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Walnuts change lipoprotein composition suppressing TNFa-stimulated cytokine production by diabetic adipocyte

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Abstract

Walnut consumption can provide both vascular and metabolic health benefits, and walnut-induced changes in lipoprotein particle chemical payloads may be responsible for these health benefits. To explore this possibility with a focus on metabolic health, this study investigated the impact of walnut consumption on lipoprotein lipid composition and changes in LDL anti-inflammatory properties, as reported by inflamed adipocyte. Hypercholesterolemic, postmenopausal females were treated with 40 g/day (i.e. 1.6 servings/day; n =15) of walnuts for 4 weeks. Fatty acids and their oxygenated metabolites, i.e. oxylipins, were quantified in isolated lipoproteins. Human primary adipocytes were exposed to LDL and TNFa-stimulated adipokine production was measured. Walnut treatment elevated α-linolenic acid and its epoxides in all lipoproteins and depleted mid-chain alcohols in VLDL and LDL, but not HDL. Walnuts also reduced TNFa-induced diabetic adipocyte production of IL-6 (−48% p =0.0006) and IL-8 (−30%p =0.01), changes inversely correlated with levels of α-linolenic acid-derived epoxides but not α-linolenic acid itself.In conclusion, modest walnut consumption can alter lipoprotein lipid profiles and enhance their ability to inhibit TNFa-dependent pro-inflammatory responses in human diabetic primary adipocytes. Moreover, this study suggests the oxylipins, rather than the parent fatty acids, mediate LDL action of adipocytes.

Keywords

adipocytes; LDL; diet; lipid mediators; dietary lipids; lipoprotein metabolism

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1. Introduction:

The consumption of walnuts and other tree nuts has been associated with a lower risk for cardiovascular disease [1]. Specifically, walnut intake has been associated with lower type-II diabetes risk [2], while tree nuts can lower LDL cholesterol and triglycerides [1, 3] and attenuate endothelial dysfunctions induced by hypercholesterolemia [4] and high-fat meals [5]. In subjects with hypercholesterolemia, walnut interventions can also decrease the production of the pro-inflammatory cytokines interleukin-6 (IL-6), IL-1b and TNFa by peripheral blood mononuclear cells [6]. Adipose inflammation is a significant consequence of obesity that negatively impacts tissue glucose handling and insulin sensitivity [7]. Importantly, elevated oxidized LDL (ox-LDL) are associated with type II diabetes [8], adipocytes actively take up ox-LDL [9, 10], the plasma levels of ox-LDL has been negatively correlated with plasma levels of the adipose generated cytokine adiponectin in humans [11] and walnut consumption can reduce LDL oxidation [4, 12, 13]. Walnuts are a rich source of linoleic acid (LA), α-linolenic acid (aLA) [14] and numerous bioactive phenolics [1, 15–19]. While each of these components have bioactive potential, it has been postulated that aLA, an omega-3 fatty acid, is a major factor responsible for the anti-inflammatory effects in a manner analogous to the anti-inflammatory effects of fish oil being attributed to the long chain omega-3 fatty acids [1].

The bioactivity of PUFAs can be associated with their direct action, as well as through the action of their metabolic products. Major products of PUFA metabolism are the oxylipins, a superclass of oxygenated metabolites. These compounds are formed by: cyclooxygenases (COX) – producing prostaglandins and thromboxanes; lipoxygenases (LOX) – producing hydroperoxides and their associated downstream products (e.g. mid-chain alcohols); cytochrome P450 (CYP) – producing epoxides and omega-hydroxides; autooxidation, producing peroxide associated products including isoprostanes and certain mid-chain alcohols [20, 21]. The structures of representative alkaline-stable arachidonic acid-derived oxylipins are presented in the Figure 1. Oxylipins are the primary mediators of PUFA action, regulating processes including inflammation, glucose metabolism and cardiovascular homeostasis (broadly reviewed in [22–29]). Different classes of oxylipins manifest distinct properties, and these effects are influenced by the parent fatty acid from which they are derived [22]. For instance, epoxides are generally anti-inflammatory [23, 24] and can suppress the production of pro-inflammatory cytokines [25, 26]. Moreover, elevated plasma concentrations of epoxides have been linked with better cardiovascular outcomes in diverse animal models [26]. On the other hand, many mid-chain alcohols are pro-inflammatory [27], with links to low-grade systemic inflammation and pro-inflammatory cytokine production in adipose tissue [30]. While much is known regarding the action of these compounds in general, their sources and mechanisms of production and delivery in vivo are as of yet poorly understood.

While oxylipins are generally considered to be autocrine and paracrine in nature, they are transported through plasma in esterified forms within lipoprotein particles at high abundances [31, 32]. The trafficking of these particle associated metabolites, and their impact on lipoprotein-metabolizing tissues are not well understood [28, 29]. Clinical trials have demonstrated that PUFA intake influences the oxylipin composition of lipoproteins.

Supplementation with marine omega-3 PUFAs results in increased docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)-derived oxylipins at the expense of arachidonic acid (AA) metabolites [31–33]. Moreover, marine-PUFAs induce changes in oxylipin composition differently among different lipoproteins classes [31]. While the effect of fish oil supplementation on the oxylipin profile has been the subject of several studies [31–33], to the best of our knowledge, the impact of tree nuts on the lipoprotein esterified oxylipin profiles has not been reported.

Previously, in a randomized controlled trial walnut treatment improved endothelial dysfunction in hyper-triglyceridemic women, an effect associated with increases in plasma non-esterified fatty acid epoxides [34]. In the current study, using samples from the study above, we investigated walnut-induced changes in the lipoprotein oxylipin and fatty acids composition. Given the known pathological association of ox-LDL, we also tested the impact of walnut-induced changes to LDL on human primary adipocytes, a model lipoprotein-metabolizing tissue which actively takes up LDL and ox-LDL [9, 10]. The production of pro- and anti-inflammatory adipokines were assessed as the primary outcome. Since walnuts have been reported to have beneficial effect in pathological conditions associated with the metabolic syndrome [4, 5, 34] and elevated ox-LDL [4, 12, 13], adipocytes with diabetic and non-diabetic phenotypes were used. Moreover, since low-grade systemic inflammation accompanies the development of obesity and diabetes and is associated with elevated pro-inflammatory cytokine production, cell culture experiments were performed with or without TNFa, as a surrogate for an inflammatory environment.

Previous studies have clearly demonstrated that dietary PUFAs can improve select health outcomes [32, 35, 36], and alter lipoprotein particles composition [32]. Moreover, lipoprotein composition has been associated with diverse physiological outcomes [37]. In the present study, we used a dietary intervention to link and examine the food-lipoprotein-inflammatory physiology axis.

2.0 Materials and methods:

2.1 Study design and analysis strategy:

This study is an ancillary study of a randomized controlled trial on the impact of walnut consumption on vascular function in hypercholesterolemic postmenopausal women [34] after 4 weeks of 40 g/day (i.e. 1.6 servings/day; n =18) or 5 g/day (i.e. 0.2 servings/day;n =12). In a subset of individuals (40 g/day, n =15 and 5 g/day n =5) samples were probed for potential changes in plasma lipids and lipoproteins (the baseline subject characteristics are presented in the Table S1). The study was registered with ClinincalTrials.gov (NCT01235390) with the study protocol approved by the Institutional Review Board of the University of California, Davis. Subject inclusion and exclusion criteria and the feeding protocol were previously described [34]. The original study observed improvements in microvascular function with the intervention of 4 weeks of 40 g/day of walnuts compared to 5 g/day. The 5 g/day level was chosen in order to provide a “placebo” comparative group that was predicted to not improve the primary outcome of microvascular function. However, in the current subset of individuals, 5 g/day of walnut showed a lipoprotein lipidomic response when analyzed as a changed from baseline (Figure S1 and Table S2). Moreover, the

low and variable number of subject in the treatment groups significantly limits the power of the analysis and the placebo-controlled approach does not allow for control of the subject effect. For those reasons the current study has been analyzed using a change from baseline approach.

