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Journal of Nutritional Biochemistry 112 (2023) 109207

Journal of Nutritional Biochemistry

RESEARCH PAPER

Dietary squalene supplementation decreases triglyceride species and modifies phospholipid lipidomic profile in the liver of a porcine model of non-alcoholic steatohepatitis

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Received 17 March 2022; received in revised form 7 September 2022; accepted 3 November 2022

Abstract

Squalene is a key minor component of virgin olive oil, the main source of fat in the Mediterranean diet, and had shown to improve the liver metabolism in rabbits and mice. The present research was carried out to find out whether this effect was conserved in a porcine model of hepatic steatohepatitis and to search for the lipidomic changes involved. The current study revealed that a 0.5% squalene supplementation to a steatotic diet for a month led to hepatic accumulation of squalene and decreased triglyceride content as well as area of hepatic lipid droplets without influencing cholesterol content or fiber areas. However, ballooning score was increased and associated with the hepatic squalene content. Of forty hepatic transcripts related to lipid metabolism and hepatic steatosis, only *citrate synthase* and a *non-coding RNA* showed decreased expressions. The hepatic lipidome, assessed by liquid chromatography-mass spectrometry in a platform able to analyze 467 lipids, revealed that squalene supplementation increased ceramide, Cer(36:2), and phosphatidylcholine (PC[32:0], PC[33:0] and PC[34:0]) species and decreased cardiolipin, CL(69:5), and triglyceride (TG[54:2], TG[55:0] and TG[55:2]) species. Plasma levels of interleukin 12p40 increased in pigs receiving the squalene diet. The latter also modified plasma lipidome by increasing TG(58:12) and decreasing non-esterified fatty acid (FA 14:0, FA 16:1 and FA 18:0) species without changes in total NEFA levels. Together this shows that squalene-induced changes in hepatic and plasma lipidomic profiles, *non-coding RNA* and anti-inflammatory interleukin are suggestive of an alleviation of the disease despite the increase in the ballooning score.
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Keywords: Squalene; Swine; NASH; Lipidomic; Cardiolipin; Triglyceride; IL-12p40; Ceramide.

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https://doi.org/10.1016/j.jnutbio.2022.109207
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1. Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized by intracellular accumulation of fat in hepatocytes, has become a burden disease in Western countries which prevalence has surpassed 40% in some epidemiology studies [1]. NAFLD induces whole genome expression changes [2] and compromises cell homeostasis through effect on diverse organelles such as mitochondria [3], endoplasmic reticulum [4] and lipid droplets [5]. NAFLD leads to a spectrum of liver pathologies such as non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma [6].

The Mediterranean dietary pattern has been proposed as a healthy choice for preventing diseases. Its main source of fat is virgin olive oil [7]. Extra virgin olive oil, as a fruit juice, is a complex mixture where triglycerides are combined with other biologically active substances (minor components) [8]. The consumption of extra virgin olive oil has positive health effects independently of its oleic acid content pointing to benefits of these minor components [9]. Squalene, is one of the most abundant of these minor components (2.4–9.3 g/kg) [10]. This cholesterol precursor has both antioxidant and anti-inflammatory properties, shown *in vitro* and *in vivo* models that have been explored in different experimental settings such as adaptive [11,12] and innate [13] immune system, cancer [14], atherosclerosis [15], and NAFLD [16].

Within the animal kingdom, sharks show one of the longest life span in vertebrates (392 ± 120 years) [17] while accumulating enormous amounts of lipids in the liver, among them squalene may represent 50–80% of liver weight [18]. This specific fatty liver represents a challenge and the effect of squalene on the progression of NAFLD deserves further attention. Studies carried out in mice and rabbits have shown that squalene accumulates in the liver and decreases hepatic cholesterol and triglycerides in a sex-dependent manner [15,19], and these actions are exerted through a complex network of gene expression changes both at transcriptional and posttranscriptional level [20–22]. These pieces of work have shown that squalene accumulates on different subcellular organelles [19] and drives specific plasma lipoprotein responses [23] depending on the species. These findings in animal models point out to a different metabolic response which could differ from the human one. In this aspect, swine is one of closest animal models to humans [24] and has emerged as a valuable model to fill the gap between rodents and humans. Recently, our group has succeeded in developing NASH in common strains of pigs [25]. Considering the squalene positive effects diminishing triglycerides in the murine liver, the present research was designed to explore the effect of squalene on the progression of NASH in the porcine liver and to search for the lipidomic changes involved.

2. Material and methods

2.1. Animals and experimental procedure

All the procedures were performed according to the European Union guidelines for the handling and care of laboratory animals (Directive 2010/63/UE), in accordance with ARRIVE guidelines and the protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza (PI43/15).

Twenty-four male Large White x Landrace pigs coming from one boar as progenitor were generated by artificial insemination at *Cooperativa Ganadera de Caspe* (Zaragoza, Spain) and transferred to the facilities of *Servicio General de Apoyo a la Investigación, División of Experimentación Animal, Facultad de Veterinaria, Universidad de Zaragoza,* where they were housed. After one month of acclimation, pigs, weighing 38 ± 2.8 kg, were fed the steatotic diet for 1 month. At this moment, the initial sampling took place and then they were split in two groups (n=12) of identical hepatic triglyceride and cholesterol levels. One group received the steatotic diet and the other, the same diet supplemented with 0.5% squalene (on average 135 mg/ kg dose of squalene/ animal/ day) to evaluate the squalene effect on NAFLD progression. Pigs had *ad libitum* access to food and water. After one month, all animals were euthanized and the biological samples collected (Fig. 1).

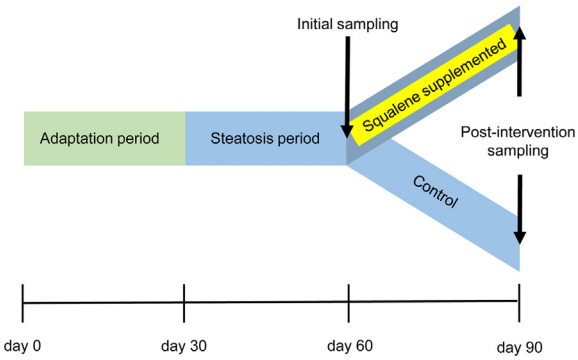

Fig. 1. Scheme displaying the used experimental setting. After an adaptation period of 30 days, 24 pigs were fed the steatotic diet for 1 month. Then, two groups of equal hepatic TG content were established. The control received the steatotic diet for another month while the squalene group was fed the squalene-supplemented steatotic diet for the same period of time.

Diets. The steatotic diet, prepared weekly at the Veterinary School facility and frozen preserved, was designed to be methionine-deficient and choline-restricted. It was also enriched in 2% cholesterol (Sigma-Aldrich, Germany), 0.5% sodium cholate (Molekula Group, Darlington, UK) and saturated fat (JL Supervía, Cuarte de Huerva, Zaragoza, Spain). This diet provided 50% of energy from hydrogenated palm fat (saturated fatty acids represented approximatively a 50%), 43% from complex carbohydrates and 7% from protein (Supplemental Table 1). Squalene, purchased from Molekula Group, was used to prepare the supplemented diet.

Biological samples. As displayed in Fig. 1, samples were collected at two timepoints: one month after the steatotic diet, and at the end of the experiment following overnight fasting. The first collection was performed under general anesthesia, induced and maintained with propofol (B/Braun-Vetcare, Rubí, Barcelona, Spain) administration. The abdomen was opened through a middle line laparotomy and blood and liver biopsies were obtained. At the end of experiment, pigs were euthanized by an anesthetic overdose and samples were taken. Pieces of the livers were stored in 4% paraformaldehyde and some immediately frozen in liquid nitrogen. The frozen samples from livers and plasma were stored at -80ºC.

