Title: Hepatic subcellular distribution of squalene changes according to the experimental setting

Short title: Hepatic subcellular distribution of squalene.

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Abstract:

Squalene is the main unsaponifiable component of virgin olive oil, the main source of dietary fat in Mediterranean diet, traditionally associated with a less frequency of cardiovascular diseases. In this study, two experimental approaches were used. The first, New Zealand rabbits fed for 4 weeks with a chow diet enriched in 1% sunflower oil for the control group, and in 1% of sunflower oil and 0.5% squalene for the squalene group. In the second, APOE KO mice received either Western diet or Western diet enriched in 0.5% squalene for 11 weeks. In both studies, liver samples were obtained and analyzed for their squalene content by gas chromatography-mass spectrometry. Hepatic distribution of squalene was also characterized in isolated subcellular organelles. Our results show that dietary squalene accumulates in the liver and a differential distribution according to studied model. In this regard, rabbits accumulated in cytoplasm within small size vesicles, whose size was not big enough to be considered lipid droplets, rough endoplasmic reticulum and nuclear and plasma membrane. On the contrary, mice accumulated in large lipid droplets, and smooth reticulum fractions in addition to nuclear and plasma membrane. These results show that the squalene cellular localization may change according to experimental setting and be a starting point to characterize the mechanisms involved in the protective action of dietary squalene in several pathologies.

Keywords: Squalene, the liver, subcellular fractions, steatosis

Introduction.

Non-alcoholic fatty liver disease (NAFLD) is now viewed as a considerable health issue since it affects about 25% of the USA population, and up to 30% of these patients will develop more severe diseases such as non-alcoholic steatohepatitis (NASH), cirrhosis, and in some cases hepatocellular carcinoma [14]. The liver, as an organ with a major role in metabolizing glucose, fatty acids, and cholesterol, seems particularly sensitive to the increased incidence of human obesity in the modern society, and is becoming a lipid-accumulating tissue as well, and as consequence, promoting insulin resistance in human peripheral tissues [12]. NAFLD does not show symptoms so, when detected in a regular analytical, it is complicated to reverse the damage for the advanced stages of the disease, it is crucial to identify the first symptoms, and to provide early effective treatment for this ailment [4].

Squalene is an isoprenoid lipid belonging to the terpene family with linear structure and six double bounds, firstly isolated from shark livers where it accumulates with no apparent damage to the organ [21]. In addition, it is also present in amaranth and virgin olive oils [21]. Squalene is the precursor of sterol biosynthesis in all cell types and is located in the midplane of the lipid bilayer [9]. Moreover, It has been proposed that squalene could be a bioactive compound [17] able to act as antioxidant [30,19,5], anti-inflammatory [6,7,23] or as antineoplastic agent against skin, colon, and lung cancer cells as well as sarcoma [22,16,18,24]. Not surprisingly, some authors have considered it as an effective therapeutic agent in treatment of age-associated disorders where free radicals are a major causative factor [1].

Using APOE-KO mice as model of spontaneous atherosclerosis and fatty liver [3], the administration of dietary squalene decreased atherosclerotic lesion, and in males, this lesion correlated with hepatic fat content, what suggested that squalene administration could be used as a safe alternative to correct hepatic steatosis and atherosclerosis, particularly in males [8]. Using high-throughput approaches, it was found that the normalization of the steatotic liver by squalene was associated with complex mechanisms involving mitochondria and endoplasmic reticulum [19,20]. Recently, it has been shown in HepG2 cells that squalene behaves as a peroxisome

proliferator-activated receptor-α agonist, decreasing cellular triacylglycerol and cholesterol concentrations, while fatty acid uptake was increased [10]. These facts together with that the liver is the main squalene body storage [28,9], make this compound an attractive agent in the field of NAFLD. In adipose tissue, two pools of squalene were shown to exist, one in lipid droplets and other in microsomes [27]. To our knowledge, no study has addressed the subcellular distribution of squalene in the liver and mechanisms controlling those pools. The present endeavor has set up to characterize the subcellular distribution of squalene in two animal models.

Material and methods.

Animals and diets.

During 4 weeks, two groups of 6 male New Zealand rabbits were fed with a chow diet enriched with 1% of sunflower oil for the control group, and with 1% of sunflower oil and 0.5% of squalene for the squalene group. After this period, the rabbits were fasted **for 18 hours and liver was obtained for assays**. The samples were stored at -80 ºC.