2.2 Lipoprotein isolation:

Lipoproteins were isolated by size exclusion chromatography as described by [38] using a 10 × 300 cm Superose 6 (GE Healthcare; Uppsala Sweden). The isocratic mobile phase, 20 mM TBS + 0.1% EDTA + 0.1% BHT, was degassed with helium and run at 0.5 mL/min. A 200 μL aliquot of plasma were injected and 500 μL fractions were collected as shown in Figure S2 for: VLDL, fractions 16–18 (3 total; 8.0 to 9.0 mL); LDL, fractions 23–26 (4 total; 11.5 to 13.0 mL); HDL, fractions 31–34 (4 total; 15.5 to 17 mL).

2.3 Lipoprotein fatty acids and oxylipins analysis:

Total fatty acids and alkaline stable oxylipins were isolated using a liquid-liquid extraction and quantified using GC-MS for fatty acids and ultra-performance LC-MS/MS for oxylipins. Lipids were isolated using a liquid-liquid extraction with cyclohexane/isopropanol/ ammonium acetate after enrichment with a suite of deuterated and rare compounds used as analytical surrogates, [39, 40]. Fatty acids were transformed into methyl esters using methanolic transesterification. The fatty acid methyl esters were separated on 30 m × 0.25 mm, 0.25 μm DB-225 ms (Agilent Technologies; Santa Clara, CA), detected with a 5973 A mass selective detector (Agilent) using electron impact ionization and selected ion monitoring, and quantified against authentic standards. Results were corrected for the recoveries of analytical surrogates as previously described [40]. Esterified oxylipins were transformed into oxylipin free acids by base hydrolysis, and isolated by subsequent hydrophilic/lipophilic interaction solid phase extraction. Alkaline stable oxylipins were separated on a 2.1 × 150, 1.7_μm Acquity BEH column (Waters Corp, Milford MA) and detected by negative mode electrospray ionization and multi-reaction monitoring on a Sciex 4000 QTRAP [40] . Concentrations were calibrated using analytical standards as previously reported [40, 41].

2.4 Adipocyte culture:

Primary human diabetic and non-diabetic subcutaneous pre-adipocytes as well as culture media and hormones used for differentiation were purchased from Lonza (Basel, Switzerland). Both diabetic and non-diabetic cells were purchased as a single batch. Pre-adipocytes were grown in a 96 well plate format in PGM-2 Basal Medium (Lonza; Basel, Switzerland) supplemented with 10% FBS,50 μg/mL of gentamicin and 37 ng/mL of amphotericin. Confluent cells were differentiated for 14 days in a PGM-2 Basal Medium (Lonza; Basel, Switzerland) supplemented with differentiation hormone mixture (PGM™−2 SingleQuots™; Lonza; Basel, Switzerland) containing insulin, indomethacin, 3-isobutyl-1-methylxanthine and dexamethasone. All assays were performed on differentiated cells, after 6 h of treatment with 30 μg/mL of LDL (based on the protein content) from the walnut study (n =5 for each treatment group) [34] or phosphate buffered saline vehicle, in the presence or absence of 3 ng/mL TNFa [42]. Each replication in the cell culture experiment represents LDL isolated from a different subject.

2.5 Measurement of adipokines levels:

The concentrations of IL-6, IL-8, MCP-1, PAI-1, IL-1b, leptin, adiponectin, and resistin were assessed in culture media using a Luminex multiplexing platform (ThermoFisher; Waltham, MA) with kits purchased from EMD Millipore.

2.6 Generation of DIL-LDL and LDL uptake assay:

Isolated LDL were combined with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DIL; Molecular Probes, Eugene, OR, USA) according to [43]. Generated Dil-LDL (30 μg/ml) were incorporated into culture media without FBS and applied on cells for 1 h. Next, the culture media was discarded and the amount of DIL dye in the culture dish was measured at 580 nm using Synergy MX plate reader (Biotek; Winooski, VT, USA).

2.7 Intracellular lipid accumulation:

Intracellular lipid accumulation was measured using Oil Red O staining (Lonza; Basel, Switzerland). Briefly, cells were fixed with 10% formalin for 30 min. Next, the formalin was removed and the cells were stained with 2 mg/mL Oil Red O in 60% isopropanol for 1 hour. Further excess Oil Red O was discarded and cells washed three times with distilled water. Bound Oil Red O was measured at 500 nm using Synergy MX plate reader (Biotek; Winooski, VT, USA).

2.8 Statistical analysis:

All statistical tests were performed using JMP Pro 13.1 (JMP, SAS institute, Carry, NC). Prior to analysis outliers were removed using the robust Huber M test and missing data were imputed using multivariate normal imputation. Data imputation was performed to facilitate multivariate data analysis and did not significantly change univariate analysis results like t-tests. Both treatment groups (5 g/day and 40 g/day) were analyzed independently as change from the baseline. Treatment effects on lipoprotein composition were evaluated by t-test, multivariate ANOVA (MANOVA) and partial least square discriminant analysis (PLS-DA). The t-test was performed separately for each metabolite with the visit as a fixed effect, adjusted for subject effect. For MANOVAs, variables were auto scaled [44] and grouped by hierarchical clustering using the Ward minimal variance method. MANOVA model was built with the metabolite in each cluster as a fixed effect and subject as a random effect. MANOVA and t-test p-values cutoffs were corrected for multiple comparisons using the false discovery rate (FDR) approach of Benjamini and Hochberg [45] with q =0.1. PLA-DA was used to facilitate visualization of the treatment effects across all lipoprotein particles. PLS-DA model was built using the nonlinear iterative partial least squares algorithm with K-Fold variation method (k =7) and included 204 variables (i.e. 68 detected fatty acids and oxylipins across the HDL, LDL and VLDL). Prior to the PLS-DA the paired data for each subject at baseline and after 4 weeks of treatment were scaled such that their means were centered on zero (i.e. subject centered) to reduce the subject effect and emphasizes the treatment effect using the following equations:

$$\text{baseline}_{\text{Subjected Centered}} = \text{baseline} - \left(\frac{\text{baseline} + \text{final}}{2}\right)$$

$$\text{final}_{\text{Subjected Centered}} = \text{final} - \left(\frac{\text{baseline} + \text{final}}{2}\right)$$

Low density lipoprotein-dependent effects on adipocyte adipokine production were evaluated using MANOVA. Prior to analysis, variables were clustered as above and a Tukey post-hoc test allowed multiple comparisons adjustment. Correlations between culture media adipokine levels and LDL metabolite concentrations were analyzed separately for TNFa exposed and non-exposed cells using either Pearson's or Spearman's analysis as indicated on associated figures. Data from both treatments as well as final and baseline time points were used for the analysis.

