**Rebuttal to Reviewer 1 Chris Barlow’s *second* set of comments. Please find our response below.**

Upon reviewing this paper for a second time I’m afraid that I’m still unable to recommend it for indexing. The main results and conclusions from the abstract are not clearly supported by the data here. For example in the result section of the abstract the authors state: “LSF treatment also increased levels of the lipid classes phosphtidylcholine, cholesteryl ester and oxo-phytodienoic acid but decreased levels of phosphatidylethanolamine lipids.” This would suggest that the protection afforded by LSF treatment operates by altering the lipid profile prior to oxidative stress. Indeed, in the conclusion section of the introduction the authors propose that retinal cells can be pre-conditioned with LSF to regulate levels of selected fatty acids and lipids. However, from page 6 “In the absence of oxidative stress, no changes in lipid levels between LSF-treated cells and untreated control were reported.” So LSF pre-treatment does not alter the lipid profile although there are differences in the lipid profile following oxidative stress between cells that were LSF pre-treatment. These differences most likely reflect the reduction in oxidative damage in these cells rather than providing a rational for the mechanism of protection.

Response: Our observations indicate that although under non-oxidative stress conditions, the addition of LSF to the cells did not result in significant changes to the lipid classes stated by Reviewer 1, there were significant changes in these lipid classes when the cells were pre-treated with LSF prior to the exposure of hydrogen-peroxide when compared with untreated cells under the same stress conditions. These observations suggest that LSF may only result in very subtle but undetectable changes in lipids which may be masked minimal changes in the level of enzymes required for lipid metabolism and transport (<https://www.sciencedirect.com/science/article/pii/S0005273617301220>). However, when cells are pre-treated with LSF and subsequently exposed to oxidative stress, the activation of genes encoding such enzymes may be triggered in the presence of stress and disease (<https://www.mdpi.com/1420-3049/24/18/3231/htm>) which may account for the said changes in the lipid levels. This phenomenon may be due to the fact that LSF is a known histone deacetylase inhibitor (<https://www.sciencedirect.com/science/article/pii/S1359644614001202?via%3Dihub>), it may induce an epigenetic memory in cells without causing any changes in gene expression under normal circumstances but actives genes when necessary (i.e. under cellular stress). Therefore, we have proposed investigations surrounding the effects of LSF on expression of genes that regulate lipid metabolism and transport to form part of our future studies as already mentioned in our manuscript. Despite this our observations still supports the conclusion made that cells can be pre-conditioned with LSF to prepare them for subsequent oxidative stress and assist their recovery should cellular insult occurs (as evidenced by our cell proliferation data). We have also now amended the final sentence in the manuscript to read “These further studies will facilitate the design of targeted therapies that can be co-administered with LSF for the management of AMD progression”.

There also appear to be discrepancies between the authors response to my previous review and the changes made in the revised version of the manuscript. For example, in their response to my previous review:

We have amended the Methods section by removing the use of calibration curves and added the statement “The data was generated using the reverse phase peak area response of each lipid species rather than the absolute concentration.”

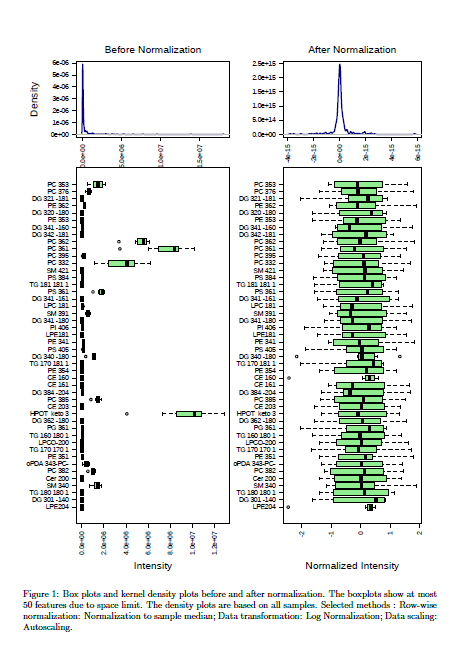
I was unable to find this statement in the revised version of the manuscript, instead on page 5 we have: “The lipidomic data was generated using the peak ion area response of each lipid series relative to an internal standard rather than absolute concentrations using reverse phase LC-MS”.

Response: The amended statement appears to be omitted during the processing and publication of the revised version of the manuscript. This statement has now been included in Version 3 of the manuscript under the subheading “Lipid analysis under LC-MS” in the Methods section.

So, did the authors use internal standards or not? d7-cholesterol is the only lipidomics standard that I could find mentioned in the text although it doesn’t seem to be included in the repository data. The repository data does include internal standards for LPC, PI, SM and TG.

