palmitic and/or oleic acid. Indeed, primary cilium is needed to activate autophagy and prevent endothelial senescence during atherosclerosis (PMID: 38152888: ATVB, 2024). On the other hand, autophagy has also been shown to be dysfunctional in response to palmitic acid linked to endothelial senescence (Cellular signaling 2022, PMID: 35843572, Curr Med sci 2022 PMID: 35896932). These data do not agree with the results described in this article indicating that palmitic acid helps to maintain ciliogenesis and protect endothelial cell. Can you investigate autophagy and senescence in response to different fatty acids in endothelial cells?

- On the other hand, the results presented on the cilium are surprising in view of the articles published recently on the role of saturated versus unsaturated fatty acids on the primary cilium (Cell Death Dis. 2022, PMID: 35902579). In this article, the authors show that unsaturated fatty acids do not modify the primary cilium, whereas incubation with saturated fatty acids induces a reduction in ciliated cells. In this article, carried out on neurons, the authors deprive the cells of serum to induce cilium formation and then incubate them with different fatty acids. the effects of both fatty acids on primary cilium under the same conditions should be performed in endothelial cells.

-The impact of a SCD1 inhibitor on atherosclerosis is also surprising. It was published in 2008 in ATVB (PMID: 19095997) that SCD1 invalidation led to an increase in atherosclerosis, independent of bone marrow cells. By using an intravenously injected inhibitor, the authors claim that they specifically target the endothelial cells... I'm not convinced that this explains the differences. A systemic inhibitor or a KO should produce the same results. One of the differences could be the diet used (western diet for the 2008 article and here HFD enriched in cholesterol). This at least needs to be discussed in greater depth.

-Finally, the results obtained in vivo with the different diets are very surprising compared with the literature that demonstrate a protection against atherosclerosis of oleic rich diet compared to palmitic rich diet (Br J of Nutr 2005, PMID: 16351765 ; ATVB 2020, PMID: 31619061:, BBRC 2012 PMID: 23058919).The diets rich in oleic or palmitic acids used here are cholesterol-free and are different from those used in figure 1. It would have been better to use a classic proatherogenic diet (identical to the one used in figure 1) adding more palmitic acid or more oleic acid in order to be able to compare the results on both

cilia and atherosclerosis as was done in PMID: 31619061.

Reviewer #2 (Remarks to the Author):

Thank you for an excellent rebuttal. I am satisfied with the author responses.

Reviewer #3 (Remarks to the Author):

The work is noteworthy in an emerging area of research of endothelial lipid droplet accumulation and vascular inflammation where there are only a few published reports. The role of endothelial ciliation and vascular health is also an area of research that warrants further investigation. The work is relevant and significant to multiple fields of research including endothelial cell biology, lipid metabolism and atherosclerosis.

The authors have submitted a revised manuscript and have addressed most of the concerns raised by the previous reviewers. I commend their efforts to the address the critiques previously raised. My main concerns arise from lack of statistical power in the quantification of the imaging performed. Since the central message of the manuscript is that cilia loss and accumulation of lipid droplets in endothelial cells contributes to vascular inflammation and atherosclerosis, the quantification of lipid and cilia must be made across many cells, especially considering the variability that is noted both in vitro and in vivo. Regarding this quantification, the methods are lacking as to details for the image analysis and the statistics used to represent variability across biological replicates.

Also, recent work indicating that endothelial SCD1 improves vascular protection should be referenced and addressed.

See specific comments below:

1. There is a general concern for lack of power for the quantification. For example, the quantification of LD in ciliated vs. non-ciliated cells in the mouse aorta utilized fluorescence intensity but does not describe how cells are segmented first to identify signal per cell. Also, not clear how BODIPY staining is quantified, per cell? The number of cells of “n = 30 cells from 10 mice for each group” would suggest 3 cells per aorta are quantified. While it states that cells were selected ‘randomly’, not clear if cells were randomly selected within acquired images? All cells within the imaging field of view should be quantified to ensure cellular variability is accurately represented.
2. For cultured ECs, a similar concern arises regarding the quantification of LipidTOX and % ciliated cells. It is not clear how many cells were quantified per condition. Figure 2 states ‘n = 10 independent experiments’ and shows 10 dots but not clear if this is 10 cells or average of some number of ECs per ‘experiment.’ This relates to accurately representing the variability within the cell culture especially the data indicates that not all cells are ciliated.
3. The above concerns should be addressed for all experiments where individual cells are quantified. To

