RESEARCH ARTICLE

REVISED Lipidomics reveal the protective effects of a vegetable-derived isothiocyanate against retinal degeneration [version 2; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Background: Age-related macular degeneration (AMD) is a leading cause of blindness in the ageing population. Without effective treatment strategies that can prevent disease progression, there is an urgent need for novel therapeutic interventions to reduce the burden of vision loss and improve patients' quality of life. Dysfunctional innate immune responses to oxidative stress observed in AMD can be caused by the formation of oxidised lipids, whilst polyunsaturated fatty acids have shown to increase the risk of AMD and disease progression in affected individuals. Previously, our laboratory has shown that the vegetable-derived isothiocyanate, L-sulforaphane (LSF), can protect human adult pigment epithelial cells from oxidative damage by upregulating gene expression of the oxidative stress enzyme Glutathione-S-Transferase μ 1. This study aims to validate the protective effects of LSF on human retinal cells under oxidative stress conditions and to reveal the key players in fatty acid and lipid metabolism that may facilitate this protection.

Methods: The *in vitro* oxidative stress model of AMD was based on the exposure of an adult retinal pigment epithelium-19 cell line to 200 μ M hydrogen peroxide. Percentage cell proliferation following LSF treatment was measured using tetrazolium salt-based assays. Untargeted fatty acid profiling was performed by gas chromatography-mass spectrometry. Untargeted lipid profiling was performed by liquid chromatography-mass spectrometry.

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Results: Under hydrogen peroxide-induced oxidative stress conditions, LSF treatment induced dose-dependent cell proliferation. The key fatty acids that were increased by LSF treatment of the retinal cells include oleic acid and eicosatrienoic acid. LSF treatment also increased levels of the lipid classes phosphatidylcholine, cholesteryl ester and oxo-phytodienoic acid but decreased levels of phosphatidylethanolamine lipids.

Conclusions: We propose that retinal cells at risk of oxidative damage and apoptosis can be pre-conditioned with LSF to regulate levels of selected fatty acids and lipids known to be implicated in the pathogenesis and progression of AMD.

Keywords

Age-related macular degeneration, fatty acid, L-Sulforaphane, lipidomics, oxidative stress, retinal pigment epithelium

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 1

The main changes reflected in Version 2 of this manuscript is in accordance with the comments made by the first reviewer, Chris Barlow:

- Font used in Figure 2 and Figure 4 have been enlarged
- Methods used to generate GCMS/LCMS data have been reworded to improve the clarity of the steps taken
- Fold changes and p values referred to the fatty acid data in the text and in Figure 3 have been removed.
- Data was processed was checked to ensure that all data was statistically analysed at a significance level of $p < 0.05$
- Typographical errors have been amended
- Duplicated lipid analysis data columns have been removed in Version 2 of the Harvard Dataverse link

Any further responses from the reviewers can be found at the end of the article

Introduction

Age-related macular degeneration (AMD) is a major cause of blindness worldwide, especially targeting the ageing population. AMD is categorised into three main stages, namely early, intermediate and late AMD. The early stage is marked by the thickening and inflammation of the Bruch's membrane, as a result of the accumulation of fatty proteins known as drusen¹. The intermediate stage proceeds with an increase in size of these drusen particles, resulting in pressure atrophy on the retinal pigment epithelium (RPE) and thinning of the macula (dry AMD), which results in the deterioration of central vision. In the late stages, the atrophic retinal tissue becomes replaced with granulation tissue consisting of abnormal leaky blood vessels (wet AMD)¹. The blood and fluid leak from these blood vessels into the retina; thus, prolonging the chronic inflammatory response and triggering further oxidative damage. Many factors contribute to AMD. One dominant factor is the increasing age of the retina, where the RPE becomes damaged due to a progressively impaired DNA repair system that fails to repair oxidative damage from prolonged exposure to visible light, ultraviolet A and reactive oxygen species (ROS) over time². Cigarette smoking is another factor that contributes to the production of ROS and oxidative damage on the RPE layer. Many studies have shown a link between excessive cigarette smoking and AMD^{3,4}. For the retina to maintain its normal physiological functions, a well-balanced diet is also necessary. Poor nutrition in the elderly influences the progression of AMD. Studies by Rochtchina *et al.* (2007) and Gopinath *et al.* (2013) showed that a deficiency of Vitamin B12 is linked to an increased risk of AMD^{5,6}. Despite recent evaluations of stem cell-derived therapeutic approaches in Phase I clinical trials, such novel methods require long-term use of immunosuppressive drugs, which may lead to other medical implications⁷. Conventional therapies include FDA-approved anti-angiogenic agents, thermal laser photocoagulation or intravitreal injection of medications to limit neovascularisation. However, each of these treatments resulted in the development of atrophic scars and haemorrhage in patients⁸. In view of the above, there is still no cure for AMD

and available therapies aim mainly to reduce patients' symptoms and target the late stages of the disease. Therefore, further studies must be carried out to find an effective preventative measure, especially in targeting early stages of the disease before the onset of vision loss.

The mentioned pathological features of AMD are known to be regulated by genes such as *Vascular Endothelium Growth Factor A (VEGFA)* and *Glutathione-S-Transferase $\mu 1$ (GSTM1)*. *VEGFA* upregulation is associated with neovascularisation, and a decrease in *GSTM1* expression is associated with an increased susceptibility to oxidative damage^{8,9}. Previously, imbalanced levels of fatty acids responsible for the abnormal function of the retina were associated with AMD progression. There are five major fatty acids in the human retina, namely, docosahexaenoic acid (DHA), arachidonic acid (ACA), stearic acid, oleic acid and palmitic acid. Both DHA and ACA are classified as long chain polyunsaturated fatty acids (LC-PUFAs)¹⁰. It was reported that a deficiency in docosahexaenoic acid and arachidonic acid interfere in neurological and visual signalling pathways, and intake of these LC-PUFAs increased the risk of AMD^{10,11}. In addition, other studies found that ROS produced during oxidative stress can damage the essential PUFAs in the retina and generate toxic lipid peroxidation end products (i.e. reactive aldehydes 4-hydroxynoneal and 4-hydroxyhexenal); thus, exacerbating the chronic-inflammatory damage in the retina. These accumulated aldehydes can in turn, inhibit redox enzyme reactions, DNA and RNA synthesis and biosynthesis of proteins¹². PUFAs are an important substrate for redox enzymes such as glutathione S transferases (*GSTs*) during oxidative stress-mediated lipid peroxidation and healthy fatty acid (FA) levels are crucial for the efficient removal of ROS from the retina^{13,14}. Dysfunctional innate immune responses to oxidative stress observed in AMD are also reported to be attributed to the formation of oxidized lipids¹⁵. Therefore, lipid and fatty acid pathways remain vital in maintaining a healthy environment in the retina. Furthermore, patients with AMD were reported to have low levels of other metabolites, such as glucose, lactate, glutamine and albumin, suggesting the possible role of a dysregulated metabolome in this disease^{16,17}. As such, the pathogenesis of AMD is likely to involve the abnormal expression of *VEGFA*, *GSTM1* and imbalanced levels of selective metabolites, such as fatty acids. This prompts the investigation of new and potential therapeutic agents that can alleviate the aberrant gene expression via chromatin remodelling processes and restore normal levels of metabolites in the retina.

