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Dietary squalene modifies plasma lipoproteins and hepatic cholesterol metabolism in rabbits

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Running title: Dietary squalene and rabbit

Abbreviations

APO, apolipoprotein

APOB, apolipoprotein B

BLAST, basic local alignment search tool

CASP1, caspase 1

CETP, cholesteryl ester transfer protein

CYP7A1, cytochrome P450 7A1 mRNA

ELISA, enzyme linked immunoassay

DMEM, Dulbecco's modified Eagle's minimum essential medium

FPLC, fast protein liquid chromatography

GC/MS, gas chromatography coupled to mass spectrometry

Gpx4, phospholipid hydroperoxide glutathione peroxidase mRNA

HDL, high density lipoproteins

HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA

HSC70, heat shock cognate 70

IDL, intermediate density lipoproteins

IL-1β, interleukin-1 beta

IL-18, interleukin-18

LDL, low density lipoproteins

LDLR, low density lipoprotein receptor mRNA

NCBI, National Center for Biotechnology Information

NLRP3, NACHT, LRR and PYD domains-containing protein 3

Ppib, peptidylprolyl isomerase B mRNA

ROS, reactive oxygen species

RT-qPCR, reverse transcriptase- quantitative polymerase chain reaction

SQLE, squalene monooxygenase mRNA

Tbp, TATA-box-binding protein mRNA

VLDL, very low density lipoproteins

Abstract:

To evaluate the effects of squalene, main unsaponifiable component of virgin olive oil, on lipid metabolism, two groups of male New Zealand rabbits were fed a 1% sunflower oil-enriched regular diet or the same diet containing 0.5% squalene for 4 weeks. Plasma triglycerides, total- and HDL-cholesterol and their lipoproteins were assayed. Analyses of hepatic lipid droplets, triglycerides, total- and non-esterified cholesterol, squalene, protein and gene expression, and cholesterol precursors were carried out. In the jejunum, squalene content, mRNA and protein APOB expressions were measured. Finally, we studied the effect of cholesterol precursors in AML12 cells. Squalene administration significantly increased plasma total cholesterol, mainly carried as non-esterified cholesterol in IDL and large LDL, and corresponded to increased number of APOB100-containing particles without accumulation of triglycerides and decreased reactive oxygen species. Despite no significant changes in APOB content in the jejunum, the latter displayed increased APOB mRNA and squalene levels. Increases in the amounts of non-esterified cholesterol, squalene, lanosterol, dihydrolanosterol, lathosterol, cholestanol, zymostenol, desmosterol and caspase 1 were also observed in the liver. Incubation of AML12 cells in the presence of lanosterol increased caspase 1. In conclusion, squalene administration in rabbits increases the number of modified APOB-containing lipoproteins, and hepatic cholesterol biosynthesis is linked to caspase1 probably through lanosterol.

Key words: non-esterified cholesterol, lanosterol, squalene, lipoproteins, liver, virgin olive oil.

Introduction

The Mediterranean dietary pattern has been associated with a reduced cardiovascular risk [1]. Virgin olive oil, the main source of fat in this diet, has been proved to reduce cardiovascular mortality [2]. The mechanism of this protective effect of virgin olive oil intake still needs to be elucidated. It was firstly attributed to its main component, oleic acid. However, its minor bioactive components play important biological actions [3, 4].

Squalene, first isolated from shark liver oil [5], is a polyunsaturated triterpene and a biochemical precursor of cholesterol [4]. In extra virgin olive oil, its content varies from 1.5 to 10.1 g/kg depending on plant cultivar, agronomical practices and fruit processing [6]. *In vitro*, it is a highly effective oxygen scavenging agent [7] and stable in virgin olive oil heated at 180ºC for 36 h [8]. For these reasons, it has been proposed that squalene rather than oleic acid could be the important active compound of virgin olive oil [9]. In humans, orally administered squalene is well absorbed (60–85%). This and the intestinal *de novo* synthesized squalene are transported by chylomicrons into circulation, being rapidly taken up by the liver, where it is converted into sterols and bile acids [10] or secreted into bloodstream [11, 12]. Others authors state that hepatic squalene, either biosynthesized or dietary, is secreted into very low density lipoproteins (VLDL) and low density lipoproteins (LDL) and distributed to various tissues. Therefore, squalene concentration in plasma lipoproteins represents an equilibrium from dietary intake, with important amounts coming from extra virgin olive oil, and intestinal or liver synthesis [13].

Rabbits, since the early work of Ignatowski showing diet-induced accumulation of cholesterol in the rabbit aorta [14], have been widely used in arteriosclerosis due to their high absorption rate of dietary cholesterol [15], rapid development of aortic lesions and relative low cost of feeding and maintenance [16, 17]. Furthermore, this species shares several features of lipoprotein metabolism with humans. In this regard, the liver does not produce apolipoprotein (APO) B48 so VLDL only contain APOB100 [18], APOB100-

containing particles show similar chemical composition, and it has high cholesteryl ester transfer protein (CETP) [19] and low phospholipid transfer activities [20]. Unlike humans, rabbits display low hepatic lipase activity [21] and do not have an analog of human APOA2 [22]. In addition to these general characteristics, some strains such as Watanabe hereditary hyperlipidemic rabbits are deficient in LDL receptors and therefore resemble human familial hypercholesterolemia [23] and St. Thomas' Hospital rabbits represent a model of human hypertriglyceridemia and combined hyperlipidemia [24]. Moreover, rabbits have been a target of genetic modifications to investigate the role of APOA1 [25], APOA2 [22], APOC3 [26], APOE [27, 28], CETP [29] and LDL receptor [28, 30] in lipoprotein metabolism and atherogenesis.

It is not clear yet squalene's role on plasma lipids in humans and animal models. While some authors observed increased plasma cholesterol after squalene intake in rats [31] and in hamsters [32], others did not in humans [33], rats [34] or *Apoe*-deficient mice [35]. We have observed changes in high density lipoproteins (HDL) following 1 g/kg squalene dose administration in *Apoe*-deficient mice, wild-type mice and *Apoa1*-deficient mice [36]. An early work in rabbit also reported an increase in plasma cholesterol in rabbits receiving squalene [37]. These discrepancies raise the questions of whether animal models or administration regimens (length and doses) are modifying squalene's effect on plasma cholesterol. In an attempt to clarify this issue, the present work has been designed to explore the effect of squalene administration in rabbits and characterize their lipoproteins.

Material and methods

Animals and diets.

During 4 weeks, 12 male New Zealand White rabbits (1.2 kg body weight) were fed with a regular diet enriched with 1% of sunflower oil for the control group (n=6), and with 1% of sunflower oil and 0.5% of squalene (Sigma-Merck, Darmstadt, Germany) for the squalene group (n=6). Sunflower oil was used to dissolve squalene and 1% was empirically found to be perfectly embedded into diet pellets without requiring milling and pelleting again. Regular diet pellets were sprayed with sunflower oil or squalene-containing sunflower oil. Diets were prepared weekly and changed every two days to reduce squalene oxidation. Intake and body weights were monitored every 2 days. At the end of the 4-week dietary intervention, food was withdrawn for 18 hours, and the rabbits were weighed and then sacrificed. This fasting time was selected according to studies carried in these animals using dietary fats [38, 39]. Venous blood samples, the livers and the jejunums were obtained for assays. The samples were immediately frozen in liquid nitrogen and stored at -80 ºC. Animals were handled and killed observing guidelines from the European Union for care and use of laboratory animals in research (Directive 2010/63/UE), and the protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza (PI47/10).

Biochemical determinations

Total plasma cholesterol and triglyceride concentrations were measured in a microtiter assay, using Infinity™ commercial kits (Thermo Fisher Diagnostics Slu, Cornella de Llobregat, Barcelona, Spain). Plasma HDL-cholesterol was quantified in the supernatant after precipitation of APOB-containing particles using phosphotungstic acid–$MgCl_2$ (Roche, Barcelona, Spain). Plasma lipoprotein profile was determined in 100 µl of pooled plasma samples from each group by fast protein liquid chromatography (FPLC)

gel filtration using a Superose 6B column (GE Healthcare, Chicago, Il, USA), and the total and non-esterified cholesterol contents in each fraction were measured as described [36, 40]. APOB were quantified by enzyme linked immunoassay (ELISA) using specific polyclonal antibodies (diluted 1/1,000, AB7616; Abcam, Cambridge, UK), as described previously [36, 40]. APOE was determined using a commercial kit (MBS700983; MyBioSource, Inc. San Diego, CA, USA).

Lipoproteins from 1 ml of plasma were fractionated by sequential ultracentrifugation at 4°C in a Beckman 60 Ti rotor, adjusting the density by addition of potassium bromide [41]. The lipoproteins in the density ranges of $d < 1.006$, $d = 1.006$-1.02 and $d = 1.02$-1.04 g/ml were isolated after centrifugation at 50,000 rpm for 16 h. The $d = 1.04$-1.06 and $d = 1.06$-1.08 g/ml fractions were centrifuged for 18 h; the $d = 1.08$-1.10 and $d = 1.10$-1.21 g/ml fractions were centrifuged for 24 h and 48 h, respectively, all at 59,000 rpm. Lipoproteins that floated at each density were collected and refloated under the same conditions to remove contaminating plasma proteins. Lipoproteins from each fraction were recovered and dialyzed in 10 mM Tris buffer, pH 7.4, 150 mM NaCl and 1 mM EDTA.

Reactive oxygen species (ROS) content in lipoproteins.

The presence of ROS was assessed by measuring the conversion of 2,7-dichlorofluorescein diacetate into fluorescent dichlorofluorescein [42] in ultracentrifuge-isolated fractions corresponding to the different lipoproteins.

Western blotting

Tissues homogenates (30 μg of protein) were loaded onto 4% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), electrophoresed, and transferred as previously described [35]. APOB bands were detected using a goat polyclonal antibody (AB7616; Abcam, Cambridge, UK, diluted 1/1,000). Equal loadings and transference

were verified by using a β-ACTIN goat polyclonal antibody in a dilution 1/2,500 (AB8229; Abcam). As secondary antibody, a 1/25,000 dilution of a donkey anti-goat Dylight 680 (NBP1-72850; Novusbio, Centenial CO, USA) was used. Image was captured and analyzed using an Odyssey® Clx (LI-COR Biosciences, Lincoln, NE, USA).

Rabbit caspase 1 (CASP1) and heat shock cognate 70 (HSC70) were assayed using 14% SDS-PAGE gels and 20 μg of protein. CASP1 bands were detected using a mouse monoclonal antibody (NB100-56565; Novusbio, diluted 1/1,000), and HSC70 with a goat polyclonal in a dilution 1/500 (SC-1059; Santa Cruz Biotechnology inc, Dallas TX, USA). As secondary antibodies, a goat anti-mouse IgG conjugated with IRDye 800CW (#926-32210; LI-COR Biosciences) at 1/30,000 dilution was used for CAPS1 and a donkey anti-goat IgG Dylight 680 (NBP1-72850; Novusbio) at 1/25,000 dilution for HSC70, the latter as loading and transfer control. For AML12 cell line proteins, the same primary antibody against CASP1 and as secondary goat anti-mouse IgG (H+L) coupled to DyLight 680 at 1/40,000 dilution (#35518; Thermo Scientific, Madrid, Spain). ACTIN was detected using a rabbit polyclonal at 1/2,000 (A2066; Sigma-Aldrich) and a goat anti-rabbit IgG (H+L) linked to DyLight 800 at 1/30,000 (SA5-35571, Thermo Scientific). Images were captured and analysed using an Odyssey® Clx (LI-COR).