ensure statistical analysis accounts for the number of experimental replicates, the quantification of individual cells from the same experimental sample should show mean per experimental replicate (see description in PMID: 32346721).
4. The authors show that SCD1 is upregulated in VECs of HFD-fed mice (Figure 5). Is this a compensatory mechanism? The authors should comment on the previous published report that loss of endothelial Scd1 increased vascular inflammation (see PMID: 38354249).

Point-by-point response to the reviewers' comments

We would like to express our gratitude for the feedback provided by the three reviewers. Reviewer #2 noted that all concerns raised have been resolved. Reviewers #1 and #3 recognized the novelty and significance of our findings and pointed out that we have adequately addressed most of the previous concerns. Reviewer #1 recommended that we incorporate additional experiments and discussions to further establish the unexpected protective effect of saturated palmitic acid in atherosclerosis. Reviewer #3 noted that the central message of the manuscript could be more effectively supported by improved quantification and statistical analysis. We sincerely appreciate the thorough analyses and constructive suggestions provided by the reviewers. In the revised version, we have further improved the manuscript by addressing all the concerns. We hope that after reading the point-by-point response, you can concur with us that we have addressed all the raised concerns in a satisfactory manner.

Reviewer #1 (Remarks to the Author):

-The authors have responded to the majority of the comments made by reviewer 1; however, there are several points that still need to be addressed in this work:

The authors demonstrate an unexpected role for palmitic acid on endothelial protection. While the majority of studies show a proatherogenic, proinflammatory role for palmitic acid, the authors demonstrate a protective role compared with a diet rich in unsaturated fatty acids. Moreover, the authors claim that this process could be due to endothelial ciliation that is maintained in palmitate enriched diet whereas oleic diet induced a decreased in ciliated endothelial cells.

The study of the endothelial function as a whole is based only on the secretion of proinflammatory cytokine (that is increased by oleic acid and not by palmitic acid correlated with the absence of cilia).

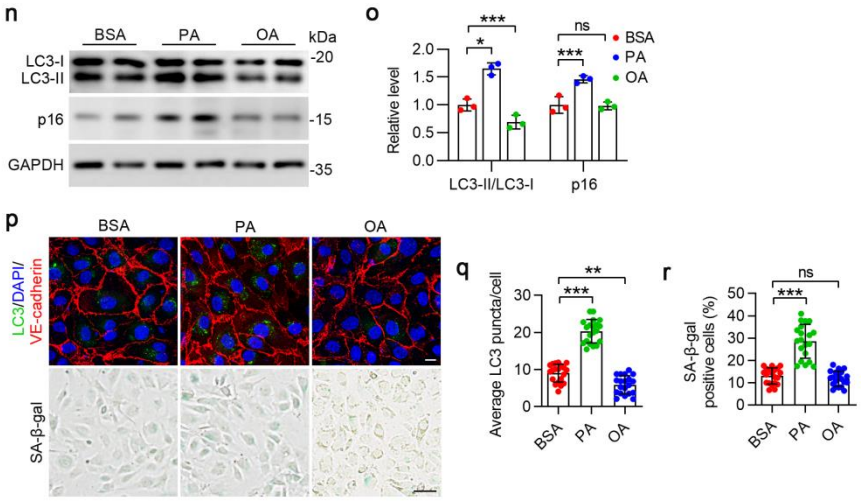
It would have been interesting to determine the level of autophagy and senescence in response to palmitic and/or oleic acid. Indeed, primary cilium is needed to activate autophagy and prevent endothelial senescence during atherosclerosis (PMID: 38152888: ATVB, 2024). On the other hand, autophagy has also been shown to be dysfunctional in response to palmitic acid linked to endothelial senescence (Cellular signaling 2022, PMID: 35843572, Curr Med sci 2022 PMID: 35896932). These data do not agree with the results described in this article indicating that palmitic acid helps to maintain ciliogenesis and protect endothelial cell. Can you investigate autophagy and senescence in response to different fatty acids in endothelial cells?