Histological analyses. Paraformaldehyde-stored liver samples were embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin or with Masson's trichrome staining and observed using a Nikon microscope. Lipid droplet and fiber areas were blindly evaluated as described [26], quantified in each section using Adobe Photoshop CS3 (Adobe, San Jose, CA, USA) and expressed as percentage of total liver section. NAFLD score (NAS) was assessed using criteria described by Kleiner et al. [27] with the standardization proposed by Liang et al. [28]. The fatty liver inhibition of progression algorithm and steatosis, activity and the fibrosis (SAF) score were also used to categorize the histological stage of pigs [29].

Lipid extraction and colorimetric measurements. Pieces of liver (20 mg) were homogenized in 1 mL of phosphate-buffered saline and lipids were extracted according to Folch's method [30], evaporated and dissolved in 200 μL of isopropanol. Cholesterol and triglyceride concentrations were measured using Infinity Reagent kits from Thermo Scientific (Thermo Fisher Scientific Waltham, MA, USA).

Squalene quantification. Squalene extraction and analyses were carried out as previously described [19].

Plasma parameters. Fasting plasma cholesterol and triglycerides were measured using Infinity Reagent from Thermo Scientific (Thermo Fisher Scientific). Plasma lipoprotein profile was determined in 100 μL of pooled plasma samples from each group by fast protein liquid chromatography gel filtration using a Superose 6B column (GE Healthcare, Chicago, Il, USA) as previously described [31]. Ketone bodies and non-esterified fatty acids were assayed by commercial colorimetric kits from FUJIFILM (Wako Pure Chemical Corporation, Tokyo, Japan). Biochemistry parameters (glucose, bilirubin, AST, ALP, ALT, and GGT) were analyzed at the Clinical Laboratory of the *Hospital Clínico Universitario Lozano Blesa* (Zaragoza, Spain). Plasma levels of IL-1beta, IL-4, IL-6, IL-8, IL-10, IL-12p40, IFN-alpha, IFN-gamma, and TNF-alpha were assayed through a procartaplex multiplex (EPX090-60829-901, Thermo Fisher Scientific) according to manufacturer's instructions. Insulin (EP0100, Finetest Wuhan, Hubei, China), leptin (EP0103, Finetest), adiponectin (EP0006, Finetest) was assayed by ELISA following manufacturers' instructions.

RNA isolation and quantification. RNA was extracted from frozen livers using TRI reagent (MRC, Cincinatti, OH, USA) following the manufacturer's instructions. DNA contamination was removed by TURBO DNAse treatment and removal kit from Ambion (Life Technologies). RNA was quantified by absorbance at 260 nm, and purity was verified regarding the A260/280 ratio (greater than 1.8) with SPECTROstar Spectrophotometer's LVis plate (BMG Labtech, Offenburg, Germany). Integrity of the 28 S and 18 S ribosomal RNAs was verified by 1% agarose gel electrophoresis followed by ethidium bromide staining.

RT-qPCR. Total RNA (500 ng) of each animal were reverse transcribed in a volume of 10 μL using the Prime Script Reagent (Perfect Real Time) from Takara (Kusatsu, Japón). PCR real time reactions were performed using 2x qPCRBIO Sgreen Mix Hi-ROX, from PCR Biosystems (London, UK), according the manufacturer's instructions using 384-well plates in a ViiA7 Real-TIME PCR System (Life Technologies). The used primers (Supplemental Table 2) were designed using NCBI primer design software and checked by BLAST analysis (National Center for Biotechnology Information and Ensembl) to verify gene specificity and exon location. The relative amount of all mRNAs was calculated using comparative $2^{-\Delta\Delta Ct}$ method and normalized with the reference gene *UBA52* (XM_013991270.1).

Liver and plasma lipidomics. Samples of 5–10 mg of the liver and 25 μL of plasma were extracted according to Folch's method in 1 mL of chloroform: methanol (2:1), 400 μL of water and 150 μL of internal standard mix (Supplemental Table 3). The organic phases (300 μL) were dried down and reconstituted in 100 μL of isopropanol:acetonitrile:water (2:1:1). Then, chromatographic separation of lipids (Virtue et al., 2018) was achieved using a Shimadzu HPLC System (Shimadzu UK Limited, Milton Keynes, UK) with the injection of 10 μL into a Waters Acquity UPLC CSH C18 column; 1.7 μm, I.D. 2.1 mm X 50 mm, maintained at 55°C. Mobile phase A was 6:4, acetonitrile and water with 10 mM ammonium formate. Mobile phase B was 9:1, propan-2-ol and acetonitrile with 10 mM ammonium formate. The flow was maintained at 500 μL per minute through the following gradient: 0.00 min 40% mobile phase B; 0.40 min 43% mobile phase B; 0.45 min 50% mobile phase B; 2.40 min 54% mobile phase B; 2.45 min 70% mobile phase B; 7.00 min 99% mobile phase B; 8.00 min 99% mobile phase B; 8.3 min 40% mobile phase B; 10 min 40% mobile phase B. The sample injection needle was washed using 9:1, 2-propan-2-ol and acetonitrile with 0.1 % formic acid. The mass spectrometer used was the Thermo Scientific Exactive Orbitrap with a heated electrospray ionization source (Thermo Fisher Scientific, Hemel Hempstead, UK). The mass spectrometer was calibrated immediately before sample analysis using positive and negative ionization calibration solution. Additionally, the heated electrospray ionization source was optimized at 50:50 mobile phase A to mobile phase B for spray stability (capillary temperature; 380°C, source heater temperature; 420°C, sheath gas flow; 60 (arbitrary), auxiliary gas flow; 20 (arbitrary), sweep gas; 5 (arbitrary), source voltage; 3.5 kV. The mass spectrometer resolution was set to 25,000 with a full-scan range of m/z 100 to 1800 Da, with continuous switching between positive and negative mode. Lipid quantification was achieved using the area under the curve of the corresponding high resolution extracted ion chromatogram (with a window of ± 8 ppm) at the indicative retention time. The ratio of area under the curves for each lipid analyte relative to its associated internal standard (Supplemental Table 3) for that lipid class was used to semi-quantify and correct for any extraction/instrument variation [32].

Statistical analyses. Results are expressed as mean ± SD. Data were analyzed by one tail Mann–Whitney's test using Prism 5 software for Windows (GraphPad, San Diego, CA, USA). Differences were considered significant when $P<.05$. Correlations among variables were tested by calculating the Spearman's correlation coefficient according to the Statistical Package for Social Sciences version 15 (SPSS, Chicago, IL, USA). The heatmaps were generated using the platform of MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

3. Results

3.1. Squalene supplementation accumulates in the liver, decreases hepatic lipid droplet areas and triglycerides while worsening ballooning score in a model of porcine NASH

In order to explore the influence of squalene on the progression of hepatic steatosis in pigs, 24 crossbred swine were made steatotic by feeding them the steatotic diet for one month. At this time point, two groups of 12 pigs were established and for an additional month one group continued receiving this diet and the other was fed a squalene-supplemented steatotic diet (Fig. 1). There were no significant changes in the body weight follow-up between both groups (Supplemental Fig. 1A) despite the significant increase in solid intake of the squalene-supplemented steatotic diet group (Supplemental Fig. 1B).

The liver histological analyses revealed significant decreases in the lipid droplet areas (Fig. 2A, B, and C) by the squalene treatment. A significant decrease was also observed for the hepatic triglyceride (Fig. 2G), but not for cholesterol content (Fig. 2H) and the fiber areas (Fig. 2D, E, and F). Using the NAFLD score [27] according to Liang et al. [28] both groups were histologically indistinguishable (Supplemental Table 4) and they had developed NASH. When FLIP algorithm and steatosis, activity, and fibrosis (SAF) score proposed by Bedossa et al. [29] was used, all of animals after consuming the steatotic diet developed NASH with equal distribution of scores 1 and 2 for ballooning. For pigs consuming the squalene-supplemented diet, there was a significant increase in the ballooning score and 100% of animals showed a score 2 corresponding to a more advanced stage of NASH (Supplemental Table 4). This parameter contributed to a significant increase in activity value considering that the latter encompasses ballooning and inflammation. As observed in other animal species, squalene accumulated in the liver of pigs fed the supplemented diet (Fig. 2I). The hepatic squalene content after the intervention was inversely correlated with the observed changes in lipid droplet area (Fig. 2J), but it was significantly and positively correlated with ballooning score (Fig. 2K). These findings indicate that squalene supplementation decreases hepatic triglycerides, and lipid droplet without changes in fiber areas despite the increase in ballooning score.