The mice experiment used two groups of 17 male APOE-KO, which were fed a purified Western diet for the control group and a purified Western diet supplemented with 0.5% of squalene for the squalene group. After the 10-week diet intervention, and four-hour fast, the animals were killed by suffocation with CO2. The livers were removed, weighed, frozen in liquid nitrogen, and stored at − 80 °C until analysis. Animals were handled and killed observing guidelines (Directive 2010/63/UE) from the European Union for care and use of laboratory animals in research, and the protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza.

Preparation of cellular fractions.

For the preparation of cellular fractions, we used 4 g of pooled livers of each experimental group and animal model and followed the protocol shown in Figure 1 adapted from [11] and [25]. In a Potter, the frozen liver was homogenized in cooled 40 ml of 0.25 M sucrose/5 mM Tris HCl, pH 7.4/ 1.0 mM $MgCl_2$ solution, with protease and phosphatase inhibitors (Sigma-Aldrich, **Saint Louis, MO, USA**), at concentrations according to manufacturer instructions. Homogenized tissue was filtered through sterile gauze, and centrifuged at 280 g for 5 minutes. The pellet was discarded, and the supernatant was centrifuged at 1500 g for 10 minutes, resulting in the pellet, which contained nuclei and membranes, and a supernatant that was again centrifuged at 19000 g for 20 minutes. After this centrifugation, the pellet contained mitochondria, lysosomes and peroxisomes, and the supernatant was centrifuged at 34000 g for 30 minutes. The latter centrifugation step resulted in a new pellet named light microsomes, and a supernatant that was centrifuged at 124000 g for 30 minutes. The

resulting pellet was dubbed heavy microsomes. Both microsomal fractions were diluted until 5 ml with 0.25 M sucrose and 0.015 M CsCl. A discontinuous gradient was generated by adding 7 ml of 1.3 M sucrose and 0.015 M CsCl, added on top of the 5 ml microsomal-containing fraction. The gradient was centrifuged at 237000 g for 120 minutes, and the resulting pellet contained the rough ER (rER) [25]. The **supernatant** was diluted in an equal volume of 0.25 M sucrose, and centrifuged at 124000 g for 60 minutes. The pellet obtained contained the smooth ER (sER). Each obtained sample was immediately frozen in liquid nitrogen, and stored at -80 ºC until sample processing.

Extraction and solid phase extraction (SPE).

In this process, all used chemicals were of CG-MS quality. We used a cellular fraction aliquot equivalent to 0.1 g of tissue, according to weight of samples and volumes used in each step. Samples was transferred to a centrifuge glass tube and homogenized in 1 ml of PBS, and 200 microliters of 932 μM 5α-cholestane in dichloromethane as internal standard for determining the efficiency of the extraction. Samples were extracted with 1 mL of hexane and vortexed for 5 seconds. Then, tubes were centrifuged at 2200 g for 10 minutes, and the organic phase was transferred to a clean tube. To improve the efficiency, this extraction process was repeated twice. The collected organic phases were combined and a further step of purification using SPE was included in order to eliminate other lipids and phospholipids and to improve chromatogram background. This is critical for samples with low squalene concentrations, so the obtained hexane extracts were passed through a SPE cartridge, EFS SiOH (Análisis Vínicos, Tomelloso, **Spain**; 200 mg/3 mL), previously equilibrated with 1.6 mL of hexane. Elution was obtained by adding 1.6 mL of hexane at a constant flow of 1.0-1.5 mL/min. Eluted samples were dried using a N_2 stream in a thermostatic bath at 55 °C. Then, they were dissolved with 200 μL of 72 μM squalane solution in dichloromethane. To facilitate complete dissolution of tube residue, they were sonicated for 3 minutes. Samples were transferred to 150 μL vial insert into a 2 mL vial and were ready for chromatographic analysis.

Gas chromatography-mass spectrometry (GC-MS).

CG analyses were carried out in an Agilent 6890 CG with a 7683B Injector and a 5975B MS acquisition parameter unit (Agilent Technologies, **Santa Clara, CA, USA)**, using a J&W122-5532 column (Agilent) with a nominal length of 30 m and a diameter of 0.25 mm and a helium flow of 1 mL/min. Oven temperature was set up to operate from 280 to 290 ºC in 15 minutes with a ramp from 5 to 13 minutes. Peak identification was done by comparison of the retention times of sample peaks with those of individual standards (squalane, squalene and 5α-cholestane with retention times of 9.8, 11.3, and 12.2 minutes), and the ion mass patron of each compound, using the m/z of 69.1 for squalene, 113 for squalane and 217.2 for 5α-cholestane. The used linear calibration curve to determinate squalene concentration was comprised between 0 and 500 µM. Concentrations of internal standards were 932 and 72 µM for 5α-cholestane and squalane, respectively.