Response: Thank you for these excellent points. As suggested, the effects of palmitic acid on autophagy and senescence in cultured vascular endothelial cells were examined. In brief, HAECs were treated with oleic acid and palmitic acid, respectively, using similar treatment conditions as described in Cellular Signaling (2022, PMID: 35843572) and Current Medical Science (2022, PMID: 35896932)[1,2]. The results showed that the palmitic acid (200 μM) treatment enhanced both autophagy and senescence in comparison to the BSA-treated control group, whereas the oleic acid treatment suppressed autophagy but had no significant effect on senescence (Supplementary Fig. 7n-r, also displayed below).

Palmitic acid, a common saturated fatty acid, has long been considered "bad fat" and is frequently utilized in *in vitro* studies to mimic HFD-induced endothelial injury. It's worth noting that most of these

studies use palmitic acid at rather high doses (0.2~1 mM). Moreover, the detrimental effects of palmitic acid are usually assessed in comparison to groups that were not treated with any fatty acids. Our research revealed that when endothelial cells are exposed to oleic acid or other stimuli that activate neutral lipid accumulation, they experience a dramatic decrease in the abundance of free palmitic acid in the cytosol, leading to reduced protein S-palmitoylation and impaired endothelial ciliation. Restoring palmitic acid availability significantly restored endothelial cilia and mitigated the progression of atherosclerosis. Thus, our findings provide evidence for the beneficial effect of palmitic acid in vascular endothelium accumulated with lipid droplets.

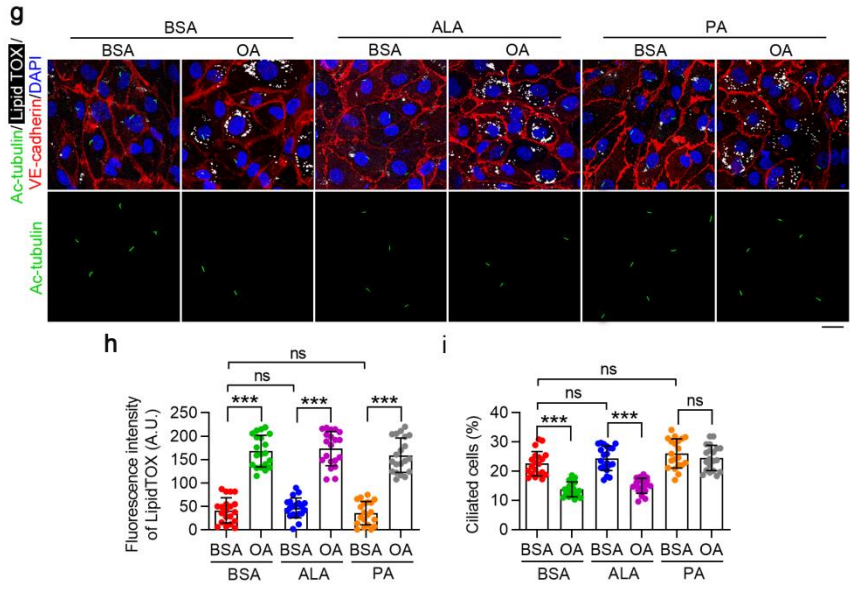
Nevertheless, when compared to the normal chow-fed mice, mice fed with the palmitic acid-rich HFD did exhibit a significant aggregation in the development of atherosclerosis (Fig. 7). In the case of *in vitro* cultured endothelial cells, the palmitic acid (200 μM) treatment itself did not result in any improvement in endothelial ciliation as compared to the control group treated with BSA (Fig. 5f-h). Besides, the treatment of palmitic acid (200 μM) resulted in a low degree of ER stress (Supplementary Fig. 7j, k) and moderate levels of autophagy and senescence (Supplementary Fig. 7n-r). Increased ER stress and senescence are considered detrimental to endothelial function/health, while enhanced autophagy is considered beneficial. Overall, our study suggests that palmitic acid has an unexpected protective effect against atherosclerosis by restoring the impaired ciliation of endothelial cells accumulated with lipid droplets. This protective effect can vary depending on the conditions, as exposure to palmitic acid may have both negative and positive effects on endothelial function through various mechanisms.