Here, we propose the use of L-Sulforaphane (LSF), a naturally occurring isothiocyanate found in many cruciferous vegetables like broccoli in the treatment of AMD¹⁸. LSF has been shown to have epigenetic properties in solid tumours by enhancing the acetylation of histones, resulting in an 'opened' chromatin state, which triggers the transcription of genes involved in cell death and restores the apoptotic potential of cancer cells^{19,20}. These anti-carcinogenic effects have also been associated with down-regulation of the pro-inflammatory marker, hypoxia inducing factor 1- α , and VEGF while increasing redox enzyme activities^{21,22}. Such antioxidant properties could be useful for

the treatment of the AMD. Whilst it has the potential to induce cell death in malignant cancer cells, it can protect retinal tissue from photoreceptor degeneration under oxidative stress conditions²³. This protection is mediated via the induction of phase II detoxification enzyme *NAD(P)H:quinoxidoreductase* and transcriptional activation of antioxidant response element; thus elevating glutathione levels in the retinal cells²³. Hence, the action of LSF is unique and seems to be disease specific. This characteristic enables LSF to be considered a potential drug candidate in targeted therapy.

In 2018, our laboratory reported the ability of LSF at micromolar concentrations (3µM and 5µM) to protect human retinal pigment epithelium from cell death and promoted the regeneration of these cells under oxidative stress conditions²⁴. This preliminary study involving gas chromatography mass spectrometry (GC-MS) analytical methods revealed that LSF treatment induced changes in the levels of FAs, such as nonanoic acid and 9,12,15- (Z-Z-Z)-Octadecatrienoic acid, and upregulated the levels of *GSTM1* gene expression²⁴. However, many of these significant changes were observed with the 5µM LSF treatment. These findings have warranted the current study to further examine lipids and fatty acids that may regulate the protective and antioxidant effects of LSF. In the current study, dose response data using LSF concentrations of 3–30µM validate the previously reported protective and regenerative properties of this compound against oxidative stress, where a dose-dependent increase in cell proliferation is observed and then plateaus at a concentration higher than 20µM. For the first time, we report the use of a lipidomic approach using liquid chromatography with triple-quadrupole mass spectrometry (LC-*QQQ*-MS) to analyse human retinal pigment epithelial (ARPE-19) cells pre-treated with 5 and 20µM LSF under oxidative stress conditions. The total pool of FAs affected by the treatment will be confirmed by gas chromatography-mass spectrometry (GC-MS) and used to putatively identify lipid classes. We hypothesize that LSF can increase the levels of lipids containing unsaturated FAs while decreasing levels of lipids with saturated FAs for the protection of ARPE-19 cells against oxidative damage.

Methods

Cell culture

The Adult Retinal Pigment Epithelium-19 (ARPE-19) cell line was purchased from the American Type Cell Collection (USA). The cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM/F12) containing 200mM L-glutamine and 15mM HEPES (Life Technologies, USA). The culture media was further supplemented with 10% foetal calf serum (FCS; Sigma Aldrich, USA) and 1% penicillin-streptomycin 10,000 U/ml (Life Technologies, USA). The ARPE-19 cells were sustained at 37°C in an atmosphere of 95% air and 5% CO₂, and phenotypic characteristics of these cells were validated in our previous publication²⁴.

Cell treatment prior to analysis

The ARPE-19 cells were starved in a serum-deprived DMEM/F12 media containing 1% FCS and 1% penicillin-streptomycin for 24 hours. For the CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) Assay, the cells were exposed to 0.025% dimethyl sulfoxide (DMSO; Sigma Aldrich, USA) as the drug

vehicle control or 3µM LSF, 5µM LSF, 10µM LSF, 20µM LSF or 30µM LSF for 24 hours. For the GC-MS/LC-MS analysis, the cells were exposed to 0.025% DMSO, 5µM LSF or 20µM LSF for 24 hours. The negative control for all analyses was untreated cells that were incubated in serum-deprived DMEM/F12 media. After 24 hours incubation, the treatments were discarded from all the wells and the cells were incubated with 200µM hydrogen peroxide (H₂O₂; Sigma Aldrich, USA) for two hours. Untreated cells or LSF-treated cells incubated in Hanks Balanced Salt Solution (HBSS; Sigma Aldrich, USA) for two hours were used as the negative control for oxidative stress. Subsequently, the H₂O₂ or HBSS was removed and the cells were allowed to recover for 24 hours in serum-deprived DMEM/F12 media before either the MTS assay or the GC-MS/LC-MS analysis were carried out²⁴.

CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS)

The ARPE-19 cells were trypsinised using 0.25% trypsin EDTA (Life Technologies, USA) and centrifuged at 200 g for three minutes, before being seeded at a density of 10⁵ cells/mL in 100µL of complete cell culture media in 96-well flat-bottom plates and treated with the agents described above. Each well contained 10,000 cells. To assess the effects of LSF treatment in the presence or absence of oxidative stress on cell proliferation, the MTS assay (catalogue number G3580; Promega, USA) was carried out according to the manufacturer's protocol and as previously described²⁴. A volume of 20µL MTS reagent was added to the cells in each well and plate was incubated for four hours at 37°C and in an atmosphere of 95% air and 5% CO₂. Absorbance readings (at 490nm) of drug-treated cells were normalised to the untreated control. As per the manufacturer's protocol, % cell proliferation = $\frac{(\text{Absorbance}_{\text{drug treatment}} - \text{Absorbance}_{\text{blank}})}{(\text{Absorbance}_{\text{untreated}} - \text{Absorbance}_{\text{blank}})} \times 100\%$. The percentage of cell proliferation was calculated as the mean of results from three independent experiments with three technical replicates per experiment.

Harvesting of treated ARPE-19 Cells for GC-MS and LC-MS analysis

The ARPE-19 cells were seeded at a density of 1.5×10⁶ per well in 6-well plates and conditioned as indicated above. The cells were removed using 0.25% trypsin EDTA, followed by centrifugation at 200 g for three minutes. The cell pellets were resuspended in ice-cold 1X phosphate-buffered saline (pH 7.4) and the number of live cells were counted by trypan blue exclusion before being transferred to microcentrifuge tubes. These tubes were centrifuged twice at 200 g for three minutes and after each spin, the pellets were resuspended in ice-cold PBS (washing step). The tubes were spun a third time, the supernatant was removed to remove any remaining dead cells and cell debris. The pellets were frozen at -80°C to be used for the extraction. Four replicates of each control and treated samples were performed.