Response: To clarify, the raw data was first normalised to d7-cholesterol using the Mass Hunter Quant V6 software and then exported into Excel for further normalisation by manual calculation using cell numbers per sample (formula: median reverse phase peak area under the chromatogram divide by cell number). The normalised data is then exported into MetaboAnalyst for further analysis and generation of heatmaps and box-whisker plots. However, the raw data presented exported using Mass Hunter will not show the d7-cholesterol peak area and hence not presented. We have removed the section in the Methods which refers to normalisation to d7-cholesterol to avoid confusion.

To reiterate, the original lipid data was further normalised to cell numbers and then median, log-transformed and autoscaled to generate results (eg heatmap). Below is an example showing lipid data not normalised to cell numbers. It can clearly be seen that even after applying post-normalisation parameters (i.e. median, log-transform and autoscaled) the lipid data is normally distributed. Please note that the graphs shown below list only a portion lipids of a non-exhaustive list and therefore the lipids you see here may not necessarily show the same lipids in the heatmap data within the manuscript which shows the top 25 lipids where differences are found between the untreated and 20µM LSF groups.



Furthermore, with the normalisation to both cell number and median reverse phase peak area, there are no changes in the significant lipid species before or after normalisation as shown in an example of data analysis below. Normalising the data to cell number is appropriate, as the cell number varies between replicates, before plotting the data using Metaboanalyst. We have not included these heatmaps here because the heatmap in the manuscript is identical before and after normalisation.

In a similar vein, my previous review made mention of several fold-changes that were in error which the authors attributed to typographical errors. The authors claim to have amended these values in the revised version of the manuscript. In the revised version of the manuscript however we find that all of the fold-changes have been altered, including those which were previously in agreement with the reviewer’s calculation. In the revised submission all values are now in disagreement with both the original submission and this reviewers’ calculations (see here). <https://f1000researchdata.s3.amazonaws.com/linked/269780.Chris_Barlow_v2_table_for_IM.JPG>

Response: We respectfully request Reviewer 1 to provide the calculation steps. We have used the open-source MetaboAnalyst Version 3 and performed the statistical analysis using the Bonferroni-adjusted values to analyse the fold-changes reported. Despite difference in the Reviewer’s calculations, the trends of lipid changes remain the same which still align with the discussion and conclusions made.

Some aspects of the data analysis continue to be unclear. For example, the lipidomic data was normalized based on the number of cells (these numbers should be provided in the repository for clarity) which seems extremely sensible. However, the authors state that the data was also normalized to the median of the peak area response. As the median peak area response would also scale with the number of cells wouldn’t this second normalization negate the normalization by the number of cells? Why is it necessary to normalize to the median value? Finally, the authors state that “An autoscale has also been applied.” What is the auto-scale and how has it been applied?

Response: We have already provided Reference 27 to support the use of an auto-scale which is routinely used by the Metabolomics community to visualise data. According to MetaboAnalyst, the auto-scale is equal to: mean-centered and divided by the standard deviation of each variable.

As there were differences between the cell numbers across each set of replicates, we normalised the data against the cell number and then against the median peak area response to limit the influence of the variable cell numbers on the data analysis.

Cell numbers are now provided in the underlying data Version 3.

The authors state in the statistical and data analysis section that a paired t-test was used for the lipidomic analysis. Given the study design it is not clear to me the basis on which the samples were “paired”.

The authors also need to adjust their analysis to account for the multiple comparisons problem. This was raised in my previous review but the authors appear to have misunderstood my meaning, discussing instead comparisons of multiple groups by ANOVA analysis.

Response: To address this concern, we have now amended the statement and specified under the subheading ‘Statistical and data analyses’: “Significant changes in cell proliferation and levels of total fatty acids or lipid species were validated by one-way analysis of variance and the post-hoc Bonferroni test and paired t-test to determine any significant differences between the treated groups (5µM, 10µM, 20µM and/or 30µM LSF) and untreated or vehicle controls”. This analysis was performed by MetaboAnalyst Version 3 as mentioned in the manuscript.

To clarify I’m talking about the problem of false positive identifications that will arise when comparing a large number of analytes. In the current example the authors consider 328 lipids, with a 5% cut-off we expect 16.4 lipids to have a p-value of <0.05 by chance alone. To combat this problem people typically employ something like a Bonferroni correction (which the authors have apparently used in their fatty acid analysis) or more often the Benjamini-Hochberg procedure. The authors need to demonstrate that the lipids reported as significantly different remain statistically significant upon application of this more stringent test. I would also recommend reporting the p-values here.

Response: The data analyses has already incorporated the Bonferroni correction. The p-values which derive from the t-test and ANOVA are now included in Version 3 of the manuscript.

I have some additional comments:

In Figure 3 plotting the points from the untreated control is misleading as all of these points have been imputed.