- On the other hand, the results presented on the cilium are surprising in view of the articles published recently on the role of saturated versus unsaturated fatty acids on the primary cilium (Cell Death Dis. 2022, PMID: 35902579). In this article, the authors show that unsaturated fatty acids do not modify the primary cilium, whereas incubation with saturated fatty acids induces a reduction in ciliated cells. In this article, carried out on neurons, the authors deprive the cells of serum to induce cilium formation and then incubate them with different fatty acids. the effects of both fatty acids on primary cilium under the same conditions should be performed in endothelial cells.

Response: Thank you for your comments. As you pointed out, the impact of palmitic acid, along with other saturated and unsaturated fatty acids, on cilia is very likely to be cell-type specific. As reported in Cell Death & Disease (2022, PMID: 35902579), palmitic acid treatment led to a reduction in the percentage of ciliated hypothalamic neurons, whereas the number of cilia in hypothalamic glial cells remained unaffected[3]. Recent studies have revealed that the lipid metabolism is tightly coupled between neurons and glial cells, particularly astrocytes[4,5]. Astrocytes are the main cells responsible for the formation of lipid droplets, which serve as storage for excess lipids[4]. In contrast, lipid droplets are rarely generated in neurons[6,7]. Consistently, neurons are generally more sensitive to lipotoxicity[4].

As suggested, we conducted additional experiments using the same treatment method described in the referenced study. In brief, HAECs were subjected to serum starvation and subsequently exposed to the indicated fatty acids. The results showed that treatment with alpha-linolenic acid (ALA) or palmitic acid (PA) alone did not have a significant effect on endothelial cilia. However, it was observed that PA, but not ALA, was able to restore the ciliary defects induced by oleic acid (OA) (Supplementary Figure 4g-i, also displayed below). These results are consistent with our previous findings and further demonstrate that PA supplementation protects endothelial cilia from oleic acid-induced disassembly.



-The impact of a SCD1 inhibitor on atherosclerosis is also surprising. It was published in 2008 in ATVB (PMID: 19095997) that SCD1 invalidation led to an increase in atherosclerosis, independent of bone marrow cells. By using an intravenously injected inhibitor, the authors claim that they specifically target the endothelial cells... I'm not convinced that this explains the differences. A systemic inhibitor or a KO should produce the same results. One of the differences could be the diet used (western diet for the 2008 article and here HFD enriched in cholesterol). This at least needs to be discussed in greater depth.

Response: Thank you for raising this concern. We fully agree with you that the involvement of SCD1 activity in atherosclerosis is complicated, and that the particular approaches and animal models utilized to investigate the function of SCD1 could significantly impact the final outcome. In our original manuscript, we briefly discussed this matter and cited the 2008 ATVB paper (PMID: 19095997)[8].

As you mentioned, besides the delivery method of the SCD1 inhibitor, the diet (and the mouse line) were also different in our study. Although the progression of atherosclerosis in humans can't be accurately simulated by any mouse model of atherosclerosis due to its artificial nature, future research that employs a "standardized" model of atherosclerosis could help in resolving this issue. As suggested, the above points have been concisely included in the discussion section of the revised manuscript. Thanks again for your suggestion.

-Finally, the results obtained in vivo with the different diets are very surprising compared with the literature that demonstrate a protection against atherosclerosis of oleic rich diet compared to palmitic rich diet (Br J of Nutr 2005, PMID: 16351765; ATVB 2020, PMID: 31619061:, BBRC 2012 PMID: 23058919).The diets rich in oleic or palmitic acids used here are cholesterol-free and are different from those used in figure 1. It would have been better to use a classic proatherogenic diet (identical to the one used in figure 1) adding more palmitic acid or more oleic acid in order to be able to compare the results on both cilia and atherosclerosis as was done in PMID: 31619061.

Response: Thank you for the valuable information. We went through the mentioned publications as well as some other papers and noted that the high-fat diets used in these studies are all quite complex, incorporating various types of fats such as palm oil, safflower oil, olive oil, etc[9-11]. In the ATVB 2020 study (PMID: 31619061), the milkfat (rich in saturated fat) in a Western diet was replaced with extra-virgin olive oil and nuts (rich in unsaturated fat)[10]. In theory, we could replace the oleic acid in the HFD with palmitic acid in order to better demonstrate our hypothesis. However, using purified fatty acids would cost far too much and pose many technical challenges, such as solidifying the food. Therefore, in our study, soybean oil-based HFD (low in palmitic acid) and palm oil-based HFD (high in palmitic acid) were used. To enable an unbiased analysis of our findings, a table listing the contents of relevant fatty acids, together with the composition of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), is provided as Supplementary Table 3.