3 Results:

3.1 Walnuts impact lipoprotein composition:

While 40 g/day of walnuts did not alter the lipoproteins triglycerides, cholesterol, phospholipids (Figure S2) or protein levels, it significantly and differentially altered the lipoprotein fatty acid and oxylipin compositions. These changes are shown in aggregate in Figure 2, and individually in tabular form for HDL (Table 1 and Table S3a), LDL (Table 2 and Table S3b) and VLDL (Table 3 and Table S3c). Of the fatty acids, aLA (C18:3n3), and LA (C18:2n6) were increased in all lipoproteins, while C22:5n3 was increased in both LDL and VLDL and C18:3n6 was increased in VLDL but decreased in LDL. The saturated fatty acids stearic acid (C18:0) and palmitic acid (C16:0) were elevated only in VLDL, while the monounsaturated palmitoleic acid (C16:1n7) and oleic acid (C18:1n9) were decreased only in LDL. Of the oxylipins, mid-chain alcohols from all parent fatty acids, apart from aLA, were decreased in VLDL (VLDL clusters 1, 4 and 7 in the Table 3). On the other hand, in LDL only AA and DGLA-derived mid-chain alcohols decreased (LDL cluster 3 in the Table 2). Epoxides of α-linolenic acid were increased across all lipoproteins (HDL cluster 1 in the Table 1, LDL cluster 1 in the Table 2 and VLDL cluster 3 in the Table 3), whereas walnuts increased AA and DHA derived epoxides specifically in the HDL compartment (Figure 3). It is important to mention that while walnut treatment did not affect fasting complex lipid levels, it significantly decreased postprandial cholesterol (−14%, $p = 0.0007$) and phospholipids (−16%, $p = 0.009$) in LDL (Data not shown).

3.2 Walnuts consumption changes functionality of low density lipoproteins in the context of adipocyte response to TNFa:

Human primary adipocytes with and without TNFa stimulation, provided a model system to probe the impact of lipoprotein particle structure on inflammatory signaling. LDL isolated after 4 weeks of 40 g/day walnut consumption prevented the TNFa stimulated IL-6 and IL-8 production in human primary diabetic adipocytes (HPDA) (Figure 4A). Notably, the non-diabetic primary adipocytes did not show a TNFa-stimulated change in IL-6 and IL-8 (data not shown). HPDA cells treated with TNFa, in the presence of LDL from the baseline (week 0), increased the levels of IL-6 and IL-8 in the media (48% and 30% respectively). LDL isolated after walnut treatment counteracted this effect, lowering IL-6 and IL-8 levels to that of uninflamed cells. Walnut treatment did not affect other measured adipokines (i.e.

adiponectin, resistin, leptin, IL-1b, MCP-1 and PAI-1), and neither walnut treatment nor TNFa effected LDL uptake or the adipocyte lipid accumulation (data not shown).

3.3 LDL oxylipins, but not their parent fatty acids, are strongly correlated with the anti-inflammatory effects:

To investigate associations between anti-inflammatory effects and altered fatty acids and their metabolites in LDL, correlations between pro-inflammatory adipokines, parent fatty acids and the sum of the metabolite classes derived from those lipids were performed. This analysis allowed the visualization of changes in the correlation structure between these variables in cells with or without TNFa stimulation (Figure 4B).

In non-inflamed adipocytes, there was no correlation between the levels of IL-6, IL-8 and aLA, LA, AA and the sum of their corresponding epoxides, diols and alcohols. On the other hand, in the inflamed adipocytes (n =14), aLA-derived epoxides (EpODEs) showed negative correlations with IL-6 ($r = -0.66$; $p = 0.0097$) and IL-8 ($r = -0.62$; $p = 0.018$), which were stronger than with aLA at $r = 0.5$; $p = 0.07$ and $r = -0.46$; $p = 0.1$, respectively. Moreover, no correlations with the aLA derived alcohols (HOTEs) were observed. Arachidonic acid-derived alcohols (HETEs) were positively correlated with the levels of IL-6 ($r = 0.54$; $p = 0.045$) and IL-8 ($r = 0.54$; $p = 0.045$), while AA-derived epoxides (EpETrEs) and AA itself showed no correlation with IL-6 or IL8. Similarly to AA metabolites, LA alcohols positively correlated with IL-6 ($r = 0.52$; $p = 0.055$) and IL-8 ($r = 0.54$; $p = 0.053$) but other LA derivatives showed no relationships.

To investigate the associations of LDL lipids and oxylipins with adipokine production in more detail, we generated a Sperman ρ correlation network between all measured adipokines from the diabetic adipocytes and all measured lipids and oxylipins, in the presence or absence of TNFa (Figure S3).

4 Discussion:

The daily consumption of walnuts has been reported to have impacts on select metabolic systems, and has been associated with diverse beneficial health outcomes including improvements in inflammatory markers and select cardiovascular outcomes (reviewed in [46]). The causal links between walnut consumption and health benefits are still poorly understood, but they have generally been associated with their high levels of the omega 3 fatty acid aLA [46]. Speculation regarding the impact of phenolic bioactives have also been made[47, 48], however the implications of in vivo metabolism on these compounds are poorly understood.

Low grade systemic inflammation is related to cardio-metabolic disorders and is characterized by increased levels of circulating pro-inflammatory cytokines like IL-6 and IL-8 (reviewed in [49, 50]). In obesity, adipocytes significantly contribute to increased pools of pro-inflammatory cytokines [50]. Moreover, lipoprotein clearance is reduced in obese individuals, increasing LDL and ox-LDL levels, which have been positively associated with the levels of adipose-derived pro inflammatory cytokines, called adipokines [51, 52]. LDL is associated with adipocyte inflammation [53–55], and the level of ox-LDL has been shown to

negatively correlate with plasma concentrations of the anti-inflammatory adipokine adiponectin in humans. Moreover, the direct treatment of 3T3-L1 adipocytes with ox-LDL induces production of adipokines including resistin and visfatin [52]. Negative effect of LDL has long been associated with the LDL cholesterol load and its ability to promote foam cell formation [56], however in the current study, a modest intake of walnuts induced positive changes in LDL function without altering cholesterol loads. Here we show that LDL isolated from subjects after 4 weeks of40 g/day (i.e. 1.6 servings/day) walnut consumption reduced the TNFa-stimulated production of the pro-inflammatory cytokines IL-6 and IL-8 by human primary diabetic adipocytes in culture. Notably, the levels of IL-6 and IL-8 in the inflamed adipocytes showed strong negative associations with the LDL composition of aLA-derived epoxy fatty acids and much less strongly with their parent fatty acid, suggesting the oxylipin load to be one of the mediators of the LDL effect. The levels of IL-6 are reported to increase with hypertriglyceridemia [57] and long-term (90 days) consumption of 15 mL/day of walnut oil has been reported to reduce triglycerides and cholesterol levels in patients with Type 2 Diabetes Mellitus [58]. Our findings suggest that walnut consumption may correct the inflammatory status without correction of hyperlipidemia. Of note, in a mouse model, IL-6 levels were reported to fluctuate between fasting and postprandial state and muscle tissue was shown to be responsible for this fluctuation [59], future studies should also focus on VLDL-mediated oxylipin delivery to the skeletal muscle and their impact on cytokine production.

We have previously reported that PUFA-rich diets as well as pathological states like metabolic syndrome or statin-resistant hypertriglyceridemia change lipoprotein oxylipins and fatty acids composition [31, 32]. Walnuts are rich in LA as well as aLA. In addition, walnuts provide a substantial amount of phenolics, vitamins, minerals, and amino acids that on their own may be of benefit, however, their specific role, let alone potential interactive effects with walnut-derived fatty acids has yet to be defined [60]. Fish oil supplementation, abundant in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), causes increases in all LOX and CYP EPA- and DHA-derived oxylipins, at the expense of oxylipins derived from other PUFAs [31–33]. On the contrary, in the current study, short term walnut treatment increased the CYP-derived epoxy-fatty acids of aLA in lipoproteins, but not aLA derived mid-chain alcohols generated by LOX metabolism. Moreover, walnut treatment reduced all PUFAs mid-chain alcohols in lipoprotein which is not accompanied by decrease in corresponding epoxy-fatty acids. Importantly, walnut treatment decreased 9-HETE, a mid-chain alcohol autooxidation marker [20], suggesting that the changes in lipoprotein oxylipins caused by walnut are more than just an effect of parent PUFA enrichment. The presence of antioxidant or lipoxygenase inhibitors within the food matrix along with the lipids are therefore likely important contributors to the observed effects [61]. Consistent with this idea, phenolic compounds that are present in walnuts, like caffeic acid, have been shown to inhibit 5-lipoxygenase [62].

