



**HAL**  
open science

## Extra Virgin Olive Oil Reduced Polyunsaturated Fatty Acid and Cholesterol Oxidation in Rodent Liver: Is This Accounted for Hydroxytyrosol-Fatty Acid Conjugation?

Yiu Yiu Lee, Céline Crauste, Hualin Wang, Ho Hang Leung, Joseph Vercauteren, Jean-Marie Galano, Camille Oger, Thierry Durand, Jennifer Man-Fan Wan, Jetty Chung-Yung Lee

### ► To cite this version:

Yiu Yiu Lee, Céline Crauste, Hualin Wang, Ho Hang Leung, Joseph Vercauteren, et al.. Extra Virgin Olive Oil Reduced Polyunsaturated Fatty Acid and Cholesterol Oxidation in Rodent Liver: Is This Accounted for Hydroxytyrosol-Fatty Acid Conjugation?. *Chemical Research in Toxicology*, 2016, 29 (10), pp.1689-1698. 10.1021/acs.chemrestox.6b00214 . hal-01622331

**HAL Id: hal-01622331**

**<https://hal.umontpellier.fr/hal-01622331>**

Submitted on 29 Mar 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Extra Virgin Olive Oil Reduced Polyunsaturated Fatty Acid and Cholesterol Oxidation in Rodent Liver: Is This Accounted for Hydroxytyrosol-Fatty Acid Conjugation?

Yiu Yiu Lee,<sup>†</sup> Céline Crauste,<sup>‡</sup> Hualin Wang,<sup>†</sup> Ho Hang Leung,<sup>†</sup> Joseph Vercauteren,<sup>‡</sup> Jean-Marie Galano,<sup>‡</sup> Camille Oger,<sup>‡</sup> Thierry Durand,<sup>‡</sup> Jennifer Man-Fan Wan,<sup>†</sup> and Jetty Chung-Yung Lee<sup>\*,†</sup>

<sup>†</sup>School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR

<sup>‡</sup>Institut des Biomolécules Max Mousseron, UMR 5247, CNRS, Université de Montpellier & ENSCM (School of Chemistry), Faculté de Pharmacie, 15 Av. Charles Flahault, 34093 Montpellier cedex 05, France

## **S** Supporting Information

**ABSTRACT:** The effects of extra virgin olive oil (EVOO) and carbon tetrachloride (CCl<sub>4</sub>) induced oxidative stress in rats were determined by the generation of isoprostanoids. These are known to be robust biomarkers to evaluate nonenzymatic and free radical related oxidation. Other oxidative stress biomarkers such as hydroxyecosatetraenoic acid products (HETEs) and cholesterol oxidation products (COPs) were also determined. The rodents received a control diet, high-fat diet (20% w/w) composed of extra virgin olive oil (EVOO), corn oil (CO), or lard, and high-fat diets with CCl<sub>4</sub> insult throughout the experimental period. The EVOO diet was found to suppress the formation of isoprostanoids and COPs compared to that of the control. EVOO also had a high total phenolic content and antioxidant activity compared to those of CO and lard and may be contributed to by the hydroxytyrosol component conjugated to fatty acids (HT-FA). This is the first study to identify HT-FA in EVOO, and it was 4-fold higher than that of olive oil, whereas none was found in corn oil. Furthermore, the EVOO diet showed reduced liver lipid vesicles in CCl<sub>4</sub> treated rats compared to that of the control. However, liver toxicity measurements of AST (aspartate transaminase) and ALT (alanine transaminase) activities showed augmentation with CCl<sub>4</sub> treatment but were not alleviated by the diets given. Our findings suggest that EVOO is a daily functional food capable of enhancing the antioxidant system for liver protection; the effect is potentially attributed to the phenolic and lipophenolic (phenol conjugated by fatty acids) content.



## 1. INTRODUCTION

Dietary extra virgin olive oil (EVOO) has shown protection against coronary heart disease (CHD) by lowering total cholesterol and low-density lipoprotein (LDL).<sup>1</sup> Also, it reduced blood pressure and insulin sensitivity in healthy and type 2 diabetic patients.<sup>2</sup> Epidemiological studies have also shown that individuals who had low saturated fats and high intake of olive oil displayed lower rates of cardiovascular disease (CVD) and cancer. In addition, an EVOO rich Mediterranean diet was more effective in primary and secondary prevention of CVD than the diet designed by the United States cholesterol education program.<sup>3,4</sup> In animal studies, 5–10% of EVOO enriched diets with low cholesterol level halted the progression of induced atherosclerosis in rabbits and in female apolipoprotein E knockout (*ApoE*<sup>-/-</sup>) mice.<sup>5–7</sup>

The fatty acid composition of EVOO is mainly oleic acid, an omega-9 monounsaturated fatty acid (MUFA), along with polar components such as polyphenols that are widely known to be involved in antioxidant activity.<sup>8–10</sup> To date, it is unknown how the polyphenol is delivered *in vivo* for its activity. It has been

shown that polyphenols in the gut increase catabolism, hydrolyze bile salt, and modify the enterohepatic circulation.<sup>11</sup> Further, it is suggested that some polyphenols and omega-3 polyunsaturated fatty acid (PUFA) together down-regulate prostaglandin production and regulate inflammation and antioxidant pathways.<sup>12</sup>

Chronic liver diseases, including chronic hepatitis virus B (HBV) and/or hepatitis virus C (HCV) infection, alcoholic liver disease, NAFLD, and primary biliary cirrhosis are major threats to human health in Asia.<sup>13,14</sup> Furthermore, chronic liver diseases will lead to liver inflammation, hepatocellular dysfunction, activation of hepatic stellate cells, and accumulation of extracellular matrix, also known as liver fibrosis and advanced cirrhosis, and even hepatocellular carcinoma.<sup>15,16</sup>

It is elucidated that hydroxytyrosol (HT) is the active polyphenol in EVOO, but the underlying mechanisms on its effect and health are still unclear. Recently, HT was found to

exert antiadipogenic effects in preadipocyte cells.<sup>17</sup> Also, studies have focused on the effect of EVOO in CHD, but those related to the liver, e.g., nonalcoholic fatty liver disease (NAFLD), are scarce. Reports suggest that liver damage arises in part from free radicals of exogenous sources such as alcohol, diet, drugs, and chemical contaminants;<sup>18,19</sup> therefore, the role of HT in EVOO may have a substantial impact.

It is known that oxidation of omega-6 PUFAs such as arachidonic acid (AA) via a nonenzymatic pathway can generate F<sub>2</sub>-isoprostanes (with its best known isomer being 15-F<sub>2t</sub>-isoprostane or 15-F<sub>2t</sub>-IsoP), while incorporation of an enzymatic pathway can release hydroxyeicosatetraenoic acid products (HETEs). Augmentations of these metabolites are toxic and highly associated with inflammation, CHD, and neurodegeneration.<sup>20–22</sup> In addition, resolvin D1 from enzymatic oxidation of docosahexaenoic acid (DHA) showed reduced inflammation in various biological models.<sup>23</sup> Moreover, nonenzymatic oxidation of omega-3 PUFAs such as eicosapentaenoic (EPA) and DHA releases F<sub>3</sub>-isoprostanes (8-F<sub>3t</sub>-isoprostane) and other F<sub>4</sub>-neuroprostane (F<sub>4</sub>-NeuroPs), for example, 4(RS)-4-F<sub>4t</sub>-neuroprostanes (4(RS)-4-F<sub>4t</sub>-NeuroP), respectively. The generation of the latter is desirable as it was recently shown to be cardioprotective.<sup>24,25</sup> Nonetheless, earlier studies indicate F<sub>4</sub>-NeuroPs to be a robust oxidative stress biomarker for neurodegeneration.<sup>26</sup>

Oxidation of cholesterol could also take place in the liver to release 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH and 7-ketocholesterol (7-ketochol) via free radical reaction, and 27-hydroxycholesterol (27-OH) via the cytochrome P450 pathway, and released into blood circulation by LDL. Elevation of these cholesterol oxidation products (COPs) can lead to lysosomal dysfunction and mitochondrial damage in human monocytes and is associated with vascular related disorder such as atherosclerosis.<sup>27</sup>

In this article, we administered CCl<sub>4</sub> to induce hepatotoxicity in a rat model to study the mechanisms of lipid peroxidation and the protective effects of EVOO (predominantly omega-9 MUFA), corn oil (mostly omega-6 PUFA), and lard (higher content of saturated fats) in rodents. It is well established that CCl<sub>4</sub> can be metabolized into a trichloromethyl radical via cytochrome P450 isoenzymes and mediate free radical reaction, and lipid peroxidation. Alteration on PUFA and cholesterol by generating unwanted oxidized metabolites leads to liver cell necrosis.<sup>28,29</sup> However, some oxidized metabolites are also recognized as lipid mediators that could exert important biological functions.<sup>26,30</sup> In addition, we identified that hydroxytyrosol is present in the form of lipophenol derivatives that are covalently conjugated to certain fatty acids in EVOO and olive oil but not corn oil.

