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Squalene: A Trove of Metabolic Actions

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Abstract

Squalene is present in high concentration in the liver of certain sharks and in small concentrations in olive oil. Previous studies showed that its administration decreases hepatic steatosis in male *Apoe*-knockout mice, but these changes might be complex. Transcriptomics, using DNA microarrays, and proteomics from mitochondrial and microsomal fractions, analyzed by 2D-DIGE and mass spectrometry, were used in these mice that received 1 g/kg/day squalene for 10 weeks. Squalene administration significantly modified the expression of genes such as lipin 1 (*Lpin1*) and thyroid hormone responsive (*Thrsp*). Changes in methionine adenosyltransferase 1 alpha (*Mat1a*), short-chain specific acyl-CoA dehydrogenase (*Acads*), and thioredoxin domain-containing protein 5 (*Txndc5*) expressions were consistent with their protein levels. Their mRNA levels were associated with hepatic fat content. These results suggest that squalene action involves changes in hepatic gene expression associated with its anti-steatotic properties. This approach shows new connections between nutrition and gene expression since *Txndc5*, a gene with unknown biological function, was upregulated by squalene administration. Overall, this nutrigenomic approach illustrates the effects of squalene and provides further support to the idea that not all monounsaturated fatty acid-containing oils behave similarly. Therefore, selection of cultivars producing olive oils enriched in this compound will be a plus.

Keywords: Apolipoprotein E-deficient mice, Virgin olive oil, Squalene, *Lpin1*, *Thrsp*, *Mat1a*, *Acads*, *Txndc5*

1. Introduction

The “Seven Countries” Study evidenced that cardiovascular mortality was the lowest in Mediterranean countries compared to other regions participating in the study [1]. The

Mediterranean dietary pattern is not only associated with lower cardiovascular mortality but also with total mortality [2]. Dietary interventions using Mediterranean diets have resulted in favorable outcomes either in primary [3] and secondary prevention by reducing the number of coronary events and death toll [4]. All these evidences have provided the scientific background to propose the Mediterranean Diet as an intangible cultural heritage of humanity (<http://www.unesco.org/culture/ich/es/RL/00394>).

In traditional Mediterranean diet, the main source of fat was olive oil [5]. Virgin olive oil, an example of oil extracted by physical means, is a functional food since it contains several components that may contribute to its overall biological properties. Known for its high levels of triacylglycerols containing monounsaturated fatty acids, it is a good source of phytochemicals such as squalene [6], phenolic compounds [7, 8], terpenes, phytosterols, and alpha-tocopherol [9, 10]. The content of squalene in virgin olive oil shows a great variability, from 1.5 to 9.6 g/kg [11], and may vary according to grove varieties [12]. In spite of this variation, squalene represents the second most abundant component of virgin olive oils and the highest in commonly consumed vegetable oils [13]. In some refinement processes, the loss of squalene may reach a 20 % [6]. However, this molecule remains stable in virgin olive oil heated at 180 °C for 36 h [14]. Its thermal stability makes squalene suitable to ensure its intake when consumed both in cooked and raw food. In vitro, it is a highly effective oxygen-scavenging agent, and it has been shown to be chemopreventive against several tumors [a detailed review of its described properties is found in Ref. [13]].

The average intake of squalene is 30 mg/day in the United States. However, when consumption of olive oil is high, the intake of squalene can reach from 200 to 400 mg/day, as observed in Mediterranean countries [15], or even can amount up to 1 g daily [16]. Despite the fact that plasma squalene levels come from endogenous biosynthesis in addition to dietary sources, its concentration is higher in those human populations consuming virgin olive oil or shark liver [17]. Its stability and bioavailability make squalene an attractive compound to characterize its biological properties.

2. The liver: an organ sensitive to diet nutrients

The liver secretes phospholipids, cholesterol, and triacylglycerols into plasma as lipoprotein complexes, which allow the transport of those lipids into the aqueous medium of blood. Apolipoproteins such as APOB100, APOA1, APOA2, and APOE are the main protein constituents of lipoproteins. Furthermore, this organ also secretes the enzymes (hepatic lipase, lecithin-cholesterol acyltransferase, and phospholipid transfer protein) involved in the plasma transformation of lipoproteins [18].

ApoE-deficient mice lack APOE, and as consequence, the elimination of lipoproteins from blood is impaired. Due to this fact, lipoproteins accumulate into vessel walls contributing to the development of spontaneous atherosclerosis [19]. When fed with high-fat diets, these mice induced changes in plasma apolipoproteins [20], as a result of hepatic apolipoprotein gene expression variations [21]. This adaptive response of the liver as an effect of different olive oil

intakes represents an ideal model to explore changes in diet composition. Using *ApoE*-deficient mice as a model of spontaneous atherosclerosis and baseline steatosis, our group showed that squalene administration decreased atherosclerotic lesion [22] and exhibited an association between hepatic fat content and atherosclerotic progression. In this study, squalene accumulated in the liver and was able to decrease the storage of hepatic triacylglycerols. We concluded that squalene was transported to the liver in an apolipoprotein E-independent way, and its mechanisms of action were complex. To address this complexity, high-throughput approaches of transcriptomics and proteomics have been employed to further characterize squalene action. The livers of *ApoE*-deficient mice fed with chow diets or the same diets supplemented with squalene were analyzed. The chapter will review our experience dealing with squalene and the use of omic technologies to explore its effects.