RNA isolation

At sacrifice, the livers and the jejunums were immediately removed and frozen in liquid nitrogen. RNA was isolated from each liver using Tri-reagent (Ambion, Austin, 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 28S and 18S ribosomal RNAs was verified by agarose gel electrophoresis and the 28S/18S ratio was greater than 2. RNA quality was also tested using an Agilent

2100 Bioanalyzer (Agilent RNA 6000 nano kit, Santa Clara, CA, USA) and the RNA integrity numbers were higher than 8.2.

Quantification of mRNA

The potential changes in mRNA expression were determined by RT-qPCR analysis of individual samples using equal amounts of DNA-free RNA from each animal. First Strand cDNA synthesis kit (Thermo Scientific) was used to generate the complementary DNA. 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 but not genomic DNA [43]. Sequences are shown in supplementary Table 1. 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 *RN18S* expression for rabbit samples, and with an average of *Tbp* and *Ppib* in AML12 samples.

Histological analyses

A sample of liver from each animal was stored in neutral formaldehyde and embedded in paraffin wax. Sections (4 µm) were stained with hematoxylin and eosin. A slide scanner Zeiss AsioScan.Z1 (Zeiss, Oberkochen, Germany) was used to record preparations. Lipid droplets were evaluated by quantifying their areas in each liver section with Adobe Photoshop CS3 (Adobe Inc. San Jose, CA, USA) and expressed as percentage of total liver section [35].

Filipin staining

The filipin staining specific for non-esterified cholesterol was carried out in a section of 4 µm of each liver with the Cholesterol Cell-Based Detection Assay Kit from Cayman

Chemical (Ann Arbour, MI, USA) [44], and the images were captured with a confocal microscope Zeiss Apotome with the program AxioVision Rel 4.6 (Zeiss).

Hepatic lipid analyses

Hepatic lipids were extracted from approximately 10 mg of liver, and cholesterol and triglyceride contents were assayed as reported [45].

Squalene extraction and analyses

Tissues were extracted and squalene analyzed by solid phase extraction, gas chromatography and mass spectrometry (GC/MS) as previously described [45].

Extraction and analyses of sterols

Sterols were extracted and analyzed by GC/MS as described [46].

AML12 cell culture

The murine hepatocyte cell line was grown in a humidified atmosphere of 5% CO_2 at 37ºC in Dulbecco's modified Eagle's minimum essential medium (DMEM) (ThermoFisher Scientific): F12-Ham's medium (GE Healthcare Life Science, South Logan, Utah) supplemented with fetal bovine serum and insulin/transferrin/selenium, as previously described [47]. When AML12 cells reached a 90-100% confluence, this medium was removed, and cells were washed twice with phosphate buffered saline prior to the addition of the medium devoid of fetal bovine serum, insulin, transferrin and selenium. Cells were incubated for 6 hours in the presence of 200 nM desmosterol, zymostenol, lanosterol or dihydrolanosterol (Avanti Polar lipids, Alabaster, AL, USA) dissolved in 0.1% ethanol. Then, media were removed and cells collected with Tri-reagent solution (Ambion). RNA isolation and cDNA synthesis were performed as described above. The effect of 200 nM lanosterol was investigated at the protein level and at different time points.

Statistical analyses

Results are shown as means and their standard deviations. The normal distribution of data was analyzed according to Shapiro–Wilk test, and homology of variances between groups using Bartlett or Levene tests. Parameters failing in any of these tests, their differences were tested using the Mann-Whitney's U test. All calculations were performed using SPSS version 15.0 software (SPSS Inc, Chicago, IL, USA) or Prism 5 for Windows (GraphPad, S. Diego, CA, USA). Significance was set at $P \leq 0.05$.

Results

Somatometric analyses

During the dietary intervention, the two groups showed similar body weight gains (Supplementary Figure 1, panel A). Nor were there statistically significant differences observed in solid intake between groups (Supplementary Figure 1, panel B). Taking into account their consumed food and body weights, it resulted that squalene was provided at a dose of 0.6 g/kg/day.

Plasma lipids and lipoproteins

Plasma total cholesterol, HDL cholesterol and triglyceride levels are shown in table 1. We observed that squalene administration induced a significant increase in total cholesterol and a trend to decrease triglycerides. The latter considered good marker of proper fasting period. In this model, dietary squalene did not change HDL cholesterol analyzed by precipitation of APOB-containing particles (Table 1). The chromatographic separation of plasma lipoproteins by FPLC confirmed the absence of changes in HDL cholesterol (Figure 1A). The chromatographic approach also unveiled an important increase of total cholesterol in VLDL and LDL (Figure 1 A) when squalene was administered. Notably, the increase corresponded to increased non-esterified cholesterol, as displayed in Figure 1 B, and absence of changes of esterified cholesterol (data not shown). Assay of APOB in lipoparticles containing this apolipoprotein showed an increase of APOB in VLDL and LDL in the squalene group (Figure 1 C). To corroborate these previous findings, isolation of plasma lipoproteins by density flotation using ultracentrifugation was carried out (Figure 2). The squalene administration resulted in a significantly increased content of total cholesterol in lipoproteins of densities lower than 1.006 (VLDL), 1.02 (intermediate density lipoproteins, IDL), 1.04 (large LDL), 1.06 (LDL) and 1.21 (mature HDL) (Figure 2 A). The increase was mainly found in IDL and

large LDL and corresponded to increased non-esterified cholesterol (Figure 2 B). Regarding triglyceride content in the different fractions (Figure 2 C), there were no significant changes in lipoproteins of densities lower than 1.06 g/ml and significant increases in particles of densities < 1.08 and 1.1 corresponding to large HDL. Significant increases in APOB were observed in lipoparticles containing this apolipoprotein (all with d<1.006) by the intake of squalene, mainly due to APOB100 (Figure 2 D and E). An increase in APOE was also observed in fractions corresponding to IDL and large LDL (Figure 2 F). These APOB-containing lipoparticles from squalene treated animals showed decreased ROS content, as shown in Figure 2 G.

Tissue analyses

In the jejunum, despite the increase in *APOB* mRNA (Figure 3 A), APOB content remained unmodified when rabbits received the squalene-supplemented diet (Figure 3 B). Significant accumulation of squalene was found in the jejunum of squalene group (Figure 3 C).

The area of the liver occupied by lipid droplets significantly augmented in rabbits receiving squalene, as displayed in Figure 4 A, B and C. Nonetheless, hepatic triglycerides and esterified cholesterol remained unmodified (Table 2) while hepatic total cholesterol, non-esterified cholesterol, and squalene significantly increased by the nutritional intervention (Table 2). The increased non-esterified cholesterol content in the liver was also confirmed by a filipin staining (Figure 4 D and E) and by GC-MS analysis (Table 3).

The increase in cellular non-esterified cholesterol content has been associated with pyroptosis mediated by Caspase 1 activation [48]. To explore this finding in our experimental setting, we assayed CASP1 by Western-blot analyses. As shown in Figure

4 F, G and H, squalene administration increased both forms (45 and 30 kDa) of caspase1. Processed forms of lower molecular weight were not observed.

The mRNA expression of crucial genes (*HMGCR*, *SQLE*, *CYP7A1 and LDLR*), involved in metabolism and uptake of cholesterol, did not experience any significant change (data not shown). To gain more insight into the increased hepatic cholesterol levels, an analysis of its intermediary metabolites was undertaken and their results are shown in Table 3. Rabbits receiving squalene showed significantly increased levels of cholestanol, lathosterol, dihydrolanosterol and lanosterol. Likewise, zymostenol and desmosterol were barely detected in the squalene-fed group, indicating the preferent use of the Kandutsch-Russell pathway for the cholesterol synthesis in the liver.

Cell culture

To explore the relevance of cholesterol metabolites on the observed action of squalene, we incubated AML12 cells in presence of lanosterol, dihydrolanosterol, desmosterol or zymostenol and explored the *Casp1* and *Gpx4* mRNA expressions. The latter has been found to be a target of squalene action [49]. As shown in Figure 5 A and B, only 200 nM lanosterol was able to significantly increase the expression of both genes. The effect of lanosterol incubation on caspase 1 was also observed at the protein levels at the different assayed time-points (Figure 5 C).

Discussion

The aims of the present study were to verify the influence of squalene administration on lipoproteins and cholesterol metabolism in rabbits. Our results indicate that rabbits consuming 0.5% squalene vehicled in sunflower oil (corresponding to a dose of 0.6 g/kg) showed an increase in total cholesterol, mainly in APOB-containing particles and without changes in HDL-cholesterol. Furthermore, the increase in plasma total cholesterol was mainly due to non-esterified cholesterol and accompanied by an increase in APOB levels, particularly APOB100, and no change in triglycerides. Squalene contents in the liver and the jejunum were also increased. The former increase was translated into increased metabolites of hepatic cholesterol biosynthesis such as lanosterol, dihydrolanosterol, lathosterol, zymostenol, desmosterol and non-esterified cholesterol. The sterol enrichment was uniformly observed in all subcellular organelles, and accompanied by lack of accumulation of hepatic triglycerides and increased caspase 1 levels. The increase in caspase 1 was also induced by lanosterol incubation in AML12 hepatic cell line. Overall, squalene appears to be an important modifier of hepatic cholesterol metabolism influencing both post-squalene biosynthetic pathways as well as plasma cholesterol lipoproteins in rabbit. It also modifies cellular responses as caspase 1, a regulator of ferroptosis.

The observed increase in total cholesterol in rabbits is in agreement with the early report of Kritchevsky et al. [37] who, using a 6-fold higher dose than the present study, reported an increase of plasma cholesterol in rabbits that was not translated in atherosclerosis development. Likewise, the increase in total cholesterol without changes in HDL-cholesterol agrees with our results using the 0.25 g/kg squalene dose in *Apoe*-deficient mice [36]. Zhang et al. observed a hypercholesterolemic effect of squalene in

hamsters [32] as well. These observations point out that the outcome of squalene administration on plasma lipids is a compromise of dose and animal model.

Our study provides a further insight regarding rabbit lipoprotein composition following a squalene dietary intervention. In this regard, the elevated total cholesterol was mainly due to the increases in VLDL and LDL non-esterified cholesterol according to FPLC data (Figure 1 B). Ultracentrifugation analysis (Figure 2) corroborated those augments in total and non-esterified cholesterol from lipoproteins (Figure 2 A and B) but its improved resolution allowed to ascertain that the most profound increases corresponded to fractions of densities 1.006-1.02 and 1.02-1.04 containing IDL and large LDL, respectively [50]. The observation of raised APOB levels in these particles (Figure 1 C and 2 D) and considering that there is a single molecule of APOB per lipoprotein is suggesting an increased number of IDL and large LDL particles with normal triglyceride content (Figure 2 C). Furthermore, characterized the molecular APOB undergoing changes, it turned out to be the APOB100 isoform (Figure 2 D). Thereby, these results indicate that squalene consumption in rabbit mainly increases the number of IDL and large LDL particles loaded with non-esterified cholesterol.