Given the variations in dietary composition and mouse models employed in the aforementioned studies, we do not possess sufficient knowledge to provide a conclusive analysis of the seemingly contradictory results. We included a concise statement acknowledging that additional research is needed to determine if increasing the amount of palmitic acid provides protection against atherosclerosis in other models. As for the cholesterol content, all diets used for the experiments in Fig. 7 contain 0.5% cholesterol. The cholesterol content in proatherogenic diets typically ranges from 0.2% to 1.25%. A cholesterol content of 0.5% was used to balance the variations across the 4 diets, making it simpler to formulate these customized diets. The cholesterol content of customized diets is also stated in Supplementary Table 3. Thank you again for your questions.

Reviewer #2 (Remarks to the Author):

Thank you for an excellent rebuttal. I am satisfied with the author responses.

Response: Thank you for your feedback. It is greatly appreciated.

Reviewer #3 (Remarks to the Author):

The work is noteworthy in an emerging area of research of endothelial lipid droplet accumulation and vascular inflammation where there are only a few published reports. The role of endothelial ciliation and vascular health is also an area of research that warrants further investigation. The work is relevant and significant to multiple fields of research including endothelial cell biology, lipid metabolism and atherosclerosis.

Response: Thanks for your recognition of the novelty and significance of our study.

The authors have submitted a revised manuscript and have addressed most of the concerns raised by the previous reviewers. I commend their efforts to the address the critiques previously raised.

Response: Thank you for the acknowledgment of our efforts. It is greatly appreciated.

My main concerns arise from lack of statistical power in the quantification of the imaging performed. Since the central message of the manuscript is that cilia loss and accumulation of lipid droplets in endothelial cells contributes to vascular inflammation and atherosclerosis, the quantification of lipid and cilia must be made across many cells, especially considering the variability that is noted both in vitro and in vivo. Regarding this quantification, the methods are lacking as to details for the image analysis and the statistics used to represent variability across biological replicates.
Also, recent work indicating that endothelial SCD1 improves vascular protection should be referenced and addressed.

Response: We sincerely apologize for the inaccurate description of the quantification in Fig. 2b, c, as well as the improper presentation of Fig. 1f, g. These issues have been resolved in the revised manuscript. In addition, we have thoroughly verified the other quantification results and incorporated additional specifics regarding the image analysis and statistical methods in the revised manuscript. The recent work (PMID: 38354249)[12] has been referenced and addressed in the revised manuscript. Thank you for the information.

See specific comments below:

1. There is a general concern for lack of power for the quantification. For example, the quantification of LD in ciliated vs. non-ciliated cells in the mouse aorta utilized fluorescence intensity but does not describe how cells are segmented first to identify signal per cell. Also, not clear how BODIPY staining is quantified, per cell? The number of cells of "n = 30 cells from 10 mice for each group" would suggest 3 cells per aorta are quantified. While it states that cells were selected 'randomly', not clear if cells were randomly selected within acquired images? All cells within the imaging field of view should be quantified to ensure cellular variability is accurately represented.

Response: Thank you for these excellent points. More details of the quantification method have been added to the revised manuscript. In brief, VE-cadherin labeling was used to segment individual endothelial cells in the mouse aorta. For certain *in vitro* cell culture experiments, randomly chosen

imaging fields containing numerous cells were used for quantification. Accordingly, the BODIPY staining in individual cells or imaging fields was quantified.

The number of cells (n = 30 cells from 10 mice for each group) is indeed misleading. We sincerely apologize for this misunderstanding. As you have pointed out, we quantified all cells within the chosen imaging fields in a "left-to-right, top-to-bottom" manner. In the previous quantification results (Fig. 1f, g), a substantial number of cells exhibit undetectable levels of ciliary signal and/or LD signal. These cells were excluded from previous analysis considering that they would be located on the Y-axis, X-axis, or at the origin (0, 0). In the revised manuscript, all quantified cells (n > 400 cells from 10 mice for each group) have been displayed in the scatter plots (Fig. 1f, g). The Source Data file now contains comprehensive information regarding these quantification results. Thank you again for pointing this out.