## 2. MATERIALS AND METHODS

**2.1. Treatments and Sampling of the Rodents.** Five month old male Fischer 344 rats (each weighing approximately 450 g) were obtained from the Animal Resources Centre, Western Australia and bred in the Animal House, School of Biological Sciences, at The University of Hong Kong (HKU) under the approval of Department of Health, Hong Kong and Committee on the Use of Live Animals in Teaching and Research, HKU. All rats were kept individually in autoclaved stainless steel cages in the animal room. The rats experienced an alternate 12 h light–dark cycle with controlled temperatures (22 °C) and humidity (50%). Animals received water ad libitum. After 1 week of acclimatization, the rats were randomly divided into either control (normal chow) or high-fat groups. The diet for high-fat groups was extra virgin olive oil (EVOO), corn oil (CO), and lard. The rats in the high-fat

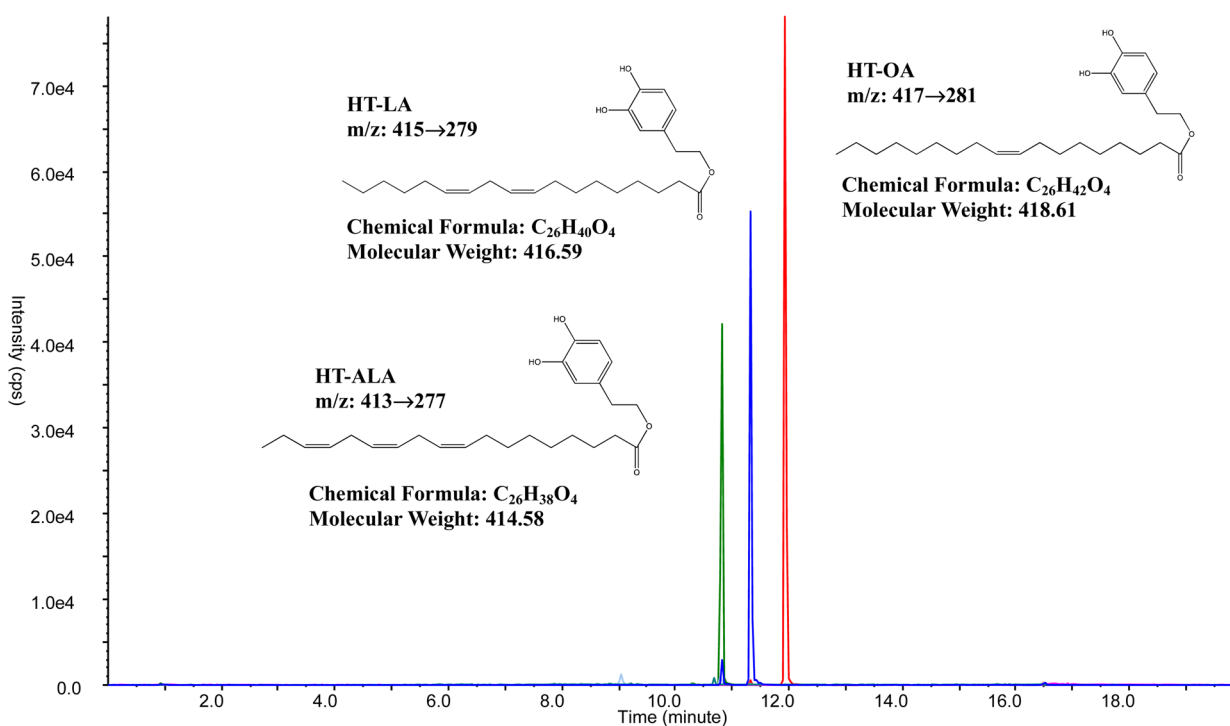
groups were fed with 20% (w/w) of respective oils mixed with normal rodent chow (5053, LabDiet, USA) ad libitum. Animals in the high-fat group were subdivided for CCl<sub>4</sub> (+ CCl<sub>4</sub>) or control (- CCl<sub>4</sub>) treatments. After 1 week of conditioning feeding, the animals in the CCl<sub>4</sub> treatment group were subcutaneously administered with CCl<sub>4</sub> (0.1 mL/100 g body weight, 1:1 mixed with soybean oil) twice a week (Monday and Thursday) for 4 weeks, at the same time of the day, in order to induce liver injury. The high-fat diets were maintained throughout the experimental period. Twenty-four hours after the last injection, all rats were sacrificed. Blood samples were collected via cardiac puncture and were allowed to clot. The serum was collected by centrifugation (1500g, 5 min, 4 °C) for hepatic toxicity analysis. The liver tissue was excised, washed with butylated hydroxytoluene (BHT) and indomethacin in PBS (pH 7.4), snap frozen in liquid nitrogen, and stored at –80 °C for future assessment of lipid and lipid mediators. The right lobe of the control and dietary oil fed groups were collected and fixed in 10% buffered formalin solution (Surgipath, Germany) for histological staining.

**2.2. Measurement of AST and ALT.** Liver toxicity tests were performed by measuring the levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) in the serum. ALT substrate solution (2 mM  $\alpha$ -ketoglutarate and 200 mM L-alanine in 0.1 M phosphate buffer, pH 7.4) and AST substrate solution (2 mM  $\alpha$ -ketoglutarate and 200 mM L-aspartate in 0.1 M phosphate buffer, pH 7.4) were freshly prepared and incubated at 37 °C. A hundred microliters of serum sample were mixed with 0.5 mL of preincubated ALT or AST substrate solution. The mixture was then incubated in a water bath at 37 °C for 30 min. Then, 0.5 mL of 1 mM 2,4-dinitrophenylhydrazine (DNP) color reagent was added to the mixture and allowed to stand at room temperature for 30 min. Finally, 5 mL of 0.4 M NaOH was added and incubated for color development. After 5 min of incubation, the mixture absorbance was measured spectrophotometrically using a UV–vis spectrophotometer (Shimadzu, Japan) at 505 nm.

**2.3. Liver Histology.** The fixed liver tissues were embedded in paraffin and sectioned at 5- $\mu$ m thickness. Sections were stained with Masson's trichrome for collagen (Sigma-Aldrich, USA). The stained slides were observed photomicrographically under a Nikon N80i microscope (Nikon, Japan) at 100 $\times$  magnification.

**2.4. Total Tissue Lipid Extraction.** Liver tissues stored in –80 °C were weighed and homogenized in Folch solution (chloroform:methanol, 2:1, v/v) spiked with 0.01% BHT, using a polytron benchtop homogenizer (T25, ULTRA-TURRAX, IKA) at 24,000 rpm in 2  $\times$  20 s bursts. Sample tubes were placed on ice during homogenization, and the blade was rinsed with Milli-Q water and methanol between samples to prevent cross-contamination. Subsequently, 2 mL of aqueous 0.9% NaCl was added and centrifuged at 3000g for 10 min at 4 °C, to introduce phase separation. The lower phase was collected and dried under nitrogen flux on a 37 °C heating block. Dried lipids were dissolved in 1 mL of methanol containing 0.005% BHT and hydrolyzed by adding 1 mL of 1 M aqueous KOH. Samples were vortexed, purged with nitrogen, and incubated overnight on a shaker at room temperature. Hydrolyzed samples were neutralized with formic acid and cleaned up using solid phase extraction (60 mg MAX SPE columns, Oasis, Waters). Cholesterol and cholesterol oxidation products were extracted with *n*-hexane during the SPE process and dried and reconstituted in 200  $\mu$ L of methanol. The final elute (hexane/ethanol/acetic acid mix) of the SPE was extracted, dried, and reconstituted in 200  $\mu$ L of methanol for the analysis of fatty acids, isoprostanoids, and lipoxygenase (LOX)-derived lipid mediators.<sup>31</sup>

**2.5. Measurement of Polyunsaturated Fatty Acids, Isoprostanoids, and LOX-Derived Lipid Mediators.** Polyunsaturated fatty acids (AA, EPA, and DHA), isoprostanoids (F<sub>2</sub>-IsoPs, F<sub>3</sub>-IsoPs, and F<sub>4</sub>-NeuroPs), and LOX-derived lipid mediators (5-, 12-, 15-HETEs, and resolvin D1) were quantified according to Leung et al. method using liquid chromatography tandem mass spectrometry (LC-MS/MS).<sup>31</sup> Briefly, a Kinetex HILIC column (30  $\times$  3 mm, 2.6  $\mu$ m, Phenomenex, USA) was connected to a 1290 Infinity LC system (Agilent Technologies, USA) and maintained at room temperature. The mobile phases were 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol (B). A stepwise gradient elution from 60% B for 5 min,



**Figure 1.** Liquid chromatography tandem mass spectrometry (LC-MS/MS) evaluation of hydroxytyrosol conjugated to fatty acids (HT-FA). Compound structure and mass fragmentation of the HT-FA are shown. HT, hydroxytyrosol; OA, oleic acid; LA, linoleic acid; ALA, alpha-linolenic acid.

followed by increasing to 70% in 1 min, and 100% for final 1 min was used. The flow rate was set to 200  $\mu\text{L}/\text{min}$ . A QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) was operated in the electrospray ionization (ESI) negative mode with a source temperature of 500  $^{\circ}\text{C}$  and capillary temperature of 250  $^{\circ}\text{C}$ . The collision energy was optimized to maximize the ion currents of the precursor to product ion dissociation.<sup>31</sup> The analytes were detected by MS/MS using multiple reactions monitoring (MRM). Quantitation of each analyte was achieved by relating the peak area with its corresponding internal standard peak. Where available, a heavy labeled isotope was used for quantitation, whereas for those without heavy labeled isotopes quantitation was achieved by relating to those deuterated standards of the closest chemical structure. All PUFAs, isoprostanoids, HETEs, and resolvin D1 standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) or synthesized (4(RS)-4-F<sub>4</sub>-neuroprostane) by the Institut des Biomolécules Max Mousseron (IBMM, Montpellier, France).<sup>32</sup>

**2.6. Measurement of cholesterol and cholesterol oxidized products (COPs).** Cholesterol and cholesterol oxidized products (COPs), namely, 7 $\beta$ -OH, 7-ketochol, and 27-OH, were measured similar to fatty acids but with different LC buffers and ESI modes. LC mobile phase of 5 mM ammonium acetate in 10% methanol (A) and 5 mM ammonium acetate in 85% methanol (B) was set to constant 200  $\mu\text{L}/\text{min}$  flow rate. The mobile phase ran at a constant of 0% B phase from 0 to 4 min and gradually increased to 100% from 4 to 5 min, and then B, 100% for subsequent 2 min. Sample injection volume was 20  $\mu\text{L}$ . Positive ESI mode was used with a QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) with a source temperature of 400  $^{\circ}\text{C}$  and a capillary temperature of 250  $^{\circ}\text{C}$ . The analytes were detected by MS/MS using MRM and monitoring the transitions as suggested by Shui et al.<sup>33</sup> Quantitation of cholesterol was achieved by relating the peak area with its corresponding internal standard peak. All native and deuterated cholesterol or COPs standards were purchased from Avanti Polar Lipids (USA).