The observed increase the number of IDL and large LDL particles loaded with non-esterified cholesterol and normal triglycerides containing APOB100 is suggesting a metabolic setting where metabolism of these lipoparticles is impaired. Hepatic lipase has been proposed to be involved in the transformation of IDL into LDL [51], and as above mentioned rabbits display low hepatic lipase activity [21]. This low hepatic lipase activity could explain the accumulation of IDL and large LDL. Something similar was also observed in double knock-out for hepatic lipase and *Apoe* [52] although in the latter model, the results were not so striking as in our conditions due to the profound influence of the absence of APOE in mice. Interestingly, in those mice the increase in plasma cholesterol

was accompanied by decreased atherosclerotic development. Likewise, the increase in total cholesterol in rabbits consuming squalene was not translated in atherosclerosis development [37]. Protective effect of 2 mg/kg squalene administration on fibrosis and endothelial activation in gingival mucosa used as a subrogate sample of cardiovascular disease was found in rabbits consuming an atherogenic diet [53]. Several interesting aspects should be considered: firstly, increased IDL and large LDL of diameter larger than 70 nm do not enter into the subendothelial space [54]. In this sense, the increase in non-esterified cholesterol due to its polar group is preferentially located in the peripheral layer and contributes to further expand the size of the lipoparticles. If there is enough APOE, they could be taken up by peripheral tissues what could resemble a delayed metabolism in the rabbit lacking hepatic lipase. Secondly, IDL and large LDL from squalene-fed rabbits showed diminished load of reactive oxygen species (Figure 2 G). Thirdly, plasma triglycerides were not modified or showed a trend to decrease by squalene. The decrease of triglycerides induced by apolipoprotein C3 deficiency has been proved to decrease atherosclerosis in the rabbit [26] and current opinion poses the deleterious effects of increased triglycerides in lipoproteins [55]. All these aspects could contribute to explain the vascular properties of squalene despite the increase of total cholesterol, although the activated reverse cholesterol transport [52] observed in mice lacking hepatic lipase cannot be excluded. This metabolic setting of rabbit could explain the findings and reinforces that the response to squalene may change in different animals depending on different genetic makeups. Furthermore, it constitutes a perfect example of nutrigenetics where hepatic lipase and squalene are influencing plasma cholesterol levels and may pose an interesting aspect to be considered into human personalised medicine.

The increased expression of jejunal *APOB* mRNA and the absence of changes in APOB48 protein in this tissue (Figure 3 A and B) could be explained by a favored release

of jejunal APOB48 into plasma. It is well known that this is a postprandial process and APO48 is essential to chylomicron particles that are particularly enriched in triglycerides [56]. However, in the case of squalene administration, there was no increase in plasma triglycerides (Table 1). This fact and the squalene accumulation in the liver (Table 2) imply that chylomicrons, main carriers of squalene [11], are properly metabolized by lipoprotein lipase and later removed from circulation at the studied fasting time. Overall, squalene administration in this model is influencing the hepatic-intestinal axis through chylomicron dynamics and a complex series of processes may participate.

Squalene administration in this model raises an interesting aspect regarding non-alcoholic fatty liver disease diagnosis. The histomorphometry was done by hematoxylin and eosin staining, although not specific for fatty vacuoles, it has the advantage of using 4 μm sections instead of thicker cryosections required for bodipy or oil red O staining. In this way, paraffin embedded sections are thinner, easier to process and better microscopical resolution is achieved [35, 47, 57] and the smallest lipid droplets (2-3 μm) can be quantified. The drawback is that microphotographs have to be measured by naked eye what represents a time-consuming task for a trained pathologist able to distinguish void vessels, glycogen deposits and lipid droplets. Our histomorphometric evaluation of the livers showed an increased area covered by lipid droplets in squalene-fed rabbits (Figure 4 C) while triglyceride content remained unchanged (Table 2). This discrepancy could be explained by squalene accumulation in lipid droplets in the rabbits fed a squalene-supplemented diet as previously described [45]. A similar discrepancy was also observed when administering oleanolic acid [58] or squalene in murine models [59]. Therefore, terpenes trend to accumulate in the liver may influence non-alcoholic fatty liver disease diagnosis. This is particularly relevant for subjects consuming diets with high squalene content such as the Mediterranean and Japanese diets [13]. This increase in hepatic squalene content

could have further prognostic implications as well, considering that some sharks particularly accumulate it in their livers, and some of them may live up to 400 years [60].

A striking result observed in this work was the accumulation of hepatic non-esterified cholesterol analyzed by enzymatic and GC-MS (Tables 2 and 3) methods and the absence of significant changes in esterified cholesterol (Table 2). According to the filipin staining (Figure 4 D and E), this increase was uniformly distributed thorough the cell. This could explain why mRNA expression of cholesterol target genes, *LDLR, HMGCR, SQLE* and *CYP7A1,* did not undergo any significant change (data not shown). However, an analysis of its metabolic precursors showed significant changes in lathosterol, dihydrolanosterol, lanosterol and its metabolic product, cholestanol, and the appearance of dihydrolathosterol, zymostenol and desmosterol (Table 3). When the values of increased metabolites are reflected on the proposed pathways of cholesterol biosynthesis (Figure 6), a selective use of lanosterol and zymosterol in detriment of desmosterol by the sterol Δ^{24}-reductase is observed what could explain the selective accumulation of some metabolites. Recently, an unexpected regulatory role of squalene for squalene monooxygenase has been described [61]. Whether this mechanism is also present in the case of sterol Δ^{24}-reductase is an interesting aspect that deserves further attention. Furthermore, inhibition of this reductase increases desmosterol and lanosterol and favors resolution of inflammation [62]. Our data indicating that desmosterol, dihydrolanosterol, lanosterol and zymostenol were particularly enriched by squalene are suggestive that they may contribute to the action of squalene.

Since increased hepatic non-esterified cholesterol content has been linked to pyroptosis mediated by CASP1 activation [48], we analyzed CASP1. As shown in Figure 4 F, G and H, squalene administration increased both forms (45 and 30 kDa) of caspase 1 in the liver of rabbits receiving squalene. Processed forms of lower molecular weight

were not observed. Activated CASP1 modulates production of mature cytokines IL-1β and IL-18, and is involved in the regulation of appetite, body weight, glucose homeostasis, and lipid metabolism through multiple mechanisms, not limited to cytokine activation [63, 64]. In addition, inactive CASP1 also plays a role in developing systemic inflammation in mice independently of the NLRP3 inflammasome cascade [65]. However, the increased CASP1 found in our work was not associated with systemic inflammation since squalene administration neutralized the LPS-induced effects [66]. This controversy needs further research to clarify the role of unprocessed CASP1 and its potential signaling cascades in different organs.

Using CASP1 as a subrogate marker of squalene administration, we explored whether desmosterol, dihydrolanosterol, lanosterol and zymostenol participate in its expression. Working with the murine hepatic cell line AML12, we observed that incubation of lanosterol significantly increased the mRNA and protein expressions of CASP1. In this case, the increase was also due to its unprocessed forms. Using characterized cell lines and genetic tools, the direct action of squalene on *Gpx4* has been proposed as a way of preventing ferroptosis [49]. When *Gpx4* was assayed following lanosterol incubation, an increased expression was equally noted. Unfortunately, no rabbit sequence matched in the blast analysis using mouse *Gpx4* gene and the current genome sequence Rabbit OryCun2.0 (https://www.ensembl.org/), so were not able to study its expression. These *in vitro* experiments provide evidence that squalene action is partly due to its metabolite lanosterol and that the action of this compound is neutral since simultaneously activates a pro- and anti-ferroptotic gene expressions. However, the absence of GPX4 in the rabbit indicates that both models are no easily exchangeable and represents an important limitation to ascertain the role of lanosterol in the rabbit liver. Present results reinforce

the increased interest in the regulatory actions of cholesterol synthesis intermediates, including squalene and lanosterol, in different physiological processes [59, 67].

In conclusion, dietary squalene modifies the rabbit plasma lipoproteins by increasing VLDL and LDL nonesterified cholesterol and APOB100, accompanied by increases in hepatic squalene and nonesterified cholesterol. In the liver, intermediate metabolites of cholesterol biosynthesis were modified, with a significant increase of lathosterol, dihydrolanosterol, lanosterol and cholestanol, and appearance of dihydrolathosterol, zymostenol and desmosterol. Squalene increased unprocessed CASP1, something that was reproduced by *in vitro* incubation of lanosterol.

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Author Contributions

RMB, MJF, JSM, CB, SG, CA, JCB, RL, RB, MAL, MJRY carried out the experiments and prepared the draft, and J.O. supervised the work and draft and prepared the final version of the manuscript. All authors read and approved the final manuscript.

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Table 1. Influence of squalene administration on plasma lipids in rabbits.

	Initial (12)	Post-intervention	
		Control (n=6)	Squalene (n=6)
Total cholesterol (mg/dl)	142 ± 55	85 ± 35[a]	366 ± 231[a,b]
HDL cholesterol (mg/dl)	24 ± 10	19 ± 5	18 ± 7
Triglycerides (mg/dl)	71 ± 26	101 ± 70	45 ± 29

Values are expressed as means ± standard deviations. Statistical analysis was done using one-tail Mann-Whitney U test. Superscripts indicate statistically significant differences. [a], $P<0.05$ vs initio and [b], $P<0.05$ vs control.

Table 2. Effect of dietary squalene supplementation on hepatic parameters.

	Control (n=6)	Squalene (n=6)
Triglycerides (mg/g of tissue)	19.2 ± 8.5	16.8 ± 4.2
Total cholesterol (mg/g of tissue)	2.6 ± 0.2	3.6 ± 1.1[a]
Non-esterified cholesterol (mg/g of tissue)	2.3 ± 0.3	2.9 ± 0.5[A]
Esterified cholesterol (mg/g of tissue)	0.3 ± 0.2	0.6 ± 0.6
Squalene (ng/mg of tissue)	124 ± 53	541 ± 476[A]

Values are expressed as means ± standard deviations. [a], $P<0.05$ and [A], $P<0.01$ vs control, respectively according to Man-Whitney's U test.

Table 3. Effect of dietary squalene supplementation on hepatic cholesterol content and its biosynthetic precursors.

	Control (n=6)	Squalene (n=6)
Cholestanol	33 ± 11	63 ± 18[a]
Cholesterol	2228 ± 224	3514 ± 706[A]
Desmosterol	0 ± 0	7 ± 6
Dihydrolathosterol	35 ± 12	34 ± 11
Lathosterol	3 ± 1	25 ± 19[A]
Zymostenol	0 ± 0	3 ± 3
Dihydrolanosterol	0.3 ± 1	18 ± 19[A]
Lanosterol	2 ± 1	63 ± 68[A]

Values are expressed as means ± standard deviations in ng/ mg of liver for each compound. [a], $P<0.05$ vs control [A], $P<0.01$ according to Man-Whitney's U test.

