Dietary squalene induces cytochromes *Cyp2b10* and *Cyp2c55* independently of sex, dose and diet in several mouse models

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Abbreviations used: PCR, polymerase chain reaction; RT, reverse transcriptase;

Running title: Squalene and gene expression

Abstract

Scope: To investigate the effects of squalene, the main hydrocarbon present in extra virgin olive oil, on liver transcriptome in different animal models and to test the influence of sex on this action and its relationship with hepatic lipids.

Methods and results: To this purpose, male C57BL/6J *Apoe*-deficient mice were fed a purified Western diet with or without squalene during 11 weeks and hepatic squalene content was assessed, so were hepatic lipids and lipid droplets. Hepatic transcriptomic changes were studied and confirmed by RT-qPCR. Dietary characteristics and influence of squalene doses were tested in *Apoe*-deficient on purified chow diets with or without squalene. These diets were also given to *Apoa1* and wild-type mice on C57BL/6J background and to C57BL/6J xOla129 *Apoe*-deficient mice. Squalene supplementation increased its hepatic content without differences among sexes and hormonal status. The *Cyp2b10* and *Cyp2c55* gene expressions were significantly up-regulated by the squalene intake in all models, with independence of sex, sexual hormones, dietary fat content, genetic background and dose, and in *Apoe*-deficient mice consuming extra-virgin olive oil.

Conclusion: Hepatic squalene increases the expression of these cytochromes and their changes in virgin olive oil diets may be due to their squalene content.

1. Introduction

The “Seven Countries” study showed that the so-called Mediterranean diet was associated with a reduced risk of cardiovascular mortality despite its high fat intake [1]. In addition, several intervention trials have indicated that the Mediterranean diet decreases the risk of coronary heart disease in a number of populations [2, 3]. Due to the favorable effects observed with this dietary pattern and since olive oil is the main source of fat calories, a great deal of research has been devoted to characterizing its role in the development of different diseases [4, 5]. Olive oil, as a fruit juice, is a complex mixture where triglycerides are combined with other biologically active substances named minor components and encompassing chemical groups such as hydrocarbons, terpenes, phytosterols and phenolic compounds [6]. Biological actions of these minor components have been documented [7-11]. Epidemiological studies indicate that olive oil exerts a remarkable preventive effect on the development of atherosclerosis [12], and several findings suggest that its minor constituents could be responsible, at least in part, for these beneficial effects. Hydrocarbons account for about 50% of the unsaponifiable fraction of olive oil, most of which is squalene**.** Recently, Martinez-Beamonte et al. [13] reported a mean value of 4.6 ± 2.1 g/kg of VOO with a wide variation from 0.4 to 10.8 g/kg. Likewise, the estimated human intake of squalene ranges from 30 up to 400 mg/day (in United States and in Mediterranean countries, respectively) [14] or even 1 g per day in some diets [15]. Due to this low toxicity, it has been successfully used to treat different ailments [16-18]. Our group has shown that a high squalene dose decreased hepatic fat content in a sex-dependent manner [19] and modified the hepatic mitochondrial and microsomal proteomes [20, 21] of *Apoe*-deficient mice on a chow diet. In wild-type mice, 1 g/kg squalene administration also remodeled high-density lipoproteins by increasing their esterified cholesterol and phosphatidylcholine contents,

accompanied by an increase in paraoxonase 1, and decreased reactive oxygen species in lipoprotein fractions without changes in apolipoproteins A1 and A4 [22]. *Apoe*-deficient mice are a well-characterized and widely used animal model which rapidly develops atherosclerotic lesions similar to those observed in humans [23]. These mice show moderate or severe hepatic steatosis when fed standard chow or a high fat diet, respectively [24, 25]. The hepatic fat content has been associated with the development of atherosclerotic lesions [26] and modulated by dietary interventions [27, 28]. Thus, the liver may undergo important metabolic changes under the influence of olive oil and the steatotic-prone liver of *Apoe*- deficient mice offers an excellent model in which to verify this effect. Mice lacking *Apoa1* as a genetic model of absence of APOA1-containing HDL [29] represent a valuable model to investigate whether squalene is delivered to the liver via these lipoparticles. To test the hypotheses that dietary squalene supplementation may significantly influence hepatic gene expression and that APOE, APOA1 and diet might condition the response, these deficient mice were fed purified Western and chow diets with or without squalene. These dietary interventions were also carried out in wild-type mice of both sexes. Gene expression was determined by microarray analysis and then confirmed by RT-qPCR.

2. Material and methods

2.1. Animals

Wild-type mice and *Apoe*-deficient mice on C57BL/6J genetic background were obtained from Charles River (Charles River Laboratories, Barcelona, Spain). *Apoa1*- on C57BL/6J and *Apoe*-deficient mice on C57BL/6JxOla129 genetic backgrounds, respectively, were generously provided by Dr. Nobuyo Maeda from University of North Carolina at Chapel Hill. To establish groups with similar initial plasma cholesterol, blood samples were taken (after four-hour fasting) from the facial vein. All animals were housed in sterile filter-top cages in rooms maintained under a 12-h light/12-h dark cycle in the *Centro de Investigación Biomédica de Aragón*. All had *ad libitum* access to food and water. Mouse experiments were carried out in accordance with the EU Directive 2010/63 on the protection of animals used for scientific purposes and the study protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza (PI36/09 and PI35/18).

All diets were prepared weekly and stored in an N_2 atmosphere at -20ºC. Fresh food was provided daily. The two-month old mice were fed the experimental diets for 11 weeks. The diets were well tolerated. Mice were euthanized by CO_2 inhalation. At sacrifice, the livers were immediately removed and frozen in liquid nitrogen.

2.2. Diets

2.2.1. Effect of dietary squalene and surgical castration on C57BL/6J Apoe-deficient mice on a Western diet

Four study groups for both sexes were established: a) one control group of mice (9 males, 8 females) that received a purified Western diet [25] containing 0.15% cholesterol and 20% refined palm oil (Gustav Heess, S.L., Barcelona, Spain); b) a treatment group (10 males, 9 females), that received the same diet but containing 1%

squalene (Sigma, Madrid, Spain), equivalent to a dose of 1 g squalene/ kg mouse assuming a daily intake of 3 g per mouse; c) a group of surgically castrated mice (9 males, 9 females) that received the purified Western diet; and d) a group of surgically castrated mice (9 males, 8 females) that received the purified Western diet supplemented with squalene.

Ovariectomy and orchiectomy were carried out using 4% or 2% isofluorane (induction and maintenance, respectively) general anesthesia in 17 females and 18 males when they were one-month old. To carry out the removal of ovaries, dorsal skin incisions were performed and the ovarian fat pads were grasped, the ovaries were removed and cauterized. Once the fat pads were introduced into the abdominal cavity, the muscle layer was sutured by a continuous stitch and the skin layer was sealed with stainless steel clips. Testicles were excised by making an incision on the skin of the medial area of the scrotum. Applying a little pressure to the lower abdomen, the testicular sac emerged. A small cut allowed to ligate the blood vessels and the vas deferens, and then the testis and the epididymis were removed. The remaining vas deferens and the fat pads were placed back into the scrotal sac. Wound closure was similar to the described for females. Ketoprofen (2-5 mg/kg) was used as analgesic to reduce postoperative pain for the following week. One-month later, they were fully recovered and used to receive the diets.

2.2.2. Effect of dietary squalene on male, C57BL/6JxOLA129 Apoe-deficient on a chow diet

Two study groups were established: a) one group consisted of 8 mice receiving a commercial chow diet, and b) the other group consisted of 9 mice receiving a commercial chow diet supplemented with 1% squalene as previously described [19].

2.2.3. Effect of dietary squalene on C57BL/6J wild-type mice on a purified chow diet

Two study groups were established: a) one group consisted of 14 mice (6 males, 8 females) receiving a purified chow diet [25], and b) the other group consisted of 17 mice (7 males, 10 females) receiving a purified chow diet supplemented with 1% squalene.

2.2.4. Effect of dietary squalene on Apoa1-deficient C57BL/6J mice fed a purified chow diet

Two study groups were established: a) one group consisted of 14 mice (7 males, 7 females) receiving a purified chow diet [25], and b) the other group consisted of 15 mice (7 males, 8 females) receiving the purified diet supplemented with 1% squalene.