3. Methodological workflow

Two-month old, male, homozygous *ApoE*-deficient mice with C57BL/6J × Ola129 genetic background were used. Two study groups of equal plasma cholesterol were established: (a) one received chow diet, and its beverage contained 1 % (v/v) of glycerol solution (n = 8) and (b) the other received the same chow diet, but its drinking solution was supplemented with squalene to provide a 1 g/kg/day dose (n = 9). For 10 weeks, mice were fed with experimental diets, which were well tolerated since there was no incidence on survival, physical appearance, and solid and liquid intakes, as described previously [22]. After this time, animals were sacrificed and the liver removed. One aliquot stored in neutral formaldehyde was used to evaluate the extent of lipid droplets, expressed as the percentage of total liver section, and the remaining, frozen in liquid nitrogen, was used to extract its total RNA and to isolate subcellular fractions.

The changes in expression of 22,690 transcripts represented on the Affymetrix GeneChip Murine Genome MOE430A array were analyzed to find out the effect of squalene. In order to do that, pooled liver samples of eight mice on the chow diet were compared with those receiving the compound, as depicted in **Figure 1**.

The huge amount of information provided by microarrays requires further processing in order to get a meaningful and manageable data to work with, such as selecting only the genes with the highest expression changes or those involved in a certain metabolic pathway [23]. In the present work, the first approach has been adopted, and only those genes whose expression was strongly modified (signal \log_2 ratio ≥ 1.5 or ≤ -1.5) were considered highly responders to the intake of squalene. Gene expression was later confirmed by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) to reinforce the validity of results.

For the preparation of mitochondrial and microsomal fractions, livers were homogenized in PBS (4 ml/g of tissue) with protease inhibitor cocktail tablets (Roche). Tissue debris was removed by centrifugation at $200 \times g$ for 10 min at 4 °C. The homogenate was spun down at $1,000 \times g$ for 15 min. The supernatant-containing mitochondria were centrifuged at full speed, $13,000 \times g$ for 2 min. The mitochondrial pellets were then washed twice, pelleted, resuspended

in PBS, and spun for 1 min. Microsomal fractions resulted from centrifugation of the post mitochondrial supernatant at $105,000 \times g$ for 90 min. These pellets were washed twice, spun at the same speed, and finally resuspended in 0.5 ml of PBS [24, 25].