Extraction of fatty acids and lipids from treated ARPE-19 Cells for GC-MS and LC-MS analysis

Upon cell harvesting, each cell pellet was washed with 200 µL of water by vigorous vortexing for 19 seconds. A volume of 250 µL of methanol and 0.01% butylated hydroxytoluene

(v/v) mixture was added to the cell pellets and to the internal standard, 10 μM d_7 -cholesterol. The samples were then frozen for five minutes in liquid nitrogen, followed by sonication for another five minutes at room temperature at 100 rpm. The freeze-sonication steps were then repeated three times to lyse the cell pellets. The lysed cells were then vortexed vigorously for one minute. A volume of 500 μL of chloroform was added to the lysate and was mixed for 30 minutes at room temperature using a shaker. Next, the samples were centrifuged at 14,100g, 5°C for 15 minutes. The supernatant from each sample was transferred into respective clean 1.5 mL Eppendorf tubes (Tube A). A mixture containing 500 μL of chloroform:methanol (2:1) (v/v) was added to the cell pellets as the second extraction step. The samples were vortexed for 30 seconds and shaken for 15 minutes at room temperature before centrifugation at 16,100g, 0°C for 15 minutes. The supernatant from the second extraction was then combined into the supernatant in the respective Tube As. The combined supernatant for each sample was dried down under a stream of nitrogen. Each dried lipid extract was resuspended in 200 μL of butanol:methanol (1:1) (v/v) with 10 mM ammonium formate for LC-MS analysis²⁵. Additionally, a 30 μL aliquot was transferred into a glass insert and dried *in vacuo* for subsequent fatty acid methyl ester (FAME) analysis on the GC-MS. All samples were stored in the dark in bags containing silica beads prior to GC-MS and LC-MS analysis.

FAME analysis using GC-MS

The dried ARPE-19 cell extracts were resuspended in chloroform:methanol (2:1 v/v) (25 μL) containing 60 μM of the internal standard (^{13}C -labelled myristic acid), followed by the addition of the derivatizing agent (5 μL) (catalogue number 11370591, Meth-Prep IITM, Grace Davison Discovery, Deerfield, IL, US.). Each sample was subsequently incubated at 37°C for 30 min, then held for 10 min at room temperature. Then, 1 μL of the derivatised ARPE-19 cell extract was injected onto the GC-MS system consisting of a Gerstel 2.5.2 autosampler (catalogue number G7368A), a 7890A Agilent gas chromatograph (catalogue number G3440B), and a 5975C Agilent quadrupole MS (catalogue number G7042A) (Agilent Technologies, Santa Clara, US). The FAME analysis was carried out using a 30 m column with a 0.25 μm film thickness, 0.25 mm inner diameter and a 10 m guard column (catalogue number CP8944, Agilent J&W Scientific VF-5MS GC Column). The following parameters were set for GC-MS FAME analysis: injection port temperature (250°C), MS transfer line (280°C), ion source temperature (230°C) and quadrupole (150°C). The carrier gas used for the analyses was helium (UHP 5.0) at a flow rate of 1.0 mL/min. For the FAME analysis, the temperature program used was: start at injection (50°C), hold for one min followed by a 15°C.min⁻¹ oven temperature ramp to 230°C, hold for three min followed by a 10°C.min⁻¹ oven temperature ramp to 325°C and a final three min heating at 325°C. Mass spectra were recorded at two scans/s with a 50–600 m/z scanning range²⁶.

Lipid analysis using LC-MS

Lipid analysis using LC-MS was carried out as published previously²⁶. Briefly, to separate the lipids, 5 μL aliquots per sample were injected onto a 50 mm $\times$ 2.1 mm $\times$ 2.7 μm Ascentis Express RP Amide column (catalogue number

53911-U, Supelco, Sigma, St Louis, USA) at 35°C using an Agilent LC 1200 (Mulgrave, Australia).

Lipid detection was carried out using Agilent 6410 triple quad (catalogue number, 6410, Mulgrave, Australia) in electrospray ionisation (ESI) mode. Lipid species were identified based on the lipid class using precursor ion and Neutral loss scanning techniques as discussed previously²⁶. Diacylglycerol and triacylglycerol species were identified based on the neutral loss of fatty acyl moiety.

Identified lipid species were quantified via multiple reaction monitoring (MRM) with a 20 ms dwell time for the simultaneous measurements of ~20 to 50 compounds and the chromatographic peak width of 30 sec to 45 secs. A minimum of 12 to 16 data points was collected across the peak. Optimised parameters for capillary, fragmented, and collision voltages were 4000 V, 140 - 380, and 15–60 V, respectively. The collision gas used was nitrogen at 7 Lmin⁻¹.

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 reversed phase LC-MS. Furthermore, to compare the lipid levels between untreated and treatment groups, the data has been normalised to the number of cells per sample, and the median of the peak area response was log₂ transformed. An auto-scale has also been applied. Detected lipid species were annotated as lipid class (sum of carbon atoms in the two fatty acid chains: sum of double bonds in the fatty acid chains).

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/Fisher tests and paired t-test. The GC-MS was plotted using MetaboAnalyst Software Version 2 (USA)²⁷. The LC-MS ESI-MRM data was processed using Agilent Mass Hunter Quantitative Analysis software (Version 6) (Mulgrave, Australia) and plotted using MetaboAnalyst Software Version 2 (USA).

Results

Effects of LSF on the proliferation of ARPE-19 cells in the presence or absence of oxidative stress

The drug vehicle control (0.025% DMSO) did not affect the percentage of proliferation regardless of exposure to oxidative stress stimulus, H_2O_2 (Figure 1; all p values > 0.05)²⁸. In the absence of H_2O_2 , 3 μM - 30 μM LSF treatments did not have a significant impact on cell proliferation (Figure 1A; all p values > 0.05). In contrast, a dose-dependent increase in the proliferation of LSF-treated cells was observed at doses of 3 μM to 20 μM under H_2O_2 conditions (Figure 1B; all p -values < 0.0001). Increasing the dose to 30 μM LSF did not induce any further increase in cell proliferation (Figure 1B: vs 20 μM , p value > 0.9999). These results validate the ability of LSF to protect ARPE-19 cells against oxidative stress by stimulating the regeneration of these cells. Henceforth, GC-MS and LC-MS analyses were performed on cells treated with the lowest and highest doses of LSF that resulted in significant increases in cell proliferation (i.e. p < 0.0001) compared

Figure 1. Effects of LSF on ARPE-19 cell proliferation in (A) without or (B) with H₂O₂. The proliferative effects of vehicle control (0.025% DMSO) and 3 µM - 30 µM LSF on cells were determined. The cells were treated with 0.025% DMSO or LSF for 24 hours prior to exposure with 200 µM H₂O₂ for two hours. The mean absorbance values for each treatment group are presented as a percentage of that of their respective untreated controls (CA) in the absence or presence of H₂O₂. n = 3; not significant (ns): $p > 0.05$, *** $p < 0.01$ and **** $p < 0.0001$. [CA, cells alone; DMSO, dimethyl sulfoxide; H₂O₂, hydrogen peroxide; LSF, L-Sulforaphane].

to the untreated cells as the control group. Since there were no significant differences in cell proliferation between 20 µM and 30 µM LSF treatment groups, 30 µM LSF was not included in the GC-MS and LC-MS analyses.