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 and observed using a Nikon microscope. A slide scanner Zeiss AsioScan.Z1 (**Zeiss, Oberkochen, Germany)** was used to record all preparations. Hepatic fat content was evaluated by quantifying the extent of lipid droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of total liver section [8].

Hepatic lipid analyses

Hepatic lipids were extracted from approximately 10 mg of liver. The tissue was homogenized in 1 ml of PBS. The homogenate was twice extracted with 2 mL of chloroform: methanol (2:1). The separated organic phases of each animal were combined and evaporated under N_2 stream. Extracts were dissolved in 100 µL of isopropanol to estimate cholesterol and triglyceride concentrations using commercial kits from ThermoFisher Scientific (**Waltham, MA, USA)** [15].

Statistical analyses

Results are expressed as median and interquartile range. Comparisons were made using the Mann-Whitney U test. Correlations between variables were sought using the Spearman's correlation test. All calculations were performed using SPSS version 15.0 software (SPSS Inc, Chicago, IL, **USA**). Significance was set at $P \leq 0.05$.

Results.

Histological analyses and chemical composition of the rabbit and mouse liver.

Figure 2A shows hepatic features of livers of control and squalene supplemented rabbits. In Figure 2B is displayed the amount of fat expressed as percentage of surface which was close to 5%. No significant differences were observed by the administration of squalene. Likewise, no changes were found for hepatic triglyceride (Figure 2C) and cholesterol (Figure 2D) contents. However, a significant increase in squalene content in those rabbits supplemented with this compound as 0.5% in their diet was observed (Figure 2E).

In Figure 2F two representative micrographs of control and squalene-supplemented APOE-KO mice are shown. As reflected the data of Figure 2G, fat extension in the liver of these mice represented a higher amount (25%) when compared to rabbits (Figure 2B), with no significant difference between control and treated mice. In agreement with this, hepatic triglycerides (Figure 2H) did not experience any significant change. Squalene administration increased significantly the hepatic cholesterol levels (Figure 2I) and squalene content (Figure 2J). The amount of squalene observed in this model was twice the amount present in rabbits receiving the same food content.

Hepatic subcellular distribution of squalene in function of treatment.

In figure 3, the main organelles experiencing changes by the administration of squalene have been represented. A differential pattern was observed according to the model studied. In this regard, treated rabbits accumulated it in the cytosolic, nuclear and plasma membrane, and rough endoplasmic reticulum fractions (Figure 3A). Interestingly, mice, with a higher degree of basal steatosis as above mentioned, showed a preferential increase in lipid droplets, nuclear and plasma membrane, and smooth reticulum fractions (Figure 3B).

Associations among hepatic parameters

To gain insight into the meaning of observed changes, a correlation analysis was carried out among different parameters. As shown in Figure 4 A and C, squalene content was directly associated with hepatic cholesterol content in both studied

models. Equally associated was found the content of triglycerides and fat assayed by the morphometric procedure in mice (Figure 4 B).

Discussion.

The present work has characterized in depth the distribution of squalene in subcellular fractions thanks to an optimization of its assay and using the combined analytical power of gas chromatography and mass spectrometry. Both animal models accumulated squalene in the liver being more sensitive the mouse model. Equally, there was a differential distribution in both models in terms of preferential distribution in endoplasmic reticulum and cytosolic compartments.

Our data using two animal models show clearly that exogenous administration of squalene *in vivo* increases the reservoir in the liver independently of studied model in agreement with classical studies [13]. Besides, the differences in accumulation may be due to experimental settings such as dietary fat percentage in used diets or the models. When we administered squalene in low fat diet to APOE-KO mice, we observed low levels of hepatic squalene [8]. Based on the latter data, it seems plausible that dietary fat content is crucial to facilitate intestinal squalene absorption. Nevertheless, it cannot be rejected the potential role of hepatic adaptation to engorge more squalene when large lipid droplets are present, such is the case of a more severe steatotic liver developed by APOE-KO mice fed Western diet.

The present experiments were designed to reduce the dose of (1 g/kg) squalene previously used [8] in an attempt to reach the moderate dietary intakes reported using virgin olive [21]. It was selected the 0.5 mg/kg because the 0.25 mg/kg dose was not particularly effective in many plasma parameters [5]. However, squalene administration failed to decrease hepatic triglyceride content at the 0.5 mg/kg dose, suggesting that squalene dose seems to be critical regarding this effect.