2. For cultured ECs, a similar concern arises regarding the quantification of LipidTOX and % ciliated cells. It is not clear how many cells were quantified per condition. Figure 2 states 'n = 10 independent experiments' and shows 10 dots but not clear if this is 10 cells or average of some number of ECs per 'experiment.' This relates to accurately representing the variability within the cell culture especially the data indicates that not all cells are ciliated.

Response: We sincerely apologize for this mistake. Similar to other panels in Fig. 2, randomly chosen imaging fields from 3 independent experiments were used for quantification shown in Fig. 2b, c. Instead of "n = 10 independent experiments", it should be "n = 10 fields from 3 independent experiments". This error has been fixed. In our response to your question #1, we clarified that for most *in vitro* cell culture experiments, quantification was carried out using randomly selected imaging fields (all imaging fields were from different wells of cell culture plates) that typically contained more than 50 cells. These details have been included in the figure legends of the revised manuscript.

3. The above concerns should be addressed for all experiments where individual cells are quantified. To ensure statistical analysis accounts for the number of experimental replicates, the quantification of individual cells from the same experimental sample should show mean per experimental replicate (see description in PMID: 32346721).

Response: Thank you for this great suggestion. We concur with you that statistical analysis should account for the number of biological replicates, and that the quantification results should indicate which individual cells originate from the same biological sample (e.g., each mouse). These details are now included in the Source Data file. Supplementary Fig. 1d is now presented in accordance with the method described in PMID: 32346721[13]. In an effort to further distinguish the data points in Fig. 1f, g, and Supplementary Fig. 1e, f from different mice (n = 10) by labeling them with varying colors, we found that the results would be very difficult to interpret. Therefore, color coding was not performed on these scatter plots. We hope you will find it acceptable. Thanks again for the suggestion.

4. The authors show that SCD1 is upregulated in VECs of HFD-fed mice (Figure 5). Is this a compensatory mechanism? The authors should comment on the previous published report that loss of endothelial Scd1 increased vascular inflammation (see PMID: 38354249).

Response: Thank you for the information. SCD1 expression is regulated by a range of nutritional, hormonal, and environmental factors[14]. Thus, in HFD-fed mice, it's probable that the excess fatty acids can stimulate the expression of SCD1 in the vascular endothelium. Enhanced SCD1 activity facilitates the conversion of saturated fatty acids to monounsaturated fatty acids, which in turn stimulates the storage of lipids in the form of lipid droplets. Our study demonstrated that the accumulation of lipid droplets disrupts endothelial ciliation and exacerbates atherosclerosis by reducing cytosolic palmitic acid. As a critical signaling hub for lipid metabolism, SCD1 plays essential roles in maintaining metabolic and tissue homeostasis. Hence, a complete lack of endothelium SCD1 may result in elevated vascular damage through various mechanisms. In our study, we employed a pharmacological approach to suppress the activity of SCD1, which is different from the mentioned study. The study (PMID: 38354249)[12] has been cited in the revised manuscript. Thank you again for the question.

References

1 Zhan, W., Tian, W., Zhang, W., Tian, H. & Sun, T. ANGPTL4 attenuates palmitic acid-induced endothelial cell injury by increasing autophagy. *Cell Signal* **98**, 110410, doi:10.1016/j.cellsig.2022.110410 (2022).

2 Xiao, X. T. *et al.* Green Tea Polyphenols Prevent Early Vascular Aging Induced by High-Fat Diet via Promoting Autophagy in Young Adult Rats. *Curr Med Sci* **42**, 981-990, doi:10.1007/s11596-022-2604-6 (2022).

3 Avalos, Y. *et al.* Palmitic acid control of ciliogenesis modulates insulin signaling in hypothalamic neurons through an autophagy-dependent mechanism. *Cell Death Dis* **13**, 659, doi:10.1038/s41419-022-05109-9 (2022).