3.2. Dietary squalene induces changes in various lipid classes in the steatotic liver

Having observed changes in hepatic TG, we aimed to know what type of triglycerides were diminished in the liver, and whether the squalene-induced decrease in hepatic lipid droplets and triglycerides were implying changes in other lipids. To answer these questions, we carried out a lipidomic assay to assess 467 different lipids: 20 ceramides (Cer), 56 cardiolipins (CL), 27 phosphatidic acids (PA), 23 lyso-phoshatidylcholines (LPC), 19 lyso-phosphatidylethanolamines (LPE), 27 non-esterified fatty acids (FA), 43 phosphatidylcholines (PC), 19 phophatidylethanolamines (PE), 19 phosphatidylinositols (PI), 34 phoshatidylglycerols (PG), 18 phosphatidylserines (PS), 89 triglycerides (TG), 28 sphingomyelins (SM), 45 sulpholipids (S). The number of lipids in each lipid class are shown in Supplemental Table 5. When analyzing the global changes in the lipid classes after the squalene treatment, only total ceramides were significantly increased (Fig. 3A) while total CL (Fig. 3B), PC (Fig. 3C) and PI (Fig. 3D) did not experienced any significant changes. With the exception of PI, all other lipid species were involved with varied number of carbons or unsaturation. Sixteen lipid species: Cer(36:2), Cer(42:1), CL(69:5), PC(32:0), PC(33:0), PC(34:0), PE(34:0), PE(38:0), PE(38:6), S(d43:1), S(d48:0), TG(36:0), TG(47:1), TG(54:2), TG(55:0), and TG(55:2), were significantly modified following the squalene supplementation (Table 1, Fig. 3E). Indeed, both ceramide and PC species increased their content and CL(69:5) and TG(36:0), TG(47:1), TG(54:2), TG(55:0), and TG(55:2) decreased. No uniform pattern was followed by PE or S lipid classes.

To establish which lipid could be associated with histological features, correlation analyses were carried out. In this sense, lipid droplet areas were significantly and positively correlated with several PC species and negatively with Cer(42:1) and other PC (Fig. 4A). Hepatic squalene was positively associated with PC(32:0) (Fig. 4B) and this lipid was also associated with ballooning (Fig. 4C). PE(38:0) was positively associated with hepatic squalene (Fig. 4B) and negatively with hepatic TG (Fig. 4D). CL(69:5) was negatively associate with hepatic squalene (Fig. 4B). Both CL(69:5) and PE(38:0) emerged as hubs of a vast range of lipid species (Fig. 4E and F).

3.3. Dietary squalene decreases Citrate synthase *and* ncRNA *expressions in the steatotic liver*

To explore which gene expressions could be involved in the hepatic changes, 40 gene transcripts participating in lipid metabolism and transport were assayed (Supplemental Table 6). Only citrate synthase (*CS)* and *ncRNA* transcripts showed significant

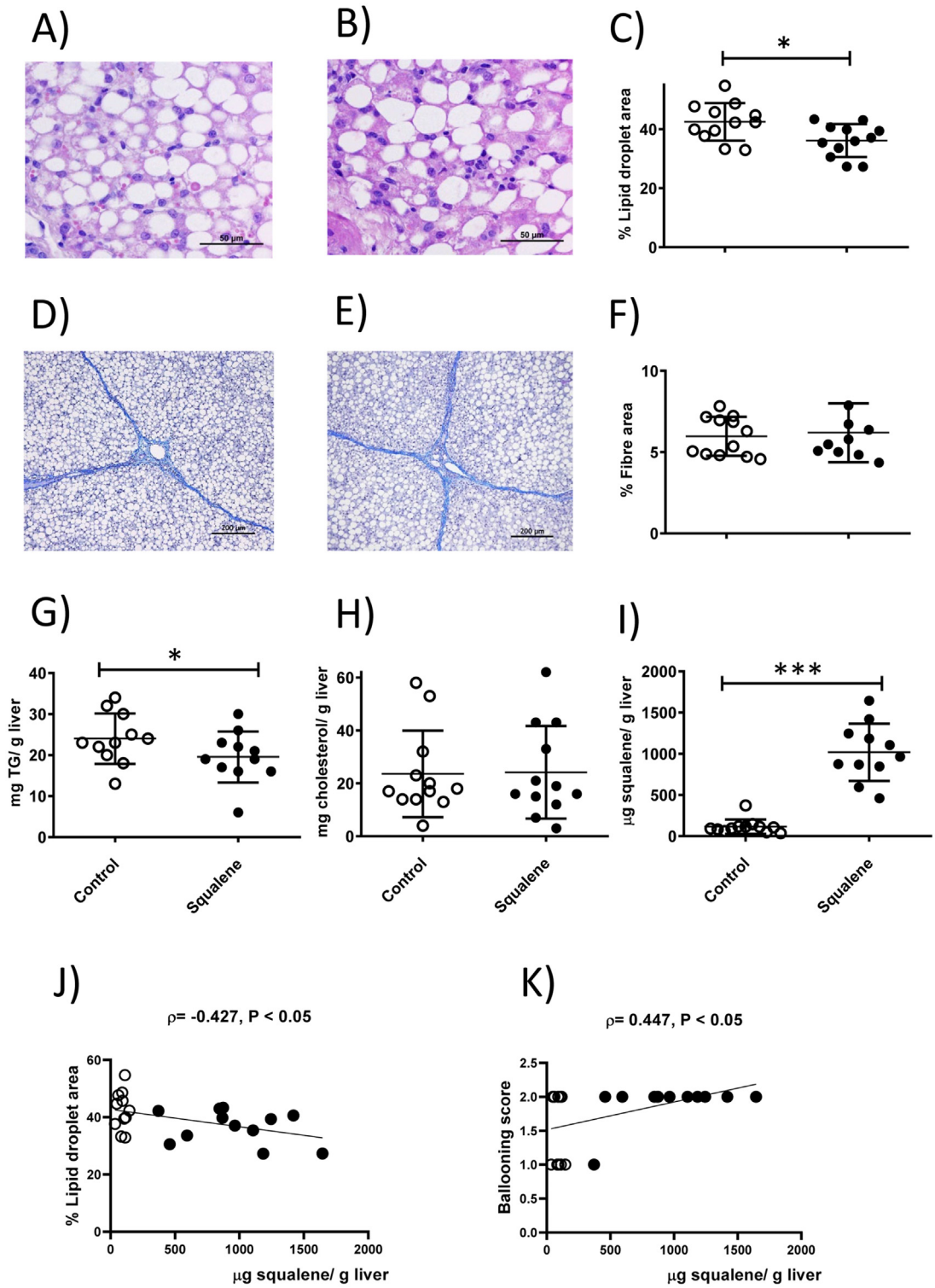

Fig. 2. Effect of squalene on hepatic features of commercial bred swine consuming a steatotic diet. Representative micrographs of hematoxylin-eosin-stained liver preparations from control (A) and squalene (B) groups. Bar denotes 50 μm. Morphometric changes in lipid droplet area expressed as percentage of total liver section (C). Masson's trichromic liver histology staining of representative liver micrographs from control (D) and squalene groups (E). Bar denotes 200 μm. Morphometric changes in fiber area (F), expressed as percentage of total liver section. Hepatic triglyceride (G), cholesterol (H), and squalene (I) levels in pigs consuming the steatotic diet or this supplemented with squalene. Individual values, means and SD are represented for each group. Statistical analyses were carried out using Mann–Whitney U test. *, $P<.05$; ***, $P<.001$. Associations among hepatic squalene content and lipid droplet area (J) and ballooning score (K). Correlations were calculated by the Spearman's rho test.