Figure 1. Effects of squalene supplementation on plasma rabbit lipoproteins separated by FPLC. Plasma lipoproteins were separated by FPLC and collected fractions were analyzed for total cholesterol (A), non-esterified cholesterol (B) and APOB (C). Fraction numbers 1–6 corresponded to VLDL/chylomicron remnants, 7–13 to LDL, 14–18 to cholesterol-rich HDL and 19–24 to cholesterol-poor HDL.

Figure 2. Effects of squalene supplementation on plasma rabbit lipoproteins isolated by ultracentrifugation and their ROS contents. Plasma lipoproteins were separated by ultracentrifugation and collected fractions were analyzed for total cholesterol (A), non-esterified cholesterol (B) and triglycerides (C). Western blot characterization of APOB in isolated fractions (D). APOB100 and position of 250 kDa marker are indicated. Lanes 1 and 2 denote fraction of d<1.006, lanes 3 and 4, fractions of d<1.02, lanes 5 and 6, fractions of d<1.04 and lanes 7 and 8, fractions of d<1.06. Odd and even numbers correspond to control and squalene, respectively. APOB (E), APOE (F) and ROS content (G) of isolated APOB-containing lipoproteins prepared of pooled plasmas. Results are shown as means and standard deviation. Statistical analyses were done according to Mann-Whitney's U test. a, P< 0.05 vs control.

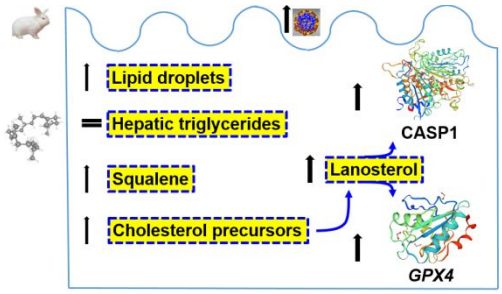
Figure 3. Apolipoprotein B expression and squalene content in the jejunum. *APOB* mRNA levels (A). Data are expressed as arbitrary fluorescence units normalized to the *18S* gene expression with the RT-qPCR analysis. APOB48 protein contents estimated as Western blot of homogenate tissue (B). Squalene content in the jejunum (C). Results are expressed as mean ± standard

deviations. Statistical analyses were done according to Mann-Whitney's U test. A, P< 0.0005 and a, P<0.05 vs control.

Figure 4. Effects of squalene supplementation on hepatic histology and hepatic caspase 1. Representative hepatic micrographs at x400 magnification from control (A) and squalene-fed rabbits (B) and morphometric quantification of areas covered by lipids (C). Representative images of liver sections stained with filipin from control (D) and squalene-fed rabbits (E). The Western blot of liver homogenate showing the 45 and 30 kDa CASP1 bands, and the 70 kDa corresponding to HSC70 used to normalize (F). Quantification of total caspase 1 (G) and of 45-kDa form caspase 1 (H). Results are shown as mean and standard deviation. Statistical analyses were done according to Mann-Whitney's U test. a, P< 0.05 vs control and A, P< 0.01 vs control.

Figure 5. *Gpx4* and *Casp1* gene expressions in AML12. Hepatic cell line of AML12 cells were incubated in the presence of 200 nM desmosterol, dihydrolanosterol, lanosterol or zymostenol dissolved in 0.1% ethanol and control containing the vehicle for 6 hours. Panel A corresponds to *Gpx4* and B to *Casp1* mRNA levels, respectively. Data are expressed as arbitrary fluorescence units normalized to the *Tbp* and *Ppib* gene expressions with the RT-qPCR analysis. Panel C shows the effect of 200 nM lanosterol on CASP1 levels at different incubation times. Results are expressed as mean ± standard deviations of n=8 replicates for each condition. [a], P<0.05 vs control [A], P<0.01 according to Man-Whitney's U test. Control (C), desmosterol (DES), dihydrolanosterol (DYH), lanosterol (LAN) or zymostenol (ZYM).

Figure 6. Impact of squalene on metabolites of the proposed cholesterol biosynthetic pathways. Red arrows denote stimulated reactions.



Squalene-enriched diet fed rabbits displayed large plasma APOB100-containing particles enriched in non-esterified cholesterol and hepatic steatosis mainly due to squalene

Manuscript ID: FO-ART-07-2020-001836

Referee: 1

We thank the Reviewer for the helpful criticisms that allowed us to improve the quality of our manuscript.

Major comments

(1) *Fasting time: In this study, the blood from the rabbits is taken after 18 hours of fasting. Thus, plasma lipid parameters are stable, and there should be little variation in plasma lipid parameters in individual rabbits. But, the variation in plasma cholesterol content is very high, especially in the Squalene group. Was 18 hours of fasting time sufficient to remove plasma chylomicrons and chylomicron remnant? I understand that fasting is usually done to remove chylomicrons and chylomicron remnant in lipoprotein study. In addition, in Figure 1, the Squalene group was showed the ApoB48 protein presents in plasma. Therefore, chylomicron remnant was showed to remain in the Squalene group. Was this insufficient time to fasting, or was there a different idea? Please provide references or reason for choosing 16 hours a fasting time. In addition, please revise the text depend on reason.*

According to this comment, the following paragraph has been included:

"At the end of the 4-week dietary intervention, food was withdrawn for 18 hours, and the rabbits were weighed and then sacrificed. This fasting time was selected according to studies carried in these animals using dietary fats [38, 39]".

38. Y. Cha, J. Y. Jang, Y. H. Ban, H. Guo, K. Shin, T. S. Kim, S. P. Lee, J. Choi, E. S. An, D. W. Seo, J. M. Yon, E. K. Choi and Y. B. Kim, Anti-atherosclerotic effects of perilla oil in rabbits fed a high-cholesterol diet, *Lab Anim Res*, 2016, **32**, 171-179.
39. A. Dorantes-Morales, D. Estrada-Luna, R. Bautista-Perez, G. Betanzos-Cabrera, M. Luna-Luna, C. Flores-Castillo, G. Vargas-Alarcon, J. M. Fragoso, O. Perez-Mendez and E. Carreon-Torres, Microencapsulated Pomegranate Modifies the Composition and Function of High-Density Lipoproteins (HDL) in New Zealand Rabbits, *Molecules*, 2020, **25**.

(2) *ApoB48 recycling (Page 19 L1-2): Will there be a report on ApoB48 recycling? Please provide references.*

This interpretation is no longer sustained once that ultracentrifugation analyses have been carried out and clear increases in APOB100 observed.

(3) *Liver non esterified cholesterol: I understand that an increase in liver cholesterol concentrations is usually as an increase in ester-type cholesterol (CE) concentrations. The increase in liver cholesterol concentration seen in the Squalene group is not reflected in CE. Is this correct?*

The observed increase was in non-esterified cholesterol as confirmed by enzymatic, GC_MS and filipin staining.

(4) *Please indicate whether there was no difference in the rabbits' growth parameters (body weight, food intake, and water intake, etc.) in Results section*

To address this suggestion, a new supplementary Figure 1 has been provided and the results commented in the following paragraph:

" **Somatometric analyses**

During the dietary intervention, the two groups showed similar body weight gains (Supplementary Figure 1, panel A). Nor were there statistically significant differences observed in solid intake between groups (Supplementary Figure 1, panel B). Taking into account their consumed food and body weights, it resulted that squalene was provided at a dose of 0.6 g/kg/day. "

(5) *The CASP1 expression in AML12 cell line has been assessed in vitro. This cell line is originated to mice liver, so there is a limitation to consider rabbit CASP1 express. Please describe the limitations and considerations to be gained from this in vitro experiment.*

This critical comment has been tackled as shown. "These *in vitro* experiments provide evidence that squalene action is partly due to its metabolite lanosterol and that the action of this compound is neutral since simultaneously activates a pro- and anti-ferroptotic gene expressions. However, the absence of GPX4 in the rabbit indicates that both models are no easily exchangeable and represents an important limitation to ascertain the role of lanosterol in the rabbit liver. Present results reinforce the increased interest in the regulatory actions of cholesterol synthesis intermediates, including squalene and lanosterol, in different physiological processes [59, 67]"

Minor comments

Introduction: Please indicate the amount of squalene in the olive oil.

Following this recommendation, the following sentence has been included: "In extra virgin olive oil, its content varies from 1.5 to 10.1 g/kg depending on plant cultivar, agronomical practices and fruit processing [6]"

RNA isolation in Materials and Methods: Liver which is used for real time PCR did not stored in RNA stabilization solutions. Was there any degradation of RNA?

To avoid degradation, more precise description of sample collection has been included in methods:

"The samples were immediately frozen in liquid nitrogen and stored at -80 ºC"

In Results, the quality was tested as follows: "The integrity of the 28S and 18S ribosomal RNAs was verified by agarose gel electrophoresis and the 28S/18S ratio was greater than 2. RNA quality was also tested using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 nano kit, Santa Clara, CA, USA) and the RNA integrity numbers were higher than 8.2."

Histological analyses in Materials and Methods: HE staining should not usually be able to determine lipid droplet surface. Since HE does not stain fat, it is difficult to tell whether they are fatty vacuoles or vacuoles for other reasons. Please provide references related to this method.

This aspect is discussed in the following paragraph: "The histomorphometry was done by hematoxylin and eosin staining, although not specific for fatty vacuoles, it has the advantage of using 4 μm sections instead of thicker cryosections required for bodipy or oil red O staining. In this way, paraffin embedded sections are thinner, easier to process and better microscopical resolution is achieved [35, 47, 57] and the smallest lipid droplets (2-3 μm) can be quantified. The drawback is that microphotographs have to be measured by naked eye what represents a time-consuming task for a trained pathologist able to distinguish void vessels, glycogen deposits and lipid droplets."

Statistical analyses in Materials and Methods: Please describe how the data is presented (means ± SD or SEM).

In agreement with this criticism, the paragraph has been written: "Results are shown as means and their standard deviations. The normal distribution of data was analyzed according to Shapiro–Wilk test, and homology of variances between groups using Bartlett or Levene tests. Parameters failing in any of these tests, their differences were tested using the Mann-Whitney's U test "

Discussion (Page 17 Line 4): mg/kg -> %

This now reads as. “Our results indicate that rabbits consuming 0.5% squalene vehicled in sunflower oil (corresponding to a dose of 0.6 g/kg) ”

Table 3: The unit is ng/mg of protein, but ng/mg liver is easier to understand.

Following this advice, Table 3 shows data in ng/mg liver.

Figure 1A: Fraction No is not shown.

This has been corrected in new Figure 1

Referee: 2

We thank the Reviewer for the helpful criticisms that allowed us to improve the quality of our manuscript.

Comments to the Author

1. *In the current study, dietary squalene seems to exert a 'worse' effect on plasma lipoprotein metabolism and hepatic histology compared with the control diet. These authors and others have shown beneficial effect of squalene in animal models. What causes this contradiction?*

This issue is addressed in the following **Discussion** paragraphs.

"The observed increase in total cholesterol in rabbits is in agreement with the early report of Kritchevsky et al. [37] who, using a 6-fold higher dose than the present study, reported an increase of plasma cholesterol in rabbits that was not translated in atherosclerosis development. Likewise, the increase in total cholesterol without changes in HDL-cholesterol agrees with our results using the 0.25 g/kg squalene dose in Apoe-deficient mice [36]. Zhang et al. observed a hypercholesterolemic effect of squalene in hamsters [32] as well. These observations point out that the outcome of squalene administration on plasma lipids is a compromise of dose and animal model."