2.2.5. Effect of dietary squalene at different doses on Apoe-deficient C57BL/6J mice on a purified chow diet

Three study groups were established: a) a control group consisted of 27 mice (13 males, 14 females) receiving a purified diet [25], b) a group consisted of 28 mice (14 males, 14 females) receiving the purified diet supplemented with 1% squalene, and the third one of 26 mice (13 males, 13 females) receiving the purified diet supplemented with 0.25% squalene; equivalent to a dose of 0.25 g/kg mouse assuming a daily intake of 3 g per mouse. Considering the higher metabolic rate of mice [30], this mouse dose of 0.25 g/kg/day would correspond to a human dose of 25 mg/kg/day and this is fairly close to the 15 mg/kg/day used in human nutritional studies [15].

2.2.6. Effect of different sources of dietary fat on male C57BL/6J Apoe-deficient mice on

a Western diet

The mice were divided into two groups: the first one (n=14) received as source of fat butter and the second (n=14) extra virgin olive oil.

2.3. Effect of squalene in HepG2 cells

For this purpose, the human hepatocyte cell line HepG2 from passage 5 was grown in a humidified atmosphere of 5% CO_2 at 37ºC in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Gibco Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 1% non essential amino acids, 1% penicillin (1000 U/ml), 1% streptomycin (1000 µg/ml), 4 mM L-glutamine and 1 mM sodium pyruvate in a 24 multiwell plate (in triplicate). Medium was changed every two days, and after one week of growth, this medium was removed, and cells were washed with phosphate buffered saline (PBS) prior to the addition of the serum-free media supplemented with 0.1% DMSO or 50 µM squalene dissolved in 0.1% DMSO. After 12-h incubation, media were removed and cells were homogenized in Tri-reagent solution (Ambion, Austin, TX, USA) to isolate RNA. Samples were further processed as indicated in *2.4* and *2.6.*

2.4. RNA isolation

RNA from each mouse liver and from HepG2 cells was isolated using Tri-reagent (AMBION, TX, USA). DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from AMBION. RNA was quantified by absorbance at $A_{260/280}$. The integrity of the 28 S and 18 S ribosomal RNAs was verified by agarose gel electrophoresis and by Bioanalyzer using Eukaryote Total RNA Nano kit (Agilent Technologies, Santa Clara, CA, USA).

2.5. Agilent oligonucleotide array hybridization and data analysis

Four hepatic RNA pools were prepared for male *Apoe*-deficient male mice receiving Western diets and another four for male *Apoe*-deficient consuming 1% squalene-enriched Western diet. Each pool, consisted of RNA from 2 or 3 animals, was hybridized to eight microarrays "SurePrint G3 Mouse Gene Expression Microarray, ID 028005" (Agilent Technologies, Santa Clara, CA, USA). Samples were labeled according to the protocol of two-color Microarray-based Gene Expression Analysis v. 6.5 (Agilent). The results from the 4 microarrays corresponding to control mice were compared to those 4 derived from squalene-receiving mice and identification of genes that were up- or down-regulated by squalene was performed by adopting a significance of $P<0.01$. Of these we selected only those whose signal $\log_2$ ratio was higher than 1.5 (up-regulated genes) or lower than –1.5 (down-regulated genes). Transcripts with signal intensities that were lower than the limit of detection estimated as background of the matrix ±3 standard deviations were not taken into account. The complete datasets were deposited in the GEO database (accession number GSE145343).

2.6. Quantification of mRNA

The differences in mRNA expression observed with the microarrays were confirmed by quantitative real-time RT-qPCR analysis of individual samples. Equal amounts of DNA-free RNA from each sample of each animal and from HepG2 cells were used in RT-qPCR analyses. First-strand cDNA synthesis was performed using the First Strand synthesis kit (Thermo Scientific, Madrid, Spain). RT-qPCR reactions were performed using the Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primers were designed using Primer Express® (Applied Biosystems) and checked by BLAST analysis (NCBI) to verify gene specificity as well as to get

amplification of the cDNA and not of genomic DNA. The sequences are shown in Supplementary Table 1. Real time RT-qPCR reactions were performed in a Step One Real Time PCR System (Applied Biosystems) following the standard procedure. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Cq}$ method and normalized to the reference Cyclophilin B *(Pipb)* mRNA expression.

2.7. Liver histology analyses

Aliquots of liver were stored in neutral formaldehyde and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin and observed with a Nikon microscope. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of total liver section [19].

2.8. Hepatic homogenate preparation

Liver was homogenized in homogenization buffer (Phosphate buffered solution with protease inhibitor cocktail) and used to estimate protein concentration and reactive oxygen species. Protein concentration was determined by the BioRad dye binding assay (BioRad, Madrid, Spain).

2.9. Determination of reactive oxygen species in the liver

Their presence was estimated by the 2', 7'-dichlorofluorescein diacetate (DCF) assay, where hepatic homogenates (7 μg of protein) were incubated with 50 μM DCF in PBS in a total volume of 50 μl and in presence of 8.3 μl of 0.12% sodium azide at 37 ºC. Fluorescence, at 485 nm excitation and 535 nm emission, was measured after 4-hour incubation.

2.10. Squalene protection against peroxidation in male mice

After 4-hour incubation with 50 μM DCF, 5 mM of oxygen peroxide was added to the liver homogenates, and incubated for 25 minutes, to estimate the capacity of squalene to protect against oxidative damage.

2.11. Hepatic lipid extraction

An aliquot (100 mg) of the liver was homogenized in 1 ml of PBS. 1 volume of homogenate was extracted with two volumes of chloroform: methanol (2:1, v/v) twice [31]. The separated organic phases were combined and evaporated under N_2 stream. Dry extracts were dissolved in 100 μl of isopropanol. Cholesterol and triglycerides were measured by colorimetric assay with Infinity kits (Thermo Scientific).

2.12. Liver fatty acid composition

Fatty acids were determined by gas chromatography (GC), as previously described [32]. The samples were saponified by heating for 25 min with 5 ml of 0.2 M sodium methylate and heated again at 80ºC for 25 min with 6% (w/v) H_2SO_4 in anhydrous methanol. The fatty acid methyl esters thus formed were extracted with hexane and analyzed in a Hewlett-Packard 5890 series II gas chromatograph equipped with flame ionization detector and using a capillary silica column Supelcowax 10 (Sulpelco, Bellefonte, PA, USA) of 60 m length and 0.25 mm internal diameter. The initial column temperature was 200ºC, which was held for 10 min, then programmed from 200–230ºC at 2ºC min.

2.13. Squalene assay

The hydrocarbon fraction of hepatic homogenates was separated by column

chromatography on silica gel and analyzed by capillary gas chromatography as previously described [33].

2.14. 16-hydroxy testosterone (16-OHT) and 19(S)-hydroxy-eicosatetraenoic acid (19-HETE) assays.

The protocol followed those described by [34, 35] with minor modifications. Briefly, 100 mg of mouse liver were homogenized in 940 µL of 0.1% acetic acid, 0.1% butylated hydroxytoluene in methanol containing internal standards (20 ppb of carbamazepine and 20-HETE-d6, respectively). The mixture was centrifuged at 9000 g for 10 minutes at 0ºC. The supernatant was collected and the pellet reextracted. Both supernatants were collected and subjected to solid phase extraction using Oasis HLB cartridges (Waters, Milkford, MA, USA). Dry eluates were dissolved in 100 µL de methanol/water (50:50) containing 0.1% formic acid. Samples were analyzed using an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with 40°C Kinetex XB-C18 (100 x 2.10 mm, 2.6 µm, 100 Å) column (Phenomenex, Torrance, CA). The chromatograph was coupled to an API3000 (AB Sciex Instruments, Toronto, Canada) triple quadrupole detector mass spectrometer. All data were acquired and processed using software Analyst versión 1.4.2.0 (Sciex). Standards of 16-OHT and 19-HETE were obtained from Steraloids (Newport, RI, USA) and Cayman (Ann Arbor, MI, USA), respectively. The selected *m/z* transitions were 304.9/109.1 and 304.9/97.2 for 16-OHT and 319.1/301.1 and 319.4/275.1 for 19-HETE. Confirmation of exact mass was carried out using LTQ-Orbitrap Velos (Thermo).