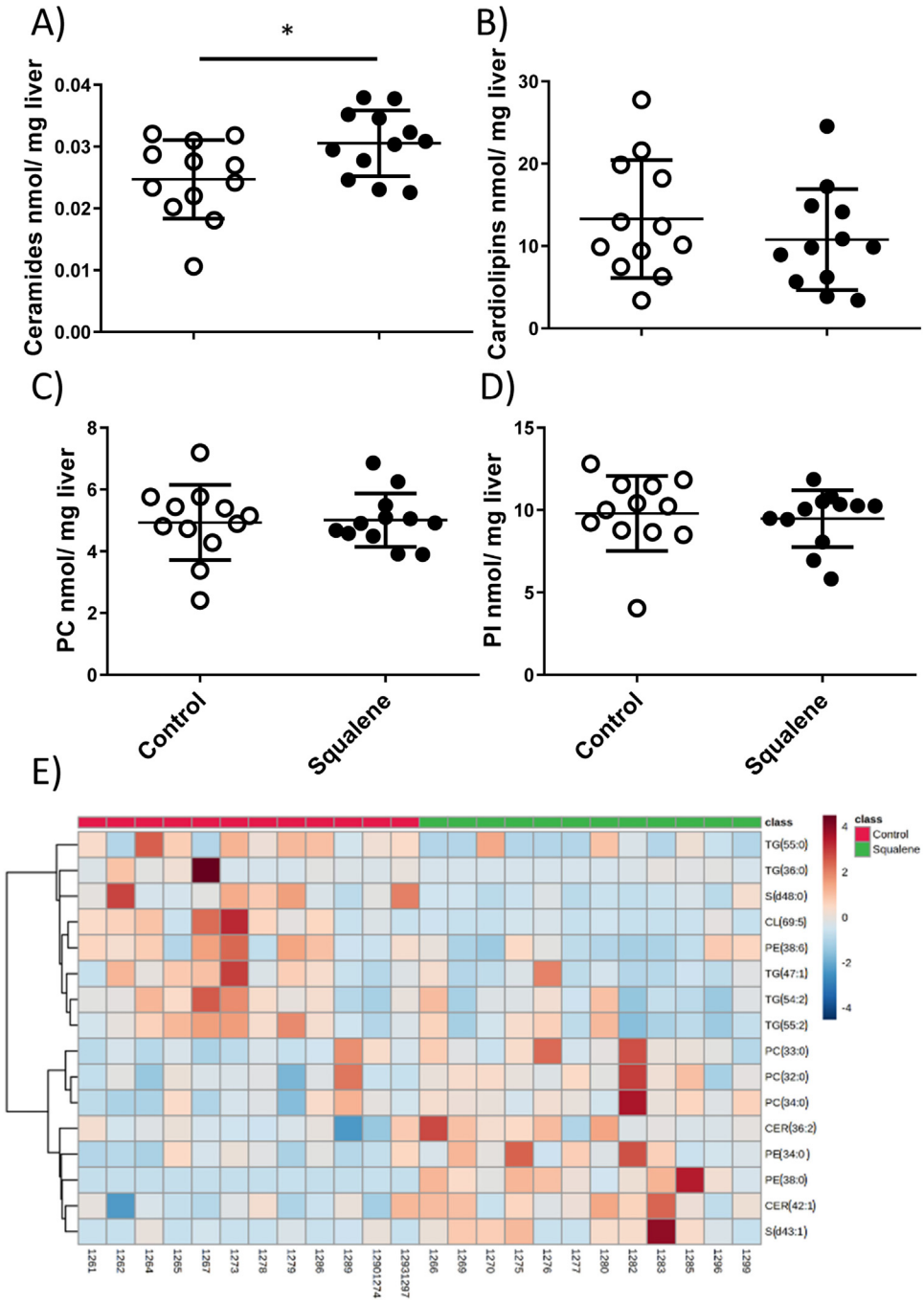

Fig. 3. Liver lipidomics of control and squalene-supplemented pigs. Controls received the steatotic diet, and squalene group the steatotic diet supplemented with squalene. Ceramides (A), CL (B), phosphatidylcholines (C) and phosphatidylinositols (D). Individual data and mean ± SD for each group is shown. Statistical analyses were carried out using Mann–Whitney U test. *, $P<.05$. Heatmap of differentially significant lipids in each animal (E).

decreases (Fig. 5A and B). Both transcript levels were positively correlated (Fig. 5C). *CS* expression was negatively correlated with Cer(42:1) (Fig. 5D). *ncRNA* expression was negatively correlated with PC(33:0) and PE(38:0) and positively with S(d38:0) (Fig. 5E).

3.4. Dietary squalene supplementation modifies some clinical biochemistry parameters and plasma lipidome

To explore which plasma parameters could be influenced by squalene supplementation, a total of 27 clinical biochemistry parameters were studied (Supplemental Table 7). Despite no plasma total cholesterol change was observed, animals consuming squalene showed larger LDL particles (Supplemental Figure 2). Plasma IL-12p40 levels significantly increased by the administration of squalene (Fig. 6A). This increase was negatively associated with hepatic lipid droplet area (Fig. 6B) and positively with hepatic PE(38:0) (Fig. 6C). Plasma ceramides (Fig. 7A) were not modified while plasma triglycerides (Fig. 7B) increased. Identified lipids of plasma lipidomics are shown in Supplemental Table 8. From them, 10 lipid species: NEFA (FA 14:0, FA 16:1 and FA 18:0), PE(34:2), S(d38:0), S(d39:1), S(d48:1), S(d48:2), TG(49:0), and TG(58:12) were significantly modified following the squalene supplementation (Table 2, Fig. 7C). Plasma triglycerides were positively associated with hepatic squalene content (Fig. 7D) and the latter was