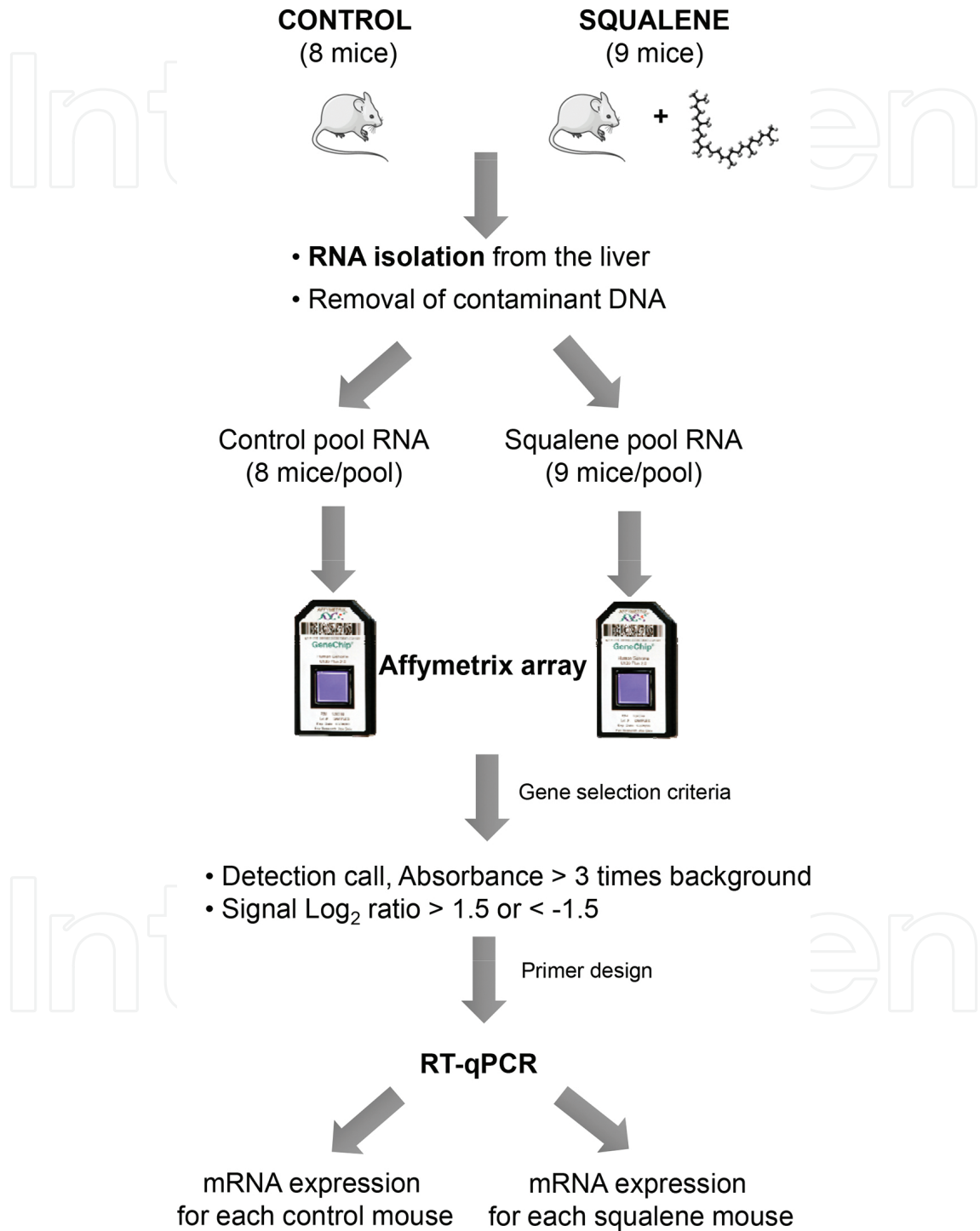


Figure 1. Graphical representation of the used approach. The process encompasses RNA preparation, microarray processing to select expression changes, and confirmation by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) of individual samples.

Differential protein expression was analyzed by DIGE analysis. Spots whose densities significantly differed between treatments were excised from the preparative gel and subjected to tryptic digestion and identification by mass spectrometry, as described [24, 25].

4. Squalene-induced global changes in hepatic gene expression

Affymetrix software identified 11,528 transcripts as expressed in the livers of chow-fed mice and 11,187 in those of squalene-fed animals. According to the Mann-Whitney ranking feature of the Affymetrix software ($P < 0.01$), squalene administration increased and reduced the expression of 413 and 428 sequences, respectively. The original data were deposited in the GEO repository (accession number GSE36932).

Biological process	GenBank	Name	Gene symbol	Chow	Squalene	Signal log ₂ ratio
Upregulated genes						
Cell signaling	NM_011267	Regulator of G-protein signaling 16	<i>Rgs16</i>	102	543	2.5
Nuclear protein	NM_009381	Thyroid hormone-responsive SPOT14 homolog (Rattus)	<i>Thrsp</i>	238	760	1.7
Lipid metabolism	NM_172950	Lipin1	<i>Lpin1</i>	423	1675	1.6
Transcription factor	NM_011575	Trefoil factor 3 intestinal	<i>Tff3</i>	200	503	1.5
Transcription factor	NM_016974	D site albumin promoter binding protein	<i>Dbp</i>	589	1547	1.5
Downregulated genes						
Cell cycle	NM_008059	G0/G1 switch gene 2	<i>G0s2</i>	3012	487	-2.5
Transcription factor	NM_007489	Aryl hydrocarbon receptor nuclear translocator-like	<i>Arntl</i>	66	11	-2.3
Cell signaling	NM_019840	Phosphodiesterase 4B	<i>Pde4b</i>	82	24	-1.8
Immunity	NM_010378	Histocompatibility 2, class II antigen A, alpha	<i>H2-Aa</i>	944	583	-1.5
Immunity	NM_010382	Histocompatibility 2, class II antigen E beta	<i>H2-Eb1</i>	357	140	-1.5

Data represent intensity of signal for each condition with the Affymetrix chip.

Table 1. Hepatic genes differentially regulated by the administration of squalene at the level of signal log₂ ratio ≥ 1.5 or ≤ -1.5 in male *ApoE*-deficient mice.

To select the most relevant, only differentially regulated genes with a signal \log_2 ratio ≥ 1.5 (for those genes upregulated) or ≤ 1.5 (for those repressed) were taken into account. **Table 1** lists the genes whose mRNAs reflected these changes. Five genes showing increased expression as a response to the administration of squalene. Two of these genes coded for transcription factors (*Dbp* and *Tff3*) and three for proteins with miscellaneous functions (one of them was involved in lipid metabolism [*Lpin1*], the second was a signaling molecule [*Rgs16*], and the third was a nuclear protein [*Thrsp*]). Five genes met the criterion of showing a reduced expression as a response to the administration of squalene (**Table 1**). Of these, two were involved in immunity (*H2-Aa* and *H2-Eb1*), one was a transcription factor (*Arntl*), one was involved in cell cycle (*G0s2*), and finally one coded for an enzyme involved in cellular signaling (*Pde-4b*).