2.15. Plasma determinations

After the experimental period, blood (0.7- 1 ml per mouse) was collected from the heart and centrifuged at 3000 rpm for 5 minutes at 4ºC to obtain plasma. Total cholesterol and triglyceride concentrations were measured in a microtitre assay, using InfinityTM commercial kits (Thermo Scientific). HDL-cholesterol was quantified in the supernatant after precipitation of APOB-containing particles with phosphotungstic acid–$MgCl_2$ (Roche, Barcelona, Spain).

2.16. Statistical analyses

The data were analyzed using the Statistical Package for Social Sciences (SPSS) program (SPSS, Chicago, IL, USA) or Instat 3.02 software for Windows (GraphPad, S. Diego, CA, USA). For parametric distributions a one-way ANOVA test, with a Tukey's post-test was employed. For non-parametric distributions, data were analyzed according to Kruskal-Wallis's test, with a Dunn's post-test or the Mann-Whitney'U test. Data are shown as mean ± standard deviation of the values. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of squalene on hepatic gene expression of Apoe-deficient mice fed on Western diet

To determine the changes in hepatic gene expression induced by squalene, the expression of 39430 transcripts and 16251 large intergenic non-coding RNAs (lncRNAs) represented on the Agilent microarray were quantified in four pooled liver samples of 9 male mice that received the purified Western diet and another four of 10 that received a squalene-enriched Western diet to provide 1g/kg dose. Using the Agilent software to determine significant differences in gene expression ($P<0.01$) and a compromised limit of detection in variations of expressions lower than background matrix ± 3 SD, the increased expression of 52 sequences plus the reduced expression of 49 sequences was identified when comparing samples from control with those of squalene-supplemented animals. To select the most relevant, only differentially regulated genes with a signal $\log_2$ ratio higher than 1.5 (for up-regulated genes) or lower than –1.5 (for down-regulated) were taken into account. Table 1 lists the genes whose mRNAs reflected these expressions. When comparing samples from control with those of squalene-supplemented mice samples, three genes were found to be up-regulated due to the dietary intervention: *Cyp2b10*, *Cyp2b9* and *Cyp2c55*, and no genes presented a significant reduced expression. This suggests that squalene supplemented in a Western diet to male *Apoe*-deficient mice elicits changes in these gene expressions.

To validate the results gathered with the microarray, the expressions of these three genes, *Cyp2b10, Cyp2b9* and *Cyp2c55* were individually studied by specific RT-qPCR assays normalized to *Pipb*. As shown in Figure 1, all of them were significantly up-regulated, thereby confirming the results obtained with the microarray. The influence of squalene on these genes was also investigated in females receiving 1 g/kg squalene

dose in Western diet. As shown in Figure 2 (A-C), mice receiving squalene showed increased expression of *Cyp2b10* and *Cyp2c55,* but no significant changes were observed for *Cyp2b9,* a gene to be low expressed in females [36]. Overall, squalene is a modifier of the expression of *Cyp2b10* and *Cyp2c55* genes in *Apoe*-deficient mice receiving Western diets in either sex.

3.2. Effect of surgical castration on hepatic gene expression of Apoe-deficient mice fed on Western diet

Due to the observed sex-differences regarding *Cyp2b9* expression [36], the influence of castration on gene expressions was carried out in males and females, Figure 2 (D-I). Interestingly, *Cyp2b9* expression was induced in orchiectomized males (Figure 2E) and decreased in ovariectomized females (Figure 2H), as *Cyp2b9* expression requires estrogens in adults [37]. Regarding *Cyp2b10* and *Cyp2c55* expressions, surgical castration had no effect in males (Figure 2D and F) but raised the mRNA levels in females (Figure 2G and I). These results indicate that the expression of these genes is influenced by sexual hormones.

3.3. Effect of squalene on hepatic gene expression of surgically castrated Apoe-deficient mice fed on Western diet

Despite the castration, the administration of squalene raised the expressions of *Cyp2b10* and *Cyp2c55* in males and females, Figure 2 (panels J-O). However, the response of *Cyp2b9* expression to squalene was lost in castrated male mice (Figure 2K vs 2E). Thus, an interaction between squalene and hormones seems to be involved particularly for *Cyp2b9* gene.

3.4. Effect of squalene on hepatic gene expression in wild-type mice on a chow diet

To verify whether the mRNA changes in response to squalene were independent of the presence of apolipoprotein E and intake of a Western diet, wild-type C57BL/6J mice were fed purified chow diets enriched in this compound and hepatic transcripts were assayed (Supplementary Fig 1 A-F). In both males and females, *Cyp2b10* and *Cyp2c55* were significantly increased by squalene administration. *Cyp2b9* expression showed no significant change in both sexes. Therefore, squalene action on *Cyp2b10* and *Cyp2c55* expressions is independent of type of diet and absence of APOE.

3.5. Effect of squalene on hepatic gene expression in livers of Apoa1-deficient mice fed a chow diet

The effect of squalene on the mRNA changes was also tested in mice lacking *Apoa1* (Supplementary Fig 1 G-L). In males, *Cyp2b10* (Panel G) and *Cyp2c55* (Panel I) response to squalene was not influenced by the absence of APOA1. *Cyp2b9* expression did not significantly change by squalene administration (Panel H). In females, while *Cyp2b10* expression (Panel J) was induced by squalene administration, this effect was not observed for *Cyp2b9* and *Cyp2c55* (Panels K and L). Thus, squalene action on *Cyp2b10* was independent of APOA1-containing HDL in both sexes, but *Cyp2c55* expression was only independent in males.

3.6. Effect of squalene doses on hepatic gene expression in Apoe-deficient mice on a chow diet

To verify whether the mRNA changes in response to squalene were observed in doses more compatible with the provided by the intake of extra virgin olive oil, C57BL/6J *Apoe*-deficient mice were fed purified chow diets enriched in this compound to provide doses of 1 and 0.25 g/kg and hepatic transcripts were assayed

(Supplementary Figure 2 A-F). At both doses and in males and females, *Cyp2b10* and *Cyp2c55* were significantly increased by squalene administration. *Cyp2b9* expression was not modified in males. In contrast, its response changed depending on the administered dose in females. Identical changes of these cytochrome expressions were obtained in male *Apoe*-deficient mice with C57BL76JxOLA129 genetic background receiving 1 g/kg squalene on chow diet in a different protocol of administration and fasting regimen [19] (Supplementary Figure 3 A and B). Therefore, the squalene action on *Cyp2b10* and *Cyp2c55* expressions is independent of type of dose, genetic background, fasting regimen or design of chow diet and is observed in the range of amount provided in Western diets containing extra virgin olive oil (EVOO). Likewise, these gene changes are *bona fide* response to squalene administration.

3.7. Effect of source of fat on gene expression in livers of Apoe-deficient mice fed on Western diet

As indicated, squalene is the main hydrocarbon of EVOO. To test whether its presence in EVOO could induce similar gene expression changes as those observed for the isolated compound, male mice were fed Western diets with two sources of fat: butter or EVOO. As shown in Figure 3 A and B, *Cyp2b10* and *Cyp2c55* expressions were significantly higher in mice receiving the EVOO compared to those on the butter diet. Collectively, our results indicate that these expressions are induced in animals receiving EVOO and that squalene may the component responsible for the observed changes of gene expressions.

3.8. Plasma lipids and hepatic squalene, lipids and oxidative status in Apoe-deficient mice on a Western diet

Apoe-deficient mouse is a model of dyslipidemia and spontaneous development of hepatic steatosis [38]. As shown in Supplementary Tables 3 and 4, dietary squalene did not modify plasma total cholesterol nor triglycerides. However, it significantly increased HDL-cholesterol levels in both sexes. Gonadectomy increased plasma total and HDL-cholesterol and decreased triglycerides. Squalene administration to castrated mice showed a trend to decrease total cholesterol and to increase triglycerides. However, dietary squalene only increased HDL-cholesterol levels in orchiectomized mice.