Walnut-induced changes in oxylipins seem to be lipoprotein specific. AA derived epoxides are uniquely enriched in HDL. HDL is involved in reverse cholesterol transport but also recirculate phospholipids and cholesterol esters [63, 64], lipid classes that are rich in oxylipins. This lipoprotein specific action of walnuts should not be surprising, considering the distinct physiological function of lipoproteins, as well as the distinct origin of their lipid

content. While VLDL is secreted by the liver, HDL obtain their lipids from tissues in the periphery [63, 64]. The enrichment of AA, aLA and LA epoxides within HDL is noteworthy, as we have previously reported that the non-esterified forms were positively associated with microvascular function [34]. Moreover, in isolation, AA derived epoxides can act as vasodilators [65]. HDL has been reported to induce nitric oxide production, and promote apoptosis and anti-inflammatory effects, while limiting LDL oxidation within the vasculature to lower cardiovascular risk, with dysfunctional HDL increasing disease risk [66]. Due in part to technological limitations, there has been limited research describing the origin of oxylipins in lipoproteins, despite their apparent physiological function. The observed specific increase in the HDL AA epoxide pool may reflect changes in peripheral tissue oxylipin metabolism. While the functional arm of this study used LDL to assess walnut-mediated impacts on peripheral tissue inflammation, an exploration of the anti-inflammatory effect of HDL on periphery is also warranted.

4.1 Limitations:

This study was originally design as a “placebo”-controlled, however, due to the apparent placebo effect, low n, and high variability of the placebo group this study was analyzed as change from the baseline. The properties of the placebo group also prevented us from analyzing the walnut treatment as a dose response, with the baseline as 0 g/day, placebo as 5 g/day and the treatment as 40 g/day. This study was performed in a group of hypercholesterolemic woman and therefore extrapolation of the findings to other subject should be done with caution. Also, cell culture experiments were performed on a single batch of primary cell line and are an approximation of the in vivo condition and therefore these results should be treated as a proof of principal, demonstrating a shift in potential interactions, and not as a demonstrated physiological response.

As epoxy PUFAs were not directly added to cells, the LDL aLA-derived epoxide levels and the interference with TNFa-stimulated cytokine production is only an association and not a demonstrated physiological action. The current findings justify future studies to evaluate the direct action of aLA-derived metabolites on adipocyte inflammatory responses. Additionally, lipoproteins consist of the complex matrix of lipids, lipid mediators and additional factors like mRNA. Therefore, focusing on one component of LDL rather than the whole composition could be misleading.

4.2 Conclusion

This study demonstrates that the consumption of a modest amount of walnuts for 4 weeks differentially changed lipoprotein fatty acid and oxylipin composition. This treatment affected LDL functionality in the context of adipocyte inflammation. Specifically, LDL oxylipin concentrations, but not those of their parent fatty acids, were strongly correlated with the inhibition of a pro-inflammatory cytokines secretion by adipocytes. These findings provide a functional demonstration of a food-lipoprotein-inflammatory physiology axis positively influenced by the daily consumption of walnuts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Kris-Etherton PM, et al., The role of tree nuts and peanuts in the prevention of coronary heart disease: multiple potential mechanisms. J Nutr, 2008 138(9): p. 1746S–1751S. [PubMed: 18716180]
2. Pan A, et al., Walnut consumption is associated with lower risk of type 2 diabetes in women. J Nutr, 2013 143(4): p. 512–8. [PubMed: 23427333]
3. Del Gobbo LC, et al., Effects of tree nuts on blood lipids, apolipoproteins, and blood pressure: systematic review, meta-analysis, and dose-response of 61 controlled intervention trials. Am J Clin Nutr, 2015 102(6): p. 1347–56. [PubMed: 26561616]
4. Ros E, et al., A walnut diet improves endothelial function in hypercholesterolemic subjects: a randomized crossover trial. Circulation, 2004 109(13): p. 1609–14. [PubMed: 15037535]
5. Cortes B, et al., Acute effects of high-fat meals enriched with walnuts or olive oil on postprandial endothelial function. J Am Coll Cardiol, 2006 48(8): p. 1666–71. [PubMed: 17045905]
6. Zhao G, et al., Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. Am J Clin Nutr, 2007 85(2): p. 385–91. [PubMed: 17284733]
7. Kohlgruber A and Lynch L, Adipose tissue inflammation in the pathogenesis of type 2 diabetes. Curr Diab Rep, 2015 15(11): p. 92. [PubMed: 26374569]
8. Njajou OT, et al., Association between oxidized LDL, obesity and type 2 diabetes in a population-based cohort, the Health, Aging and Body Composition Study. Diabetes-Metabolism Research and Reviews, 2009 25(8): p. 733–739. [PubMed: 19780064]
9. Kuniyasu A, Hayashi S, and Nakayama H, Adipocytes recognize and degrade oxidized low density lipoprotein through CD36. Biochemical and Biophysical Research Communications, 2002 295(2): p. 319–323. [PubMed: 12150950]
10. Zhong QQ, et al., High-density Lipoprotein Increases the Uptake of Oxidized Low Density Lipoprotein via PPAR gamma/CD36 Pathway in Inflammatory Adipocytes. International Journal of Biological Sciences, 2015 11(3): p. 256–265. [PubMed: 25678844]
11. Tavridou A, et al., Association of Plasma Adiponectin and Oxidized Low-Density Lipoprotein with Carotid Intima-Media Thickness in Diabetic Nephropathy. J Diabetes Res, 2015. 2015: p. 507265.
12. Zambon D, et al., Substituting walnuts for monounsaturated fat improves the serum lipid profile of hypercholesterolemic men and women. A randomized crossover trial. Ann Intern Med, 2000 132(7): p. 538–46. [PubMed: 10744590]
13. Iwamoto M, et al., Serum lipid profiles in Japanese women and men during consumption of walnuts. Eur J Clin Nutr, 2002 56(7): p. 629–37. [PubMed: 12080402]
14. Chung KH, et al., Chemical composition of nuts and seeds sold in Korea. Nutr Res Pract, 2013 7(2): p. 82–8. [PubMed: 23610599]
15. Persic M, et al., Red Walnut: Characterization of the Phenolic Profiles, Activities and Gene Expression of Selected Enzymes Related to the Phenylpropanoid Pathway in Pellicle during Walnut Development. Journal of Agricultural and Food Chemistry, 2018 66(11): p. 2742–2748. [PubMed: 29494766]