**2.7. Extraction of Polyunsaturated Fatty Acids and Lipophenols from Dietary Oils.** Fatty acids (FA), oleic acid (OA), linoleic acid (LA), and alpha-linolenic acid (ALA) and their respective hydroxytyrosol (HT) conjugated FA, namely, HT-OA, HT-LA, and HT-ALA from dietary oils, were extracted separately as the extraction of

lipophenols requires the absence of alkaline hydrolysis because of the instability of the ester linkage in basic conditions. Briefly, 50  $\mu\text{g}$  of each oil sample was weighed and mixed with 10 mL of Folch solution, and incubated for 10 min on a shaker. Subsequently, 2 mL of normal saline was added in to introduce phase separation, and the lower layer was collected after centrifuging at 3000g for 10 min at 4  $^{\circ}\text{C}$ . The extractions were repeated, and the chloroform phases were pooled together and dried at 37  $^{\circ}\text{C}$  under a stream of nitrogen. The dried extracts were redissolved in 200  $\mu\text{L}$  of methanol that spiked with AA-d<sub>8</sub> as internal standard for the analysis of lipophenols. Any impurities were removed by passing through a 0.45  $\mu\text{m}$  polytetrafluoroethylene (PTFE) mini-disc syringe filter.

Similar to the liver tissue total lipid extraction, an additional step of alkaline hydrolysis was needed for the analysis of dietary oil for free FA analysis. One milliliter of methanol containing 0.01% BHT and 1 mL of 1 M aqueous KOH were added to the dried extracts from Folch extraction and incubated for an hour at 50  $^{\circ}\text{C}$ . The samples were acidified and purified with solid phase extraction as described in section 2.4. The final elute was collected, dried, and reconstituted in 200  $\mu\text{L}$  of methanol, containing 3 ng/ $\mu\text{L}$  of AA-d<sub>8</sub> as internal standard.

**2.8. Measurement of Dietary Oil Fatty Acids (FA) and Lipophenols.** The extracted FA samples and their respective hydroxytyrosol conjugates were quantified in EVOO, olive oil (OO), and corn oil (CO). Lard was not analyzed for HT-FA as the main component is saturated fatty acids. Briefly, a Kinetex C18 column (150  $\times$  21 mm, 2.6  $\mu\text{m}$ , Phenomenex, USA) was linked to a 1290 Infinity LC system (Agilent Technologies, USA) and warmed to 30  $^{\circ}\text{C}$ . The mobile phases are 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B), with a flow rate of 300  $\mu\text{L}/\text{min}$ . A gradient elution was developed as follows: 30% B for the first 2 min, gradually increased to 98% in 8 min, and maintained for 2 min. The analytes were detected by MS/MS using MRM and monitoring the transitions as shown in Figure 1. Negative ESI mode was applied with the source temperature set to 750  $^{\circ}\text{C}$ . Quantitation of lipophenols was achieved by relating the peak area with the internal standard. The standards for lipophenols HT-OA, HT-LA, and HT-ALA were synthesized starting from commercially available dimethoxy-hydroxytyrosol and fatty acids (see Supporting Information for the synthesis description). They were fully synthesized and characterized (using NMR analysis) by Institut des Biomolécules

Max Mousseron (IBMM, Montpellier, France) and were used for analysis in dietary lipids for the first time (Figure 1). FA were measured by the concentration ratio using their respective deuterated labeled FA.

**2.9. Extraction of Dietary Lipids for Total Polyphenol and Antioxidant Activity Measurement.** Lard was prepared from fresh pig fat tissues by heating in the oven at 80 °C for 30 min. Crude lard was obtained by centrifugation at top speed for 2 min and subsequently stored at -20 °C. Extraction of polyphenol and total antioxidants was performed according to Ciafardini et al. with modifications.<sup>34</sup> Briefly, 1 g of oil sample was mixed vigorously with 1 mL of hexane and 2 mL of methanol/water solution (60:40, v/v) for 1 min. The lower hydro-alcoholic phase was collected after centrifugation at 800g for 3 min. All extractions were repeated twice, and the acquired hydro-alcoholic phases were pooled together. Residual oil present in extracts was washed with hexane. Final purified extracts were dried under a stream of nitrogen gas placed on a 30 °C heating block. All concentrated extracts were purged with nitrogen and stored at -20 °C and resuspended in 2 mL of absolute ethanol just before use. All centrifugations were performed at room temperature.

**2.10. Measurement of Total Antioxidant Capacity and Total Phenolic Compounds in Dietary Oil and Lard.** Total antioxidant capacity from dietary oil and lard was accessed via ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) discoloration assay.<sup>35</sup> Potassium persulfate was added into 7 mM ABTS solution and dissolved in water at a final concentration of 2.45 mM. The mixture was incubated in the absence of light for at least 12 h before being diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Ten microliters of the sample solution or 2.5 mM Trolox standard was added to 1 mL of diluted ABTS solution for absorbance measurements. The absorbance was measured at 0, 1, and 4 min using a UV/vis spectrophotometer (Genesys 5, Spectronics, Sweden). All determinants were carried out in triplicate. Total antioxidant capacity was calculated in reference to the area under the curve of the Trolox standard and oil samples.

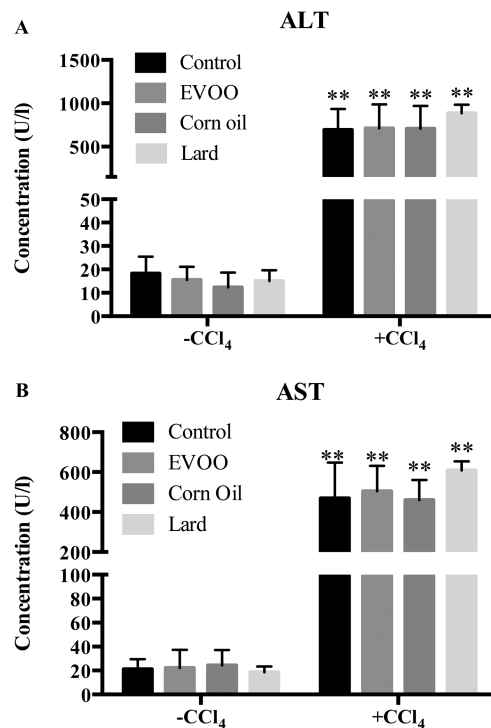
Procedure for polyphenol measurement was adapted from Marinova et al. with a slight modification.<sup>36</sup> Briefly, 1 mL of sample solution was added to 5 mL of Folin-Ciocalteu's phenol reagent (1:10 dilution with water) and incubated for 4 min. Then, 4 mL of 7% sodium carbonate solution was added and was further incubated for 40 min in darkness. The absorbance was measured at 725 nm using a UV/vis spectrophotometer (Genesys 5, Spectronics, Sweden). All determinants were carried out in triplicate. The standard curve of gallic acid was constructed from 0–100 mg/L, and the results are expressed as mg gallic acid equivalent (GAE)/g of oil.

**2.11. Statistical Analysis.** All data were analyzed by GraphPad Prism (version 6 for Mac, USA) and reported as mean ± SD. One-way ANOVA or Student's *t* test analysis was tested for statistical significance between control and dietary fat groups. *P* values of 0.05 or less were considered statistically significant.

### 3. RESULTS

**3.1. Carbon Tetrachloride (CCl<sub>4</sub>) Induced Hepatic Toxicity and Was Unaffected by Dietary Lipids.** The degree of hepatic injury with or without CCl<sub>4</sub> in the rodents with different lipid diets was determined by serum ALT and AST activity. Serum ALT and AST were not significantly different in rats fed with different dietary oils in the presence or absence of CCl<sub>4</sub> as compared to the control (Figure 2). Nevertheless, the elevation of AST and ALT in CCl<sub>4</sub> treated rats confirmed the effect of CCl<sub>4</sub> in liver injury but was not affected by the different dietary lipid supplementations.

**3.2. Hepatic Histology Displayed Less Modification by EVOO.** Hepatic histology with CCl<sub>4</sub> treatment in the rodents supplemented with all dietary lipids remarkably showed the formation of adipose vesicles and fibrous septa. In particular, corn oil and lard supplementation were the two most prominent effectors. In contrast, EVOO supplemented rats showed less adipose vesicles and fibrous septa development than those receiving the corn oil or lard diet (Figure 3).