Effects of LSF on the total fatty acid and lipidome in ARPE-19 cells in the absence or presence of oxidative stress

In the absence of oxidative stress, 5 µM LSF treatment resulted in higher levels of the fatty acid, *cis*-oleic acid (18:1) while 20 µM LSF treatment led to increased levels of *trans*-oleic acid, *cis*-oleic acid and eicosatrienoic acid (ETA) (20:3) in comparison to the untreated control (Figure 2 and Figure 3). It is noteworthy that the levels of fatty acids in the untreated control fell below the level of detection and thus the missing values for the control were imputed, a common practice in metabolomics data analysis. In the presence of oxidative stress, there were no consistent differences in the fatty acid levels between the replicate samples within the 5 µM LSF or 20 µM LSF treatment groups and those within the untreated control group (see *Underlying data*)²⁸.

In the absence of oxidative stress, no changes in lipid levels between LSF-treated cells and the untreated control were reported (all p values > 0.05 ; see *Underlying data*)²⁸. In the presence of oxidative stress, significant changes (p values < 0.05) were observed in the 20 µM LSF treatment groups. This study showed that LSF treatment increased levels of phosphatidylcholine (PC 33:3) by 2.133-fold and cholesteryl ester (CE 18:2 and CE 20:2) lipids containing unsaturated FAs by 5.619-fold and 8.155-fold respectively, and oxo-phytodienoic acid (oPDA 34:3-PC 16:0) by 3.040-fold. However, LSF treatment decreased levels of phosphatidylethanolamine (PE 34:0) consisting of saturated FAs by 0.359-fold (Figure 4B and Figure 5) was also observed. Other PE lipids containing

unsaturated FAs (PE 38:5) were also decreased by 0.427-fold. In contrast, treatment with 5 µM LSF did not result in any statistically significant changes in lipid levels (all p values > 0.05 ; Figure 4A).

Discussion

In 2019, the World Health Organisation has classified AMD as one of the top 10 priority eye diseases and leading cause of blindness in the ageing population²⁹. Therefore, without a current cure, there is an urgent need for better prevention, treatment and management strategies to reduce the burden of vision loss and improve patients' quality of life. Oxidative stress and abnormal neovascularization are processes known to promote the pathological changes observed in the retina of AMD patients. The underlying molecular mechanisms triggering these processes involve aberrant downregulation of *GSTM1* and upregulation of *VEGFA*^{8,9}. More recently, deficient levels of dietary PUFAs have shown to increase the risk of AMD and disease progression in affected individuals³⁰. Previously, our laboratory has shown that the cruciferous vegetable-derived compound, LSF, can protect human adult pigment epithelial cells from oxidative damage by upregulating *GSTM1* expression and modulating levels of selected PUFAs²⁴. Here, we validated the protective effects of LSF on human retinal cells under oxidative stress conditions and revealed the key fatty acids and lipids that may facilitate this protection.

A dose-dependent increase in cell proliferation was observed in LSF-treated ARPE-19 cells exposed to H₂O₂-induced oxidative damage but no changes in cell proliferation were detected in the absence of stress. This finding demonstrates that LSF is not harmful at the investigated micromolar doses when oxidative stress is absent but can induce regeneration of retinal cells in an oxidative stress environment. Thus, LSF

Figure 2. Total fatty acid levels in (A) 5µM or (B) 20µM LSF-treated ARPE-19 cells without H₂O₂. Four replicate samples for each treatment group were compared with that of the untreated controls. Data in red and blue indicate an increase and decrease in FA levels, respectively. Total fatty acids (FACs) highlighted in red boxes show consistent changes in levels between the replicates within the treatment groups and those within the untreated control group. [5_LSF: 5µM LSF; 20_LSF: 20µM LSF. FA, fatty acid; FAC, total fatty acid; H₂O₂, hydrogen peroxide; LSF, L-Sulforaphane; UN, untreated].

Figure 3. Changes in total fatty acid (FAC) levels in LSF-treated ARPE-19 cells without H₂O₂ compared to the untreated control. Four replicate LSF samples were compared with that of the untreated controls. Sample variation is depicted by the error bars. The y-axis values are automatically generated as arbitrary units by MetaboAnalyst software. [FAC, total fatty acid; FAC 18:1 n9c, *cis*-oleic acid; FAC 18:1 n9t, *trans*-oleic acid; FAC 20:3, eicosatrienoic acid; H₂O₂, hydrogen peroxide; LSF, L-Sulforaphane].

may be beneficial in the treatment of AMD without causing unwanted cellular toxicity and downstream side effects.

Fatty acids are freed from the triglyceride state by a process called lipolysis. During this process, glycerol is removed from the triglycerides by lipases to release free fatty acids³¹. The free fatty acids are then broken down to acetyl-coA in the mitochondria in the presence of nicotinamide adenine dinucleotide and the reduced form of flavin adenine dinucleotide to generate energy in a reaction known as beta oxidation³¹. Many free fatty acids are key components of phospholipids, which stabilise the cell membranes of various cells including those

of the retina. These phospholipids are cleaved into several metabolites, such as 1-palmitoyl-2-oleoyl-glycerol, which consists of side-chains derived from palmitic acid and oleic acid³¹. Patients with AMD have demonstrated dysregulated levels of such fatty acids, which may contribute in the impairment of the retinal pigment epithelial cells seen in this disease³².

To determine the types of total fatty acids possibly implicated in LSF's impact on ARPE-19 cells, GC-MS was performed. We showed that LSF treatment increased the levels of *trans*- and *cis*-oleic acid and ETA. Oleic acid is one of the most abundant monosaturated fatty acids (MUFAs) of the omega-9

Figure 4. Lipid levels in **(A)** 5µM LSF or **(B)** 20µM LSF-treated ARPE-19 cells exposed to H_2O_2 . Four replicate samples across the treatment groups were compared with that of the untreated [UN] controls. Data in red and blue indicate an increase and decrease in lipid levels, respectively. Lipids highlighted in red boxes show statistically significant changes in levels between treated groups and untreated controls (p values < 0.05). [CE, cholesteryl ester; oPDA, oxo-phytodieneic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 5LSF, 5µM LSF; 20LSF, 20µM LSF; H_2O_2 , hydrogen peroxide; LSF, L-Sulforaphane].