To test the influence of squalene on the fatty liver, the hepatic squalene content was quantified (Figure 4A). Significant increases were observed in groups of mice consuming squalene, irrespective of sex and surgical castration. When histological analysis of liver was carried out (Figure 4B), males receiving the squalene-supplemented diet showed no significant changes on surface of tissue occupied by lipid droplets. In contrast, females receiving the same diet had a significantly higher percentage of hepatic fat than their control group. Surgical castration induced an increased presence of lipid droplets in males and females and administration of squalene did not have any effect. Chemical analysis of hepatic triglycerides showed that squalene intake decreased them in both control and orchiectomized male mice (Table 2). On the other hand, a significant increase in triglycerides was observed in squalene-supplemented females and in ovariectomized mice (Table 2). In the latter group, squalene administration did not have an additive effect. Hepatic cholesterol content was significantly lower in males receiving squalene (Table 2) and significantly higher in females compared to their respective control groups. In the latter sex, surgical castration also raised hepatic cholesterol content and this effect was partially abolished by squalene administration. These results further confirm the squalene sex-dependent

response on hepatic lipids. The increase in squalene levels found in male and female mice was directly associated with *Cyp2b10* and *Cyp2c55* gene expressions (ρ=0.748, P<0.0 and ρ=0.853, P<0.0, respectively). These results indicate a strong relationship between squalene liver content and these two cytochromes in *Apoe*-deficient mice.

To verify whether *Cyp2b10*, *Cyp2c55* and lipid changes were modifying oxidative stress, hepatic levels of ROS were determined in males and no significant differences in the initial levels of reactive oxygen species among groups were found in *Apoe*-deficient mice. However, after H_2O_2-induced exposure, squalene was able to act as an antioxidant in both control and castrated male mice, reducing ROS levels (Figure 5). Consequently, these results suggest the ability of squalene to control the influx of ROS generated by H_2O_2 exposure, corroborating previous studies on the ability of squalene to inhibit oxidative stress in the liver.

To further understand the relation between lipids and cytochromes, fatty acid composition of the hepatic lipid extracts was undertaken and results are shown in supplementary Table 2. A slight increase in the monounsaturated fatty acid (MUFA) content and a decreased level of PUFA characterized the livers' fatty acid composition of orchiectomized *Apoe*-deficient mice compared to controls. Therefore, the ratio of MUFA to PUFA increased significantly in the liver of orchiectomized compared to control mice (3.04 vs 2.23, respectively, $P < 0.01$). These changes were not reverted by squalene administration. Interestingly, a decrease in arachidonic acid (20:4 n-6) levels was found in squalene and ovariectomized females compared to their respective controls, and this reduction was inversely correlated with hepatic TG content in control non-ovariectomized females, Figure 6B.

According to supplementary figure 4, the method employed for the measurement of 16-OHT and 19-HETE did not allow the detection of these compounds in the liver

after the oral administration of 1 g/kg of squalene since the concentrations of these compounds were below the limit of detection.

3.9. Hepatic lipids in wild-type and Apoa1-deficient mice on a chow diet

A significant increase in triglyceride levels was observed in both male and female wild type mice, but not in *Apoa1*-deficient mice, consuming the squalene-supplemented diet (Table 3). The previous finding of increased hepatic cholesterol in females consuming squalene was also observed in these two mice models. Overall, these results emphasize the different response of hepatic lipids to squalene administration depending on genetic make-up and sex.

3.10. CYP2B6 mRNA expression is a direct target of squalene in HepG2 cell culture

To investigate whether squalene alone was able to induce *CYP2B6* expression, the human orthologous of mouse *Cyp2b10* gene, HepG2 cells were incubated in presence of 50 µM squalene in DMSO during 12 h. As shown in Figure 7, a significant increased expression was observed following the 12-hour incubation. This reinforces the notion of a key role of *Cyp2b10* in the hepatic effect of squalene. The human ortholog of *Cyp2c55 (CYP2C18*) was not expressed in this cell line, nor squalene induced its expression.

4. Discussion

This nutrigenomic approach was aimed to determine the hepatic transcriptomic changes taking place after eleven weeks of 1 g/kg squalene supplementation in a Western diet. Using a high restrictive approach, we found that in male *Apoe*-deficient mouse liver, squalene mainly upregulated *Cyp2b10*, *Cyp2b9* and *Cyp2c55* expressions. In these conditions, a decrease in hepatic triglycerides and oxidative stress was observed. With the exception of *Cyp2b9*, the effect was also observed in females and surgically castrated males and females. To test our hypothesis that squalene could be transported in HDL particles, *Apoa1*-deficient mice, as a model of absence of HDL, also received the diet supplemented with squalene. In this setting, *Cyp2b10* and *Cyp2c55* expressions were also induced by squalene administration, so were they in wild-type and *Apoa1*- deficient mice receiving a chow diet supplemented with squalene. The gene changes were also assessed in *Apoe*-deficient mice receiving chow diet enriched in squalene at two different doses (1 and 0.25 g/kg). Once again, squalene administration raised *Cyp2b10* and *Cyp2c55* expressions with independence of dose. Furthermore, squalene incubation also elicited the expression of *CYP2B6*, the human ortholog of *Cyp2b10*, in HepG2 cells. These experimental conditions indicate that *Cyp2b10* and *Cyp2c55* expressions are targets of squalene with independence of diet and used model, and that APOA1-containing HDL is not relevant to deliver the compound to the liver. Mice receiving extra virgin olive oil also raised *Cyp2b10* and *Cyp2c55* expressions suggesting that the squalene content of this oil may be responsible for these actions.

In the present work, the microarray assay was carried out on a semi-individual approach. In this regard, liver samples from *Apoe*-deficient mice were divided into four pools for control and another four for squalene-treated mice, each pool consisting in RNA from 2 or 3 individuals. RNA samples are often pooled in microarray experiments

to reduce the cost and complexity of analysis of transcript profiling. The present approach had an important impact on reducing the number of significant changes in gene expression. Not surprisingly, since this approach is partly considering biological variability and reinforces our previous experience [24, 27, 39-41] using a restrictive step, selecting only the genes with the highest expression changes, according to our previous setting the cut point on a signal $\log_2$ ratio of ± 1.5. As previously discussed [41], the biological variation of mRNA, when individually studied, is high enough so that only genes with a certain threshold of change would be candidates to experience significant biological response to a dietary intervention. With this criterion, only three gene expressions were found to be remarkably up-regulated (*Cyp2b10*, *Cyp2c55* and *Cyp2b9)* and none was significantly down-regulated. Indeed, these microarray-suggested changes were confirmed by RT-qPCR of individual samples (Figure 1). In our experience, this low number is quite common using such a high restrictive criterion and a single dietary component [24, 39, 41]. With a high restrictive criterion and an herbal complex preparation, 24 important gene changes were reported [42] and we reported 54, using an unsaponifiable-enriched fraction [27]. Thus, the more dietary components are added, the higher number genes are involved.

Cytochrome P450s monooxygenases enzymes (P450s) represent a large class of heme-containing enzymes that catalyze the metabolism of various endogenous compounds such as steroids and bile acids. In addition, they metabolize a wide variety of xenobiotics, including many drugs, carcinogens, food constituents, and environmental chemicals [43-45]. In this work, three of them, *Cyp2b9*, *Cyp2b10* and *Cyp2c55*, were targets of squalene administration when animals received a Western diet. This pattern of gene induction seems to be specific for this compound because no changes in expression were observed for other cytochromes such as *Cyp3a11* and

Cyp2a4, modified in low fat diets [46]; *Cyp3a11* and *Sult2a1* induced following 24-nor ursodeoxycholic [47]; *Cyp2a5* and *Cyp2d9* increased in mice lacking HNF1alpha [48]; *Cyp2b10*, *Cyp2c29*, *Cyp3a11*, *Ugt1a1*, *Mrp4*, *Mrp2* and *c-Myc* induced in response to constitutive androstane receptor (CAR) [49] or *Cyp3a11*, *Ces2*, *Insig2* and *Abcc3*, CAR and pregnane X receptor activated genes [50]. The effect of squalene on *Cyp2b9* required from high fat diet and cholesterol and was sex-dependent because it was lost in animals receiving purified chow diets, in females and in orchiectomized mice. However, squalene up-regulation of *Cyp2b10* and *Cyp2c55* mRNA expressions in all tested experimental conditions shows that the effect is independent of sex, sexual hormones, squalene dose, dietary fat content, genetic background and mouse model. This effect was also observed when mice received a Western diet containing extra virgin olive oil as the source of fat. These data suggest that squalene is an important contributor of the hepatic effects of extra virgin olive oil and that *Cyp2b10* and *Cyp2c55* are two crucial genes in the response to squalene and oils containing this compound.