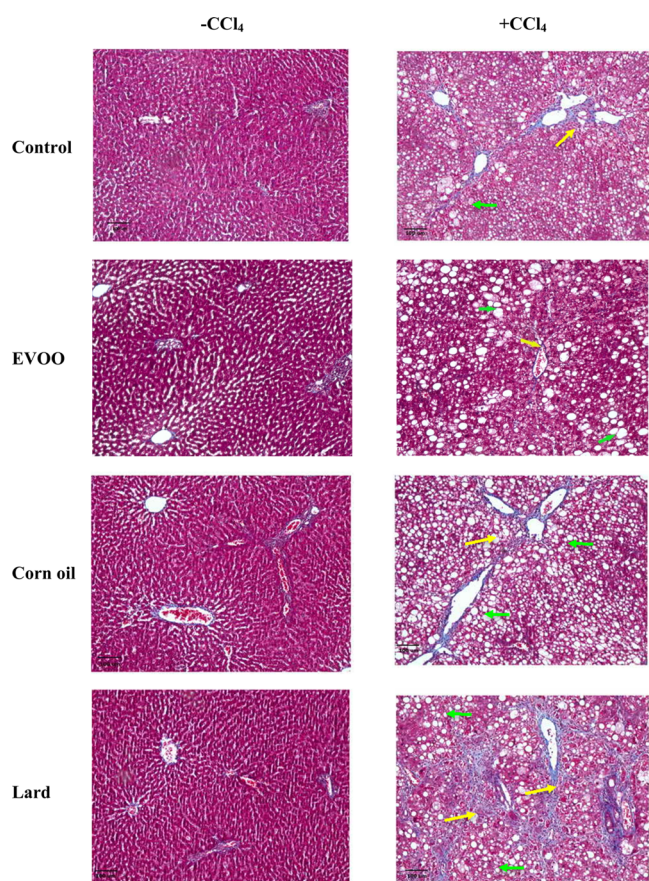


**Figure 2.** Serum AST (A) and ALT (B) levels in rats that received dietary oils. Values are expressed as the mean ± SD, *n* = 4–6 rats. AST, aspartate transaminase; ALT, alanine transaminase; EVOO, extra virgin olive oil; CCl<sub>4</sub>, carbon tetrachloride. \*\**p* < 0.01 -CCl<sub>4</sub> vs + CCl<sub>4</sub> for the respective diets.

**3.3. Different Dietary Lipids Modified Liver Lipid Metabolism.** Supplementation of different dietary lipids appeared to have an effect on the liver lipid metabolism (Table 1). It was surprising to find EVOO to augment the AA level, whereas the corn oil and lard diet reduced AA levels. However, treatment of CCl<sub>4</sub> reduced AA concentration, but the lowering effect was less for those fed with the dietary lipids. It was also interesting to observe suppressed EPA levels in the liver with or without CCl<sub>4</sub> treatment for all diets, whereas only the corn oil and lard diet suppressed DHA levels after treatment with CCl<sub>4</sub>. The unusually high levels of DHA in both control and treatments regardless of supplemented dietary lipids was noted. This may be attributed to the PUFA content in the basic rodent chow where the ratio of omega-6 to omega-3 PUFA was found to be approximately 3:1. This ratio is significantly lower when compared to that in the modern Western diet (10–20:1).

**3.4. EVOO Reduced Isoprostanoids and HETEs, and the Effect Persisted Even with CCl<sub>4</sub> Challenge.** As indicated per se, the elevation of lipid mediators F<sub>2</sub>-IsoPs *in vivo* is toxic, while increase in F<sub>4</sub>-NeuroPs is potentially beneficial.<sup>20–22,24,25</sup> Supplementations of EVOO and corn oil but not lard reduced the levels of F<sub>2</sub>-IsoPs compared to that of the control, and only corn oil lowered F<sub>3</sub>-IsoPs levels compared to that of the control. However, F<sub>4</sub>-NeuroPs concentration increased regardless of the type of dietary lipid feeding (Figure 4A). When the rats were treated with CCl<sub>4</sub>, only EVOO and not corn oil or lard was able to effectively suppress F<sub>2</sub>-IsoPs and F<sub>3</sub>-IsoPs but also F<sub>4</sub>-NeuroPs compared to the control (Figure 4B).

Measurement of pro-inflammatory (HETEs) and anti-inflammatory (RvD1) lipoxygenase lipid mediators revealed the influence of dietary oils in enzymatic oxidation. Consistently, levels of 5-, 12-, and 15-HETEs were reduced considerably by the

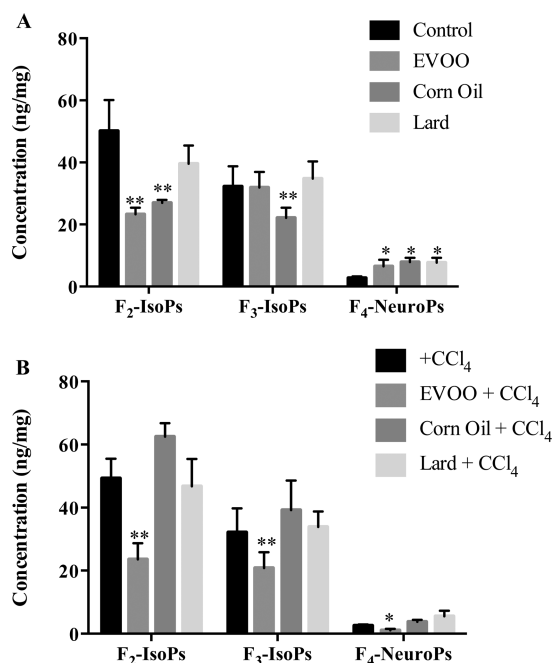


**Figure 3.** Liver biopsy specimen sections stained with Masson's trichrome (100 X). Cell nuclei were stained in dark brown, cytoplasm were stained in red, and collagen fibers were stained in blue. Adipose vesicles were denoted with yellow arrows and fibrous septa in green arrows. EVOO, extra virgin olive oil; CCl<sub>4</sub>, carbon tetrachloride.

EVOO diet but not RvD1 compared to other dietary lipids and control (Figure 5A). In the presence of CCl<sub>4</sub>, the presence of EVOO in the diet consistently showed a lower amount of 5-, 12-, and 15-HETEs productions compared to that of the control (Figure 5B), whereas RvD1 was reduced by EVOO and corn oil.

These observations indicate that EVOO could potentially have a strong antioxidant property even under acute and severe oxidative stress.

**3.5. EVOO Modulated Cholesterol and COPs Generation.** Elevated cholesterol level was observed in rats fed with all dietary lipids, and it is likely due to the high lipid diet (20%) given to the rats. However, EVOO significantly lowered the cholesterol production in CCl<sub>4</sub> challenged rats, suggesting that EVOO might



**Figure 4.** Effect of nonenzymatic lipid peroxidation after dietary oils and lard supplementation (A) and treatment with carbon tetrachloride (CCl<sub>4</sub>) (B). Values are the mean  $\pm$  SD,  $n = 4-6$  rats. The oxidized metabolites are annotated as F<sub>2</sub>-IsoPs, 15-F<sub>2</sub>-isoprostanes from AA; F<sub>3</sub>-IsoPs, 15-F<sub>3</sub>-isoprostanes from EPA; and F<sub>4</sub>-NeuroPs, 4(RS)-4-F<sub>4</sub>-neuroprostanes from DHA. EVOO: extra virgin olive oil. \* $p < 0.05$  and \*\* $p < 0.01$  compared to the control.

be able to reverse the cholesterol overproduction or deposition in the liver in the presence of CCl<sub>4</sub>. Supplementation of EVOO also reduced 7 $\beta$ -OH and 7-ketochol in the presence and absence of CCl<sub>4</sub> compared to those in the control. Dietary corn oil elevated 7 $\beta$ -OH only in the absence of CCl<sub>4</sub>. Our data also showed lard to reduce 7-ketochol and 27-OH in the presence and absence of CCl<sub>4</sub> but not 7 $\beta$ -OH (Figures 6A and B).

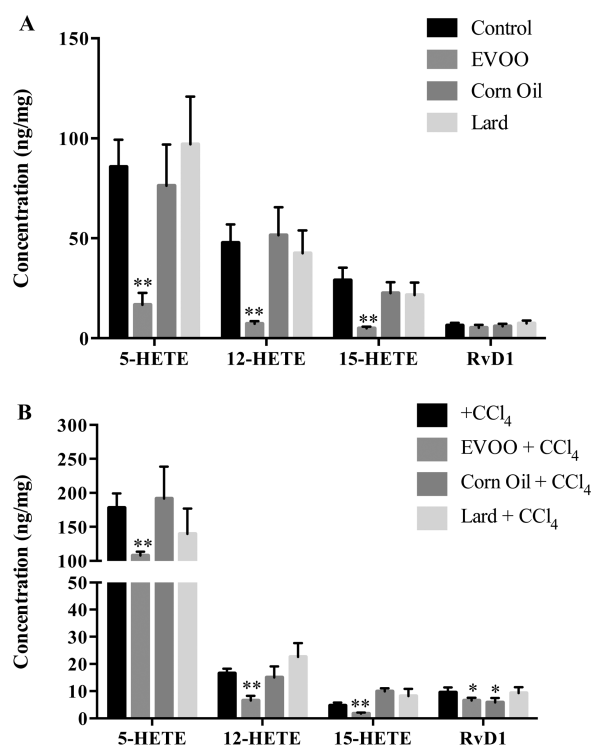
The evaluation of liver COPs in this study further substantiates EVOO as being a strong antioxidant.

**3.6. EVOO Had High Total Phenolic Content and Strong Antioxidant Capacity.** The potent inhibition of producing oxidized lipid products under CCl<sub>4</sub> injury by EVOO led us to investigate the total phenolic content and antioxidant capacity of the actual dietary oils that we used. Figure 7A and B illustrates that EVOO possessed the highest concentrations of total phenolic content and antioxidant capacity when compared to that of corn oil and lard. Intriguingly, lard contains a higher amount of total phenolic content and antioxidant capacity than corn oil.