Figure 5. Significant changes in lipid levels in 20µM LSF-treated ARPE-19 cells exposed to H_2O_2 . Four replicate LSF samples were compared with that of the untreated controls. Sample variation is depicted by error bars. The y-axis values are automatically generated as arbitrary units by the Agilent Mass Hunter Quantitative Analysis software. [CE, cholesteryl ester; oPDA, oxo-phytodieneic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; H_2O_2 , hydrogen peroxide; LSF, L-Sulforaphane].

fatty acid family, while common omega-3 PUFAs include ETA, eicosapentaenoic acid and docosahexaenoic acid, found in fish oil³³. These fatty acids contribute to several biological processes, including visual pathways signalling in the retina, anti-inflammatory properties and protection against metabolic diseases. The benefits of a high dietary intake of omega-3 and omega-9 fatty acids in alleviating the risk of AMD by about 30% to 40% and neovascularisation have been extensively reviewed by van Leeuwen *et al.* (2018)³⁰. The action of LSF appears to be cell-type specific. Pasko *et al.* (2018) revealed that the pro-apoptotic effect of LSF on hepatocellular carcinoma and colorectal cancer cell lines was correlated with increased levels of oleic acid found in the cancer cells³⁴. This is in contrast to our findings, where no toxicity was seen in LSF-treated ARPE-19 cell line despite increased oleic acid levels. The lack of harmful effects and the evident protective effects of LSF on human retinal cells shown here can be mirrored by findings from an association study that demonstrated a correlation between a high MUFA diet and significantly reduced risk of AMD³⁵. This protective effect of MUFAs against AMD may involve anti-atherogenic pathways, as discussed by Parekh *et al.* (2009)³⁵.

Many studies have shown that an increased dietary intake of the selected omega-3 PUFAs lowers the risk of dementia, improves cognition and aids age-related degenerative disorders³⁶. Connor *et al.* used a hypoxia-induced animal model of retinopathy to show that an omega-3 PUFA diet suppressed retinal expression of the inflammatory cytokine tumour necrosis factor (TNF)- α and macrophage-induced inflammatory responses in retinal cells^{10,37}. This anti-inflammatory phenomenon promoted a suppression of neovascularisation of comparable magnitude to that induced by VEGF inhibitors^{37,38}. Interestingly, AMD patients demonstrated lower levels of oleic acid and omega-3 PUFAs in their red blood cells compared to their age-matched healthy controls³². Furthermore, a good distribution of omega 3-PUFAs in the retina is said to be protective against photo-sensitised oxidation and peroxidation of lipids (e.g. 7-ketocholesterol) in the eyes of aging adults^{15,32}. Oxidised lipids can induce the migration and activation of retinal microglia into an M1 pro-inflammatory phenotype, which triggers the expression of pro-angiogenic cytokines and subsequent choroid neovascularisation seen in advanced AMD. Therefore, the findings from these reports support the potential use of LSF as a naturally-occurring enhancer of omega-3 levels in RPE cells to protect RPE cells from inflammation and abnormal neovascularisation observed in AMD patients and with possibly less risk of side effects caused by conventional VEGF inhibitors³⁹. The direct relationship between the action of LSF, omega-3 PUFAs and anti-oxidative pathways has yet to be elucidated but it is known that omega-3 PUFAs, when oxidised, can protect cells against free radical superoxide and H₂O₂ by activating the nuclear factor erythroid-derived-2 like-2 (*Nrf2*) pathway⁴⁰. It has been reported that ageing impairs *Nrf2* responses to oxidative stress⁴¹. As discussed in our recent publication, LSF acts as a potent *Nrf2* activator, which further promotes its use as a therapeutic agent in chronic inflammatory conditions such as AMD^{24,42}. Future studies arising from our GC-MS data may include investigations into the possible synergistic effects of LSF and omega-3 PUFA combination treatment

on the suppression of oxidative stress, neovascularisation and VEGF expression in RPE cells and choroid-derived endothelial cells.

To identify the lipid classes that are affected by LSF treatment of ARPE-19 cells, LC-MS was performed. In the presence of oxidative stress, LSF treatment decreased levels of PE lipids but increased levels of levels CE, oPDA and PC lipids. Lipofuscin, a type of pigment granule, accumulates in the ageing retina as a result of light-associated vitamin A recycling⁴³. A major component of lipofuscin is A2E, which has the capacity to destabilise cell membranes of RPE cells and compromise their viability. The creation of A2E within retinal cells involves condensation reactions between PE lipids and all-trans-retinal⁴⁴. The photo-oxidation of such lipids in RPE cells can be initiated via sensitisation of A2E, triggered by blue light exposure over time. Consequently, H₂O₂ is generated and complement is activated via C3-dependent pathways, leading to oxidative stress, inflammation and apoptosis⁴⁵. This supports the use of H₂O₂ as an ideal stimulant of both photo-oxidation and oxidative stress seen in the ageing retina of AMD patients and validates our *in vitro* model reported here. Other studies have shown that phytochemicals including anthocyanin and LSF can reduce A2E photo-oxidation and confer RPE cell protection by increasing expression of oxidative pathway phase II enzyme NAD(P)H:quinone reductase⁴⁶. This aligns with our previous findings where we showed that LSF treatment of ARPE-19 cells can confer protection against H₂O₂-induced oxidative stress by upregulating another phase II enzyme, GSTM1²⁴. In this present study, we demonstrate that LSF treatment of ARPE-19 cells in the presence of H₂O₂ can downregulate levels of PE lipids (i.e. PE 34:0 and PE 38:5). Since PE lipids are precursors of A2E, we propose that retinal cells experiencing oxidative stress can benefit from LSF treatment, since this compound can reduce PE levels and, consequently, a smaller amount of PE lipids is available for the biosynthesis of A2E, which may attenuate the risk of photo-oxidation leading to retinal cell death.

In patients with early AMD, pathological observations include the accumulation of drusen particles containing lipoproteins in the Bruch's membrane, accompanied by apoptosis of RPE cells. The RPE is responsible for controlling lipoprotein uptake into the retina and their distribution to photoreceptors for the replacement of shed membrane disks. These lipoproteins mainly consist of CEs but when these lipids are oxidised, they become cytotoxic to retinal cells⁴⁷. The levels of CEs can also be upregulated by oxidative stress stimuli, and treatment of ARPE-19 cells with lipoproteins containing oxidised lipids can increase levels of CEs consisting of oleic acid⁴⁷. Here, we report that LSF upregulates levels of CEs containing omega 6-PUFAs linoleic acid (18:2) and eicosadienoic acid (20:2) in the presence of H₂O₂. Since H₂O₂ is an oxidative stress stimulus, it is possible that the increased CE levels we observe in LSF-treated cells may be attributed, to some extent, to the exposure of cells to H₂O₂. It is noteworthy that omega 6-PUFAs are more prone to lipid peroxidation due to the increased risk of attacks to their double bonds by reactive oxygen species and because accumulation of peroxidised lipids in retinal cell membranes over time can trigger AMD progression³⁵. However, the relationship between LSF-induced mechanisms and

oxidised/peroxidised lipids is not well known. Hence, a future study stemming from this work may include evaluating the oxidation/peroxidation status of lipids in LSF-treated ARPE-19 cells using well-established assays.