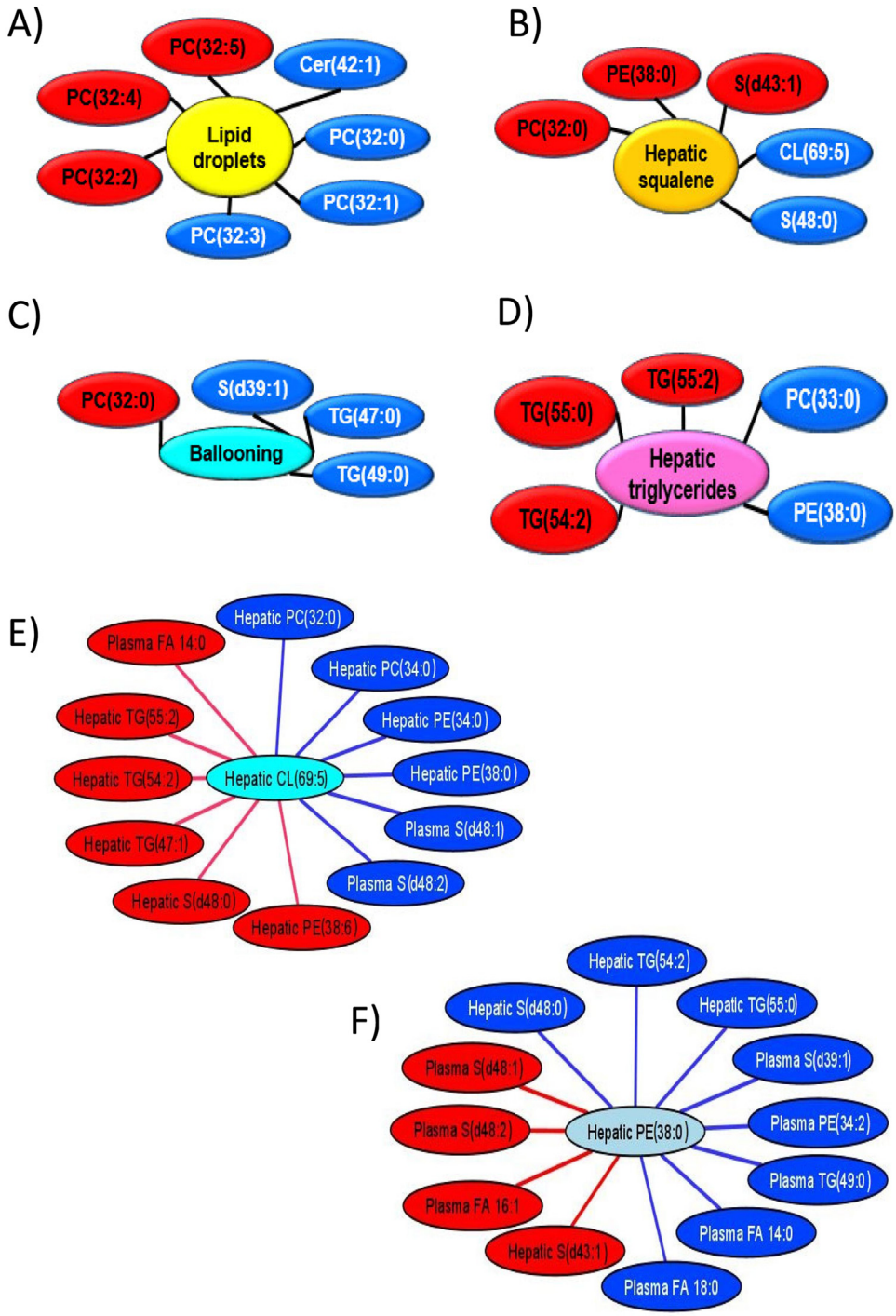

Fig. 4. Association among lipid variables. Associations of hepatic lipid species with lipid droplet area (A), squalene content (B), ballooning score (C) or triglyceride content (D). Main association of two hepatic species: cardiolipin CL(69:5) (E) and phosphatidylethanolamine, PE(38:0), (F) with other lipid species. Red text boxes denote positive associations while blues negative ones. Correlations were calculated according to the Spearman's rho test and only significant ($P<.02$) associations are shown. Cytoscape 9.0 was used to display the networks. Cer, Ceramide; FA, non-esterified fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine, S, sulphatide; TG, triglyceride.

positively associated with plasma NEFA (FA 16:1), S(d48:2), and TG(58:12) and negatively with NEFA (FA 14:0 and FA 18:0) (Fig. 7E).

4. Discussion

The aim of the present study was to test the influence of squalene administration on the progression of hepatic steatosis. Our results indicate that pigs consuming the 0.5% squalene-supplemented steatotic diet (corresponding to a dose of 135 mg/kg of squalene/animal/day) showed decreased hepatic triglycerides and lipid droplet areas without changes in fiber areas. However, this group of pigs showed an increased NASH due to an increase in the ballooning score. Squalene accumulated in the liver and its effect was studied at two different levels. On the one hand, hepatic gene transcripts involved in lipid metabolism or porcine steatosis were assayed and only the expressions of *CS* and *ncRNA* were

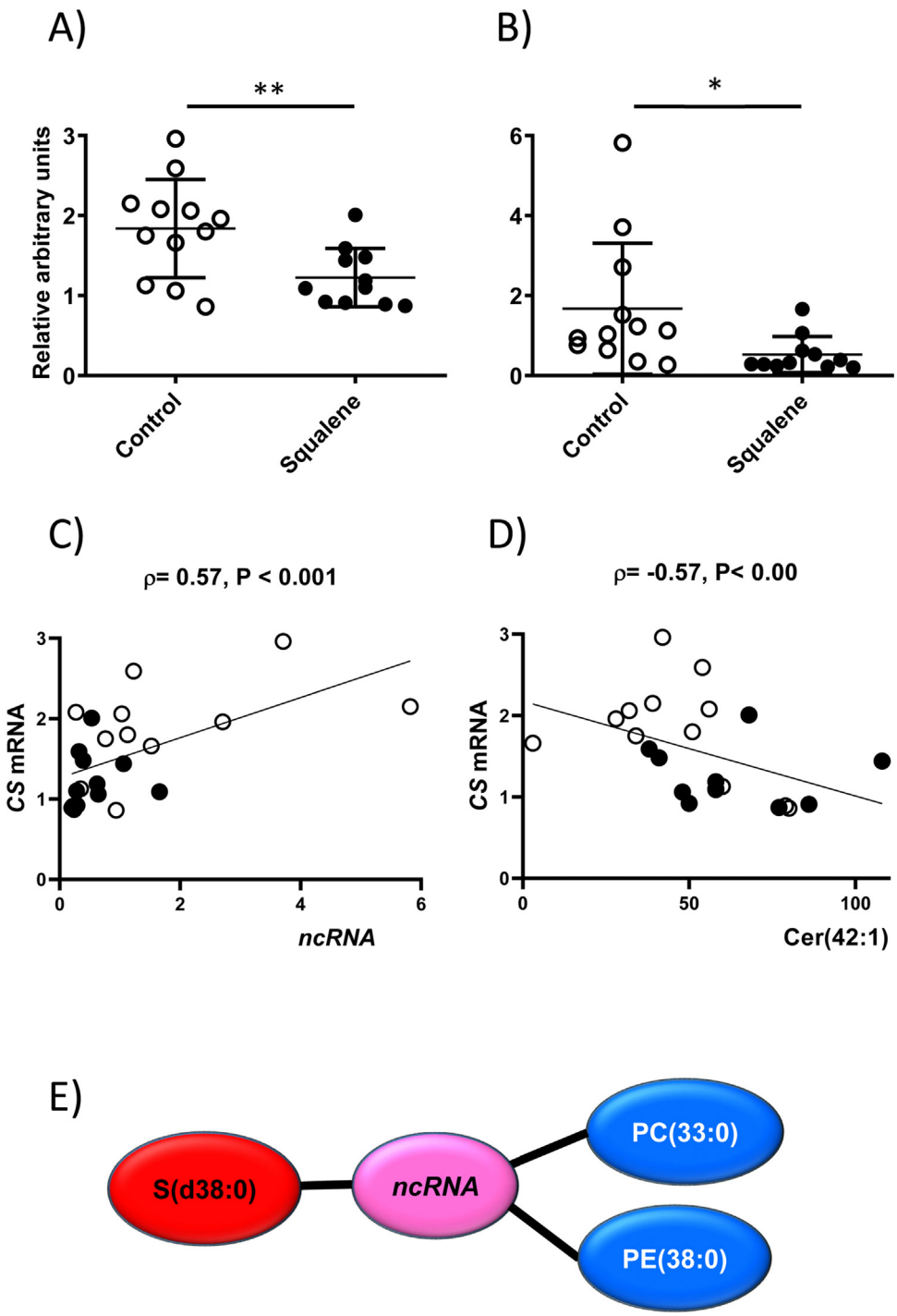

Fig. 5. Effect of squalene on selected hepatic transcripts of pigs fed a steatotic diet. Citrate synthase (CS) (A) and non-coding RNA (ncRNA) located in intron 4 of ZBTB16 (B) gene expressions analyzed by RT-qPCR in both experimental groups. Data, as relative arbitrary units (RAU), are expressed as individual values with their mean ± SD for each group normalized to UBA52 expression. Statistical analyses were carried out using Mann–Whitney U test. *, $P<.05$ and **, $P<.01$. Associations of CS expression with hepatic ncRNA expression (C) and ceramide (42:1) species (D). Significant ($P<.02$) associations among hepatic ncRNA expression and several lipid species (E). Correlations were calculated according to the Spearman's rho test. Red text boxes reflect positive associations while blues negative ones. PC, phosphatidylcholine; PE: phosphatidylethanolamine; S, sulphatide.

significantly diminished. On the other hand, the hepatic lipid composition was assessed by LC-MS, revealing a decrease in triglycerides and CL 69:5 and an increase of ceramide and PC species. In addition, among plasma clinical biochemistry parameters studied, significant increases in IL-12p40 and TG were found. When plasma lipidomics was carried out, only NEFA (FA 14:0, FA 16:1, and FA18:0) species showed a consistent decrease. Overall, squalene appears to be an important modifier of hepatic lipid metabolism with translation into histological features of liver pathology (Fig. 8).