CYP2B10 has been shown to own a pentoxyresorufin O-deethylase activity, to be expressed in both sexes and be induced by phenobarbital (PB) in the livers of all mouse strains, whereas inductive effects on the CYP2B9 were not observed in female C57BL/6 and DBA/2NCrj mice [51]. Hepatic CYP2B10 has also been proposed to metabolize testosterone into 16α-hydroxytestosterone (16-OHT) [52]. Based on this fact, it could be hypothesized that loss of testosterone in castrated mice would influence this gene expression in response to squalene. In fact, orchietomized mice did not experience any significant change, whereas ovariectomized females showed increased hepatic *Cyp2b10* expressions. In both surgically castrated *Apoe*-deficient male and female mice, *Cyp2b10* gene expression remained up-regulated following squalene consumption. Unfortunately, when we tested hepatic 16-OHT levels in *Apoe*-deficient

mice and due to low amount of remaining tissue, we were not able to detect changes in this metabolite. These results suggest that the basal level of expression of this gene is influenced by estradiol levels but that the effect of squalene on *Cyp2b10* expression is independent on testosterone and estradiol levels. The squalene induction was also observed in *CYP2B6* expression (Figure 7), the human analog of the mouse gene, in HepG2, something that was not observed for human ortholog of *Cyp2c55 (CYP2C18*). The latter finding is compatible with the observations that *in vitro* hepatic models using immortalized hepatocyte-like cell lines or hepatoma derived cell lines are not considered to be fully representative of hepatic functions in the view of their drug-metabolizing enzymes [53]. These findings indicate that *CYP2B10* or its analogues are important targets of squalene action, the cascade of this gene activation in response to squalene is preserved in HepG2 and squalene *per se* is capable of exerting the action. Our results add further evidence to the recent notion that specific nutrients may reprogram the lack of cytochromes P-450 activities in cell lines reported for amino acids [54]. Human CYP2B6 is involved in biotransformation of drugs used to control platelet aggregation (clopidogrel [55] and prasugrel [56]), hypercholesterolemia (atorvastatin) [57] and smoking cessation (bupropion) [58]. A change in CYP2B6 gene expression induced by squalene could compromise the response to these drugs and represents a new example of potential dietary component-drug interaction.

Mouse CYP2C55 has been characterized as an enzyme that catalyzes the biosynthesis of 19-hydroxyeicosatetraenoic acid (19-HETE) from arachidonic acid in mouse hepatic and extrahepatic tissues [59]. In agreement with this fact, when the *Cyp2c55* mRNA levels were upregulated in squalene-treated mice (Figures 1 and 2), arachidonic acid levels were reduced (Supplementary Table 2), although they only reached statistical significance in females (Figure 6). When hepatic *Cyp2c55* was

induced by phenobarbital or pregnenolone 16alpha-carbonitrile in mouse, 19-HETE serum levels were raised [60]. These results and our data suggest that squalene could induce the conversion of arachidonic into 19-HETE through the CYP2C55 pathway. Another dietary component, alpha-tocopherol, has been reported to increase the *Cyp2c55* gene expression in wild-type in a PXR-independent way [61]. It has been proposed that food derived xenobiotics might up-regulate CYP2C55 [62] and differences between purified and commercial chow diets may influence CYP2C55 expression in mice [45]. In this way, our work may explain the dietary discrepancies since squalene is an important modifier of this gene expression and dietary fats containing it as well. 19-HETE has been reported to affect vascular tone and ion transport in the kidney and brain [63, 64]. Plasma 19-HETE levels were found associated with better prognosis in patients with acute coronary syndrome [65], a finding that could be related to its properties of inducing vasorelaxation and platelet inhibition [66] and normalizing blood pressure [67]. Interestingly, inverse and significant associations were found among arachidonic acid levels and hepatic triglyceride content in *Apoe*-deficient females (Figure 8). These findings are suggesting a role for arachidonic acid and its metabolite, 19-HETE, in management of lipid metabolism in liver and the relevance of some dietary components in modulating C*yp2c55* expression. However, 19-HETE levels are particularly low in the liver and, due to limitations in tissue sample, we did not detect changes in hepatic content of 19-HETE by the administration of squalene. Another possibility could be that this metabolite could be released from the liver. The enzyme activity of CYP2C18, the human ortholog of CYP2C55, is involved in metabolizing two antihyperglycaemic agents, tolbutamide [68] and tofogliflozin [69]. Recently, variants of CYP2C18 have also been found as major regulators of clopidogrel response [70]. Once again, the

induction of this cytochrome by squalene represents another example of potential new nutrient-drug interactions.

Cytochrome P450 system as a source of reactive oxygen species (ROS) is believed to play the important role in various pathological conditions and diseases [71]. In this way, it has been suggested that the lower susceptibility to hyperoxia-induced oxidative stress in females was due to their lower *Cyp2b9* expression [72]. In this work, three cytochromes, *Cyp2b9*, *Cyp2b10* and *Cyp2c55*, were targets of squalene administration when animals received a Western diet. Despite this fact, squalene administration exerted a protective effect against oxygen peroxide-induced damage in hepatic homogenates from both control and orchiectomized *Apoe*-deficient male mice (Figure 5). Since ROS content is a balance of pro-oxidant and antioxidant elements, the increased defense observed by squalene administration is indicative of an enhanced antioxidant status either provided by squalene itself or induction of antioxidant defense. According to the present results, both aspects may be involved, first an important increase in hepatic squalene content was observed (Figure 4) and several works have reported antioxidant properties of this compound [17] and we have described increases in antioxidant proteins such as catalase, thioredoxin domain-containing protein 5 and pyridine nucleotide-disulfide oxidoreductase domain-containing protein 2 following squalene administration [21]. In lymphomas, squalene also prevents oxidative stress [73]. These data indicate that induction of P450 system does not necessarily convey into increased ROS if conveniently buffered by antioxidant compounds or elicited endogenous antioxidant defenses.

In conclusion, a highly restrictive scheme of gene selection has enabled us to characterize *Cyp2b10*, *Cyp2b9* and *Cyp2c55* gene expressions as changes due to squalene in Western diets. In chow diets, *Cyp2b10* and *Cyp2c55* induction represents a

specific response to the prolonged intake of squalene with independence of sex, animal and dose. Furthermore, the presence of this compound in extra virgin olive oil containing diets may explain the induction of *Cyp2b10* and *Cyp2c55* observed after consuming these diets. The induction of these cytochrome P450s monooxygenases (*Cyp2b10* and *Cyp2c55*) was not translated into increased ROS content since it was buffered by squalene itself and its antioxidant induction.

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Author Contributions

CGR, EJR, ASO, ER, R MB, MAN, JCS, CA, MJRY, CAL carried out the experiments and prepared the draft, and J.O. supervised the work and draft and prepared the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure 1. Effect of squalene on hepatic gene expression in male *Apoe*-deficient mice on a Western diet. A) *Cyp2b10*, B) *Cyp2b9*, C) *Cyp2c55.* Data are expressed as means ± standard deviation and represent arbitrary units normalized to the cyclophilin B expressions according to the RT-qPCR assay for the hepatic genes significantly up-regulated in *Apoe*-deficient mice receiving 1 g/kg squalene. Statistical analysis was carried out by Mann–Whitney U test. **, P<0.01.

Figure 2. Effect of squalene intake and surgical castration on hepatic gene expression in *Apoe*-deficient mice on a Western diet. Females consuming 1 g/kg squalene (A-C). The effect of surgical castration in males (D-F) and in females (G-I) on gene expression. The influence of 1 g/kg squalene on these gene expressions in castrated males (J-L) and females (M-O). Data are expressed as means ± standard deviation and represent arbitrary units normalized to the cyclophilin B expression for control (left bar) and experimental conditions (right bar) according to the RT-qPCR assay for the genes selected. Statistical analysis was carried out by Mann–Whitney U test. **, P<0.01; *, P<0.05.

Figure 3. Effect of extra virgin olive oil on the hepatic gene expression of male *Apoe*-deficient mice on a purified Western diet, compared to those on a butter-enriched diet. Data in bars, expressed as means ± standard deviation, represent arbitrary units normalized to the cyclophilin B expression in butter (white bars) and EVOO (black bars) animals according to the RT-qPCR assay for the genes previously confirmed to be up-regulated by squalene administration. Statistical analysis was carried out by Mann–Whitney U test. **, P<0.01; *, P<0.05.

Figure 4. A) Liver squalene content in *Apoe*-deficient mice on a Western diet.