**Table 1. Concentration of Precursors of Isoprostanoids Measured in Liver Samples before (-CCl<sub>4</sub>) and after (+CCl<sub>4</sub>) Carbon Tetrachloride Treatment<sup>a</sup>**

	group	control	EVOO	corn	lard
AA	-CCl <sub>4</sub>	410.43 $\pm$ 130.62	865.23 $\pm$ 75.01 <sup>c</sup>	273.33 $\pm$ 52.23 <sup>c</sup>	296.62 $\pm$ 74.47 <sup>c</sup>
	+CCl <sub>4</sub>	78.05 $\pm$ 13.73 <sup>b</sup>	298.68 $\pm$ 63.35 <sup>b,c</sup>	181.76 $\pm$ 17.17 <sup>b,c</sup>	173.17 $\pm$ 18.74 <sup>b,c</sup>
EPA	-CCl <sub>4</sub>	204.47 $\pm$ 49.61	88.78 $\pm$ 19.45 <sup>c</sup>	77.47 $\pm$ 19.36 <sup>c</sup>	118.75 $\pm$ 22.7 <sup>c</sup>
	+CCl <sub>4</sub>	215.35 $\pm$ 54.23	96.81 $\pm$ 19.41 <sup>c</sup>	68.60 $\pm$ 4.32 <sup>c</sup>	98.39 $\pm$ 11.42 <sup>c</sup>
DHA	-CCl <sub>4</sub>	331.66 $\pm$ 74.64	485.01 $\pm$ 15.83	302.21 $\pm$ 43.78	433.02 $\pm$ 76.34
	+CCl <sub>4</sub>	452.82 $\pm$ 87.64	441.60 $\pm$ 100.11	212.36 $\pm$ 46.08 <sup>c</sup>	152.83 $\pm$ 20.40 <sup>b,c</sup>

<sup>a</sup>Values are the mean  $\pm$  SD (ng/mg tissue),  $n = 4-6$ . Significant difference. <sup>b</sup> $p < 0.01$  - CCl<sub>4</sub> vs + CCl<sub>4</sub>. <sup>c</sup> $p < 0.01$  compared to control of respective group. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

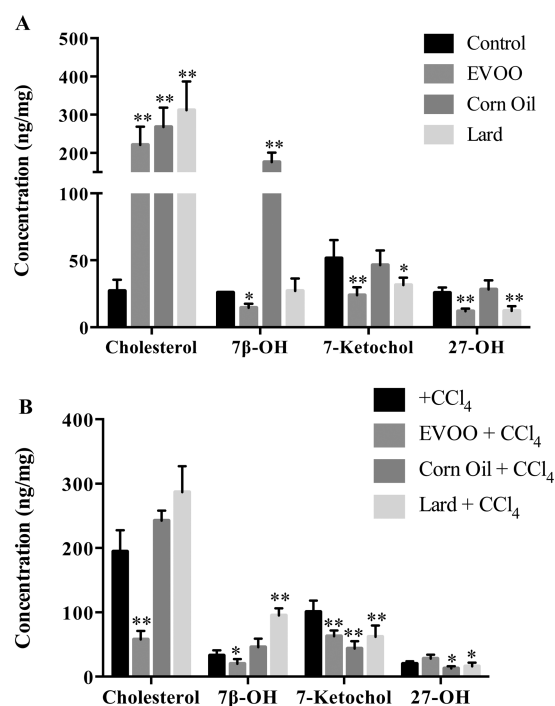


**Figure 5.** Effect of enzymatic lipid peroxidation after dietary oils and lard supplementation (A) and treatment with carbon tetrachloride (CCl<sub>4</sub>) (B). Values are the mean  $\pm$  SD,  $n = 4-6$  rats. The oxidized metabolites are annotated as HETE, hydroxyeicosatetraenoic acid from AA; and RvD1, resolvin D1 from DHA. EVOO, extra virgin olive oil. \* $p < 0.05$  and \*\* $p < 0.01$  compared to control.

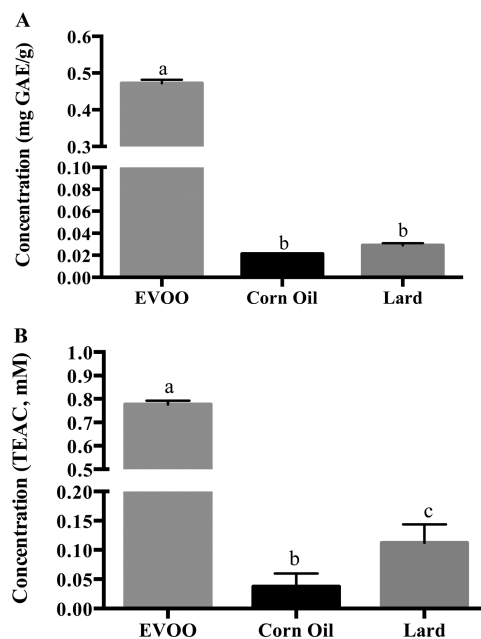
**3.7. High Amounts of Hydroxytyrosol Conjugated FA Are Present in EVOO.** Despite the high total phenolic compounds and strong antioxidant capacity *in vitro*, the bioavailability of EVOO phenolic compounds in humans is extremely poor.<sup>37</sup> Hence, to better understand the potent antioxidant property *in vivo*, we investigated the proportion of FA (Figure 8A) and the amount of lipophenols (HT-FA) (Figure 8B) in EVOO and OO with respect to CO. Our data (Figure 8A) showed that the major FA profile of both EVOO and OO are similar but different from that of corn oil. Expectedly, OA contributed as the major FA present in EVOO and OO, whereas LA was the main component in CO. Furthermore, high levels of HT-OA, HT-LA, and HT-ALA were found in EVOO and olive oil (Figure 8B) but absent in corn oil. HT-LA in EVOO had the highest concentration among those evaluated, and the concentration of total HT-FA in EVOO was approximately four times more than OO. This suggests that the high content of lipophenols in EVOO and to a lesser extent in OO might increase the bioavailability of hydroxytyrosol *in vivo* by conjugating with the FA present.

#### 4. DISCUSSION

This study clearly demonstrates the bioactivity of EVOO in the liver. Foremost, it is able to reduce oxidative stress even when challenged by CCl<sub>4</sub>. The measurement of isoprostanes, particularly F<sub>2</sub>-IsoPs, is often used to unveil the *in vivo* status of oxidative stress,<sup>38</sup> in which elevated plasma was observed in male and female subjects with NALFD,<sup>39</sup> and the reduction by EVOO in this study indicates a protection mechanism of the liver that may be attributed to lipophenols (HT-FA). A recent study

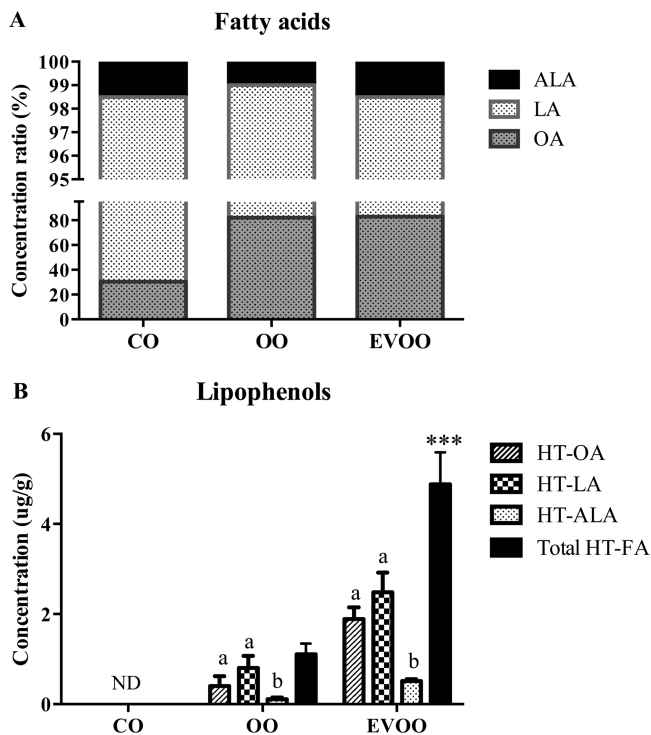


**Figure 6.** Effect of cholesterol and cholesterol oxidation after dietary oils and lard supplementation (A) and treatment with carbon tetrachloride (CCl<sub>4</sub>) (B). Values are the mean  $\pm$  SD,  $n = 4-6$  rats. The oxidized metabolites are annotated as 7 $\beta$ -OH, 7 $\beta$ -hydroxycholesterol; 7-ketochol, 7-ketocholesterol; and 27-OH, 27-hydroxycholesterol. EVOO, extra virgin olive oil. \* $p < 0.05$  and \*\* $p < 0.01$  compared to control.



**Figure 7.** Total phenolic content (A) and antioxidant capacity (B) of EVOO, corn oil, and lard supplemented to the experimental rats. Values are the mean  $\pm$  SD,  $n = 3$ . Columns sharing different alphabets are significantly different at least  $p < 0.05$ . EVOO, extra virgin olive oil; GAE, gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity.

showed the consumption of a Mediterranean diet rich in EVOO for one year reduced urinary F<sub>2</sub>-IsoPs in females with metabolic syndrome.<sup>40</sup>



**Figure 8.** Percentage of fatty acids (FA) (A) and concentration of lipophenols (B) from EVOO, olive oil, and corn oil. All experimental data were repeated three times and reported as the mean (A) or mean  $\pm$  SD (B). HT, hydroxytyrosol; CO, corn oil; OO, olive oil; EVOO, extra virgin olive oil; OA, oleic acid; LA, linoleic acid; ALA, alpha-linolenic acid; HT-FA, total sum of HT-OA + HT-LA + HT-ALA. Columns (HT-OA, HT-LA, and HT-ALA) sharing differ superscript letters indicate significant difference  $p < 0.05$ . \*\*\* $p < 0.001$  HT-FA in OO vs HT-FA in EVOO.