The employed model displays a reproducible and reversible NASH stage using the commercial bred pig through manipulating its diet for a short period of time [25]. This required not only

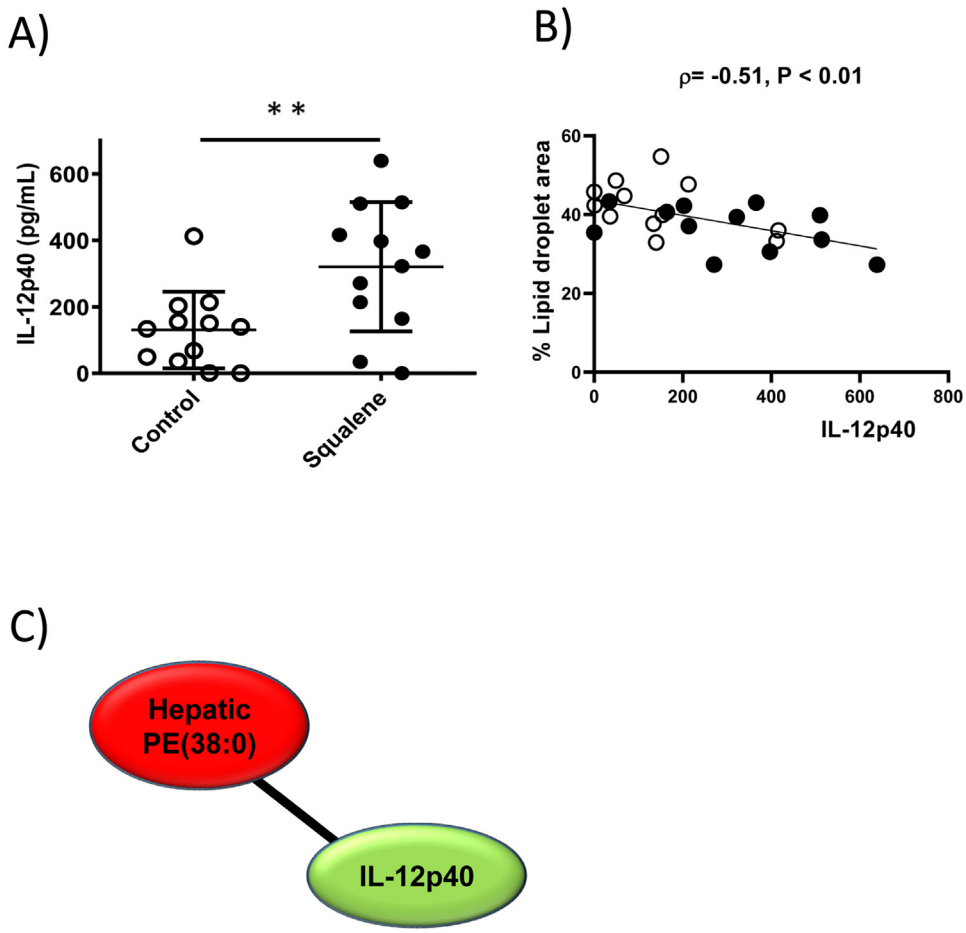

Fig. 6. Effect of squalene on plasma levels of interleukin-12 subunit beta (IL12p40) in pigs receiving a steatotic diet. Plasma concentration of IL12p40 in both experimental groups (A). Association between plasma IL12p40 and hepatic lipid droplet area (B) and between IL12p40 and hepatic PE(38:0) (C). Correlations were calculated according to the Spearman's rho test and only significant (P<.02) associations are shown. Red text box denotes positive association. PE: phosphatidylethanolamine.

the use of increased contents of common hepatic stressors such as cholesterol, saturated fat and fructose but also un-physiological dietary modifications such as inclusion of cholate and lowering methionine and choline contents (Supplemental Table 1). Lee et al. and Panasevich et al. [33,34] proposed the use of such extreme dietary modifications to cause liver injury in Ossabaw miniature pigs. Likewise, the latter modifications were crucial to overcome a natural resistance to develop NAFLD in Large White x Landrace bred pig due to a long pursue of breeding focused on selecting muscular pigs and force the liver metabolism to fit into this objective. The characterization of this model reinforced the notion that dyslipidemia in the absence of metabolic syndrome was crucial for the development of NAFLD and NASH [25]. In this regard, this model resembles the NASH phenotype observed in some non-obese subjects [35] and may increase the translational knowledge of a model that could be a potential source for liver xenotransplantation [36].

Squalene administration in this model confirms that this compound accumulates in the porcine liver as observed in mice [15,20–22] and rabbits [23]. Previous experience in mice had already shown that dietary squalene was able to reduce the hepatic triglyceride content accompanied or not by reduction in lipid droplet areas depending on the dietary conditions such as the use of a low-fat chow [15,20,21] or a high-fat Western diets [22]. In squalene-fed rabbits, an increased hepatic area of lipid droplets was found while triglyceride content remained unchanged [23]. In the present research both TG and lipid droplet areas were significantly decreased in pigs fed the squalene-supplemented steatotic diet. This discrepancy among models was a consequence of the subcellular compartment accumulating squalene: reticulum vs lipid droplets [19]. In the current setting, squalene would not be accumulated in large lipid droplets. A similar discrepancy was also observed when administering another terpene, oleanolic acid [37].

Four features are considered hallmarks to categorize NASH (macrosteatosis, ballooning, inflammation and fibrosis) [29]. While inflammation and fibrosis were not modified by the intervention, both hepatic TG and lipid droplet areas were significantly decreased in pigs fed the squalene-supplemented steatotic diet. However, the macrosteatosis index was not sensitive enough to detect the changes in TG and lipid droplet areas. It should be taken into account that it represents a qualitative approach of a quantitative variable (score 0, <5%; score 1, between 5% and 33%; score 2, between 34% and 66% and score 3, >67% of hepatic area occupied by lipid droplets). In fact, most of the control pigs (9 out of 12) were categorized as score 2 while 6 out of 12 pigs consuming the squalene-supplemented steatotic diet displayed score 2. The latter group also showed an increase in the ballooning score. Interestingly, the squalene accumulation was significantly associated with the ballooning score, consequently due to the latter increase, a more severe stage of NASH was assigned to pigs consuming the squalene-supplemented steatotic diet despite the decreased TG and lipid droplet areas. Clearly, this terpene accumulates in the liver and is likely to influence NAFLD diagnosis. This could challenge the