Results are expressed as media ± SD. Statistical analysis was carried out with one-way ANOVA test for parametric distributions, with Tukey´s post-test; and Kruskal-Wallis test with Dunn´s post-test for non-parametric distributions. **, P<0.01. **B) Liver histology and hepatic fat content.** Morphometric changes in hepatic fat content in *Apoe*-deficient mice consuming a Western diet, quantified with Adobe Photoshop 7.0 and expressed as percentage of area of total liver section. Data are expressed as mean ± SD for each group. Statistical analyses were done according to Mann-Whitney test. *P<0.05. C, control; S, squalene; +, gonad presence and -, gonad absence.

Figure 5. Hepatic ROS levels in liver homogenates of male *Apoe*-deficient mice on a Western diet after hydrogen peroxide exposure. ROS levels in *Apoe*-deficient and orchiectomized-*Apoe*-deficient male mice consuming a Western diet with or without squalene. Results are expressed as arbitrary fluorescence units after 25 minutes incubation with 5 mM hydrogen peroxide. C, control; S, squalene and -, gonad absence.

Figure 6. Arachidonic acid in liver lipid fractions. A) Arachidonic acid content in female *Apoe*-deficient mice on a Western diet, expressed as arbitrary units. Statistical analysis was performed according to one-way ANOVA test, with a Tukey's post-test. Statistical significance was set at P< 0.05. B) Inverse correlation between arachidonic acid in lipid liver fractions and liver hepatic triglycerides. Correlation analysis was performed with SPSS package, according to Spearman's test.

Figure 7. Direct action of squalene in a human hepatocyte cell line. HepG2 cells were incubated in presence of 0.1% DMSO (control) and 50 μM squalene dissolved in 0.1% DMSO for 12 hours. The experiment was performed in triplicate, with n=6 in each

experiment for control and treated cells. *CYP2B6* expression was quantified relative to cyclophilin B by RT-qPCR. Results are expressed as mean and standard deviation, and statistical analysis was done according to the Mann-Whitney test. *, $P<0.05$.

Table 1. Hepatic genes differentially regulated by the administration of squalene at the level of signal log_2 ratio >1.5 or <-1.5 in male *Apoe*-deficient mice consuming purified Western diet according to Agilent microarray.

Biological process	GenBank ID	Agilent ID	Name	Gene symbol	Signal log_2 ratio
Up-regulated genes					
Drug metabolism	NM_009999	A_55_P2044653	Cytochrome P450, 2b10	*Cyp2b10*	3.3
Drug metabolism	NM_010000	A_52_P256914	Cytochrome P450, 2b9	*Cyp2b9*	1.7
Drug metabolism	NM_028089	A_51_P447785	Cytochrome P450, 2c55	*Cyp2c55*	1.6

Table 2. Hepatic lipid contents in *Apoe*-deficient mice consuming purified Western diets.

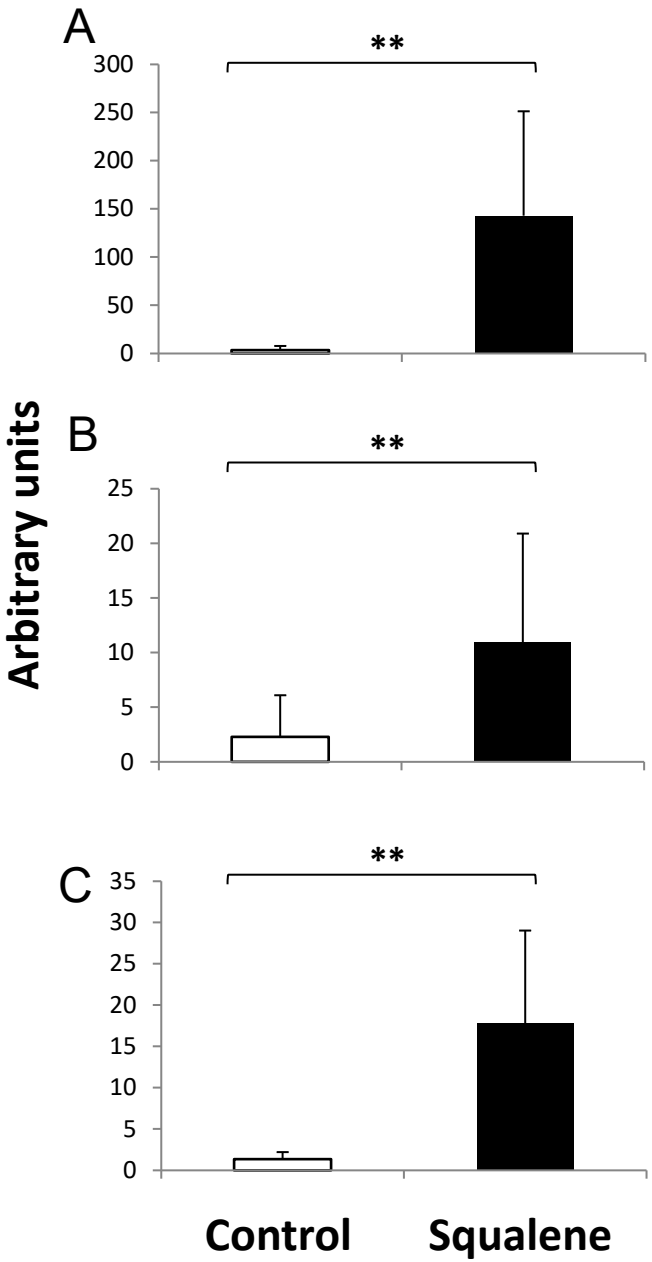
	Males				Females			
	Control	Squalene	Orchiectomized control	Orchiectomized squalene	Control	Squalene	Ovariectomized control	Ovariectomized squalene
Triglycerides	83 ± 27	61 ± 13[a]	87 ± 27	74 ± 15[b]	46 ± 9	73 ± 10[a]	73 ±15[a]	78 ±19
Cholesterol	10 ± 2	7 ± 1[a]	10 ± 3	8 ± 2	7 ± 1	9 ± 1[a]	9 ± 2[a]	8 ± 1[c]

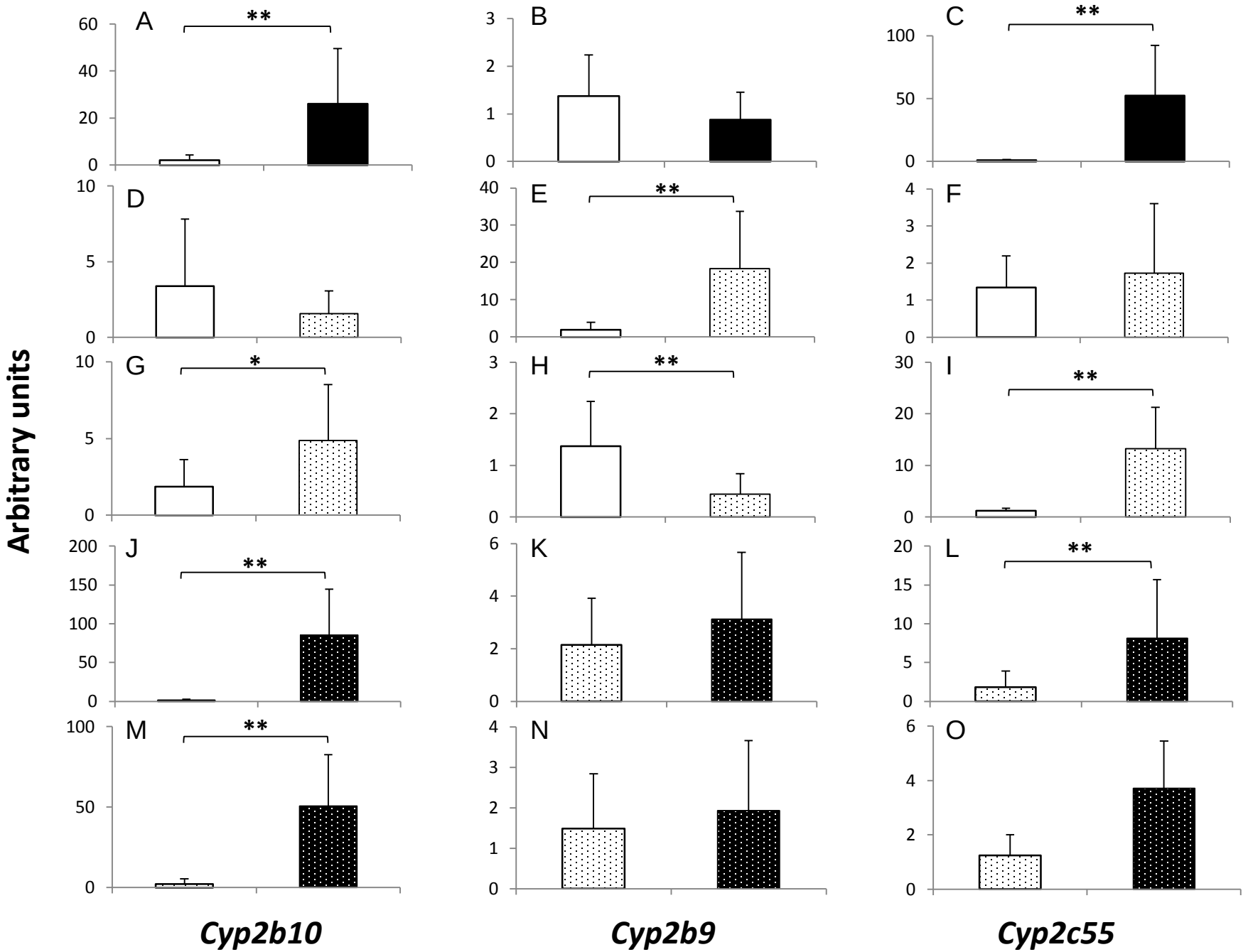
Data (mean ± sd) represent mg of lipid/g of liver tissue. Statistical analysis was carried out with Kruskal-Wallis one-way ANOVA and Mann-Whitney-U as post hoc test. a, $P<0.05$ *vs* control; b, $P<0.05$ *vs* orchiectomized control and c, $P<0.05$ *vs* squalene.