16. Shi BB, et al., Seasonal variations of phenolic profiles and antioxidant activity of walnut (Juglans sigillata Dode) green husks. International Journal of Food Properties, 2018 20: p. S2635–S2646.
17. Trandafir I, Cosmulescu S, and Nour V, Phenolic Profile and Antioxidant Capacity of Walnut Extract as Influenced by the Extraction Method and Solvent. International Journal of Food Engineering, 2017 13(1).
18. Amaral JS, et al., Phenolic profile in the quality control of walnut (Juglans regia L.) leaves. Food Chemistry, 2004 88(3): p. 373–379.
19. Hayes D, et al., Walnuts (Juglans regia) Chemical Composition and Research in Human Health. Crit Rev Food Sci Nutr, 2016 56(8): p. 1231–41. [PubMed: 25747270]
20. Mallat Z, et al., The relationship of hydroxyeicosatetraenoic acids and F2-isoprostanes to plaque instability in human carotid atherosclerosis. J Clin Invest, 1999 103(3): p. 421–7. [PubMed: 9927504]
21. Picklo MJ and Newman JW, Antioxidant supplementation and obesity have independent effects on hepatic oxylipin profiles in insulin-resistant, obesity-prone rats. Free Radical Biology and Medicine, 2015 89: p. 182–191. [PubMed: 26398714]
22. Gabbs M, et al., Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. Advances in Nutrition, 2015 6(5): p. 513–540. [PubMed: 26374175]
23. Christmas P, Role of Cytochrome P450s in Inflammation. Adv Pharmacol, 2015 74: p. 163–92. [PubMed: 26233907]
24. He J, et al., Soluble epoxide hydrolase: A potential target for metabolic diseases. J Diabetes, 2016 8(3): p. 305–13. [PubMed: 26621325]
25. Fleming I, The pharmacology of the cytochrome P450 epoxygenase/soluble epoxide hydrolase axis in the vasculature and cardiovascular disease. Pharmacol Rev, 2014 66(4): p. 1106–40. [PubMed: 25244930]
26. El-Sherbeni AA and El-Kadi AO, The role of epoxide hydrolases in health and disease. Arch Toxicol, 2014 88(11): p. 2013–32. [PubMed: 25248500]
27. Mashima R and Okuyama T, The role of lipoxygenases in pathophysiology; new insights and future perspectives. Redox Biol, 2015 6: p. 297–310. [PubMed: 26298204]
28. Shearer GC and Newman JW, Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. Curr Atheroscler Rep, 2009 11(6): p. 403–10. [PubMed: 19852880]
29. Hammond VJ and O'Donnell VB, Esterified eicosanoids: generation, characterization and function. Biochim Biophys Acta, 2012. 1818(10): p. 2403–12.
30. Cole BK, et al., 12- and 15-lipoxygenases in adipose tissue inflammation. Prostaglandins Other Lipid Mediat, 2013 104–105: p. 84–92.
31. Newman JW, et al., Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects. PLoS One, 2014 9(11): p. e111471. [PubMed: 25393536]
32. Shearer GC, et al., Abnormal lipoprotein oxylipins in metabolic syndrome and partial correction by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids, 2018 128: p. 1–10. [PubMed: 29413356]
33. Keenan AH, et al., Basal omega-3 fatty acid status affects fatty acid and oxylipin responses to high-dose n3-HUFA in healthy volunteers. J Lipid Res, 2012 53(8): p. 1662–9. [PubMed: 22628615]
34. Holt RR, et al., Effects of short-term walnut consumption on human microvascular function and its relationship to plasma epoxide content. J Nutr Biochem, 2015 26(12): p. 1458–66. [PubMed: 26396054]
35. Dwyer JT, et al., Creating the Future of Evidence-Based Nutrition Recommendations: Case Studies from Lipid Research. Adv Nutr, 2016 7(4): p. 747–55. [PubMed: 27422509]
36. Bowen KJ, Harris WS, and Kris-Etherton PM, Omega-3 Fatty Acids and Cardiovascular Disease: Are There Benefits? Curr Treat Options Cardiovasc Med, 2016 18(11): p. 69. [PubMed: 27747477]
37. Sun C, et al., IRF-1 and miRNA126 modulate VCAM-1 expression in response to a high-fat meal. Circ Res, 2012 111(8): p. 1054–64. [PubMed: 22874466]

38. Scheffer PG, et al., Measurement of low-density lipoprotein particle size by high-performance gel-filtration chromatography. Clin Chem, 1997 43(10): p. 1904–12. [PubMed: 9342011]
39. Smedes F, Determination of total lipid using non-chlorinated solvents. Analyst, 1999 124(11): p. 1711–1718.
40. Grapov D, et al., Type 2 diabetes associated changes in the plasma non-esterified fatty acids, oxylipins and endocannabinoids. PLoS One, 2012 7(11): p. e48852. [PubMed: 23144998]
41. Agrawal K, et al., Sweat lipid mediator profiling: a noninvasive approach for cutaneous research. J Lipid Res, 2017 58(1): p. 188–195. [PubMed: 27875258]
42. Lee MJ and Fried SK, Glucocorticoids antagonize tumor necrosis factor-alpha-stimulated lipolysis and resistance to the antilipolytic effect of insulin in human adipocytes. American Journal of Physiology-Endocrinology and Metabolism, 2012 303(9): p. E1126–E1133. [PubMed: 22949029]
43. Rutledge JC, et al., Direct visualization of lipid deposition and reverse lipid transport in a perfused artery : roles of VLDL and HDL. Circ Res, 2000 86(7): p. 768–73. [PubMed: 10764410]
44. van den Berg RA, et al., Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics, 2006 7: p. 142. [PubMed: 16762068]
45. Benjamini Y and Yekutieli D, Quantitative trait loci analysis using the false discovery rate. Genetics, 2005 171(2): p. 783–789. [PubMed: 15956674]
46. de Souza RGM, et al., Nuts and Human Health Outcomes: A Systematic Review. Nutrients, 2017 9(12).
47. Lee J, et al., Walnut Phenolic Extract and Its Bioactive Compounds Suppress Colon Cancer Cell Growth by Regulating Colon Cancer Stemness. Nutrients, 2016 8(7).
48. Koh SJ, et al., Walnut phenolic extract inhibits nuclear factor kappaB signaling in intestinal epithelial cells, and ameliorates experimental colitis and colitis-associated colon cancer in mice. Eur J Nutr, 2018.
49. Bastard JP, et al., Recent advances in the relationship between obesity, inflammation, and insulin resistance. European Cytokine Network, 2006 17(1): p. 4–12. [PubMed: 16613757]
50. Maurizi G, et al., Adipocytes properties and crosstalk with immune system in obesity-related inflammation. J Cell Physiol, 2018 233(1): p. 88–97. [PubMed: 28181253]
51. Song G, et al., High-density lipoprotein inhibits ox-LDL-induced adipokine secretion by upregulating SR-BI expression and suppressing ER Stress pathway. Sci Rep, 2016 6: p. 30889. [PubMed: 27468698]
52. Chen Y, et al., Ox-LDL induces ER stress and promotes the adipokines secretion in 3T3-L1 adipocytes. PLoS One, 2013 8(10): p. e81379. [PubMed: 24278099]
53. Schaiff RA, Moe RM, and Krichbaum DW, An overview of cholesterol management. Am Health Drug Benefits, 2008 1(9): p. 39–48.
54. Maiolino G, et al., The role of oxidized low-density lipoproteins in atherosclerosis: the myths and the facts. Mediators Inflamm, 2013. 2013: p. 714653.
55. Berliner JA, et al., Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation, 1995 91(9): p. 2488–96. [PubMed: 7729036]
56. Chehade JM, Gladysz M, and Mooradian AD, Dyslipidemia in type 2 diabetes: prevalence, pathophysiology, and management. Drugs, 2013 73(4): p. 327–39. [PubMed: 23479408]
57. Jung UJ and Choi MS, Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease. International Journal of Molecular Sciences, 2014 15(4): p. 6184–6223. [PubMed: 24733068]
58. Zibaeenezhad MJ, et al., Effects of walnut oil on lipid profiles in hyperlipidemic type 2 diabetic patients: a randomized, double-blind, placebo-controlled trial. Nutr Diabetes, 2017 7(4): p. e259. [PubMed: 28394361]
59. Wueest S, et al., Interleukin-6 contributes to early fasting-induced free fatty acid mobilization in mice. Am J Physiol Regul Integr Comp Physiol, 2014 306(11): p. R861–7. [PubMed: 24694381]
60. Ros E and Hu FB, Consumption of plant seeds and cardiovascular health: epidemiological and clinical trial evidence. Circulation, 2013 128(5): p. 553–65. [PubMed: 23897849]