The vast majority of phospholipids that make up the membranes of cells in the retina are PC lipids, with omega-3 PUFAs making up about 20% of the fatty acids in this lipid class⁴⁵. Perhaps, the upregulation of ETA fatty acids resulting from LSF treatment observed here is reflected in the elevated levels of PC 33:3 lipids. PC and CE lipids are commonly found in drusen particles but they also accumulate in the Bruch's membrane in normal healthy eyes throughout adulthood⁴⁸. Lipid accumulation in the Bruch's membrane eventually forms a "lipid wall" that prevents the normal exchange of oxygen and nutrients between the RPE and the choroid¹⁵. In addition, the higher the content of PC and CE lipids in the Bruch's membrane, the higher the risk of lipid peroxidation and oxidation, complement activation, inflammation and generation of toxic metabolites with age. If these lipids are retained at higher levels in the RPE cells, there is a lower tendency for lipids to be shed into the Bruch's membrane or accumulate in drusen particles; thus, lowering the risk of toxic metabolite production and apoptosis⁴⁸. Since LSF treatment can increase the levels of PCs and CEs in ARPE-19 cells in the current study, this suggests that this compound may have the potential to restore or maintain healthy levels of such lipids within the retinal cells by interfering with the biosynthesis or transportation of major drusen components. Genome-wide association studies have identified risk variants in genes (e.g. ATP-binding cassette transporter, cholesteryl ester transfer protein, apolipoprotein E4, etc.) that regulate lipid metabolism and transportation that may confer a protective status against AMD pathophysiology⁴⁹. Thus, investigating the changes in the expression of such genes may help to further dissect the lipid pathways responsible for the LSF-mediated regeneration of RPE cells under oxidative stress conditions.

Lipids are major components of plant stress hormones. An example is oPDA, which is the key precursor of the oxylipin stress hormone, jasmonate. oPDA lipids can activate genes involved in oxidative stress pathways and a correlation between oPDA signalling and decreased hydrogen peroxide levels has been reported in plants^{50,51}. Interestingly, administering the stress hormone jasmonate to broccoli sprouts increased levels of LSF⁵². This suggests that LSF may be a by-product of a compensatory mechanism found in plants that maintains cellular redox homeostasis in stressful environments. Additionally, oPDA treatment of human neuroblastoma cells can prevent harmful effects from oxidative stress and apoptosis by activating the Nrf2 pathway⁵³. The redox activity of oPDA is also evidenced in its capacity to regulate the expression of *GST* genes⁵⁴. Taken together, oPDA behaves as a Nrf2 activator like LSF. Therefore, LSF's antioxidant effects on ARPE-19 cells shown here may either involve: 1) independent activation of Nrf2; 2) an upregulation of oPDA, which in turn triggers the Nrf2 pathway;

or 3) a synergistic activation of this pathway mediated by the combined action of LSF and oPDA signalling.

Although we attempt to discuss the possible relationship between the observations arisen from the total fatty acid analysis (GC-MS) and lipidomic data (LC-MS), drawing a correlation between fatty acid data and the LC-MS lipid profile in this study proved to be challenging, since the methods used here could not explicitly identify the source of the fatty acids (i.e. free/circulating or conjugated to lipids) implicated in LSF's protection of the ARPE-19 cell line. Despite this limitation, this study revealed the ability of LSF to alter levels of selected fatty acids and lipid classes involved in mechanisms that can promote AMD processes in human RPE cells.

In conclusion, we propose that RPE cells at risk of apoptosis can be pre-conditioned with LSF to regulate levels of selected fatty acids and lipids known to be implicated in downstream pathways of photo-oxidation, inflammation and oxidative stress for the generation of a protective state against the ageing process and AMD progression. This work warrants future investigations, such as trialling LSF treatment in co-culture models of ARPE-19 and choroid-derived cells, and animal models of AMD. Performing high throughput transcriptomics methods will also help to identify key genes that mediate LSF's effects on fatty acid and lipid metabolism, biosynthesis and translocation in RPE cells under AMD-like pathological conditions⁵⁵. These further studies will facilitate the design of targeted therapies that can be co-administered with LSF for the prevention of AMD progression.

Data availability

Underlying data

Harvard Dataverse: Lipidomics reveal the protective effects of a vegetable-derived isothiocyanate against retinal degeneration. <https://doi.org/10.7910/DVN/C9VCBX>²⁸

This project contains the following underlying data:

- GCMS Fatty Acid Analysis Data.tab (raw fatty acid analysis data)
- LCMS Lipid Analysis Data.tab (raw lipid analysis data)
- MTS Raw Data_Kwa.tab (raw cell proliferation assay data)

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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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.
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 cells¹². 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 cycle³.
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?
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. 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?

Other concerns:

1. In the figure legends, authors should clarify the treatment conditions, although the information has been mentioned somewhere else.
2. In fig. 4, authors should list lipid species following the order of lipid classification, but not in the current mixed pattern.
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.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell biology, lipid metabolism and lipidomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Feb 2020

Faith Kwa, Royal Melbourne Institute of Technology University, Bundoora, Australia

Dear Reviewers 1 and 2,

Thank you for the time you have taken to provide comments and suggestions. Please see our rebuttal to your comments below or via [this document](#).

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 phosphatidylcholine, 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 rationale 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 activates 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".

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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). [Here](#) 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 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 (5 μ M, 10 μ M, 20 μ M and/or 30 μ M 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 20 μ M 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 200 μM H₂O₂ in Figure 2 of this previous paper. In addition, other studies have also used H₂O₂ concentrations of 100 μM 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>). 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-H2O2 groups and LSF+H2O2 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.

Competing Interests: No competing interests were disclosed.

Reviewer Report 19 November 2019

<https://doi.org/10.5256/f1000research.22941.r55259>

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Chris Barlow

Monash University, Melbourne, Vic, Australia

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 phosphatidylcholine, 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 rationale for the mechanism of protection.

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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.

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Are all the source data underlying the results available to ensure full reproducibility?

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Are the conclusions drawn adequately supported by the results?

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Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 13 Feb 2020

Faith Kwa, Royal Melbourne Institute of Technology University, Bundoora, Australia

Dear Reviewers 1 and 2,

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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 activates 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".

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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). [Here](#) 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 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 (5 μ M, 10 μ M, 20 μ M and/or 30 μ M 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 ([Page 27 of 37](https://iopscience.iop.org/article/10.1088/1752-</p></div><div data-bbox=)

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>). 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-H₂O₂ groups and LSF+H₂O₂ 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.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 08 August 2019

<https://doi.org/10.5256/f1000research.21489.r52230>

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Chris Barlow

Monash University, Melbourne, Vic, Australia

Overview:

This paper seeks to build on previous work published by this group which has demonstrated that pre-treatment of ARPE-19 cells with L-Sulforaphane (LSF) leads to protection against oxidative damage as assessed using a cell proliferation assay. In particular, here the authors seek to identify any changes in fatty acids and more complex lipids following pre-treatment with LSF and then H₂O₂. The authors report that no fatty acids (total fatty acid analysis) were significantly altered under conditions of oxidative stress. However, they report extremely large fold-changes for *cis*-oleic, *trans*-oleic and eicosatrienoic acids upon treatment with LSF in the absence of oxidative stress. For the lipidomic portion of the study the authors report that several lipids showed statistically significant differences under oxidative stress following pre-treatment with LSF. Unfortunately, the paper suffers from problems associated with data analysis making it unsuitable for indexing at this time.