Table 3. Hepatic lipid contents in wild-type and *Apoa1*-deficient mice consuming purified chow diets.

	Wild-type mice				*Apoa1*-deficient mice			
	Male		Female		Male		Female	
	Control	Squalene	Control	Squalene	Control	Squalene	Control	Squalene
Triglycerides	15 ± 3	21 ± 7[a]	21 ± 5	28 ± 6[a]	15 ± 9	14 ± 8	24 ± 6	26 ± 8
Cholesterol	8 ± 1	7 ± 2	7 ± 1	10 ± 1[a]	6 ± 1	7 ± 1	7 ± 1	9 ± 2[a]

Data (mean ± sd) represent mg of lipid/g of liver tissue. Statistical analysis was carried out with Kruskal-Wallis one-way ANOVA and Mann-Whitney-U as post hoc test. a, $P<0.05$ vs control.


A
**
300
250
200
150
100
50
0
B
**
25
20
15
10
5
0
C
**
35
30
25
20
15
10
5
0
Arbitrary units
Control
Squalene


Arbitrary units
A
B
C
D
E
F
G
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I
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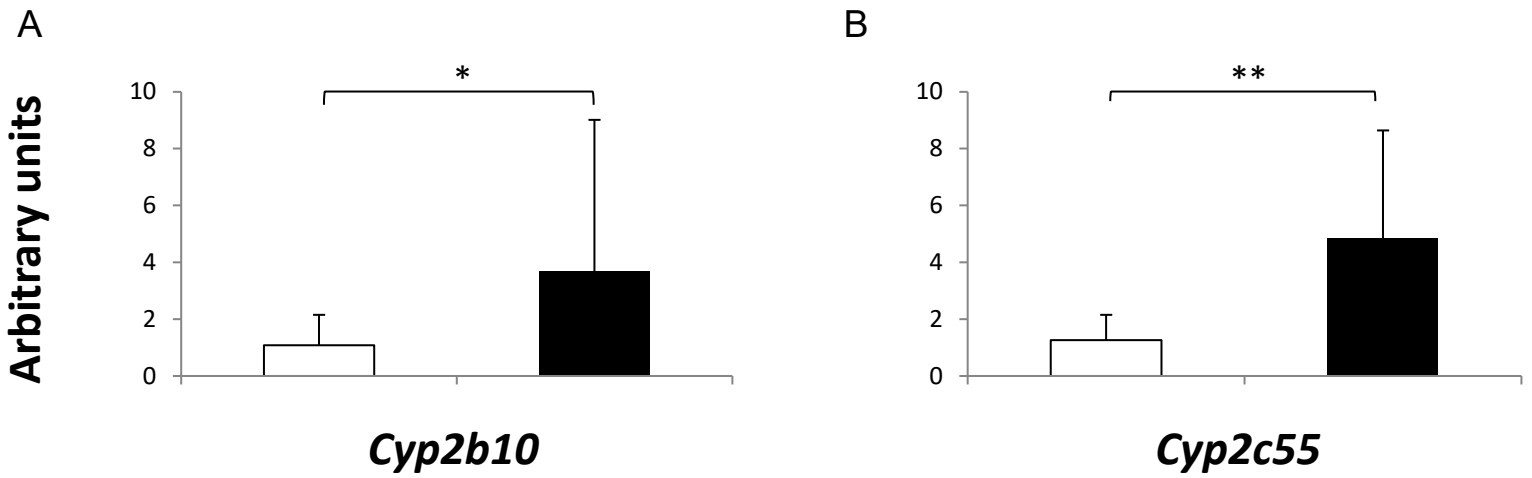
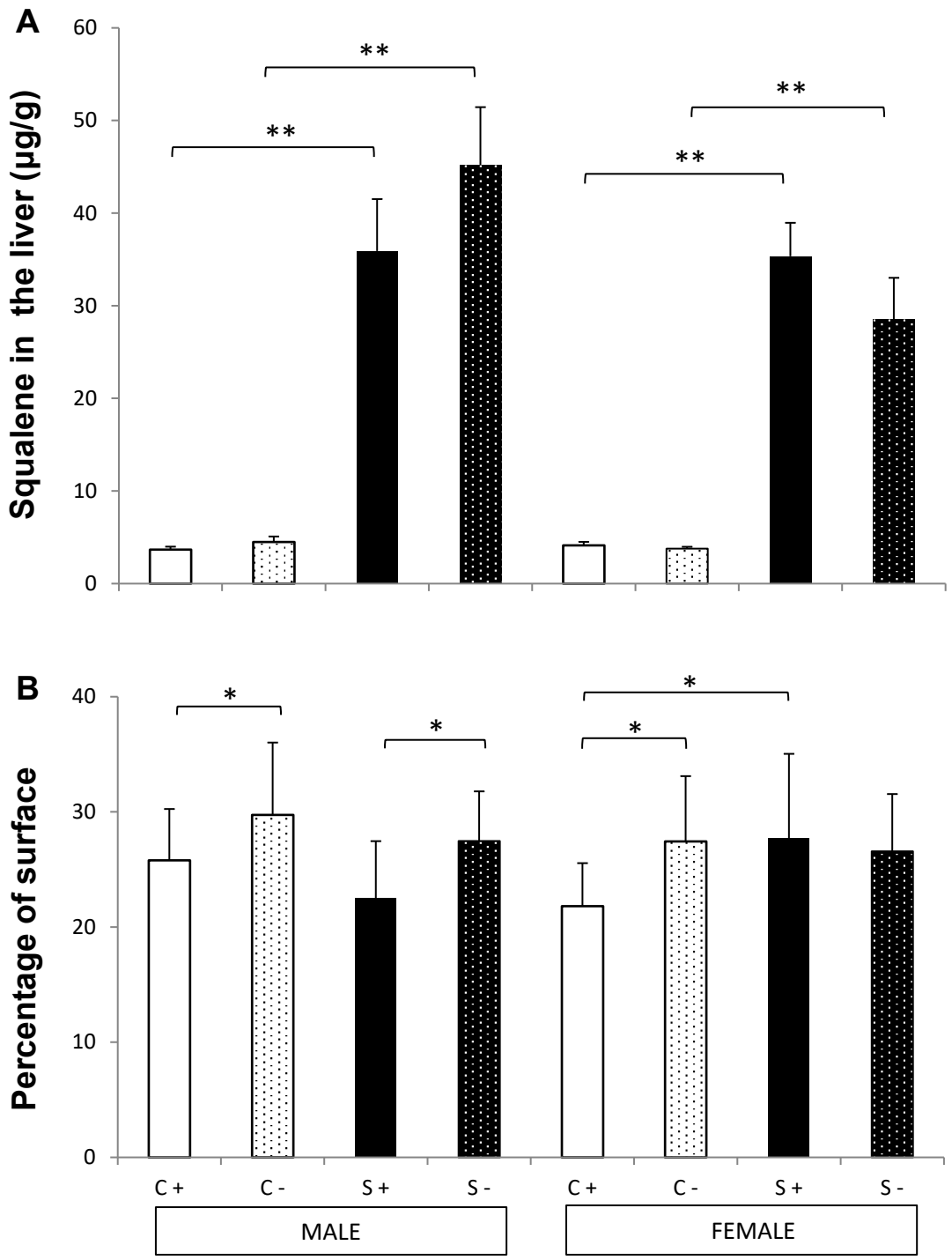
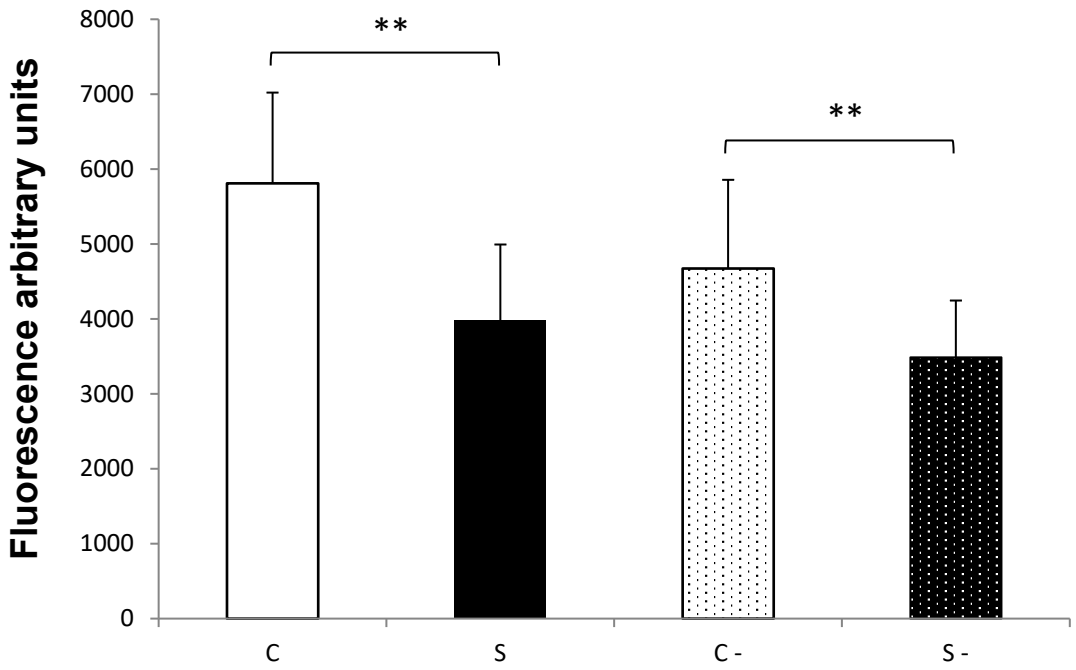
K
L
M
N
O
**
*
Cyp2b10
Cyp2b9
Cyp2c55