"The observed increase the number of IDL and large LDL particles loaded with non-esterified cholesterol and normal triglycerides containing APOB100 is suggesting a metabolic setting where metabolism of these lipoparticles is impaired. Hepatic lipase has been proposed to be involved in the transformation of IDL into LDL [51], and as above mentioned rabbits display low hepatic lipase activity [21]. This low hepatic lipase activity could explain the accumulation of IDL and large LDL. Something similar was also observed in double knock-out for hepatic lipase and Apoe [52] although in the latter model, the results were not so striking as in our conditions due to the profound influence of the absence of APOE in mice. Interestingly, in those mice the increase in plasma cholesterol was accompanied by decreased atherosclerotic development. Likewise, the increase in total cholesterol in rabbits consuming squalene was not translated in atherosclerosis development [37]. Protective effect of 2 mg/kg squalene administration on fibrosis and endothelial activation in gingival mucosa used as a subrogate sample of cardiovascular disease was found in rabbits consuming an atherogenic diet [53]. Several interesting aspects should be considered: firstly, increased IDL and large LDL of diameter larger than

70 nm do not enter into the subendothelial space [54]. In this sense, the increase in non-esterified cholesterol due to its polar group is preferentially located in the peripheral layer and contributes to further expand the size of the lipoparticles. If there is enough APOE, they could be taken up by peripheral tissues what could resemble a delayed metabolism in the rabbit lacking hepatic lipase. Secondly, IDL and large LDL from squalene-fed rabbits showed diminished load of reactive oxygen species (Figure 2 G). Thirdly, plasma triglycerides were not modified or showed a trend to decrease by squalene. The decrease of triglycerides induced by apolipoprotein C3 deficiency has been proved to decrease atherosclerosis in the rabbit [26] and current opinion poses the deleterious effects of increased triglycerides in lipoproteins [55]. All these aspects could contribute to explain the vascular properties of squalene despite the increase of total cholesterol, although the activated reverse cholesterol transport [52] observed in mice lacking hepatic lipase cannot be excluded. This metabolic setting of rabbit could explain the findings and reinforces that the response to squalene may change in different animals depending on different genetic makeups. Furthermore, it constitutes a perfect example of nutrigenetics where hepatic lipase and squalene are influencing plasma cholesterol levels and may pose an interesting aspect to be considered into human personalised medicine"

2. *As the authors mention in the discussion, apoB48 upregulation without triglycerides elevation is challenging. The authors should provide robust evidence for their claims. FPLC profile monitored by triglycerides need to be shown. Lipoprotein analysis by ultracentrifugation is also needed.*

Accordingly, ultracentrifugation has been carried out. To describe this aspect, the following paragraphs have been included:

In methods

"Lipoproteins from 1 ml of plasma were fractionated by sequential ultracentrifugation at 4°C in a Beckman 60 Ti rotor, adjusting the density by addition of potassium bromide [41]. The lipoproteins in the density ranges of d< 1.006, d = 1.006-1.02 and d = 1.02-1.04 g/ml were isolated after centrifugation at 50,000 rpm for 16 h. The d = 1.04-1.06 and d = 1.06-1.08 g/ml fractions were centrifuged for 18 h; the d = 1.08-1.10 and d= 1.10-1.21 g/ml fractions were centrifuged for 24 h and 48 h, respectively, all at 59,000 rpm. Lipoproteins that

floated at each density were collected and refloated under the same conditions to remove contaminating plasma proteins. Lipoproteins from each fraction were recovered and dialyzed in 10 mM Tris buffer, pH 7.4, 150 mM NaCl and 1 mM EDTA."

In Results

" To corroborate these previous findings, isolation of plasma lipoproteins by density flotation using ultracentrifugation was carried out (Figure 2). The squalene administration resulted in a significantly increased content of total cholesterol in lipoproteins of densities lower than 1.006 (VLDL), 1.02 (intermediate density lipoproteins, IDL), 1.04 (large LDL), 1.06 (LDL) and 1.21 (mature HDL) (Figure 2 A). The increase was mainly found in IDL and large LDL and corresponded to increased non-esterified cholesterol (Figure 2 B). Regarding triglyceride content in the different fractions (Figure 2 C), there were no significant changes in lipoproteins of densities lower than 1.06 g/ml and significant increases in particles of densities < 1.08 and 1.1 corresponding to large HDL. Significant increases in APOB were observed in lipoparticles containing this apolipoprotein (all with d<1.006) by the intake of squalene, mainly due to APOB100 (Figure 2 D and E). An increase in APOE was also observed in fractions corresponding to IDL and large LDL (Figure 2 F). These APOB-containing lipoparticles from squalene treated animals showed decreased ROS content, as shown in Figure 2 G. "

In Discussion

"Our study provides a further insight regarding rabbit lipoprotein composition following a squalene dietary intervention. In this regard, the elevated total cholesterol was mainly due to the increases in VLDL and LDL non-esterified cholesterol according to FPLC data (Figure 1 B). Ultracentrifugation analysis (Figure 2) corroborated those augments in total and non-esterified cholesterol from lipoproteins (Figure 2 A and B) but its improved resolution allowed to ascertain that the most profound increases corresponded to fractions of densities 1.006-1.02 and 1.02-1.04 containing IDL and large LDL, respectively [50]. The observation of raised APOB levels in these particles (Figure 1 C and 2 D) and considering that there is a single molecule of APOB per lipoprotein is suggesting an increased number of IDL and large LDL particles with normal triglyceride content (Figure 2 C). Furthermore, characterized the molecular APOB undergoing changes, it turned

out to be the APOB100 isoform (Figure 2 D). Thereby, these results indicate that squalene consumption in rabbit mainly increases the number of IDL and large LDL particles loaded with non-esterified cholesterol."

3. *Plasma apoB100 and apoB48 levels should be calculated and performed statistical analysis (Figure 1D). The total amount of apoB measured by ELISA (Figure 1C) and Western blotting (Figure 1D) is not matched. Why are apoB48 protein levels in the liver higher than those in the jejunum (20 vs 2.5 arbitrary units, Figure 2C and D), although apoB48 protein is generally not expressed in the liver in rabbits?*

Following this criticism, a scrupulous revision of results has been done. A previous problem in transference of APOB100 has been solved and statistical analyses of APOB100 is shown in new Figure 2 corresponding to ultracentrifuge fractions.

4. *In the current model, plasma triglycerides levels are relatively high [10 mmol/L (887 mg/dL) in the control rabbits. Is it the effect of sunflower oil supplementation? Please provide plasma lipids on the standard normal diet.*

We apologize for the raised confusion regarding units. The mistake in units has been solved and levels of plasma triglycerides are correctly shown. The values of rabbits at the beginning of the study are also shown in new Table 1.

5. *Hepatic caspase 1 expression levels should be calculated with statistical analysis (Figure 4E).*

Following this critical comment, statistical analysis has been included.

Other concerns:

Why did you use 1% sunflower oil? How did you prepare the diet?

These critical comments have addressed as follows:

"Sunflower oil was used to dissolve squalene and 1% was empirically found to be perfectly embedded into diet pellets without requiring milling and pelleting again. Regular diet pellets were sprayed with sunflower oil or squalene-containing sunflower oil. "

Statistical methods are problematic. Why did you use U test?

In agreement with this criticism, the paragraph has been written: "The normal distribution of data was analyzed according to Shapiro–Wilk test, and homology of

variances between groups using Bartlett or Levene tests. Parameters failing in any of these tests, their differences were tested using the Mann-Whitney's U test "

The word 'chow" should not be used because it is a commercial name. You need to use regular or normal diet.

Following this advice, regular diet has been used.

During 4 weeks, 12 male New Zealand White rabbits (1.2 kg body weight) were fed with a **regular** diet enriched with 1% of sunflower oil for the control group (n=6), and with 1% of sunflower oil and 0.5% of squalene (Sigma-Merck, Darmstadt, Germany) for the squalene group (n=6).

There are many abbreviations. You should spell out all names before you use abbreviations. A table of abbreviations is needed.

Accordingly, a table of abbreviations has been added.

Abbreviations

APO, apolipoprotein
APOB, apolipoprotein B
BLAST, basic local alignment search tool
CASP1, caspase 1
CETP, cholesteryl ester transfer protein
CYP7A1, cytochrome P450 7A1 mRNA
ELISA, enzyme linked immunoassay
DMEM, Dulbecco's modified Eagle's minimum essential medium
FPLC, fast protein liquid chromatography
GC/MS, gas chromatography coupled to mass spectrometry
Gpx4, phospholipid hydroperoxide glutathione peroxidase mRNA
HDL, high density lipoproteins
HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA
HSC70, heat shock cognate 70
IDL, intermediate density lipoproteins
IL-1β, interleukin-1 beta
IL-18, interleukin-18
LDL, low density lipoproteins
LDLR, low density lipoprotein receptor mRNA
NCBI, *National Center for Biotechnology Information*
NLRP3, NACHT, LRR and PYD domains-containing protein 3
Ppib, peptidylprolyl isomerase B mRNA
ROS, reactive oxygen species
RT-qPCR, reverse transcriptase- quantitative polymerase chain reaction
SQLE, squalene monooxygenase mRNA
Tbp, TATA-box-binding protein mRNA
VLDL, very low density lipoproteins

Droplet surface is confusing. Do you mean perimeter or area?

This issue has been dealt as follows: Lipid droplets were evaluated by quantifying their areas in each liver section with Adobe Photoshop CS3 (Adobe Inc. San Jose, CA, USA) and expressed as percentage of total liver section [35]

ApoB-48 and -100 are labeled with exact size. This is not honest. Please show the marker you used.

Following this critical point, in Figure 2, the size of the marker is denoted.

References are pretty old and you should refer to latest references.

The vibrant research of rabbit as research model is now updated in the following paragraph. "Moreover, rabbits have been a target of genetic modifications to investigate the role of APOA1 [25], APOA2 [22], APOC3 [26], APOE [27], [28], CETP [29] and LDL receptor [28, 30] in lipoprotein metabolism and atherogenesis."

22. T. Koike, Y. Koike, D. Yang, Y. Guo, O. Rom, J. Song, J. Xu, Y. Chen, Y. Wang, T. Zhu, M. T. Garcia-Barrio, J. Fan, Y. E. Chen and J. Zhang, Human apolipoprotein A-II reduces atherosclerosis in knock-in rabbits, *Atherosclerosis*, 2021, **316**, 32-40.

23. M. Shiomi, The History of the WHHL Rabbit, an Animal Model of Familial Hypercholesterolemia (I) - Contribution to the Elucidation of the Pathophysiology of Human Hypercholesterolemia and Coronary Heart Disease, *J Atheroscler Thromb*, 2020, **27**, 105-118.

24. T. H. Beaty, V. L. Prenger, D. G. Virgil, B. Lewis, P. O. Kwiterovich and P. S. Bachorik, A genetic model for control of hypertriglyceridemia and apolipoprotein B levels in the Johns Hopkins colony of St. Thomas Hospital rabbits, *Genetics*, 1992, **132**, 1095-1104.