61. Jacobs DR Jr. and Tapsell LC, Food, not nutrients, is the fundamental unit in nutrition. Nutr Rev, 2007 65(10): p. 439–50. [PubMed: 17972438]

62. Sudina GF, et al., Caffeic Acid Phenethyl Ester as a Lipoxygenase Inhibitor with Antioxidant Properties. Febs Letters, 1993 329(1–2): p. 21–24. [PubMed: 7689063]

63. Oram JF and Vaughan AM, ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins. Current Opinion in Lipidology, 2000 11(3): p. 253–260. [PubMed: 10882340]

64. Quazi F and Molday RS, Differential Phospholipid Substrates and Directional Transport by ATP-binding Cassette Proteins ABCA1, ABCA7, and ABCA4 and Disease-causing Mutants. Journal of Biological Chemistry, 2013 288(48): p. 34414–34426. [PubMed: 24097981]

65. Imig JD, Epoxides and soluble epoxide hydrolase in cardiovascular physiology. Physiol Rev, 2012 92(1): p. 101–30. [PubMed: 22298653]

66. Riwanto M and Landmesser U, High density lipoproteins and endothelial functions: mechanistic insights and alterations in cardiovascular disease. J Lipid Res, 2013 54(12): p. 3227–43. [PubMed: 23873269]

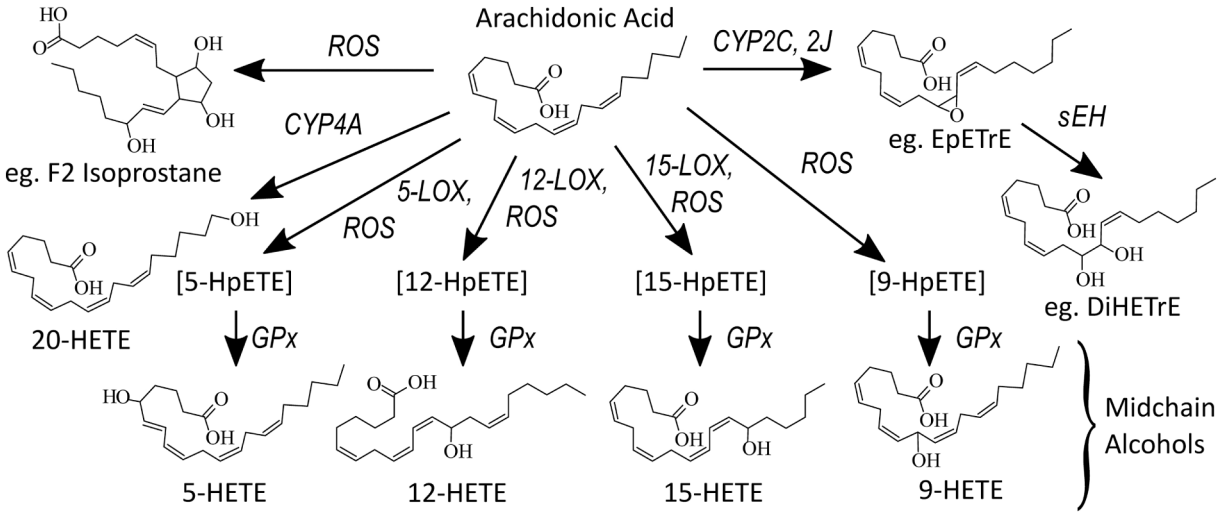

Figure 1: Representative alkaline-stable arachidonic acid-derived oxylipins which can be released from and measured in esterified oxylipin pools.

Unsaturated fatty acids (e.g. arachidonic acid) can be transformed into a variety of structures through interactions with cytochrome P450s (CYPs), epoxide hydrolases (EH), lipoxygenases (LOX), glutathione peroxidases (Gpx), and reactive oxygen species (ROS), as well as other enzymes. These reactions result in the formation of epoxides (e.g. epoxyeicosatrienoic acids (EpETrEs), vicinal or 1,2-diols (e.g. dihydroxyeicosatrienoic acids (DiHETrEs), hydroperoxides (e.g. hydroperoxyeicosatrienoic acids (HpETEs), mono-alcohols (e.g. hydroxyeicosatrienoic acids (HETEs), and isomeric prostaglandins (e.g F2 isoprostanes). These pathways and additional structures are well reviewed elsewhere.

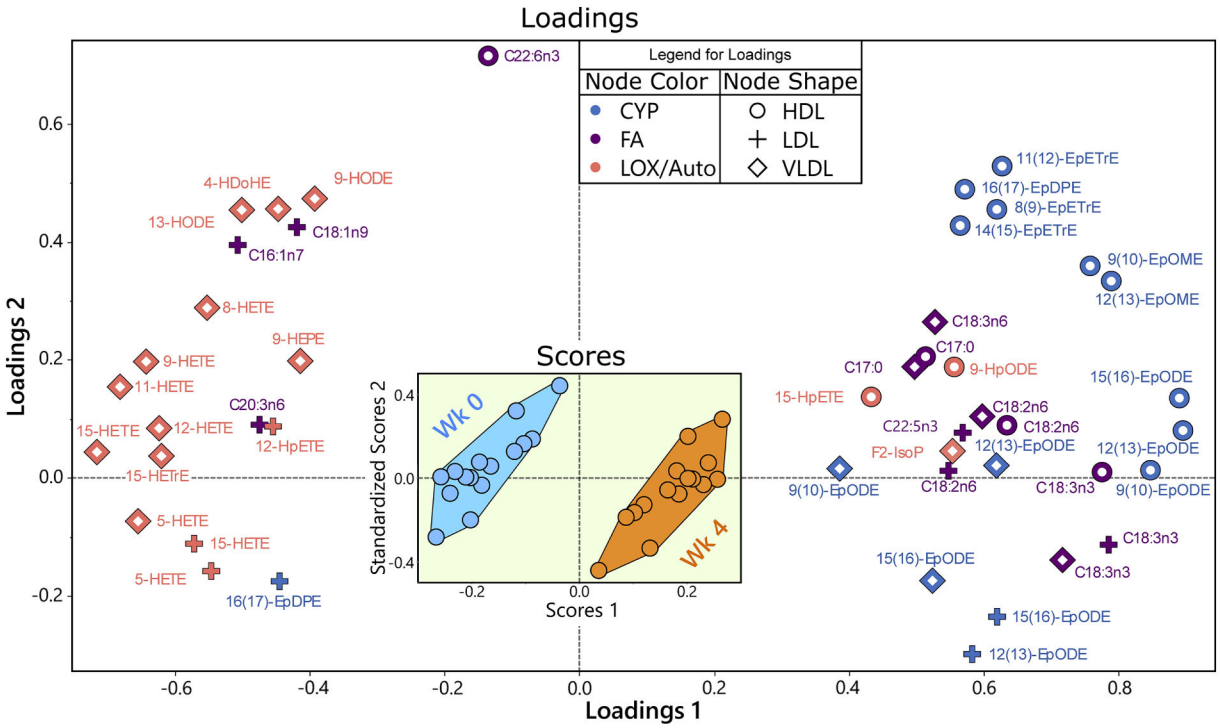

Figure 2: Partial least squares-discriminant analysis of the impact of the 40 g/day walnut treatment on the level of fatty acids and oxylipins in VLDL, LDL and HDL.