Interestingly, regardless of the type of dietary lipid, it appears to modify the PUFA metabolism, in particular the modulation of desaturase/elongase activity. We notably observed that dietary EVOO augmented the liver AA level but not in the presence of  $\text{CCl}_4$ . The rationale for this observation is not clear as we did not measure desaturase/elongase activities; however, the increment of liver cholesterol by the lipid diet may regulate the fatty acids, act on  $\Delta^9$ -desaturase, and lead to competition for  $\Delta^6$ -desaturase of the omega-6 and omega-3 PUFA metabolism; perhaps an EVOO diet favored the activation of  $\Delta^6$ -desaturase of the AA pathway irrespective of the  $\text{CCl}_4$  effect.<sup>41</sup> Nevertheless, an inverse relationship was reported between OA and AA in healthy human serum phospholipids. It is postulated that the inverse relationship may be attributed to the OA suppression of  $\Delta^5$ -desaturase and  $\Delta^6$ -desaturase, together with elongase-5 to reduce the formation of AA. Moreover, there is a possibility that AA might suppress the formation of OA by the inhibition of  $\Delta^9$ -desaturase by gene transcription regulated by omega-3 and omega-6 PUFA.<sup>41,42</sup> Further, our findings may be related to increased nonesterified fatty acid production by EVOO by the modulation of hepatic adipophilin. Down-regulation of adipophilin protein causes the channelling of fatty acids and leads primarily to  $\beta$ -oxidation.<sup>43</sup>

It has been recently reported that dietary long chain PUFA with EVOO diet reduced liver steatosis induced by a high fat diet. The report also indicated the suppression of oxidative stress and inflammation by the same diet. However, the study determined an approximate value of TBARS and  $\text{F}_2$ -IsoPs for oxidative

stress using the kit. Also,  $\text{F}_2$ -IsoPs is only related to AA.<sup>44</sup> Our study is the first to measure multiple oxygenated lipid mediators related to oxidative stress including  $\text{F}_3$ -IsoPs and  $\text{F}_4$ -NeuroPs from EPA and DHA, respectively, in the liver with or without  $\text{CCl}_4$  challenge and different lipid diets. EVOO was the only dietary lipid in the study to reduce these lipid mediators. Whether this decrease is beneficial or damaging is not clear as major reports focused on the release of these lipid mediators as biomarkers of oxidative stress associated with the brain or the effect of a fish oil diet; both are concentrated in EPA and DHA. However, endogenous elevation of  $\text{F}_3$ -IsoPs after fish oil supplementation attenuated inflammation in mice, whereas increased  $\text{F}_4$ -NeuroPs were shown in the plasma of neurodegenerative disease patients.<sup>26,30</sup> On the contrary, recent studies indicate that  $\text{F}_4$ -NeuroPs in  $\text{LDL}^{-/-}$  mouse livers was associated with lower risk of atherosclerosis, and an *in vitro* and *in vivo* study showed  $\text{F}_4$ -NeuroPs to have antiarrhythmic properties.<sup>24,45</sup>

Hydroxyeicosatetraenoic acids (HETEs) are oxidized end products nonenzymatically and enzymatically derived from AA. The substrate AA is transformed into 5-, 12-, and 15-HETEs by 5-, 12-, and 15-lipoxygenases (LOX), respectively, or by the action of free radicals. The expressions of 5- and 12-LOX but not 15-LOX have been implicated in carcinogenesis.<sup>46</sup> The forming of 5- and 12-HETEs, are also known to aid in cancer cell proliferations and tumor development, and induce inflammation.<sup>47</sup> In contrast, 15-LOX and 15-HETE appear to inhibit cancerous cell growth.<sup>48</sup> Here, the moderation of HETEs by EVOO supplementation has been suppressed consistently in both the absence and presence of  $\text{CCl}_4$  injury. This result suggests that the bioactive EVOO has lessened lipid oxidation and generation of LOX-mediated products in rodents. This is in line with previous reports showing the reduced expression of LOX and their downstream metabolites, HETEs, in the presence of phenolic contents from EVOO. For example, the expression of 5-LOX from a leukocyte was inhibited by hydroxytyrosol (HT), oleuropein, caffeic acid, and tyrosol found in virgin olive oil, in which HT had the strongest effect.<sup>49</sup> Similarly, it was also reported that HT suppressed platelet aggregation and the production of 12-HETE.<sup>50</sup>

LDL oxidation (ox-LDL) is associated with the development of atherosclerosis, and dietary EVOO has shown to lower ox-LDL.<sup>51,52</sup> Notwithstanding, total cholesterol and its oxidized products are important regulators in the lipidome but are often missing in the studies of EVOO in the liver. An earlier study showed that EVOO and other lipid diets increased hepatic cholesterol in  $\text{ApoE}^{-/-}$  mice and may be accounted for by the type; EVOO is reported to up-regulate hepatic HDL. Similar to PUFA oxidized metabolites, cholesterol oxidized products (COPs) are both enzymatically and nonenzymatically derived. The  $7\beta$ -OH and 7-ketocholesterol are interchangeable COPs that arise by free radical reactions from cholesterol,<sup>53</sup> whereas 27-OH is produced enzymatically via a cytochrome P450 system. The production of these COPs is generally disadvantageous as they are being pointed to be disruptive *in vivo* modulators and biomarkers of LDL oxidation.<sup>54,55</sup> Here, the type of diets provided to the rats did not alter the liberation of specific COPs in both normal and  $\text{CCl}_4$ -injured rat groups.

Collectively, profiling of oxidized lipid and cholesterol products from dietary oil supplemented rats against hepatotoxicity induced by  $\text{CCl}_4$  showed that EVOO, as compared to the control, consistently reduced the levels of most oxidized lipid products derived via enzymatic and nonenzymatic pathways. This suggests that EVOO supplementation is far more superior



in reducing the rate of *in vivo* oxidation as compared to other dietary lipids. The reduction of *in vivo* autoxidation by EVOO is mostly recognized to be attributed to the presence of phenolic compounds, instead of its omega-9-rich MUFA content.<sup>56</sup> Indeed, measurement of total phenolic content and antioxidant capacity showed EVOO to have higher phenolic amount and higher rate of antioxidant capacity consistently than corn oil and lard. In addition, for the first time we identified that HT may exist as a conjugated form in these dietary oils and potentially link covalently with FA through the primary alcohol of the phenolic derivative. The conjugation with FA may facilitate the bioavailability of HT endogenously through catabolic action and hydrolysis of the intestinal microbiota on the HT-FA ligand.<sup>11</sup> Depending on the polyphenol structure and their flexibility, the molecule should be able to adjust differently in the position of FA and allow the phenolic component to obtain better interaction with the active site of the enzyme during hydrolysis. Moreover, the number of double bonds in the FA chain could alter the flexibility of the lipophenol conjugate and thus influence its conformation. Take note, if the lipophenol structure contains PUFA, peroxidation may be prevented by the presence of the phenolic residue conjugated, thereby increasing the bioavailability and functionality of the fatty acid as well as the polyphenol when metabolized.<sup>57</sup>

Recently, vanillyl alcohol conjugated to DHA displayed neuroprotective properties in rat primary neurons exposed to amyloid- $\beta$  oligomers, and the study suggests that the two molecules synergize for the beneficial effects.<sup>58</sup> Moreover, when phloroglucinol ring structure was conjugated with DHA, it had a cytoprotective effect on the retinal epithelial cells under carbonyl stress.<sup>59</sup> In addition, it has been shown that treatment of genistein with omega-3 PUFA in macrophages reduced cyclooxygenase A<sub>2</sub> and subsequently inflammatory transcription factor nuclear factor  $\kappa$ B<sup>5</sup> and that omega-3 PUFA and quercetin supplementation down-regulated intestinal inflammation via an antioxidant effect by quercetin in colitis rats.<sup>60</sup> As a consequence, in this study, we postulate that the presence of phenolic compounds as a conjugated form with FA (lipophenols) and its antioxidant capacity had a greater effect in reducing oxidative stress.

Several reports have shown EVOO to have a protective mechanism for CVD and reduce inflammation and breast cancer,<sup>4,56,61</sup> but studies related to the liver are scarce. The liver filters and detoxifies harmful substances in the bloodstream with the help of specific enzymes, ALT and AST, when hepatic cells are damaged or destroyed. To date, ALT and AST activities are regarded as reliable and sensitive markers for liver damage. Intoxication of CCl<sub>4</sub> in animals of different species is a well-known model for inducing acute and chronic liver injury by prohibiting exportation of lipids away from the liver causing fatty liver and the generation of several radical species via the cytochrome P450 system leading to lipid peroxidation.<sup>62</sup> Our study employed the use of CCl<sub>4</sub> to induce acute liver injury and supplementing different dietary lipids for liver injury protection or resolution. The initial observation of supplementing 20% of EVOO, corn oil, and lard with normal chow diet to the rats did not modify the morphological features of the rat liver, whereas a liver damaging effect was observed by CCl<sub>4</sub> treatment. Only EVOO appeared to reduce but not prevent the formation of adipose vesicles and fibrous septa triggered by CCl<sub>4</sub> activation in this study. Indeed, this finding opposes a previous report, which proposed that hepatic lipid droplets are enhanced by an EVOO diet and is associated with high levels of hepatic adipophilin

protein.<sup>43</sup> However, a recent report has described the intake of EVOO to be linked with enhanced expression of two cholesterol efflux regulatory proteins that thereby mediate cholesterol efflux.<sup>63</sup> This probably suggests that EVOO acts as an enhancer or effector in the exportation of accumulated lipids in the liver, thereby reducing lipid deposition, but is not capable of preventing liver cell death.

Taken together, EVOO has shown to deliver molecular benefits in hepatocytes, preventing and rectifying enzymatic and nonenzymatic lipid peroxidation when compared to corn oil and lard. However, as to whether the high portion of OA or LA or ALA especially its conjugation with HT in EVOO has a role to play in counteracting oxidative stress *in vivo* remains to be elucidated. Also, because EVOO, CO, and lard are commonly used as cooking oil, the bioactivity of these dietary oils might not necessarily be the same after heating and further processing. Further evaluation is necessary especially of the concentration of HT, OA, LA, and ALA as well as its lipophenol circulating after EVOO supplementation to correspond with those found in EVOO.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.chemres-tox.6b00214](https://doi.org/10.1021/acs.chemres-tox.6b00214).