Response: We have addressed this point in our earlier rebuttal. We have removed any mention of fold changes in fatty acid levels in the manuscript and in Figure 3, but indicated that a comparison in the detection levels between untreated and treated groups. We have also removed any references to p values when reporting fatty acid data. Figure 3’s aim is to depict the trends in different levels of fatty acids between the treated and untreated groups.

In Figure 5 I note that PC 33:3 is plotted twice and somewhat surprisingly the plots do not match.

Response: We thank Reviewer 1 for detecting this anomaly. PC33:3.1 was a replicate in the set of 20µM LSF + treatment group. Subtle differences between the replicate and the averaged data point may not be apparent in the heatmap. The data for this replicate has been now been removed and Figures 4B and 5 have been updated.

**Rebuttal to Reviewer 2 Jacob Qi’s comments. Please find our response below.**

LSF is a major antioxidant from cruciferous vegetables. Kwa et al. aim to validate if LSF protects human retinal epithelial cells from H2O2-induced oxidative damage and to identify the underlying molecular basis by employing lipidomic analyses. It may result in a better understanding of the pathogenesis of AMD. However, the following concerns should be better addressed.

Major concerns: 1.In general, I don’t reckon that authors should split data interpretation into -/+ H2O2. It results in the loss of comparisons between – H2O2 and + H2O2 (in the absence of LSF) that is fundamental to demonstrate proper modelling of oxidative damage.

Response: The in vitro oxidative stress model we have used is widely validated in the ARPE-19 cell line (<https://ekjo.org/DOIx.php?id=10.3341/kjo.2003.17.1.19> and <https://www.ncbi.nlm.nih.gov/pubmed/29376497>) . The purpose of splitting the data into -/+ H2O2 are to assess the net effects of LSF on the cells under normal conditions and oxidative stress conditions. It is important to establish that LSF does not exert any toxicity on the cells under normal circumstances and if it was toxic, this would challenge our hypothesis that LSF can be used as a supplement in people at risk of AMD or have early stages of the disease where it is characterised by oxidative damage in retinal cells. Therefore, for the consistency of data presentation throughout the manuscript, all data reported have been presented in this format.

2.Fig. 1B. I cannot judge that effects of LSF result from protection against H2O2-induced cell death or an increased proliferation. 24 h treatment with H2O2 has been shown to cause toxicity in ARPE-19 cells 12. The underlying raw data also demonstrate that the absorbance was reduced from 2.0+ to 1.0+ between CA-H2O2 (fig.1A) and CA+H2O2 (Fig.1b), if comparable. Did 2 h treatment with H2O2 cause any toxicity?  Following LSF pre-treatment, why the addition of H2O2 for only 2 h induced such dramatic proliferation? What is the doubling time of ARPE-19 cells? Please note, MTS assay measures the mitochondrial activity. To draw your conclusion of “increase in proliferation”, other assays should be carried out, e.g. BrdU incorporation and Ki67 staining. LSF may also result in the phenotype in Fig. 1b, via the regulation of the cell cycle 3.

Response: As the study outlined in this manuscript is an extension of our first paper published in 2018 (<https://www.ncbi.nlm.nih.gov/pubmed/29376497>), we have already reported the toxic effects of 200µM H2O2 in Figure 2 of this previous paper. In addition, other studies have also used H2O2 concentrations of 100µM to low mM concentrations in oxidative stress cell-based models (<https://iopscience.iop.org/article/10.1088/1752-7163/ab1fc4/pdf>). As referenced in our earlier paper, the ARPE-10 cells have been starved in a serum-deprived DMEM/F12

media containing 1% FCS and 1% penicillin-streptomycin for 24 hours so that all the cells are synchronized at the same cell cycle phase and to remove time as a factor for cell proliferation. This is common practice in *in vitro* assays (<https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0120587&type=printable>, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3329488/> and [file://rmit.internal/USRHome/eh2/E02862/Downloads/z7g00906004098.pdf](file:///\\rmit.internal\USRHome\eh2\E02862\Downloads\z7g00906004098.pdf)). The MTS assay used in this study is a commercially available Cell Proliferation Assay from Promega and has been widely used to determine proliferation and cytotoxicity based on the measurement of lactate dehydrogenase enzymes in the metabolically active cells. Further references are provided in the manufacturer’s protocol (<https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-96-aqueous-one-solution-cell-proliferation-assay-system-protocol.pdf>).

3.Fig 2 and 3 show that in the absence of H2O2, LSF increased FAs C18:1 and C20:3. In the related discussion, authors believe that these increases contribute to LSF-mediated protection. However, as shown in the underlying data, the levels of C18:1 and C20:3 was higher or, at least, similar in H2O2-treated cells, as compared to LSF-treated samples. How to explain both LSF and H2O2 caused changes in the same way, whereas they were claimed to have opposite biological effects?