	Chow (n = 8)	Squalene (n = 9)	Fold change	SL ₂ R
Upregulated genes				
<i>Rgs16</i>	0.91 ± 0.16	11.64 ± 1.5**	12.8	3.7
<i>Thrsp</i>	0.92 ± 0.13	4.00 ± 0.65**	4.3	2.1
<i>Lpin1</i>	0.96 ± 0.19	9.77 ± 2.00**	10.2	3.3
<i>Tff3</i>	0.85 ± 0.11	1.26 ± 0.29	1.6	0.6
<i>Dbp</i>	0.92 ± 0.20	3.76 ± 0.72**	4.08	2.0
Downregulated genes				
<i>G0s2</i>	0.56 ± 0.19	0.13 ± 0.02*	0.2	-2.3
<i>Arntl</i>	1.19 ± 0.25	0.46 ± 0.07**	0.4	-1.3
<i>Pde4b</i>	1.11 ± 0.17	1.22 ± 0.27	1.1	0.1
<i>H2-Aa</i>	0.94 ± 0.18	0.92 ± 0.18	1.0	0.0
<i>H2-Eb1</i>	1.01 ± 0.16	0.75 ± 0.23	0.7	-0.5

Data (means ± SEM) represent arbitrary units normalized to the *Cyclophilin B* expression for each condition with the RT-qPCR. Statistical analysis was carried out by the Mann-Whitney *U* test. ** $P \leq 0.01$ vs chow, * $P \geq 0.05$ vs chow.

Table 2. Effect of squalene on the hepatic gene expression in male *Apoe*-deficient mice.

To validate the results obtained with the microarray, the expressions of the above genes—*Arntl*, *Dbp*, *G0s2*, *H2-Aa*, *H2-Eb1*, *Lpin1*, *Pde-4b*, *Rgs16*, *Tff3*, and *Thrsp*—that were up- or downregulated were individually analyzed by specific RT-qPCR assays. *Cyclophilin B* was the reference gene used to normalize the results (**Table 2**).

Four out of the five upregulated genes included in the validation analysis—*Rgs16*, *Thrsp*, *Lpin1*, and *Dbp*—were confirmed to be significantly increased in their expressions by the squalene administration. Two of the five downregulated genes selected—*G0s2* and *Arntl*—were significantly decreased in male mice receiving squalene. Good agreement between these procedures was obtained ($r = 0.94$, $P < 0.007$), and all samples except the two were correctly classified, although the magnitude of the response differed between both methods. These

results indicate that pooled samples can be successfully used to provide an initial screening of gene expression, with the economic and timesaving benefits and with the limitation of no information on biological variability.

To further explore the significance of these changes, correlation analyses between hepatic fat and gene expressions were studied. Two genes, *Lpin1* and *Thrsp*, showed significant inverse associations (Figure 2).