Synthesis of lipophenols and NMR characterizations (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Fax: +852-2559-9114. E-mail: [jettylee@hku.hk](mailto:jettylee@hku.hk).

### Funding

We thank The University of Hong Kong, Seed Funding for Basic Research (Nos. 201211159156 and 201310159020) and Small Project Funding (No. 201409176019) for the support of this work.

### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

27-OH, 27-hydroxycholesterol; 7-ketochol, 7-ketocholesterol; 7 $\beta$ -OH, 7 $\beta$ -hydroxycholesterol; AA, arachidonic acid; ALA, alpha-linolenic acid; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ALT, alanine transaminase; AST, aspartate transaminase; BHT, butylated hydroxytoluene; CCl<sub>4</sub>, carbon tetrachloride; CO, corn oil; COPs, cholesterol oxidation products; CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DNP, 2,4-dinitrophenylhydrazine; EPA, eicosapentaenoic acid; ESI, electrospray ionization; EVOO, extra virgin olive oil; F<sub>2</sub>-IsoP, 15-F<sub>2t</sub>-isoprostane; F<sub>3</sub>-IsoP, 8-F<sub>3t</sub>-isoprostane; F<sub>4</sub>-NeuroP, 4(RS)-4-F<sub>4t</sub>-neuroprostane; FA, fatty acid; HBV, hepatitis virus B; HCV, hepatitis virus C; HETEs, hydroxyeicosatetraenoic acids; HT, hydroxytyrosol; HT-ALA, hydroxytyrosol conjugated to alpha-linolenic acid; HT-FA, hydroxytyrosol conjugated fatty acid; HT-LA, hydroxytyrosol conjugated to linoleic acid; HT-OA, hydroxytyrosol conjugated to oleic acid; LA, linoleic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDL, low-density lipoprotein; LOX, lipoxygenase; MRM, multiple reactions monitoring; MUFA, monounsaturated fatty acid; NAFLD, nonalcoholic fatty liver disease; OA, oleic acid;

OO, olive oil; PUFA, polyunsaturated fatty acid; RvD1, resolvin D1

## ■ REFERENCES

- (1) Covas, M. I. (2007) Olive oil and the cardiovascular system. *Pharmacol. Res.* 55, 175–186.
- (2) Garg, A. (1998) High-monounsaturated-fat diets for patients with diabetes mellitus: a meta-analysis. *Am. J. Clin. Nutr.* 67, 577S–582S.
- (3) Estruch, R., Ros, E., Salas-Salvado, J., Covas, M. I., Corella, D., Aros, F., Gomez-Gracia, E., Ruiz-Gutierrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R. M., Serra-Majem, L., Pinto, X., Basora, J., Munoz, M. A., Sorli, J. V., Martinez, J. A., Martinez-Gonzalez, M. A., and Investigators, P. S. (2013) Primary prevention of cardiovascular disease with a Mediterranean diet. *N. Engl. J. Med.* 368, 1279–1290.
- (4) Guasch-Ferre, M., Hu, F. B., Martinez-Gonzalez, M. A., Fito, M., Bullo, M., Estruch, R., Ros, E., Corella, D., Recondo, J., Gomez-Gracia, E., Fiol, M., Lapetra, J., Serra-Majem, L., Munoz, M. A., Pinto, X., Lamuela-Raventos, R. M., Basora, J., Buil-Cosiales, P., Sorli, J. V., Ruiz-Gutierrez, V., Martinez, J. A., and Salas-Salvado, J. (2014) Olive oil intake and risk of cardiovascular disease and mortality in the PREDIMED Study. *BMC Med.* 12, 78.
- (5) Aguilera, C. M., Ramirez-Tortosa, M. C., Mesa, M. D., Ramirez-Tortosa, C. L., and Gil, A. (2002) Sunflower, virgin-olive and fish oils differentially affect the progression of aortic lesions in rabbits with experimental atherosclerosis. *Atherosclerosis* 162, 335–344.
- (6) Calleja, L., Paris, M. A., Paul, A., Vilella, E., Joven, J., Jimenez, A., Beltran, G., Uceda, M., Maeda, N., and Osada, J. (1999) Low-cholesterol and high-fat diets reduce atherosclerotic lesion development in ApoE-knockout mice. *Arterioscler., Thromb., Vasc. Biol.* 19, 2368–2375.
- (7) Eilertsen, K. E., Maehre, H. K., Cludts, K., Olsen, J. O., and Hoylaerts, M. F. (2011) Dietary enrichment of apolipoprotein E-deficient mice with extra virgin olive oil in combination with seal oil inhibits atherogenesis. *Lipids Health Dis.* 10, 41.
- (8) Abuznait, A. H., Qosa, H., Busnena, B. A., El Sayed, K. A., and Kaddoumi, A. (2013) Olive-oil-derived oleocanthal enhances beta-amyloid clearance as a potential neuroprotective mechanism against Alzheimer's disease: in vitro and in vivo studies. *ACS Chem. Neurosci.* 4, 973–982.
- (9) Beauchamp, G. K., Keast, R. S., Morel, D., Lin, J., Pika, J., Han, Q., Lee, C. H., Smith, A. B., and Breslin, P. A. (2005) Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. *Nature* 437, 45–46.
- (10) LeGendre, O., Breslin, P. A., and Foster, D. A. (2015) (–)-Oleocanthal rapidly and selectively induces cancer cell death via lysosomal membrane permeabilization. *Mol. Cell Oncol.* 2, e1006077.
- (11) Tuohy, K. M., Fava, F., and Viola, R. (2014) 'The way to a man's heart is through his gut microbiota'—dietary pro- and prebiotics for the management of cardiovascular risk. *Proc. Nutr. Soc.* 73, 172–185.
- (12) Horia, E., and Watkins, B. A. (2007) Complementary actions of docosahexaenoic acid and genistein on COX-2, PGE2 and invasiveness in MDA-MB-231 breast cancer cells. *Carcinogenesis* 28, 809–815.
- (13) Fan, J. G., and Farrell, G. C. (2009) Epidemiology of non-alcoholic fatty liver disease in China. *J. Hepatol.* 50, 204–210.
- (14) Yuen, M. F., Hou, J. L., Chutaputti, A., and Asia Pacific Working Party on Prevention of Hepatocellular, C. (2009) Hepatocellular carcinoma in the Asia Pacific region. *J. Gastroenterol. Hepatol.* 24, 346–353.
- (15) Bataller, R., and Brenner, D. A. (2005) Liver fibrosis. *J. Clin. Invest.* 115, 209–218.
- (16) Parsons, C. J., Takashima, M., and Rippe, R. A. (2007) Molecular mechanisms of hepatic fibrogenesis. *J. Gastroenterol. Hepatol.* 22, S79–84.
- (17) Tutino, V., Orlando, A., Russo, F., and Notarnicola, M. (2016) Hydroxytyrosol inhibits cannabinoid CB1 receptor gene expression in 3T3-L1 preadipocyte cell line. *J. Cell. Physiol.* 231, 483–489.
- (18) Muriel, P. (2009) Role of free radicals in liver diseases. *Hepatol. Int.* 3, 526–536.
- (19) Poli, G. (1993) Liver damage due to free radicals. *Br. Med. Bull.* 49, 604–620.
- (20) Basu, S. (2003) Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. *Toxicology* 189, 113–127.
- (21) Morrow, J. D., Awad, J. A., Kato, T., Takahashi, K., Badr, K. F., Roberts, L. J., 2nd, and Burk, R. F. (1992) Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. *J. Clin. Invest.* 90, 2502–2507.
- (22) Musiek, E. S., Milne, G. L., McLaughlin, B., and Morrow, J. D. (2005) Cyclopentenone eicosanoids as mediators of neurodegeneration: a pathogenic mechanism of oxidative stress-mediated and cyclooxygenase-mediated neurotoxicity. *Brain Pathol.* 15, 149–158.
- (23) Sun, Y. P., Oh, S. F., Uddin, J., Yang, R., Gotlinger, K., Campbell, E., Colgan, S. P., Petasis, N. A., and Serhan, C. N. (2007) Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation. *J. Biol. Chem.* 282, 9323–9334.
- (24) Gladine, C., Newman, J. W., Durand, T., Pedersen, T. L., Galano, J. M., Demougeot, C., Berdeaux, O., Pujos-Guillot, E., Mazur, A., and Comte, B. (2014) Lipid profiling following intake of the omega 3 fatty acid DHA identifies the peroxidized metabolites F4-neuroprostanes as the best predictors of atherosclerosis prevention. *PLoS One* 9, e89393.
- (25) Roy, J., Le Guennec, J. Y., Galano, J. M., Thireau, J., Bultel-Ponce, V., Demion, M., Oger, C., Lee, J. C., and Durand, T. (2016) Non-enzymatic cyclic oxygenated metabolites of omega-3 polyunsaturated fatty acid: Bioactive drugs? *Biochimie* 120, 56–61.
- (26) Galano, J. M., Lee, J. C., Gladine, C., Comte, B., Le Guennec, J. Y., Oger, C., and Durand, T. (2015) Non-enzymatic cyclic oxygenated metabolites of adrenic, docosahexaenoic, eicosapentaenoic and alpha-linolenic acids; bioactivities and potential use as biomarkers. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 1851, 446–455.
- (27) Garenc, C., Julien, P., and Levy, E. (2010) Oxysterols in biological systems: the gastrointestinal tract, liver, vascular wall and central nervous system. *Free Radical Res.* 44, 47–73.
- (28) Slater, T. F., Cheeseman, K. H., and Ingold, K. U. (1985) Carbon tetrachloride toxicity as a model for studying free-radical mediated liver injury. *Philos. Trans. R. Soc., B* 311, 633–645.
- (29) Tomasi, A., Albano, E., Banni, S., Botti, B., Corongiu, F., Dessi, M. A., Iannone, A., Vannini, V., and Dianzani, M. U. (1987) Free-radical metabolism of carbon tetrachloride in rat liver mitochondria. A study of the mechanism of activation. *Biochem. J.* 246, 313–317.
- (30) Vigor, C., Bertrand-Michel, J., Pinot, E., Oger, C., Vercauteren, J., Le Faouder, P., Galano, J. M., Lee, J. C., and Durand, T. (2014) Non-enzymatic lipid oxidation products in biological systems: assessment of the metabolites from polyunsaturated fatty acids. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 964, 65–78.
- (31) Leung, K. S., Chen, X., Zhong, W., Yu, A. C., and Lee, C. Y. (2014) Microbubble-mediated sonoporation amplified lipid peroxidation of Jurkat cells. *Chem. Phys. Lipids* 180, 53–60.
- (32) de La Torre, A., Lee, Y. Y., Oger, C., Sangild, P. T., Durand, T., Lee, J. C., and Galano, J. M. (2014) Synthesis, discovery, and quantitation of dihydro-isofurans: biomarkers for in vivo adrenic acid peroxidation. *Angew. Chem., Int. Ed.* 53, 6249–6252.
- (33) Shui, G., Cheong, W. F., Jappar, I. A., Hoi, A., Xue, Y., Fernandez, A. Z., Tan, B. K., and Wenk, M. R. (2011) Derivatization-independent cholesterol analysis in crude lipid extracts by liquid chromatography/mass spectrometry: applications to a rabbit model for atherosclerosis. *J. Chromatogr. A* 1218, 4357–4365.
- (34) Ciafardini, G., and Zullo, B. A. (2015) Effect of lipolytic activity of *Candida adriatica*, *Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content. *Food Microbiol.* 47, 12–20.
- (35) Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* 26, 1231–1237.
- (36) Marinova, D., Ribarova, F., and Atanassova, M. (2005) Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *J. Univ. Chem. Technol. Metal.* 40, 255–260.