A
Arbitrary units
10
8
6
4
2
0
*
Cyp2b10
B
10
8
6
4
2
0
**
Cyp2c55


A
Squalene in the liver (µg/g)
60
50
40
30
20
10
0
**
**
**
**
B
Percentage of surface
40
30
20
10
0
*
*
*
*
C +
C -
S +
S -
C +
C -
S +
S -
MALE
FEMALE

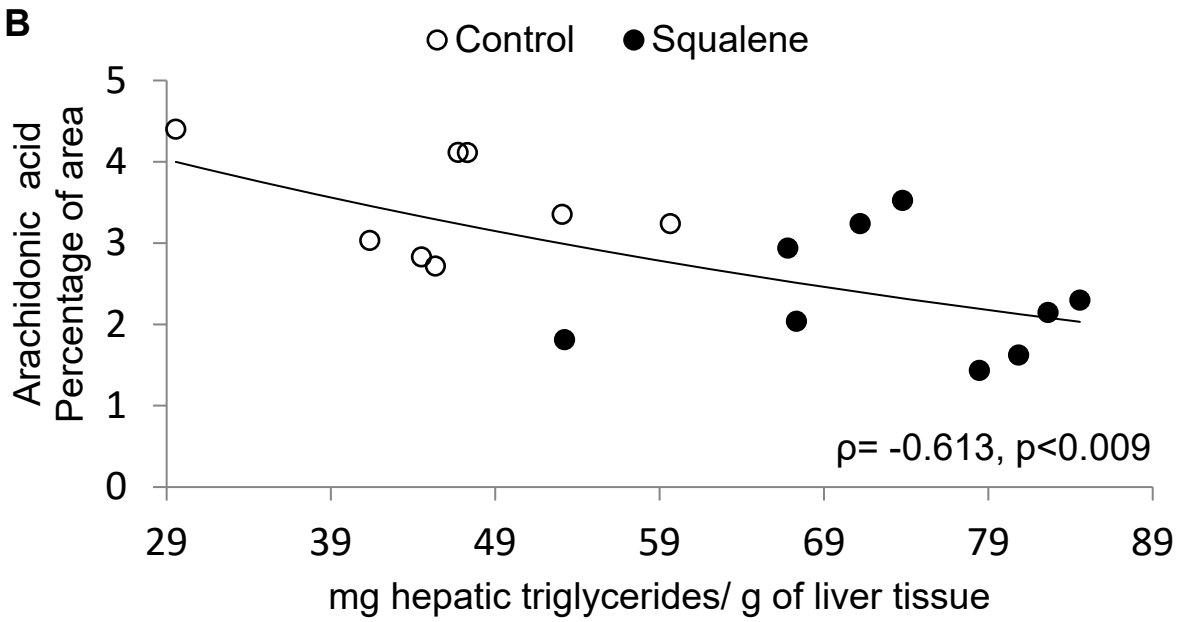

Fluorescence arbitrary units
8000
7000
6000
5000
4000
3000
2000
1000
0
**
**
C
S
C -
S -

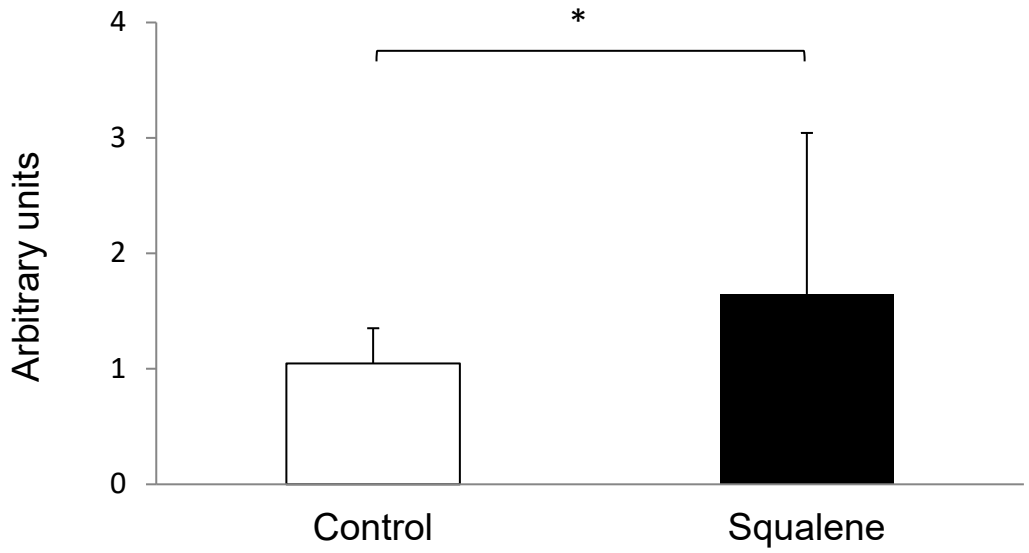
A

Female

	Control	Squalene
20:4 n-6	3.5 ± 0.2	2.3 ± 0.2*

B




*
4
3
2
1
0
Arbitrary units
Control
Squalene