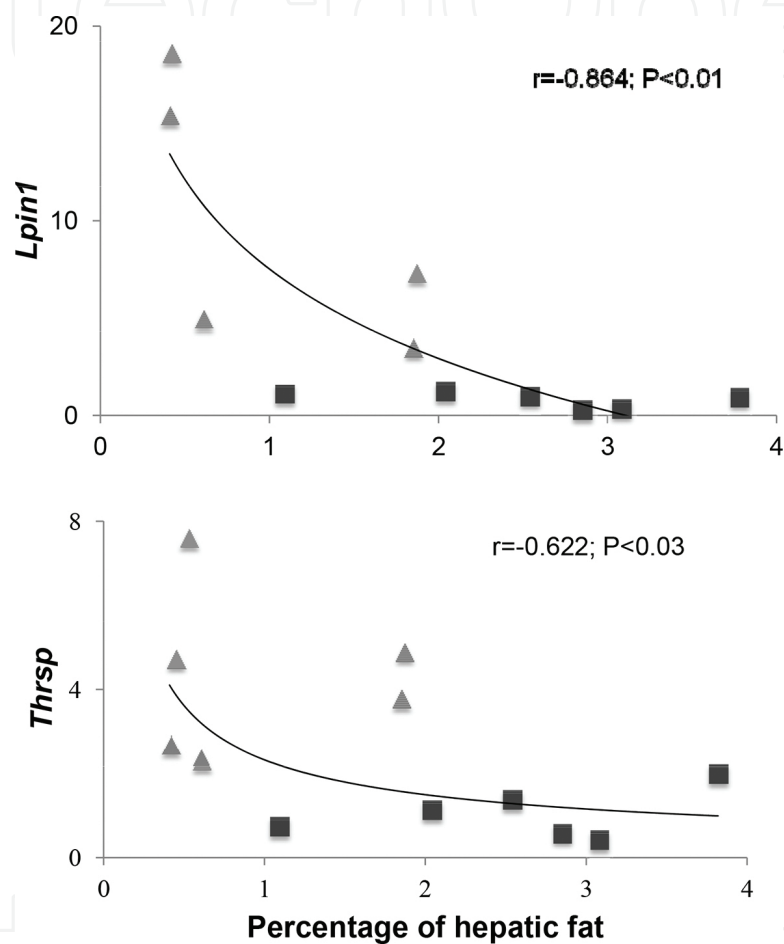


Figure 2. Association analysis among liver fat content and hepatic mRNA levels in male *ApoE*-deficient mice. Correlations were calculated according to Spearman's test, and values corresponding to all experimental groups have been included. Squares and triangles correspond to chow and squalene groups, respectively.

Squalene modulated these genes and could modulate hepatic lipid metabolism. In fact, LPIN1 (LIPIN1) plays a dual function in lipid metabolism by (1) catalysis of the conversion of phosphatidate to diacylglycerol, required for triacylglycerol and phospholipid biosynthesis, and (2) by acting as a transcriptional regulator. Through its 3-sn-phosphatidate phosphatase activity, this protein favors triacylglycerol biosynthesis [26]. Conversely, acting as a transcriptional regulator, it suppresses the lipogenic program [27]. Accordingly, a hypothetical increase in nuclear LPIN1 protein levels induced by the action of squalene may explain the strong negative association with hepatic fat content (Figure 1). THRSP is also a nuclear protein that

participates in the regulation of lipid synthesis by modulating the levels of lipogenic enzymes such as ATP citrate lyase, fatty acid synthase, and malic enzyme [28]. However, *Thrsp*-deficient mice showed enhanced lipogenesis, which led to the finding of its paralog, called S14R [29]. THRSP and S14R might have an overlapping role in this metabolism [30]. Interestingly, *Lipin1* and *Thrsp* expressions showed a strong positive association ($r = 0.84$, $P < 0.001$), suggesting that they both play a role in lipid metabolism and are influenced by squalene administration.

5. Squalene-induced changes in mitochondrial proteins

The mitochondrial proteome analysis unveiled caused induction of methionine adenosyltransferase 1 alpha and decreased short-chain specific acyl-CoA dehydrogenase levels [24]. Both changes were associated with lipid droplet area ($r = -0.661$ and 0.721 , $P < 0.05$). These changes in proteins were due to changes in their mRNAs (**Figure 3**), and these mRNA changes were associated with lipid droplet content, as well. In fact, squalene reverted changes in ACADS to values present in wild-type mice without baseline steatosis. This protein could be a marker of hepatic steatosis. These results point out that changes in MAT1A and ACADS levels are influenced by squalene, being the former a target of squalene administration, while the latter is associated with its anti-steatotic properties [24].

Two genes, MAT1A and MAT2A, codify for methionine adenosyltransferases, which catalyze the generation of S-adenosyl-L-methionine (SAME), the main biological methyl donor. The mammalian liver is the main organ in the regulation of serum methionine since more than 85 % of all methylation reactions and up to 48 % of methionine metabolism take place in hepatocytes. MAT1A is the isoform present in adult liver, and mice lacking the *Mat1a* gene exhibit a chronic reduction in hepatic SAME levels and spontaneous development of nonalcoholic steatohepatitis [31] and hepatocellular carcinoma [32]. Recently, *Mat1a* deficiency has been associated with fatty liver, by regulating phosphatidylcholine-mediated processing of sterol regulatory element-binding protein 1 [33], required for very-low-density lipoprotein (VLDL) assembly and plasma lipid homeostasis in mice. Therefore, the relevant role of MAT1A in VLDL metabolism [34] may explain its increased expression in squalene-treated mice and the significant correlation with hepatic fat content.

A family of acyl-CoA dehydrogenases, including ACADS, whose function is exerted on short-chain acyl-CoA [35], catalyzes the initial step in fatty acid β -oxidation. A genome-wide association study found that some variants of this gene were associated with impaired fatty acid β -oxidation and seemed to be a marker of hepatic steatosis [36]. Thus, ACADS changes may play a role in this condition's amelioration induced by squalene. These findings regarding these two proteins, MAT1A and ACADS, as targets of squalene action and their role in advanced liver diseases suggest that squalene could have a role in preventing these pathologies.