4 Ioannou, M. S. *et al.* Neuron-Astrocyte Metabolic Coupling Protects against Activity-Induced Fatty Acid Toxicity. *Cell* **177**, 1522-1535 e1514, doi:10.1016/j.cell.2019.04.001 (2019).

5 Mi, Y. *et al.* Loss of fatty acid degradation by astrocytic mitochondria triggers neuroinflammation and neurodegeneration. *Nat Metab* **5**, 445-465, doi:10.1038/s42255-023-00756-4 (2023).

6 Kis, V., Barti, B., Lippai, M. & Sass, M. Specialized Cortex Glial Cells Accumulate Lipid Droplets in Drosophila melanogaster. *PLoS One* **10**, e0131250, doi:10.1371/journal.pone.0131250 (2015).

7 Liu, L., MacKenzie, K. R., Putluri, N., Maletic-Savatic, M. & Bellen, H. J. The Glia-Neuron Lactate Shuttle and Elevated ROS Promote Lipid Synthesis in Neurons and Lipid Droplet Accumulation in Glia via APOE/D. *Cell Metab* **26**, 719-737 e716, doi:10.1016/j.cmet.2017.08.024 (2017).

8 MacDonald, M. L. *et al.* Despite antiatherogenic metabolic characteristics, SCD1-deficient mice have increased inflammation and atherosclerosis. *Arterioscler Thromb Vasc Biol* **29**, 341-347, doi:10.1161/ATVBAHA.108.181099 (2009).

9 Sato, M. *et al.* Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats. *Br J Nutr* **94**, 896-901, doi:10.1079/bjn20051409 (2005).

10 Lian, Z. *et al.* Replacing Saturated Fat With Unsaturated Fat in Western Diet Reduces Foamy Monocytes and Atherosclerosis in Male Ldlr(-/-) Mice. *Arterioscler Thromb Vasc Biol* **40**, 72-85, doi:10.1161/ATVBAHA.119.313078 (2020).

11 Jin, F. *et al.* Acipimox attenuates atherosclerosis and enhances plaque stability in ApoE-deficient mice fed a palmitate-rich diet. *Biochem Biophys Res Commun* **428**, 86-92, doi:10.1016/j.bbrc.2012.10.011 (2012).

12 Cavallero, S. *et al.* Exercise mitigates flow recirculation and activates metabolic transducer SCD1 to catalyze vascular protective metabolites. *Sci Adv* **10**, eadj7481, doi:10.1126/sciadv.adj7481 (2024).

13 Lord, S. J., Velle, K. B., Mullins, R. D. & Fritz-Laylin, L. K. SuperPlots: Communicating reproducibility and variability in cell biology. *J Cell Biol* **219**, doi:10.1083/jcb.202001064 (2020).

14 Sun, Q. *et al.* SCD1 is the critical signaling hub to mediate metabolic diseases: Mechanism and the development of its inhibitors. *Biomed Pharmacother* **170**, 115586, doi:10.1016/j.biopha.2023.115586 (2024).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Thnak you for the rebuttal. I am satisfied with the authors response.

Reviewer #3 (Remarks to the Author):

The authors have done a good job in responding to the questions raised and critiques with improvements and clarifications in the manuscript text/figures.

A request still remaining is to provide a full description of the cell segmentation procedure and the ImageJ analysis pipeline used for cell quantification:

Currently, the methods state: "To quantify the fluorescence intensity per cell, individual endothelial cells in the mouse aorta were segmented according to VE-cadherin staining, and then the fluorescence intensity of BODIPY and cilia was determined using ImageJ, following the instructions of the ImageJ User Guide."

Please add a description of segmentation procedure performed and the fluorescence intensity quantification (not sure what 'User Guide' means here).

To ensure reproducibility across investigators, also provide the quantification procedures used for the in vitro culture. Currently, the methods state:

"Finally, the cells were stained with BODIPY and DAPI and observed using a fluorescent microscope. The percentage of ciliated cells was calculated with Image J (National Institutes of Health). The fluorescence intensity of lipid droplets was measured using Fiji software (National Institutes of Health)."

Were the in vitro cultured ECs segmented in the same methods as the en face aorta imaging or different method? Providing the image analysis steps will be useful not only for proper interpretation of the imaging data but also for other researchers to reproduce results.