Fatty acid analysis:

On page 5 the authors report very large fold-changes on the order of 10⁷ to 10⁹ for oleic (*cis* and *trans*) and ETA upon treatment with LSF in the absence of oxidative stress. Upon inspection of the underlying data I found that for all four of the "Untreated -" samples, the intensity for these fatty acids was 1x10⁻⁷ for both *cis*- and *trans*- oleic acid and 1x10⁻⁹ for ETA (see table [here](#)). It would appear that these fatty acids were not detected in these samples and subsequently values have been imputed automatically. While imputation is common in metabolomic analysis it is not valid to report a fold-change or p-value where all values for the control group have been imputed.

Lipidomic Analysis:

Here the authors report that several lipids were significantly different (p < 0.001) upon pre-treatment with 20 µM LSF relative to the untreated controls under conditions of oxidative stress.

Careful examination of the underlying data suggests that a couple of errors may have been made in data processing. Specifically, the fold-change for PC 33:3 was 1.946 and not 2.33 as reported. Similarly, PE 34:0 and PE 38:5 had fold-changes of 0.723 and 0.684 respectively and not the reported 0.394 and 0.292 reported in the paper. I have reproduced the relevant data from the underlying data in the table [here](#) for clarity. I'm also confused about the statistical analysis. Using a two-tailed t-test none of the lipids reported as significant had a p-value < 0.001 as reported in the text, more generally it is unclear how the authors have dealt with the issue of multiple comparisons. The experimental section of lists several statistical tests but it is unclear which test was used for each analysis. Greater clarification as to how the statistical analysis was performed is necessary.

Experimental Design:

The authors have used $n = 4$ for each group. While $n = 4$ is probably fine for using assays with a single metric such as the proliferation assay it is probably insufficient for lipidomic analysis. Indeed, the lipid data seems to be highly variable. For example, the total amount of lipid seems to vary substantially more than I would have expected. A crude measure of this variation is reflected in the median intensity of all the lipid measured for each sample which I have plotted [here](#). From the experimental section on page 4 it appears that cells were seeded at a density of 1.5×10^6 before being conditioned as described in the "Cell treatment prior to analysis" section. If I'm reading this correctly then there was no adjustment for the number of cells following the conditioning but immediately before lipid extraction. Were an equivalent number of cells extracted (as opposed to seeded) in each sample, and if so, do the authors have any insight into why such a high degree of variability was observed in the lipidomics data? Similarly, I assume that treatment with hydrogen peroxide resulted in some cell death, what steps were taken to ensure that dead cells were not being extracted along with the live cells? Finally, I would suggest that some important comparisons have not been analysed. Presumably the hypothesis is that LSF treatment leads to changes in lipid profile which are then somehow protective against oxidative damage. I would suggest that the authors need to compare the lipid profiles of the LSF 20 μM – against the untreated – group. This should capture lipidomic differences associated with LSF treatment without the confounding effect of H_2O_2 treatment.

Additional notes and suggestions:

- On page 3 the authors state that all five major fatty acids in the human retina are long chain polyunsaturated fatty acids (PUFAs). This is incorrect, docosahexaenoic and arachidonic acids are PUFAs, oleic acid is monounsaturated and stearic and palmitic are saturated.
- Figures 2 and 4: The annotations are too small to read. The graphs need to be re-drawn with a larger font.
- Figure 3 and 5: The authors state that the y-axis values are automatically generated as arbitrary units by the software used. The authors need to clearly state how the data is being processed. (In this case it seems likely that the data is being $\log_2$ transformed.) Is a box and whisker plot appropriate for four data points?
- Page 5: The text here indicates that lipid quantitation was performed using external calibration curves however no concentrations are given throughout the text or underlying data.

- In the "LCMS Lipid Analysis Data.xlsx" file from the underlying data, five lipids are included in duplicate; PC 31:1, PC 33:3, PC 33:2, PC 34:4 and PC 37:4.

Conclusion:

Due to the problems with the data analysis outlined above I'm unable to recommend this paper for indexing. If the authors are able to address these problems however, this paper should be considered as a fresh submission.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 04 Oct 2019

Faith Kwa, Royal Melbourne Institute of Technology University, Bundoora, Australia

Rebuttal to Reviewer 1 Chris Barlow's comments. Please find our response below.

Overview:

This paper seeks to build on previous work published by this group which has demonstrated that pre-treatment of ARPE-19 cells with L-Sulforaphane (LSF) leads to protection against oxidative damage as assessed using a cell proliferation assay. In particular, here the authors seek to identify any changes in fatty acids and more complex lipids following pre-treatment with LSF and then H₂O₂. The authors report that no fatty acids (total fatty acid analysis) were significantly altered under conditions of oxidative stress.

However, they report extremely large fold-changes for cis-oleic, trans-oleic and eicosatrienoic acids upon treatment with LSF in the absence of oxidative stress. For the lipidomic portion of the study the authors report that several lipids showed statistically significant differences under oxidative stress following pre-treatment with LSF. Unfortunately, the paper suffers from problems associated with data analysis making it unsuitable for indexing at this time.

Response: We thank Reviewer 1's concise summary of the work presented in our manuscript. However, we have to respectfully disagree that the data analysis using one-way ANOVA and paired t-test is not appropriate for the nature of the work here. The data was always analysed at the significance level of $p < 0.05$ but where p values generated were indeed less than 0.001, we have indicated this as $p < 0.001$. We have now clearly indicated a statistically significant level of $p < 0.05$ in the Methods section. This significance level and both statistical tests used in the fatty acid and lipid data here are standard statistical packages recommended by MetaboAnalyst Version 2 which we have already referenced in Reference 27. For the fatty acids where fold-changes for cis-oleic, trans-oleic and eicosatrienoic acids upon treatment with LSF in the absence of oxidative stress were reported, the untreated groups had an amount of fatty acids that were below the level of detection and hence for the purpose of performing univariate statistics, the missing values were imputed in alignment with common practice in metabolomics analysis as acknowledged by the Reviewer below. Taking the reviewer's comments into consideration, we have edited the Result section to indicate that the levels of these fatty acids were only detected in the respective LSF treatment groups but not in the untreated controls. We have also 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.

On page 5 the authors report very large fold-changes on the order of 107 to 109 for oleic (cis and trans) and ETA upon treatment with LSF in the absence of oxidative stress. Upon inspection of the underlying data I found that for all four of the "Untreated -" samples, the intensity for these fatty acids was 1×10^{-7} for both cis- and trans- oleic acid and 1×10^{-9} for ETA (see table here). It would appear that these fatty acids were not detected in these samples and subsequently values have been imputed automatically. While imputation is common in metabolomic analysis it is not valid to report a fold-change or p-value where all values for the control group have been imputed.

Response: Kindly see our response to the previous comment.