The distribution of squalene among cytosolic and reticulum in both models is confirmatory for the liver of results observed by Tilvis et al [27] in adipocytes. However, a profound analytical development has unveiled a new source (nuclei and plasma membranes) with important consequences regarding transcriptional changes induced by squalene action [19,20]. This nuclear location could also be the responsible

for the protection of DNA damage reported in MCF10A human mammary epithelial cells [30]. Equally remarkable is the differential accumulation in potential small vesicles isolated from cytosol for rabbits or the large lipid droplet isolated at lower centrifugal force for mice. This fact may be related to the higher amount of squalene in the latter model and it would have required an expansion of lipid droplets in terms of phospholipids and proteins [26]. In yeast, it has been proposed that squalene is lipotoxic if not adequately sequestered in lipid droplets [29]. Surprisingly, it seems that the liver can handle it with ease due to the low toxicity observed in sharks, which accumulate [21].

New unsuspected information gained through meticulous analyses of reticulum adds a complexity that could be species-specific. No much is known for proteins carrying squalene in the aqueous cell environment. In this regard, supernatant protein factor has been proposed as a candidate [2] but how its mechanism of action is not well known. Selective accumulation of squalene in specific domains of reticulum and the positive association with cholesterol content observed in our models (Figure 4) are suggestive of different location of squalene epoxidase, the enzyme using squalene as substrate. Besides, considering the ability of this compound to be intercalated into the layer of membranes [9] is may just merely represent the existence of these specific domains able to accrue it.

In conclusion, the present results provide a suggestive framework to further advance in the knowledge of protective action of squalene in the liver by opening unsuspected locations of squalene in nuclei and the cellular dynamics of squalene between vesicles/lipid droplets and endoplasmic reticulum. These facts warrant further research to unveil them.

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Conflicts of interest: The authors declare no conflict of interest.

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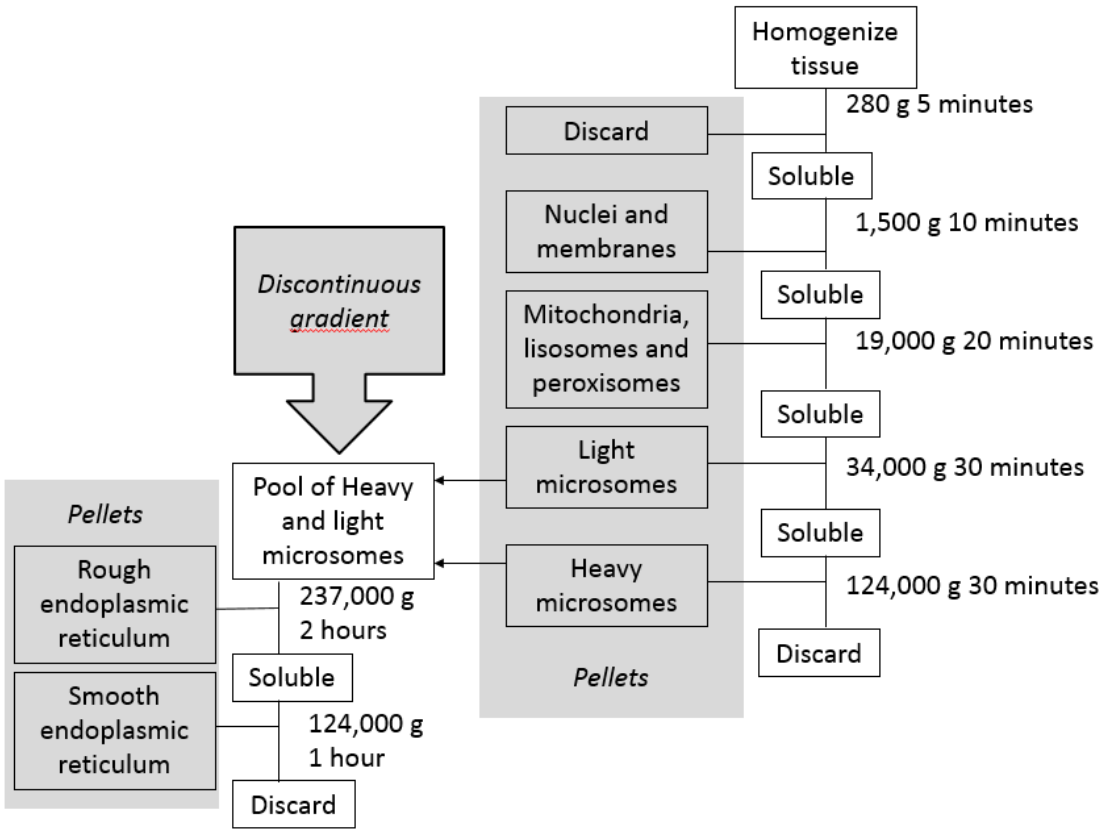
Figure 1. Flow chart displaying steps required to isolate different subcellular fractions.