Response: Figures 2 and 3 show the trend differences in fatty acid levels between LSF treated cells and the untreated controls in the absence of oxidative stress. The underlying data shows that the raw values of the replicates between LSF treated cells and untreated controls in the presence of oxidative stress are relatively similar and may account for any lack in significant trend differences in the “+H2O2” groups. I compared the underlying values for LSF-H202 groups and LSF+H202 groups. Although it appears that values of the LSF-treated cells in the “+H2O2” groups are slightly higher to that of those in the “-H2O2” groups, there is some variation in cell numbers (version 3 of data in repository) across the replicates within each of these “+H2O2” groups. The “increased values” could possibly be due to the effects of hydrogen peroxide since the variable between the two groups is the presence of H2O2 but without rigorous statistical analysis, this conclusion cannot be made. It is important to note that the aim of Figures 2 and 3 is to show if the variable in this case, LSF treatment, changes the levels of the fatty acids in comparison to the untreated control and this is indeed what we observed which supports our hypothesis that LSF treatment can precondition cells to adapt to subsequent oxidative stress which may involve regulating levels of fatty acids that are known to play an important role in oxidative damage recovery. The direct relationship of how these fatty acids regulate the protective effects of LSF needs to be investigated in future studies as outlined in the manuscript.

4.Fig. 4 and 5. In Fig. 5, authors need to double-check the fold change of PE 34:0, PE 38:5 and oPDA 34:3, which do not match the underlying data.  In addition, if you normalised the data over the untreated control, it is better to set the untreated control as 1, and show 20uM LSF in fold change.

Response: Kindly note that the underlying data depicts the raw values generated by the mass spectrophotometer before any data analysis is performed where the figures show data normalised to cell numbers and then to median peak area. The heatmaps and boxplots were automatically generated by MetaboAnalyst Software Version 3 using the normalised data. Thus, the values shown in the figures do not match that of the underlying data. The fold changes were determined by: Median peak area of the lipid in the treatment group/median peak area of the lipid in the respective control group.

In the related discussions, 1) PE. Indeed, PE is the precursor for A2E production (ref 44). However, based on your lipidomics results, PE 34:0 is a less abundant PE species. Will the reduction of PE 34:0 “reduce the PE level” and thus determine the A2E production? Did you measure the A2E level? 2) PC. Similarly, the authors discussed the biofunction of overall PC in Bruch’s membrane. But the most abundant PC species in the retina are C 16:0/18:1, 18:0/18:1, 18:0/22:6, 18:2/20:4, etc 4. What is the physiological role of PC 33:3?

Response: We would like to respectfully ask how Reviewer 2 came to a conclusion that PE34:0 is a less abundant PE species and in comparison to which other PE species? The underlying data indicate the values of some PE species such as PE34:3 are even lower than that of PE34:0. We have not measured the levels of A2E in this study this can be included as a future study to validate if reducing PE34:0 and PE38:5 can reduce A2E levels. We have now included this as a future study in the revised manuscript. Unfortunately, the relationship between PC33:3 and retinal function or AMD is not well established and hence we discussed the general biofunction of PCs in the Bruch’s membrane. However, according to the Metabolomics Innovation Centre website, PC33:3 which can be made up of (15:0/18:3) and is said to be involved in phospholipid metabolism, lipid transport, lipid metabolism, lipid peroxidation etc. Please see here: <http://www.hmdb.ca/metabolites/HMDB0007941> .

Other concerns: 1.In the figure legends, authors should clarify the treatment conditions, although the information has been mentioned somewhere else.

Response: Although the treatment conditions are often found in the figure title, the figure legends have now been expanded to include information on the treatment conditions, where previous information is vague.

2.In fig. 4, authors should list lipid species following the order of lipid classification, but not in the current mixed pattern.

Response: As mentioned above, the heatmaps were automatically generated by MetaboAnalyst Software Version 3 using the analysed data. We are not able to change the way this data is presented.

3.I suggest plotting the selected subspecies from all 6 experimental groups in Fig. 3 and 5. Then the ANOVA should be applied for multi-comparisons. T-tests on two groups are less critical.

Response: The boxplots were automatically generated by MetaboAnalyst Software Version 3 using the analysed data. However, boxplots were only generated by the software where statistically significant changes between the treated and untreated groups were detected. Reference 27 which has been provided in the manuscript supports the routine use of MetaboAnalysis Software Version 3 in analysing metabolomic-based data. Pairwise T test compares two groups (i.e. treatment group versus untreated group) while the ANOVA was performed for multiple comparison of all groups. Both types of statistical analyses have been done in the study. The p-values generated by the ANOVA analysis have now been included in the respective Results section of the revised manuscript.