- (37) Tuck, K. L., and Hayball, P. J. (2002) Major phenolic compounds in olive oil: metabolism and health effects. *J. Nutr. Biochem.* 13, 636–644.
- (38) Halliwell, B., and Lee, C. Y. (2010) Using isoprostanes as biomarkers of oxidative stress: some rarely considered issues. *Antioxid. Redox Signaling* 13, 145–156.
- (39) Raszeja-Wyszomirska, J., Safranow, K., Milkiewicz, M., Milkiewicz, P., Szykowska, A., and Stachowska, E. (2012) Lipidic last breath of life in patients with alcoholic liver disease. *Prostaglandins Other Lipid Mediators* 99, 51–56.
- (40) Mitjavila, M. T., Fandos, M., Salas-Salvado, J., Covas, M. I., Borrego, S., Estruch, R., Lamuela-Raventos, R., Corella, D., Martinez-Gonzalez, M. A., Sanchez, J. M., Bullo, M., Fito, M., Tormos, C., Cerda, C., Casillas, R., Moreno, J. J., Iradi, A., Zaragoza, C., Chaves, J., and Saez, G. T. (2013) The Mediterranean diet improves the systemic lipid and DNA oxidative damage in metabolic syndrome individuals. A randomized, controlled, trial. *Clin. Nutr.* 32, 172–178.
- (41) Ntambi, J. M., and Bene, H. (2001) Polyunsaturated fatty acid regulation of gene expression. *J. Mol. Neurosci.* 16, 273–278 discussion 279–284.
- (42) Hostmark, A. T., and Haug, A. (2013) Percentages of oleic acid and arachidonic acid are inversely related in phospholipids of human sera. *Lipids Health Dis.* 12, 106.
- (43) Arbones-Mainar, J. M., Ross, K., Rucklidge, G. J., Reid, M., Duncan, G., Arthur, J. R., Horgan, G. W., Navarro, M. A., Carnicer, R., Arnal, C., Osada, J., and de Roos, B. (2007) Extra virgin olive oils increase hepatic fat accumulation and hepatic antioxidant protein levels in APOE<sup>-/-</sup> mice. *J. Proteome Res.* 6, 4041–4054.
- (44) Valenzuela, R., Espinosa, A., Llanos, P., Hernandez-Rodas, M. C., Barrera, C., Vergara, D., Romero, N., Perez, F., Ruz, M., and Videla, L. A. (2016) Anti-steatotic effects of an n-3 LCPUFA and extra virgin olive oil mixture in the liver of mice subjected to high-fat diet. *Food Funct.* 7, 140–150.
- (45) Roy, J., Oger, C., Thireau, J., Roussel, J., Mercier-Touzet, O., Faure, D., Pinot, E., Farah, C., Taber, D. F., Cristol, J. P., Lee, J. C., Lacampagne, A., Galano, J. M., Durand, T., and Le Guennec, J. Y. (2015) Nonenzymatic lipid mediators, neuroprostanes, exert the antiarrhythmic properties of docosahexaenoic acid. *Free Radical Biol. Med.* 86, 269–278.
- (46) Ghosh, J., and Myers, C. E. (1997) Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem. Biophys. Res. Commun.* 235, 418–423.
- (47) Shureiqi, I., and Lippman, S. M. (2001) Lipoxygenase modulation to reverse carcinogenesis. *Cancer Res.* 61, 6307–6312.
- (48) Shureiqi, I., Chen, D., Lotan, R., Yang, P., Newman, R. A., Fischer, S. M., and Lippman, S. M. (2000) 15-Lipoxygenase-1 mediates nonsteroidal anti-inflammatory drug-induced apoptosis independently of cyclooxygenase-2 in colon cancer cells. *Cancer Res.* 60, 6846–6850.
- (49) de la Puerta, R., Ruiz Gutierrez, V., and Houlst, J. R. (1999) Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* 57, 445–449.
- (50) Petroni, A., Blasevich, M., Salami, M., Papini, N., Montedoro, G. F., and Galli, C. (1995) Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thromb. Res.* 78, 151–160.
- (51) Aruoma, O. I., Deiana, M., Jenner, A., Halliwell, B., Kaur, H., Banni, S., Corongiu, F. P., Dessì, M. A., and Aeschbach, R. (1998) Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low-density lipoprotein oxidation. *J. Agric. Food Chem.* 46, 5181–5187.
- (52) Wiseman, S. A., Mathot, J. N., de Fouw, N. J., and Tijburg, L. B. (1996) Dietary non-tocopherol antioxidants present in extra virgin olive oil increase the resistance of low density lipoproteins to oxidation in rabbits. *Atherosclerosis* 120, 15–23.
- (53) Larsson, H., Bottiger, Y., Iuliano, L., and Diczfalusy, U. (2007) In vivo interconversion of 7 $\beta$ -hydroxycholesterol and 7-ketocholesterol, potential surrogate markers for oxidative stress. *Free Radical Biol. Med.* 43, 695–701.
- (54) Leonarduzzi, G., Sottero, B., and Poli, G. (2002) Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects. *J. Nutr. Biochem.* 13, 700–710.
- (55) Umetani, M., Domoto, H., Gormley, A. K., Yuhanna, I. S., Cummins, C. L., Javitt, N. B., Korach, K. S., Shaul, P. W., and Mangelsdorf, D. J. (2007) 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. *Nat. Med.* 13, 1185–1192.
- (56) Bogani, P., Galli, C., Villa, M., and Visioli, F. (2007) Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* 190, 181–186.
- (57) Crauste, C., Rosell, M., Durand, T., and Vercauteren, J. (2016) Omega-3 polyunsaturated lipophenols, how and why? *Biochimie* 120, 62–74.
- (58) Roby, M. H., Allouche, A., Dahdou, L., De Castro, V. C., da Silva, P. H., Targino, B. N., Huguet, M., Paris, C., Chretien, F., Gueant, R. M., Desobry, S., Oster, T., and Humeau, C. (2015) Enzymatic production of bioactive docosahexaenoic acid phenolic ester. *Food Chem.* 171, 397–404.
- (59) Crauste, C., Willand, N., Villemagne, B., Flipo, M., Willery, E., Carette, X., Dimala, M. M., Drucbert, A. S., Danze, P. M., Deprez, B., and Baulard, A. R. (2014) Unconventional surface plasmon resonance signals reveal quantitative inhibition of transcriptional repressor EthR by synthetic ligands. *Anal. Biochem.* 452, 54–66.
- (60) Camuesco, D., Comalada, M., Concha, A., Nieto, A., Sierra, S., Xaus, J., Zarzuelo, A., and Galvez, J. (2006) Intestinal anti-inflammatory activity of combined quercitrin and dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, in rats with DSS-induced colitis. *Clin. Nutr.* 25, 466–476.
- (61) Sirianni, R., Chimento, A., De Luca, A., Casaburi, I., Rizza, P., Onofrio, A., Iacopetta, D., Puoci, F., Ando, S., Maggiolini, M., and Pezzi, V. (2010) Oleuropein and hydroxytyrosol inhibit MCF-7 breast cancer cell proliferation interfering with ERK1/2 activation. *Mol. Nutr. Food Res.* 54, 833–840.
- (62) Diaz Gomez, M. I., de Castro, C. R., D'Acosta, N., de Fenos, O. M., de Ferreyra, E. C., and Castro, J. A. (1975) Species differences in carbon tetrachloride-induced hepatotoxicity: the role of CCl<sub>4</sub> activation and of lipid peroxidation. *Toxicol. Appl. Pharmacol.* 34, 102–114.
- (63) Helal, O., Berrougui, H., Loued, S., and Khalil, A. (2013) Extra-virgin olive oil consumption improves the capacity of HDL to mediate cholesterol efflux and increases ABCA1 and ABCG1 expression in human macrophages. *Br. J. Nutr.* 109, 1844–1855.