25. M. E. Brousseau and J. M. Hoeg, Transgenic rabbits as models for atherosclerosis research, *J Lipid Res*, 1999, **40**, 365-375.

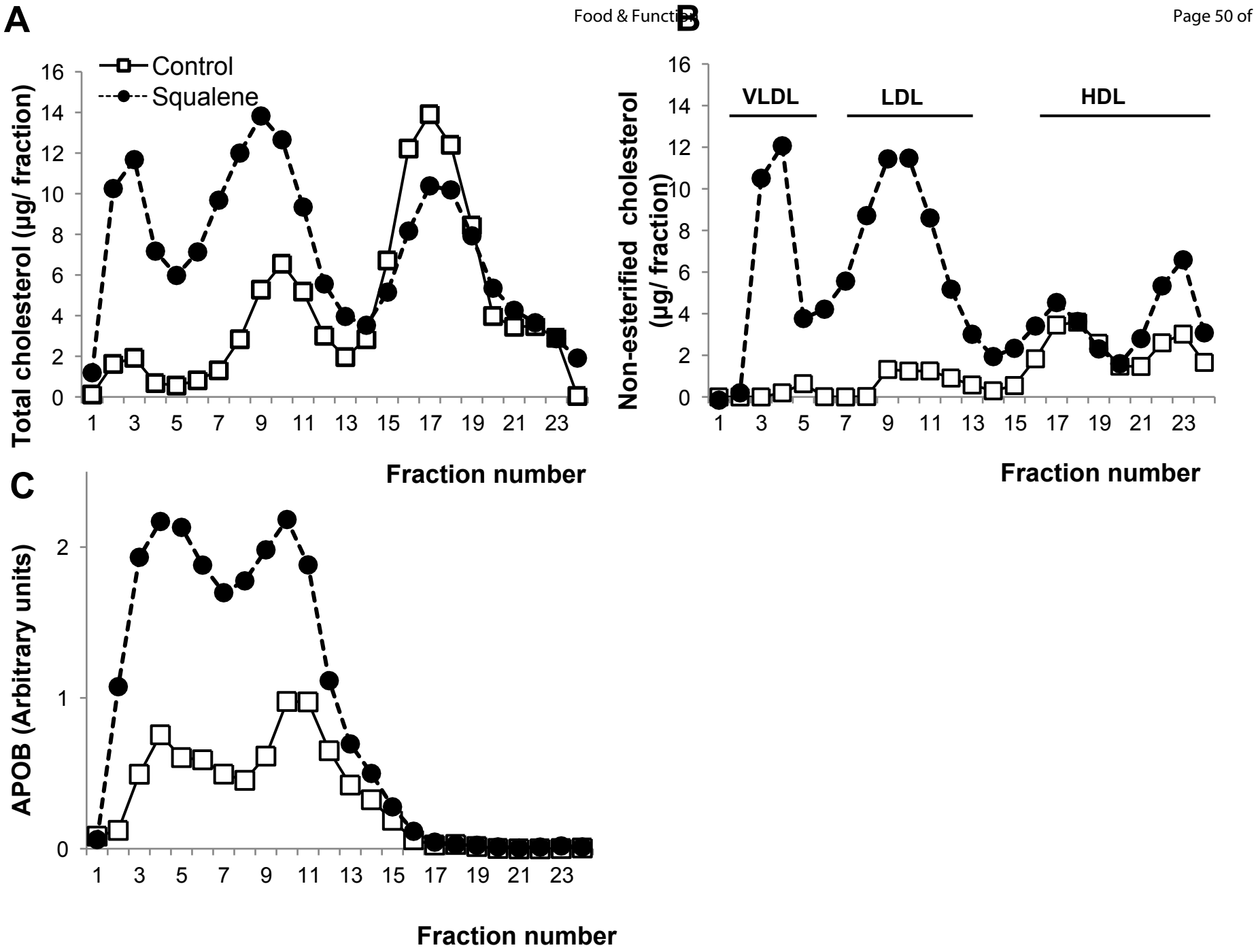
26. H. Yan, M. Niimi, F. Matsuhisa, H. Zhou, S. Kitajima, Y. Chen, C. Wang, X. Yang, J. Yao, D. Yang, J. Zhang, M. Murakami, K. Nakajima, Y. Wang, E. Liu, J. Liang, Y. E. Chen and J. Fan, Apolipoprotein CIII Deficiency Protects Against Atherosclerosis in Knockout Rabbits, *Arterioscler Thromb Vasc Biol*, 2020, **40**, 2095–2107.

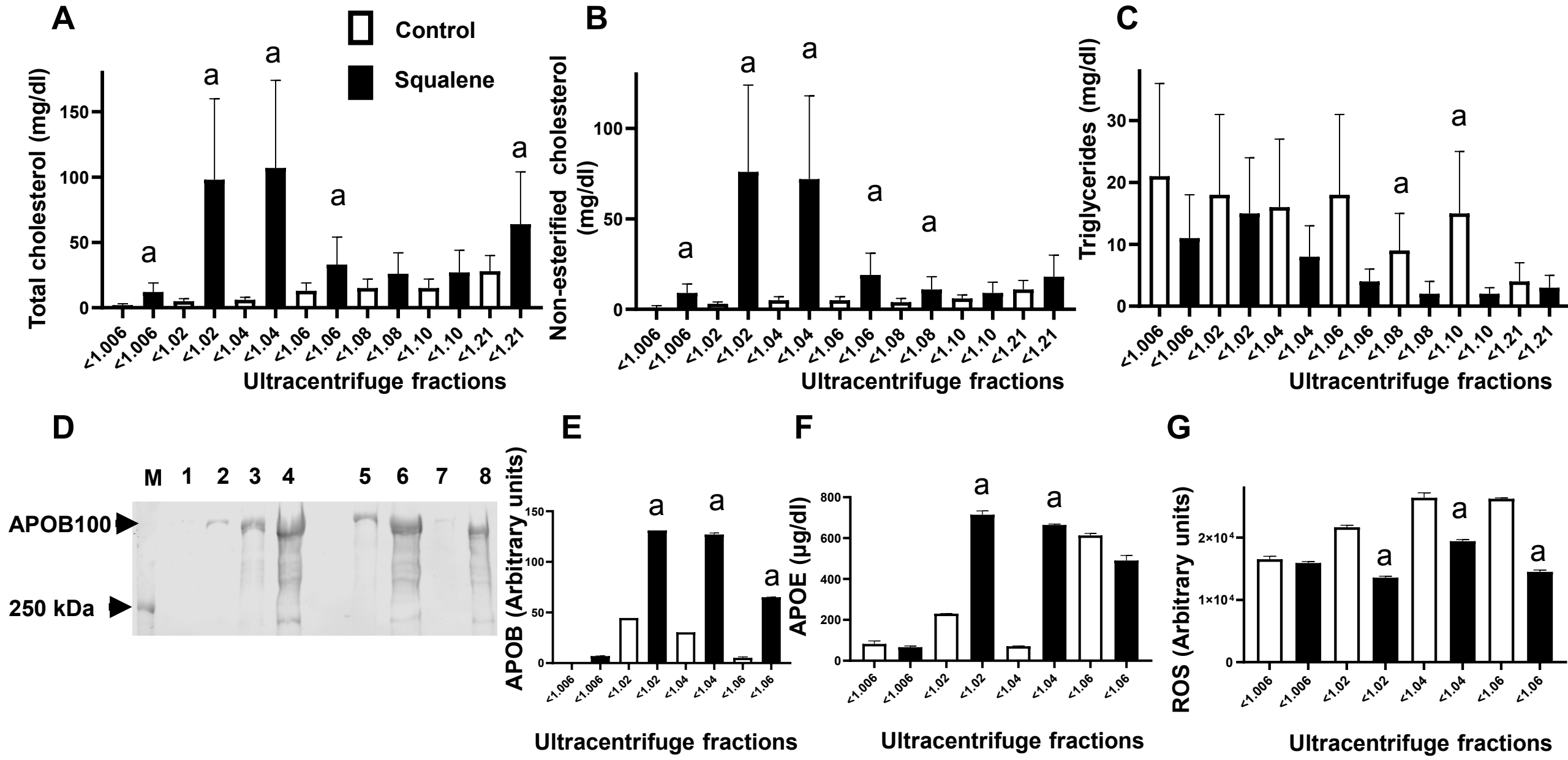
27. M. Niimi, D. Yang, S. Kitajima, B. Ning, C. Wang, S. Li, E. Liu, J. Zhang, Y. Eugene Chen and J. Fan, ApoE knockout rabbits: A novel model for the study of human hyperlipidemia, *Atherosclerosis*, 2016, **245**, 187-193.

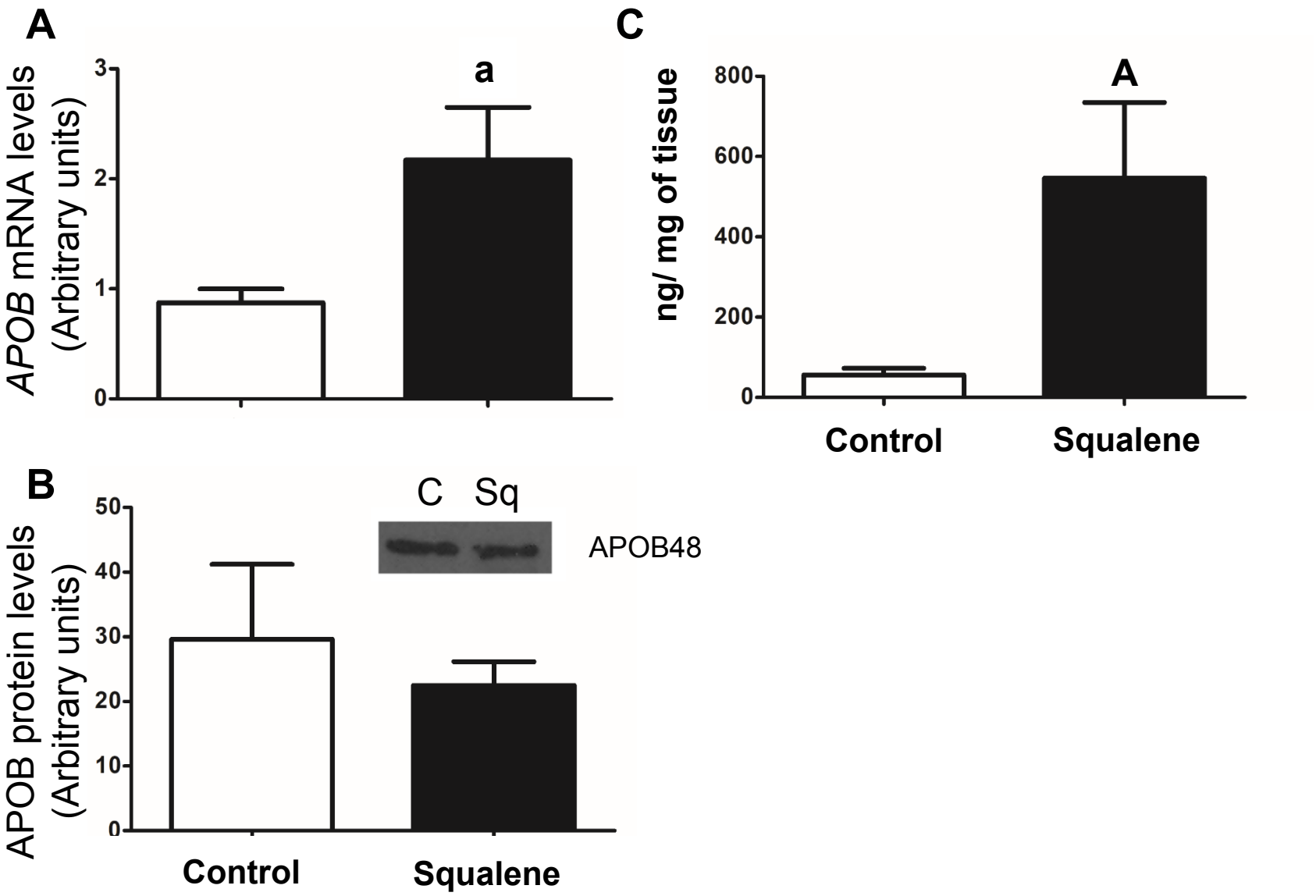
28. T. Yuan, Y. Zhong, Y. Wang, T. Zhang, R. Lu, M. Zhou, Y. Lu, K. Yan, Y. Chen, Z. Hu, J. Liang, J. Fan and Y. Cheng, Generation of hyperlipidemic rabbit models using multiple sgRNAs targeted CRISPR/Cas9 gene editing system, *Lipids Health Dis*, 2019, **18**, 69.