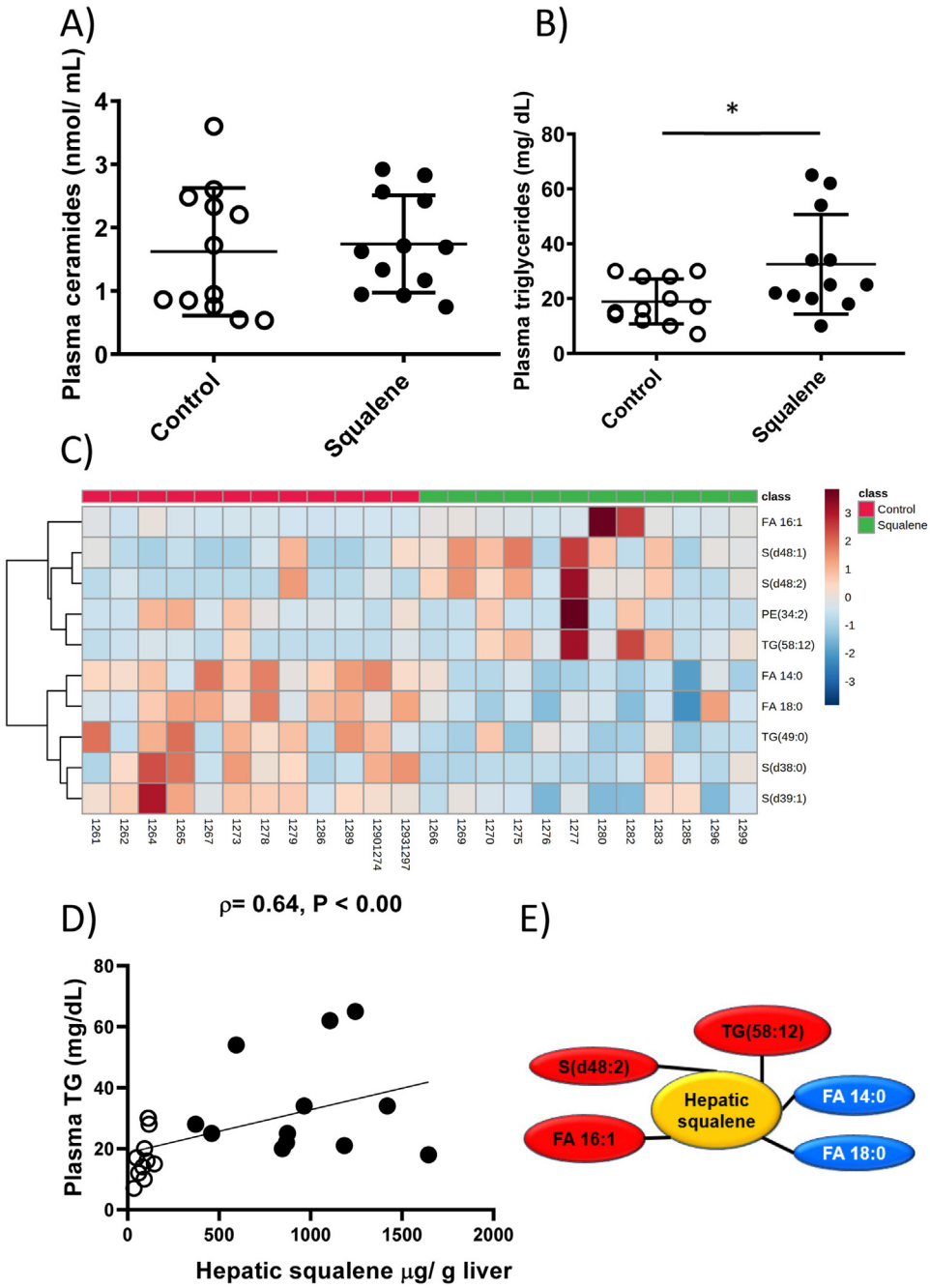

Fig. 7. Effect of squalene administration on plasma lipidomics of pigs fed a steatotic diet. Concentrations of plasma ceramides (A) and triglycerides (B) in both experimental groups. Data are expressed as individual values with their mean ± SD for each group. Statistical analyses were carried out using Mann–Whitney U test. *, $P<.05$. Heatmap of significantly changed plasma lipids in each animal (C). Associations of plasma triglycerides with hepatic squalene content (D) and the latter with plasma lipid species (E). Correlations were calculated according to the Spearman's rho test and only significant ($P<.02$) associations are shown. Red text boxes reflect positive associations while blues negative ones. FA, non-esterified fatty acid; S, sulphatide; TG, triglyceride.

impact of ballooning as marker of severity in populations consuming diets with high squalene content such as the Mediterranean and Japanese diets [38]. Furthermore, it should bear in mind that some sharks particularly accumulate this terpene in their livers, and they may live up to 400 years [17]. In light of these findings, the exact role of ballooning needs to be better understood to correctly establish diagnosis with prognostic implications.

The squalene effect was also explored at the hepatic transcriptional level by assaying forty gene transcripts involved in squalene response, lipid metabolism and porcine steatosis. Regarding the first aspect, fourteen transcripts (*ACADS, ACOX1, ARNTL1, CASP1, CPT1A, CYP2B6B, CYP2C42, CYP51A1, DBP, GPX4, MAT1A, SCD1, THRPS,* and *TXNDC5*) were selected based on their previously described changes in mice [20–22]. In the present experimental setting, the reported murine effects were not observed. Two reasons could explain it, there could be a specie-specific effect or maybe these gene changes require a longer time. While experiments in mice lasted 8–11 weeks, the present study only explored the effects of one-month squalene supplementation. Seventeen genes (*ACACA, ACACB, ACLY, CAV1, CD36, CIDEB, CIDEC, CS, FABP1, FASN, FATP2,*

Table 1
Significant changes in molecular species of hepatic lipids by the influence of squalene administration in pigs developing NASH.

Name carbon atoms: db	Control (n=12)	Squalene (n=12)
Cer(36:2)	1.2±0.5	1.7±0.6*
Cer(42:1)	4.2±1.8	6.2±1.9*
CL(69:5)	3.5±3.4	0.4±0.5***
PC(32:0)	175.6±42.6	204.7±43.2**
PC(33:0)	1.2±0.5	1.7±0.8*
PC(34:0)	34.5±10.1	44±13.2*
PE(34:0)	0.9±0.8	2±1.5*
PE(38:0)	0.002±0.005	0.6±0.5*
PE(38:6)	11.7±6.1	6.3±4.5***
S(d43:1)	0.1±0.1	0.5±0.6**
S(d48:0)	0.2±0.2	0.05±0.05***
TG(36:0)	7.8±16.5	0.8±1.3*
TG(47:1)	90.5±55.8	53.9±43.3*
TG(54:2)	6128±3152	3933±2447*
TG(55:0)	0.6±0.4	0.2±0.3*
TG(55:2)	29.4±11.8	18.4±11.2*

Results are mean ± SD and expressed as pmol/ mg of liver. Statistical analyses were carried out using the Mann-Whitney's U test.
* P<.05;
** P<.01 and
*** P<.001.
Abbreviations: Cer, ceramide; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; S, sulphatide; TG, triglyceride.

Table 2
Significant changes in molecular species of plasma lipids by the influence of squalene administration in pigs developing NASH.

Name carbon atoms: db	Control (n=12)	Squalene (n=12)
FA 14:0	69,129±1,386	39,960±10,016***
FA 16:1	5,386±4,183	26,707±40,105***
FA 18:0	619,127±93,022	469,226±106,217***
PE(34:2)	429±411	437±876*
S(d38:0)	97±77	25±36*
S(d39:1)	154±64	66±51***
S(d48:1)	8.6±10.9	26±19**
S(d48:2)	8.7±21	41.3±38**
TG(49:0)	499±275	167±165***
TG(58:12)	143±241	762±841**

Results are mean ± SD and expressed as pmol/ mL of plasma. Statistical analyses were carried out using the Mann-Whitney's U test.
* P<.05;
** P<.01 and
*** P<.001.
Abbreviations: FA, non-esterified fatty acids; PE, phosphatidylethanolamine; S, sulphatide; TG, triglyceride.