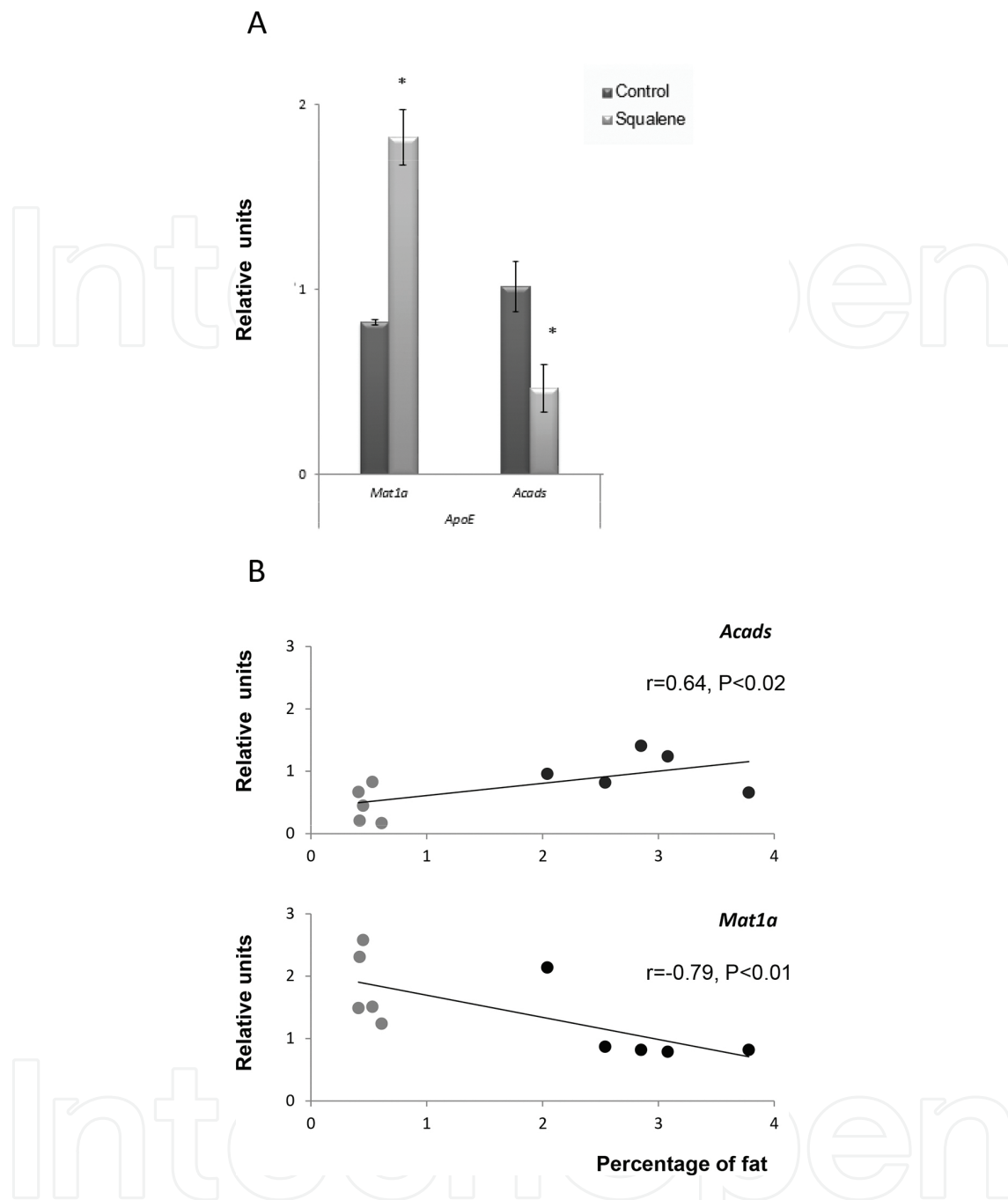


Figure 3. Effect of squalene on hepatic *Mat1a* and *Acads* mRNA levels in male *ApoE*-deficient mice. (A) Hepatic mRNA levels. Data, expressed as arbitrary absorbance units referred to *Cyclophilin B* gene expression obtained by RT-qPCR analysis, are presented as mean \pm SEM. Statistical analyses were carried out using the Mann-Whitney *U* test. ** $P < 0.01$; * $P < 0.05$. (B) Association among hepatic mRNA levels and liver fat content in *ApoE*-deficient mice.

6. Squalene-induced changes in microsomal proteins

Analysis of microsomal proteome showed that squalene induced the expression of proteins involved in lipid (MUP8 and SCP2) and vesicular transport (NIPSNAP1 and VCP),

protein quality control (PSMA7, PDIA3, HYOU1, and HSPA5), calcium storage (CALR), and redox homeostasis (TXNDC5 and PYROXD2). While the role of PDIA3 in intracellular dynamics of VLDL has been proved, this is not the case for proteins such as GRP78/HSPA5 and TXNDC5 [25]. However, TXNDC5 protein and mRNA levels showed an inverse and statistically significant correlation with the area of lipid droplets, as reflected in **Figure 4**.