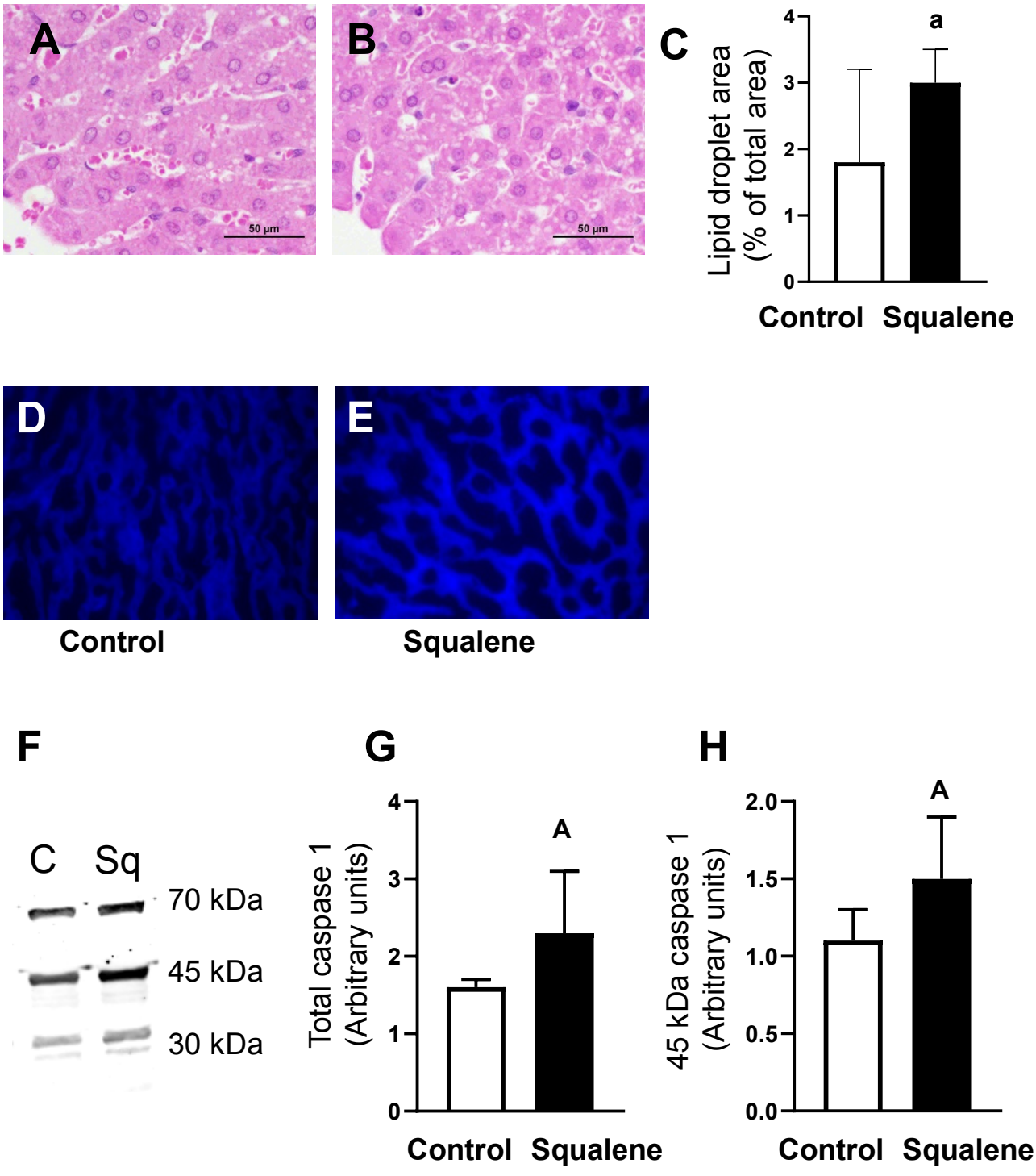
29. J. Zhang, M. Niimi, D. Yang, J. Liang, J. Xu, T. Kimura, A. V. Mathew, Y. Guo, Y. Fan, T. Zhu, J. Song, R. Ackermann, Y. Koike, A. Schwendeman, L. Lai, S. Pennathur, M. Garcia-Barrio, J. Fan and Y. E. Chen, Deficiency of Cholesteryl Ester Transfer Protein Protects Against Atherosclerosis in Rabbits, *Arterioscler Thromb Vasc Biol*, 2017, **37**, 1068-1075.

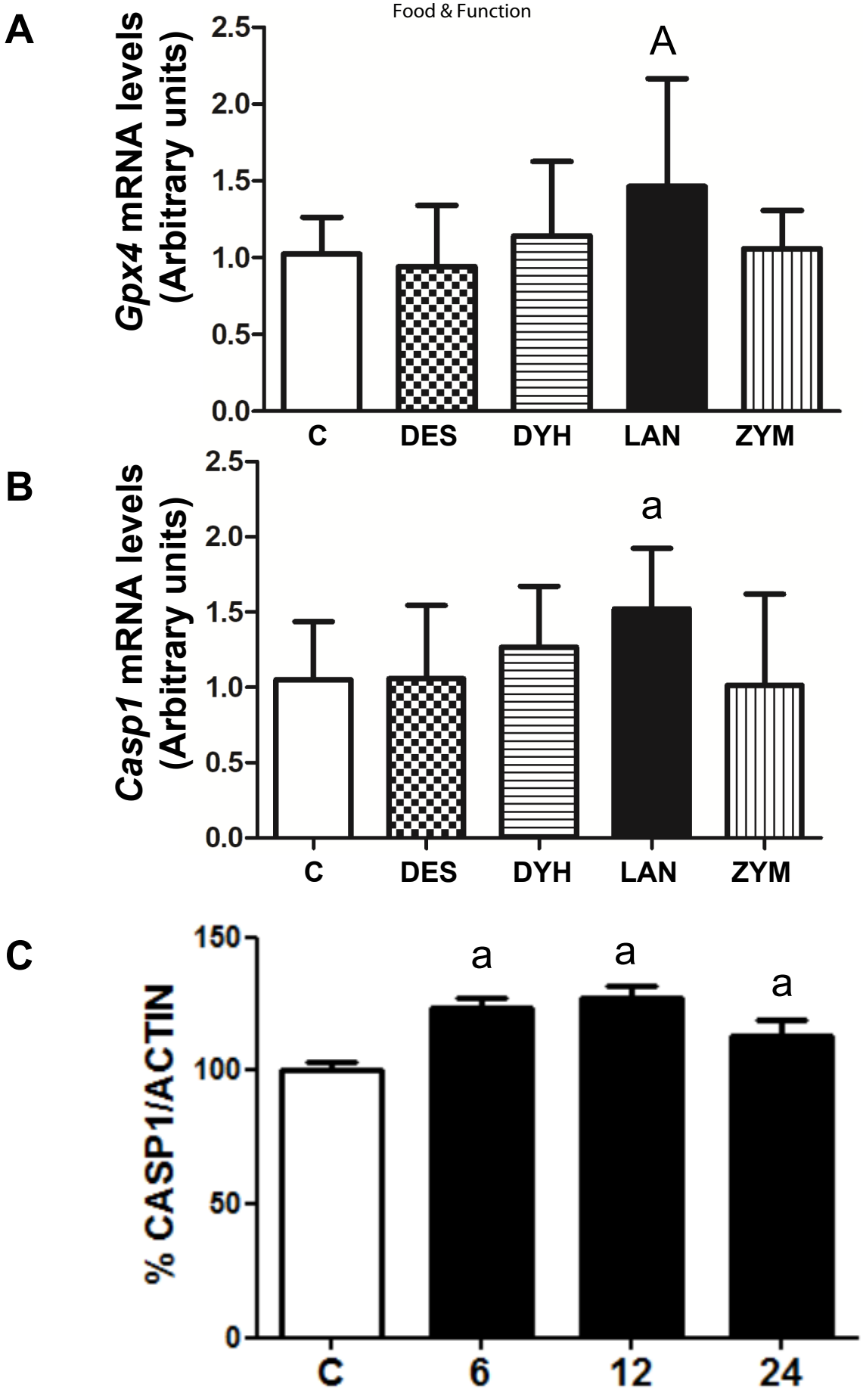
30. R. Lu, T. Yuan, Y. Wang, T. Zhang, Y. Yuan, D. Wu, M. Zhou, Z. He, Y. Lu, Y. Chen, J. Fan, J. Liang and Y. Cheng, Spontaneous severe hypercholesterolemia and atherosclerosis lesions in rabbits with deficiency of low-density lipoprotein receptor (LDLR) on exon 7, *EBioMedicine*, 2018, **36**, 29-38.