Treatment group discrimination is shown by the Scores Plot (inset), while metabolite weighting in group discrimination is shown by the Loadings Plot. Loading node colors indicate enzymatic pathways, while shapes indicate the lipoprotein particle in which the metabolite was found. Variables with Standardized Loading 1 values >0 are elevated at week 4 relative to baseline. Analysis was performed with all measured variables but only those with variable importance in projection (i.e. VIP) ≥1 are displayed. CYP – cytochrome P450; FA – fatty acid; LOX/Auto – lipoxygenase/autooxidation; sEH – soluble epoxide hydrolase; Wk 0 – subject scores at baseline; Wk 4 – subject scores after 4 weeks of walnut treatment.

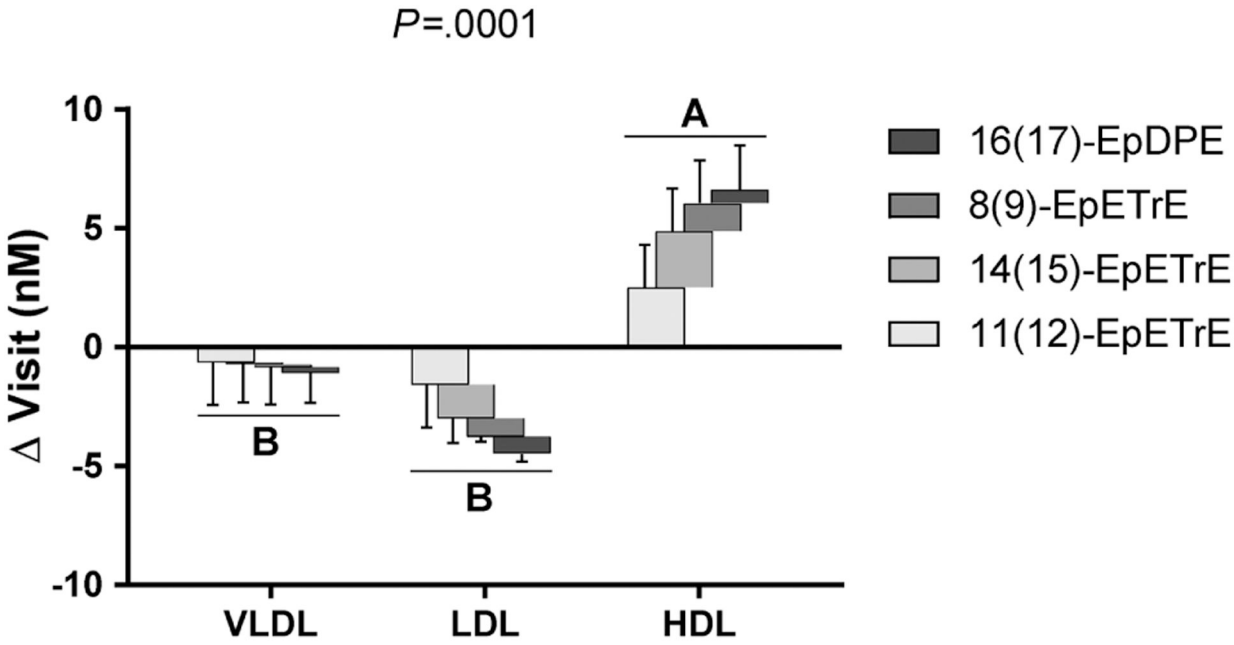

Figure 3: Lipoprotein specific changes in long chain epoxy fatty acids induced by 40 g/day walnut treatment.

A multivariate ANOVA was performed on the walnut induced change from baseline (i.e. Δ Visit = value at baseline – value after 4 weeks of walnut treatment) in long chain epoxy fatty acids. EpETrE -epoxyeicosatrienoic acid, EpDPE – epoxydocosapentaenoic acid. Lipoproteins with different annotations are different at $p < 0.05$ by a Tukey post-test. Error bars represent standard deviation.

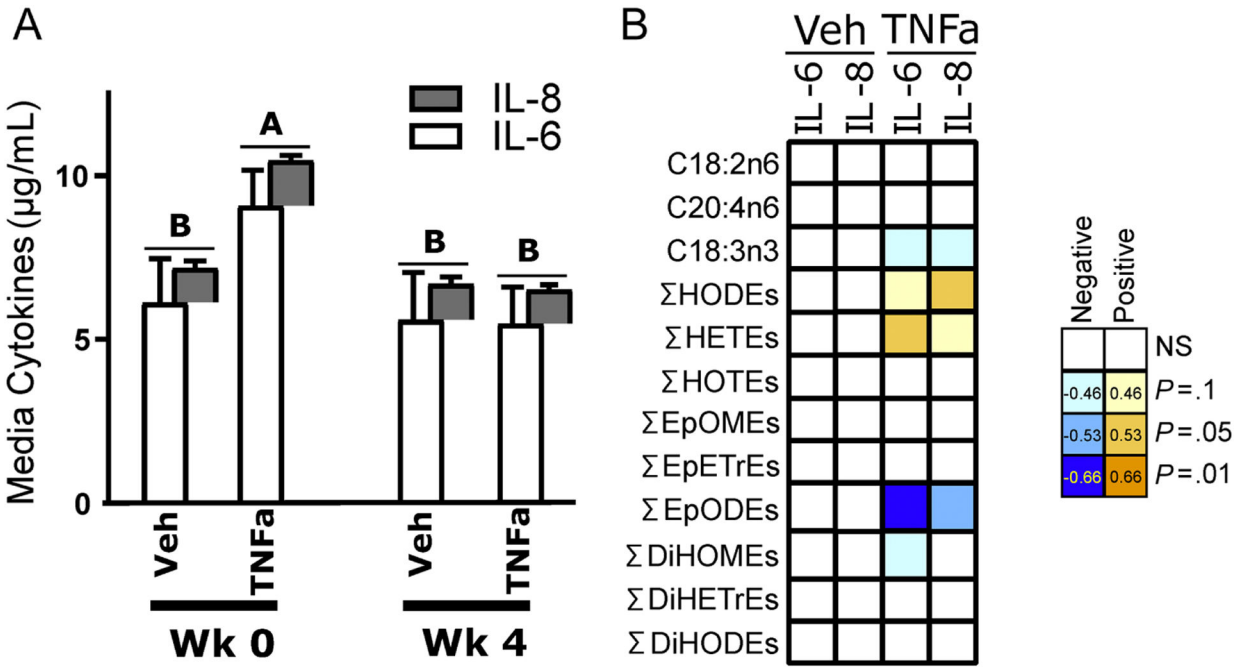

Figure 4: The effect of LDL from subjects treated with 40 g/day of walnut on TNFa- stimulated IL-6 and IL-8 production by primary human diabetic adipocytes.

A) Adipocyte culture media IL-6 and IL- 8 concentrations after 6 h incubation with LDL (n =5) and either 3 ng/mL TNFa or vehicle control (Veh). B) Correlation of IL-6 and IL-8 levels with the levels of LDL oxylipins and their corresponding parent fatty acids. HODEs - hydroxyoctadecadienoic acids, HETEs - hydroxyeicosatetraenoic acids, HOTEs - hydroxyoctadecatrienoic acids, EpOMEs - epoxyoctadecanoic acids, EpETrEs - epoxyeicosatrienoic acids, EpODEs - epoxyoctadecadienoic acids, DiHOMEs - dihydroxyoctadecanoic acids, DiHETrEs - dihydroxyeicosatrienoic acids, DiHODEs - dihydroxyoctadecadienoic acids. Wk 0 – cells treated with LDL isolated at baseline, Wk 4 – cells treated with LDL isolated after 4 weeks of walnut supplementation. Error bars represent standard error. Letters represent significant difference according to MANOVA analysis with the p =0.05.