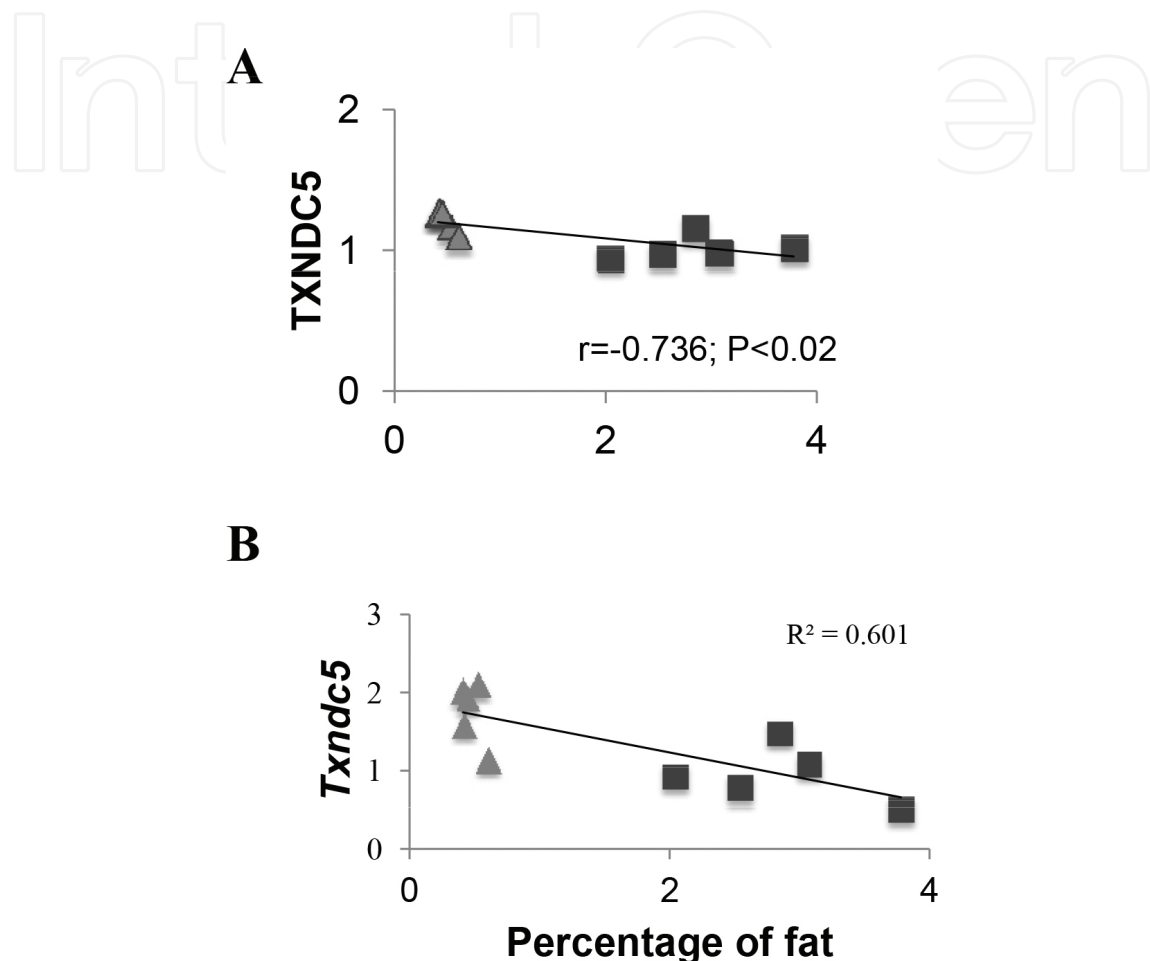


Figure 4. Association analyses among liver fat content and hepatic mRNA and protein levels of control and squalene-treated *Apoe*-deficient mice. (A) Correlation analysis between liver fat content and protein levels, (B) correlation analysis between *Txndc5* mRNA levels and liver fat content. Black squares denote chow-fed mice and gray triangles squalene-treated mice.

TXNDC5, a member of the thioredoxin family, is considered to catalyze disulfide formation in protein folding, to protect proteins against oxidative damage, and to prevent endoplasmic reticulum stress [37]. A decrease in oxidative stress, evaluated as 8-isoprostaglandin $F_{2\alpha}$ was found after squalene administration in mice [22], in agreement with other authors [38]. In this study, the observed TXNDC5 changes could contribute to lower oxidative stress. Considering that the latter is a factor inducing APOB degradation [39] and consequently decreases VLDL secretion, the increase in TXNDC5 could stabilize APOB and favor VLDL secretion. This hypothetical mechanism could explain the observed association between TXNDC5 levels and the degree of fatty liver and represents a new role for this protein. Furthermore, the action of

squalene was exerted at mRNA level. TXNDC5 seems to be a marker of the hepatic steatosis developed in the absence of APOE and may play a role in this condition's amelioration induced by squalene. This role of TXNDC5 in terms of lipid metabolism and lipid droplets needs to be defined.