Lipidomic Analysis:

Here the authors report that several lipids were significantly different ($p < 0.001$) upon pre-treatment with 20 μM LSF relative to the untreated controls under conditions of oxidative stress. Careful examination of the underlying data suggests that a couple of errors may have been made in data processing. Specifically, the fold-change for PC 33:3 was 1.946 and not 2.33 as reported. Similarly, PE 34:0 and PE 38:5 had fold-changes of 0.723 and 0.684 respectively and not the reported 0.394 and 0.292 reported in the paper. I have reproduced

the relevant data from the underlying data in the table here for clarity. I'm also confused about the statistical analysis. Using a two-tailed t-test none of the lipids reported as significant had a p-value < 0.001 as reported in the text, more generally it is unclear how the authors have dealt with the issue of multiple comparisons. The experimental section of lists several statistical tests but it is unclear which test was used for each analysis. Greater clarification as to how the statistical analysis was performed is necessary.

Response: We thank Reviewer 1 for pointing out the typographical errors which have been amended in the revised version of the manuscript. Regarding the statistical analyses, we used one-way ANOVA to analyse the GCMS and LCMS data presented in the heat maps that illustrates how the expression of fatty acids or lipids differ with each treatment or oxidative stress/normal conditions. Therefore, a multiple comparison was made determine the effect of LSF treatment and oxidative stress across the various groups. In contrast, a paired t test was used to analyse the GCMS and LCMS data represented in the box plots. This was a direct comparison between the fold change levels seen in the untreated control and those in the groups treated with 20 μ M LSF. We did not use the two-tailed t-test. We would also like to clarify that we used the statistical significance level of $p < 0.05$ and not $p < 0.001$ in MetaboAnalyst Version 2. The revised manuscript has been corrected to indicate where p values are less than 0.05 instead of 0.001.

Experimental Design:

The authors have used $n = 4$ for each group. While $n = 4$ is probably fine for using assays with a single metric such as the proliferation assay it is probably insufficient for lipidomic analysis. Indeed, the lipid data seems to be highly variable. For example, the total amount of lipid seems to vary substantially more than I would have expected. A crude measure of this variation is reflected in the median intensity of all the lipid measured for each sample which I have plotted here.

Response: Due to the complexity of the study, it is challenging to generate a higher number of replicates for this study. A minimum of 1.5 million cells were seeded for each of the four replicates per control and treatment groups (i.e. a minimum of 36 million cells used in the metabolomics investigations). There are many papers reporting lipidomic/ LCMS studies which analyse data from less than 4 repeat experiments. These include recent articles published in Q1 journals such as Oncology Reports and PLOS Biology. Examples can be found in the links below:

<https://www.spandidos-publications.com/10.3892/or.2018.6510>

<https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.2002214>

From the experimental section on page 4 it appears that cells were seeded at a density of 1.5×10^6 before being conditioned as described in the "Cell treatment prior to analysis" section. If I'm reading this correctly then there was no adjustment for the number of cells following the conditioning but immediately before lipid extraction. Were an equivalent number of cells extracted (as opposed to seeded) in each sample, and if so, do the authors have any insight into why such a high degree of variability was observed in the lipidomics

data? Similarly, I assume that treatment with hydrogen peroxide resulted in some cell death, what steps were taken to ensure that dead cells were not being extracted along with the live cells?

Response: Although not apparent in our Methods section of the manuscript, during cell harvesting prior to lipid extraction, we washed the wells containing the adherent cells with PBS (pH 7.4) to remove any dead cells and cell debris. Following trypsinisation of the adherent cells per well, we performed a live cell count using the trypan blue exclusion method. Furthermore, the data has been normalised to both the number of cells per sample and the median of the reverse phase peak area response using the MetaboAnalyst Software Version 2 which will reduce any impact of variation by any differences in cell numbers. We have added these details in the revised version of the manuscript.

Finally, I would suggest that some important comparisons have not been analysed. Presumably the hypothesis is that LSF treatment leads to changes in lipid profile which are then somehow protective against oxidative damage. I would suggest that the authors need to compare the lipid profiles of the LSF 20 μ M – against the untreated – group. This should capture lipidomic differences associated with LSF treatment without the confounding effect of H₂O₂ treatment.

Response: We thank Reviewer 1 for his recommendation. We have indeed compared fatty acid and lipid levels between LSF 20 μ M and untreated groups in the presence and absence of H₂O₂. This is already stated in the manuscript in the first line of the relevant paragraph: "In the absence of oxidative stress, no changes in lipid levels between LSF-treated cells and the untreated control were reported (all *p* values > 0.05; see *Underlying data*)". However, we only had presented figures where significant differences were established using the statistical tests described.

Additional notes and suggestions: •On page 3 the authors state that all five major fatty acids in the human retina are long chain polyunsaturated fatty acids (PUFAs). This is incorrect, docosahexaenoic and arachidonic acids are PUFAs, oleic acid is monounsaturated and stearic and palmitic are saturated.

Response: We have amended this statement to "There are five major fatty acids in the human retina, namely, docosahexaenoic acid (DHA), arachidonic acid (ACA), stearic acid, oleic acid and palmitic acid. Both DHA and ACA are classified as long chain polyunsaturated fatty acids (LC-PUFAs)."

•Figures 2 and 4: The annotations are too small to read. The graphs need to be re-drawn with a larger font.

Response: We have enlarged the font in these figures in the revised version of the manuscript.

•Figure 3 and 5: The authors state that the y-axis values are automatically generated as arbitrary units by the software used. The authors need to clearly state how the data is being processed. (In this case it seems likely that the data is being log₂ transformed.) Is a box and

whisker plot appropriate for four data points?

Response: All the data has been normalised to the median of the reverse phase peak area response and log2 transformed and number of cells per sample. An auto-scale has also been applied. These statements have been added to the Methods section of the revised manuscript. A box and whisker plot is one of the standard ways to present data generated by Metaboanalyst 2.0.

•Page 5: The text here indicates that lipid quantitation was performed using external calibration curves however no concentrations are given throughout the text or underlying data.

Response: All the figures were generated using reverse phase peak area response of each lipid species rather than the absolute concentration. We did not have standards for some of the lipid classes and therefore, to be consistent, we have used such responses to make comparison between the untreated and treated groups. 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 concentrations”.

•In the “LCMS Lipid Analysis Data.xlsx” file from the underlying data, five lipids are included in duplicate; PC 31:1, PC 33:3, PC 33:2, PC 34:4 and PC 37:4.

Response: We have removed the duplicated columns and reuploaded the underlying data onto the Version 2 of the Harvard Dataverse link.

Conclusion:

Due to the problems with the data analysis outlined above I’m unable to recommend this paper for indexing. If the authors are able to address these problems however, this paper should be considered as a fresh submission.

Response: We believe that our revised manuscript following the inclusion of the recommended edits deserves another peer-review and approval for publication in F1000 Research. The data has been statistically validated and does support the conclusions made in this manuscript.

Competing Interests: No competing interests were disclosed.

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