Table 1:

HDL fatty acids and lipid mediator before and after 40 g/day walnut treatment.

Only affected clusters ($p < 0.1$) are presented, with unaffected clusters shown in Table S3a.

Cluster	Unit	Metabolite	Baseline (Mean ± SD)	4 weeks (Mean ± SD)	t-test p-value	MANOVA p-value
HDL1 C:18 Epoxides	nM	12(13)-EpODE	0.199 ± 0.113	0.397 ± 0.173	0.0001	0.0001
	nM	15(16)-EpODE	0.665 ± 0.448	1.69 ± 0.856	0.0001	
	nM	9(10)-EpODE	0.658 ± 0.407	1.5 ± 0.817	0.0001	
	nM	12(13)-EpOME	30.8 ± 11.8	45.9 ± 19.3	0.0002	
	nM	9(10)-EpOME	28.5 ± 11.2	41.2 ± 18.4	0.0005	
HDL 2 C:18FA	μM	C18:3n3	0.878 ± 0.262	1.75 ± 0.77	0.0001	0.0001
	μM	C18:2n6	75.3 ± 22.1	96.9 ± 28.4	0.0007	
HDL 3 C:18 −00H	%	9-HpODE	2.04 ± 0.703	2.78 ± 1.13	0.0028	0.011
	%	13-HpODE	2.05 ± 1.09	2.58 ± 1.06	0.049	
HDL 4 AA Epoxides	nM	8(9)-EpETrE	6.03 ± 2.46	7.2 ± 2.4	0.012	0.012
	nM	14(15)-EpETrE	9.69 ± 3.71	12.1 ± 5.33	0.019	
	nM	11(12)-EpETrE	12.4 ± 4.91	14.9 ± 5.63	0.022	
HDL 5	nM	5-KETE	0.919 ± 0.454	1.12 ± 0.522	0.038	0.038

% – Percent composition. Semi-quantitative data generated based on MS area count, without standard curve.

Table 2:

LDL fatty acids and lipid mediator before and after 40 g/day walnut treatment.

Only affected clusters ($p < 0.1$) are presented, with unaffected clusters shown in Table S3b.

Cluster	Unit	Metabolite	Baseline (Mean ± SD)	4 weeks Mean ± SD	t-test p-value	MANOVA p-value
LDL1 aLA and its Epoxides	µM	C18:3n3	1.28 ± 0.498	2.56 ± 0.895	0.0001	0.0002
	nM	15(16)-EpODE	1.04 ± 0.307	1.88 ± 1.04	0.0002	
	nM	12(13)-EpODE	0.267 ± 0.13	0.508 ± 0.3	0.0004	
	nM	9(10)-EpODE	1.05 ± 0.582	1.5 ± 0.845	0.05	
LDL2 Fatty acids	µM	C16:1n7	6.08 ± 2.71	4.88 ± 2.18	0.0002	0.0002
	µM	C18:1n9	47.9 ± 14.5	41.1 ± 10.1	0.002	
	µM	C20:3n6	3.78 ±1.42	3.11 ± 1.38	0.0091	
LDL3 AA and DGLA mid-chain alcohols	nM	15-HETE	11 ± 9.26	7.31 ± 6.19	0.0032	0.014
	nM	5-HETE	11 ± 11.1	7.37 ± 8.35	0.0073	
	nM	9-HETE	9.53 ± 10.9	6.14 ± 6.54	0.023	
	nM	11-HETE	9.32 ± 9.61	6.05 ± 6.24	0.013	
	nM	8-HETE	9.41 ± 8.73	6.44 ± 5.93	0.013	
	nM	12-HETE	10.2 ± 9.31	7.31 ± 6.28	0.034	
	nM	15-HETrE	1.56 ± 1.31	1.17 ± 0.91	0.033	
LDL4 DHA and its Epoxides	nM	16(17)-EpDPE	1.85 ± 1.27	1.12 ± 0.676	0.0093	0.07
	nM	19(20)-EpDPE	2.31 ± 1.66	1.84 ± 1.14	--	
	µM	C22:6n3	2.77 ± 1.27	2.46 ± 1.19	--	
LDL5 PUFAs	µM	C18:2n6	108 ± 32.7	129 ± 34.1	0.0037	0.07
	µM	C22:5n3	0.781 ± 0.281	0.968 ± 0.315	0.0043	
	µM	C20:4n6	20.9 ± 6.63	20 ± 5.83	--	

Table 3:

VLDL fatty acids and lipid mediator before and after 40 g/day walnut treatment.

Only affected clusters ($p < 0.1$) are presented here, with unaffected clusters presented in Table S3c.

Cluster	Unit	Metabolite	Baseline Mean ± SD	4 weeks Mean ± SD	t-test p-value	MANOVA p-value
VLDL1 nAA and DGLA mid-chain alcohols	nM	15-HETE	4.11 ± 2.51	2.53 ± 1.53	0.0001	0.0001
	nM	9-HETE	2.23 ± 1.47	1.51 ± 1.11	0.0001	
	nM	11-HETE	2.65 ± 1.65	1.73 ± 1.33	0.0004	
	nM	15-HETrE	1.02 ± 0.613	0.681 ± 0.418	0.0009	
	nM	5-HETE	2.8 ± 1.58	2 ± 1.52	0.0012	
	nM	8-HETE	3.78 ± 2.11	2.6 ± 1.8	0.0021	
	nM	12-HETE	3.01 ± 1.81	2.18 ± 1.89	0.0024	
VLDL2 PUFAs and saturated fatty acids	µM	C18:3n3	1.71 ± 1.13	3.6 ± 2.43	0.0001	0.0006
	µM	C18:2n6	51.4 ± 34.2	67.3 ± 42	0.0019	
	µM	C17:0	0.59 ± 0.504	0.797 ± 0.477	0.013	
	µM	C18:3n6	1.27 ± 1.01	1.64 ± 1.27	0.022	
	µM	C22:5n3	0.705 ± 0.629	0.941 ± 0.676	0.024	
	µM	C16:0	64.3 ± 49.9	73.3 ± 44.4	0.029	
VLDL3 aLA Epoxides	nM	12(13)-EpODE	0.437 ± 0.301	0.794 ± 0.699	0.0001	0.0009
	nM	15(16)-EpODE	1.59 ± 1.16	2.66 ± 2.32	0.0004	
	nM	9(10)-EpODE	1.49 ± 1.11	2.14 ± 2.07	0.017	
VLDL4 LA and DHA mid-chain alcohols	nM	13-HODE	78 ± 36.4	59.4 ± 29.8	0.0014	0.0012
	nM	4-HDoHE	2.5 ± 1.74	1.42 ± 1.13	0.0046	
	nM	9-HODE	30.6 ± 14.8	24.8 ± 11.9	0.012	
VLDL5	nM	F2-IsoProstanes	0.167 ± 0.113	0.269 ± 0.19	0.019	0.019
VLDL6	µM	C18:0	12.7 ± 8.17	16.8 ± 9.48	0.032	0.032
VLDL7 EPA and DHA mid-chain alcohols	nM	9-HEPE	0.642 ± 0.553	0.402 ± 0.216	0.013	0.036
	nM	15-HEPE	1.35 ± 1.03	0.965 ± 0.58	0.038	
	nM	12-HEPE	0.943 ± 1.02	0.497 ± 0.315	0.05	
	nM	17-HDoHE	5.9 ± 7.12	3.29 ± 3.18	0.06	
	nM	5-HEPE	0.681 ± 0.638	0.459 ± 0.28	0.048	
	nM	14-HDoHE	2.31 ± 2.5	1.17 ± 1	0.033	
VLDL8	nM	5-KETE	0.597 ± 0.361	0.428 ± 0.21	0.048	0.048