7. A tentative model of squalene action

Overall, squalene is decreasing the hepatic content of lipids by facilitating the output of triacylglycerols in VLDL and promoting fatty acid oxidation, as displayed in **Figure 5**. These mechanisms were observed in male mice showing basal hepatic steatosis, as is the case of apolipoprotein E deficiency.

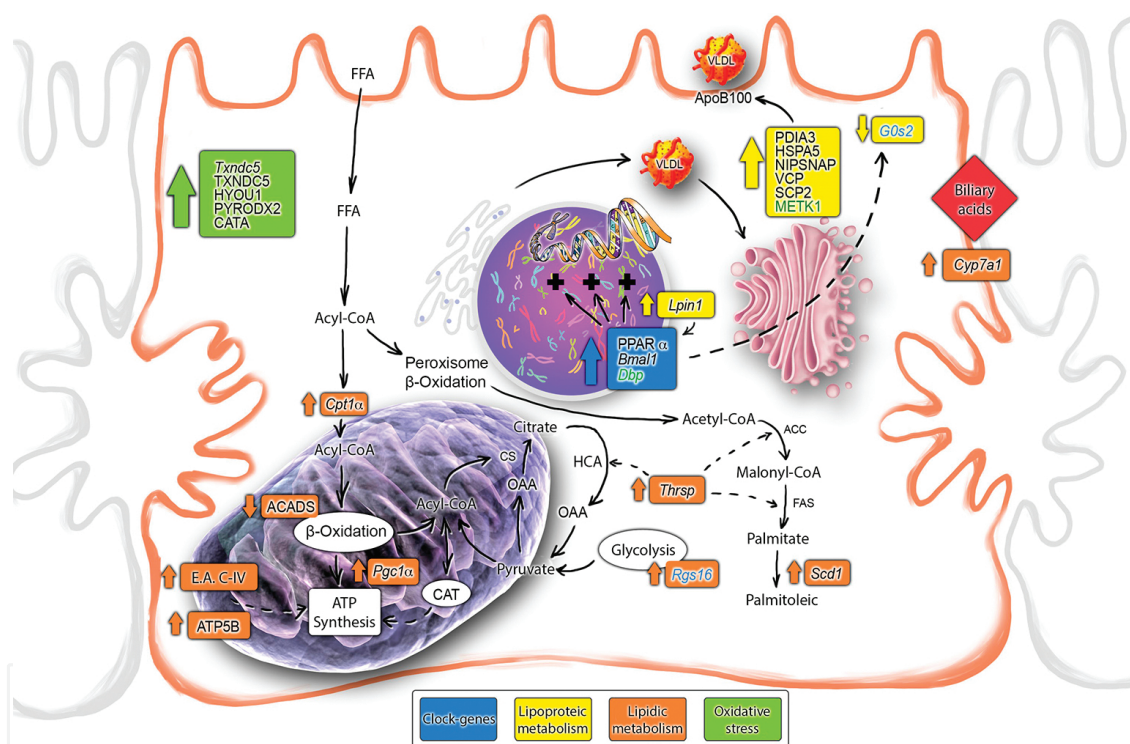


Figure 5. Squalene action in hepatocytes of *ApoE*-deficient mice. Squalene decreases fatty liver extent by favoring the secretion of VLDL and stimulating mitochondrial β -oxidation.

In addition, the complex role of dietary administered squalene is contributing to better understand hepatic lipid dynamics. The action of squalene may help to explain the protective role of virgin olive oil, where steatosis was observed with lower oxidative stress [40] and lesser atherosclerosis development compared to mice receiving palm oil [41].

In acute toxicology, a no-observed-adverse-effect level (NOAEL) of 58 g/kg was detected after a single oral dose and of 29 g/kg after intramuscular administration in mice [42]. Using 20 g/kg/day for four days, Gajkowska et al. reported the development of encephaloneuropathy in rats [43]. In mice, the lethal dose 50 is considered 5 g/kg/day [44], and a NOAEL of 2 g/kg/day

was found in 10-day administration regimen [42]. The 1 g/kg/day squalene dose used in our work is perfectly safe, and in fact, no secondary effects were noted. As mice display a higher metabolic rate than humans [45], this dose would correspond to a human dose of 100 mg/kg/day. Clearly, this dose is higher than the reported in human nutritional studies (15 mg/kg/day) [46] but does not reach the doses of 185 and 385 mg/kg/day used in women [47]. Therefore, the present study explores an attractive dose able to be reached in fortified foods and suggests a potential squalene dose to be used as functional food or therapy in fatty liver.

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