FATP4, FATP5, FDPS, PGC1A, PLIN2, and *PLIN5)* were selected to analyze lipid metabolism and transport [26,39] and with the exception of *CS,* there were no statistically significant changes. Thus, squalene effect on lipid metabolism is not executed at transcriptional level of these genes. Regarding porcine steatosis [25], nine gene expressions *(APC5, APCS, ISG15, LGALS3, PARQ7, SMPDL3A, SPP1, SRGN, and ncRNA)* were analyzed, and only *ncRNA* showed a significant decrease by squalene administration. The *ncRNA,* (LNG010034H01) according to Ensembl database, is located on an intron of *ZBTB16* gene and seems to be a porcine-specific transcript

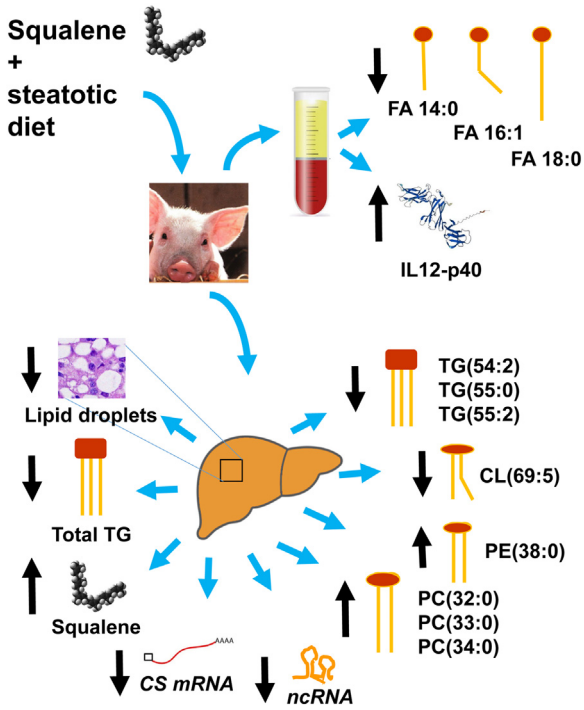

Fig. 8. Comprehensive scheme displaying the observed findings. The plasma and hepatic changes are summarized.

since a BLAST at NCBI reported no such RNA in other species. As previously observed [25], this transcript increased in the induction of hepatic steatosis and decreased when it reverted. Following the squalene administration, the trend followed the regression effect.

Lipidomic analyses revealed that squalene administration increased some hepatic ceramides [Cer(36:2) and Cer(42:1)]. The latter is likely to correspond to a dihydroceramide that do not induce the lipotoxicity induced by ceramides [40]. Moreover, Turner et al. have proven that the inhibition of ceramide biosynthesis does not protect against insulin resistance [41]. Probably, the key to the ceramides' homeostasis is that it should always be considered regarding their balance with the sphingosine-1-phosphate (S1P), a related molecule of ceramides which has opposite effects and whose function in NAFLD still remains poorly understood [42]. Further investigation on liver ceramides should be undertaken to decipher the paper of the sphingolipid rheostat in liver steatosis and whether the contribution of ceramides to the liver state depends on their subcellular location as well as on the S1P role. Squalene administration did not change total PC content, something also observed in the diabetic pig [43]. However, specific PC(32:0), PC(33:0), PC(34:0) species increased in the livers of pigs receiving squalene. Their increase has been proposed as a positive effect in delaying the progression to steatohepatitis [42,44] since they have a lower peroxidation index due to their saturated fatty acids [45]. The increase also represents a specific pattern for squalene considering that a high-fat diet provided to Ossabaw pigs increased hepatic PC(36:3), PC(38:5) and PC(38:6) [46], PC(32:2) was found increased following porcine hepatectomy [47] and hepatic PC(38:6) and PC(40:6) decreased in pigs consuming insect proteins [48]. Regarding the observed decrease in CL(69:5) by administration of squalene, it has been proposed that steatosis may increase CL [49], and blocking CL biosynthesis prevented hepatic steatosis through an improved electron flux between mitochondrial respiratory complexes [50]. The decrease in hepatic TG(54:2), TG(55:0), and TG(55:2) by administration of squalene could be the

main contributors to the decrease in total hepatic TG, as their significant correlations suggest. These species contain mostly saturated and short chain fatty acids and are considered markers of *de novo* lipogenesis. Their increase was associated with hepatic steatosis [51–53]. In this sense, squalene would reduce *de novo* lipogenesis and ameliorate hepatic steatosis. Overall, the changes in hepatic lipidomic profile induced by squalene administration are suggestive of an alleviation of the disease and specific of this compound.

Among plasma clinical biochemistry parameters studied, significant increases in plasma IL-12p40 and TG were found in the squalene group. IL-12p40 is an antagonist of the pro-inflammatory protein IL12 [54]. Thereby, this effect reinforces the anti-inflammatory role of squalene. Circulating levels of IL-12p40 were found inversely associated with hepatic lipid droplets in progression and regression of porcine steatohepatitis [25]. The present results further reinforce this inverse relationship with hepatic lipid droplet area and show a positive association with hepatic PE(38:0). The latter was found to be a hub with multiple connections with other lipid classes. The increase in plasma triglycerides could be due to the increase in TG(58:12). In fact, both plasma total-TG and TG(58:12) were significantly associated with hepatic squalene content. The scarcity of changes in plasma lipids in contrast to the hepatic is something also observed by Hanhineva et al. [46] and may be related to the fact that lipid is compartmentalized in plasma lipoproteins and exosomes with different roles [55]. In this sense, changes in HDL PC as those reported by Padro et al. [56] in hypercholesterolemic pigs may be masked at the plasma global level. Plasma lipidomics also evidenced a consistent decrease in NEFA (FA 14:0, FA 16:1, and FA 18:0) species without changes in total NEFA levels in pig receiving squalene. These results point out that individual species of FA are more sensitive parameters of metabolic changes that total NEFA levels. Plasma FA are transported into albumin and usually released from adipocytes [57]. Little attention has received the effect of squalene on adipocyte [16] but in light of these results, more attention should be addressed. A decrease in plasma NEFA specific species involves selective actions on adipocyte TG hydrolysis, NEFA release or enhanced hepatic uptake. In support of the latter possibility, it should be taken into account the increase in hepatic PC(32:0) that would be using FA 14:0 and FA 18:0 and would decrease their plasma levels. These findings reinforce a role for squalene in the modulation of lipid metabolism in several tissues that requires further attention.

5. Conclusion

In line with previous results of accumulating squalene in the liver of other experimental models, pigs do accumulate as well (Fig. 8). This was translated into decreased hepatic lipid droplet area and TG levels. However, the ballooning score worsened in a direct relationship with hepatic squalene content. The changes in hepatic lipidomic profile induced by squalene were specific in terms of decreasing CL(69:5) and increasing of Cer(36:2) and PC(32:0), PC(33:0) and PC(34:0) species. According to the current literature, these data are suggestive of an alleviation of the disease. This observation was also reinforced by the results of hepatic *ncRNA* and plasma levels of IL-12p40. The plasma lipidome underwent an increase in TG(58:12) associated with an increase in plasma triglycerides and decreases in NEFA (FA 14:0, FA 16:1 and FA 18:0) species without changes in total NEFA levels. These results confirm previous observations of lack of sensitivity of plasma versus hepatic lipidomics, but it is also suggestive of a role for squalene in the regulation of lipid metabolism in several tissues considering the adipocyte origin of those NEFA.

Author contributions

Designing and experimental work (LVHM, RMB, CA, CB, JJPL, THC, JMLB, GGR, GM, BJ, JCS, MJRY, JCB, RL, AGG, AG, AK, and JO). Preparation of draft (LVHM and JO). Search for funding (AK, AG, and JO). All authors edited and approved the final version of the manuscript.

Acknowledgments

We thank the University of Zaragoza Veterinary Hospital for their anesthesia support, the *Servicio General de Apoyo a la Investigación, División of Experimentación Animal, Universidad de Zaragoza* for their help in maintaining the animals and Larisa Richardson for her lab support. This research was funded by grants from the *Ministerio de Economía y Competitividad-Fondo Europeo de Desarrollo Regional* (SAF2016-75441-R), *Ministerio de Ciencia e Innovación* (MCIN/AEI/10.13039/501100011033, PID2019-104915RB-I00), "ERDF A way of making Europe", by the European Union and the *Fondo Social Europeo-Gobierno de Aragón* (B16_20R). CIBER *de Fisiopatología de la Obesidad y Nutrición* (CIBEROBN, CB06/03/1012) is an initiative of ISCIII. L.V.H.M. was recipient of a *Fondo Social Europeo-Gobierno de Aragón* contract.

Declaration of Competing Interests

The authors declare that there are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2022.109207.

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