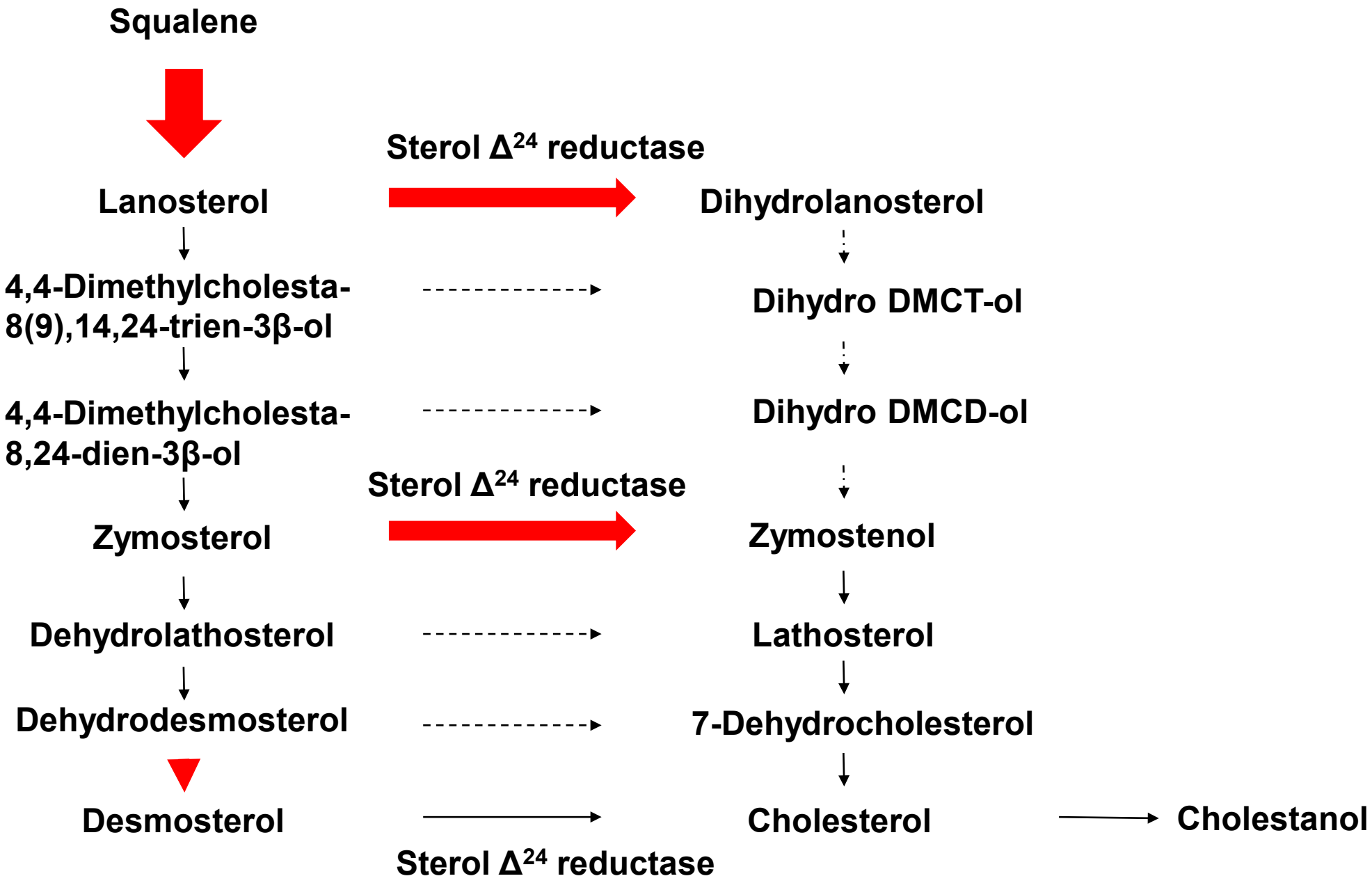

A
Control
Squalene
Total cholesterol (µg/ fraction)
Fraction number
B
VLDL
LDL
HDL
Non-esterified cholesterol (µg/ fraction)
Fraction number
C
APOB (Arbitrary units)
Fraction number


A
Control
Squalene
Total cholesterol (mg/dl)
Ultracentrifuge fractions
B
Non-esterified cholesterol (mg/dl)
Ultracentrifuge fractions
C
Triglycerides (mg/dl)
Ultracentrifuge fractions
D
M 1 2 3 4 5 6 7 8
APOB100
250 kDa
E
APOB (Arbitrary units)
Ultracentrifuge fractions
F
APOE (µg/dl)
Ultracentrifuge fractions
G
ROS (Arbitrary units)
Ultracentrifuge fractions


A
APOB mRNA levels (Arbitrary units)
3
2
1
0
a
C
ng/ mg of tissue
800
600
400
200
0
A
Control
Squalene
B
APOB protein levels (Arbitrary units)
50
40
30
20
10
0
C Sq
APOB48
Control
Squalene

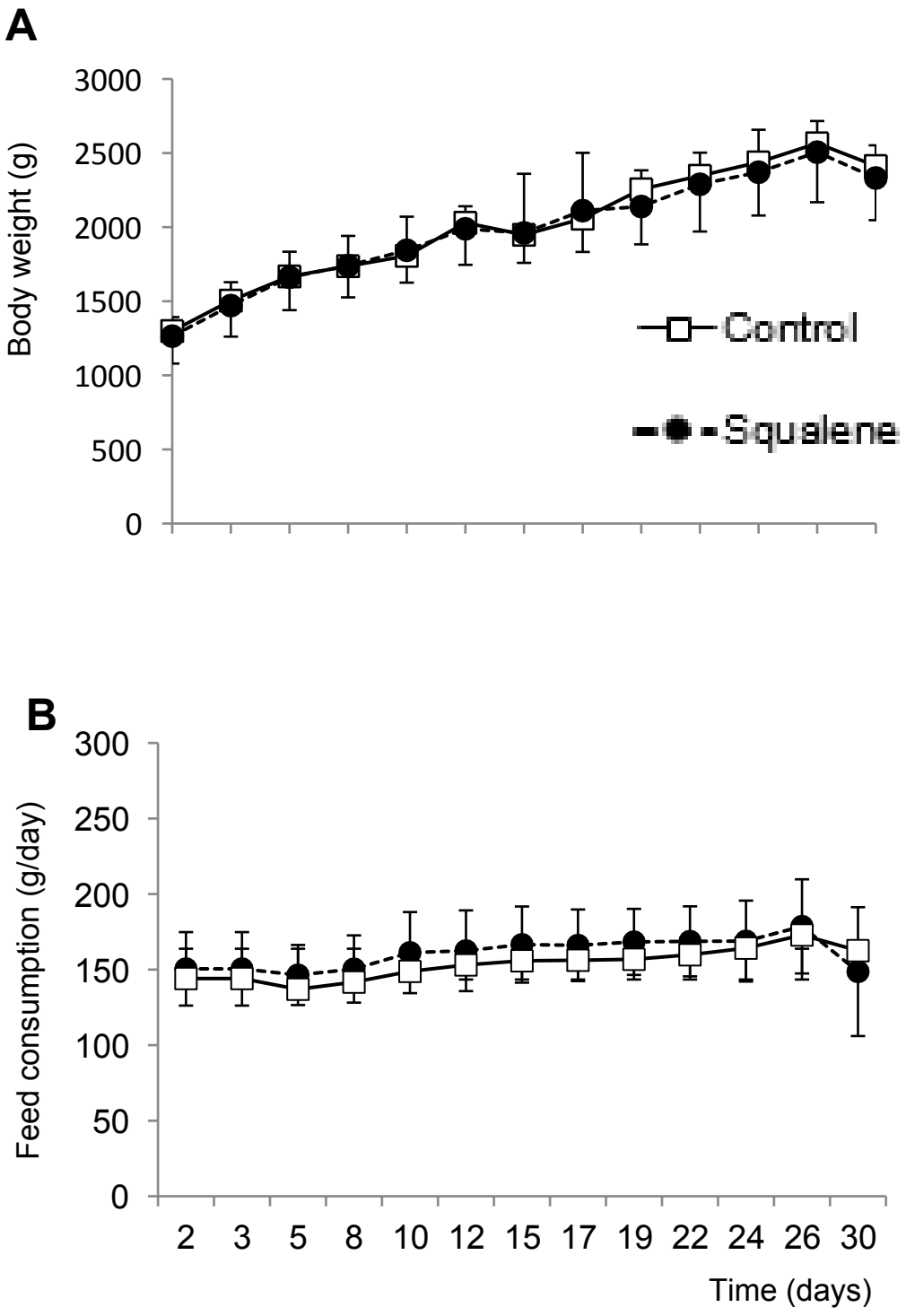

A
50 µm
B
50 µm
C
Lipid droplet area (% of total area)
a
Control
Squalene
D
E
Control
Squalene
F
C
Sq
70 kDa
45 kDa
30 kDa
G
Total caspase 1 (Arbitrary units)
A
Control
Squalene
H
45 kDa caspase 1 (Arbitrary units)
A
Control
Squalene


A
Gpx4 mRNA levels (Arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
A
C
DES
DYH
LAN
ZYM
B
Casp1 mRNA levels (Arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
a
C
DES
DYH
LAN
ZYM
C
% CASP1/ACTIN
150
100
50
0
a
a
a
C
6
12
24


Squalene
Lanosterol
Sterol Δ24 reductase
Dihydrolanosterol
4,4-Dimethylcholesta-8(9),14,24-trien-3β-ol
Dihydro DMCT-ol
4,4-Dimethylcholesta-8,24-dien-3β-ol
Dihydro DMCD-ol
Sterol Δ24 reductase
Zymosterol
Zymostenol
Dehydrolathosterol
Lathosterol
Dehydrodesmosterol
7-Dehydrocholesterol
Desmosterol
Cholesterol
Cholestanol
Sterol Δ24 reductase

Supplementary Table 1. Characteristics of primers used in RT-qPCR according to MIQE guidelines.

Gene symbol	Accession	Sequence		Amplicon length	Exons	Primer nM	Efficiency
Rabbit							
APOB	XM_008254783.2	Sense:	CTGTCCAAAAGATGCCACGC	181	2/4	100	105%
		Antisense:	GATGCACTGGTTGGTCCTCA				
HMGCR	XM_017339036.1	Sense:	GTGCGCCAGCTTGAAATCAT	97	7	100	102%
		Antisense:	ACACCAAGGACACACAAGCA				
LDLR	NM_001278865.1	Sense:	AGCAACCGCATCTACTGGTC	101	10/11	100	108%
		Antisense:	TGCTGATGACGGTGTCGTAG				
CYP7A1	NM_001170929.1	Sense:	GTTCAGAGACACCCTTGCTTT	179	3/4	100	103%
		Antisense:	CATCCTTTCTCCAGAGGTGGT				
SQLE	XM_017341522.1	Sense:	TATGGCAGAGCCCAACACAA	157	3/6	100	99%
		Antisense:	GGAGAAAAGCCCATCTGCAA				
RN18S	NR_033238.1	Sense:	ACTCAACACGGGAAACCTCA	114	1	100	102%
		Antisense:	AATCGCTCCACCAACTAACA				
Mouse							
Casp1	NM_009807.2	Sense:	CTATGGACAAGGCACGGGAC	107	2	200	91%
		Antisense:	TCCTGCCAGGTAGCAGTCTT				
Gpx4	NM_001367995.1	Sense:	ATGGATGAAAGTCCAGCCCAAG	148	4/7	100	104%
		Antisense:	CGGCAGGTCCTTCTCTATCAC				
Pipb	NM_011149	Sense	GGAGATGGCACAGGAGGAA	72	3/4	300	103%
		Antisense	GTAGTGCTTCAGCTTGAAGTTCTCAT				
Tbp	NM_013684.3	Sense	GTGAGTTGCTTGCTCTGTGC	359	8	200	104%
		Antisense	GCTGCGTTTTTGTGCAGAGT				



Supplemental Figure 1. Follow-up of body weight during diet intervention (A) and solid intake (B). Data are means ± SD for each group. Statistical analyses were carried out by t-test.