Figure 2. **Histological analyses and chemical composition of the liver**. A and F, Representative micrographs, bar denotes 20 µm; B and G, hepatic fat expressed as percentage of surface; C and H, hepatic triglycerides mg/ g; D and I, hepatic cholesterol mg/ g and E and J, µg of squalene/ g. Results are shown as median and interquartile ranges **of n=6 for each rabbit group, and n=10 and n=9 for mouse control and squalene groups.** Statistical analysis was carried out by Mann-Whitney U –test. *, $P<0.05$ and **, $P<0.01$.

Figure 3. **Subcellular distribution of squalene in function of treatment**. A, rabbit and B, mouse. Results are shown as average of three analytical determinations of the pooled samples **of n=6 for each rabbit group, and n=10 and n=9 for mouse control and squalene groups.**

Figure 4. **Significant associations among hepatic parameters**. A, relationship between squalene and cholesterol contents in rabbits. B, relationship between triglyceride and morphometric analysis of lipid content in mice. C, relationship between squalene and cholesterol contents in mice.

Fig. 1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Soluble
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Light microsomes
Soluble
124,000 g 30 minutes
Heavy microsomes
Discard
Pellets
Discontinuous gradient
Pool of Heavy and light microsomes
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Soluble
124,000 g 1 hour
Discard
Pellets
Rough endoplasmic reticulum
Smooth endoplasmic reticulum

Fig. 2

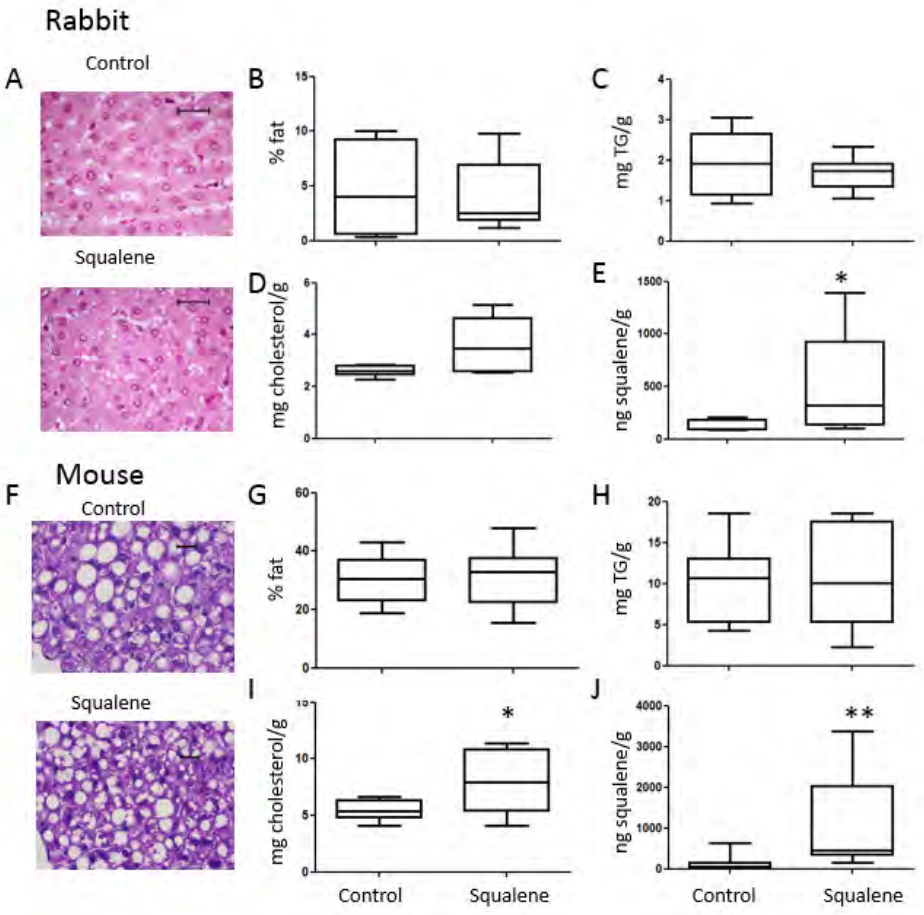
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Fig. 3

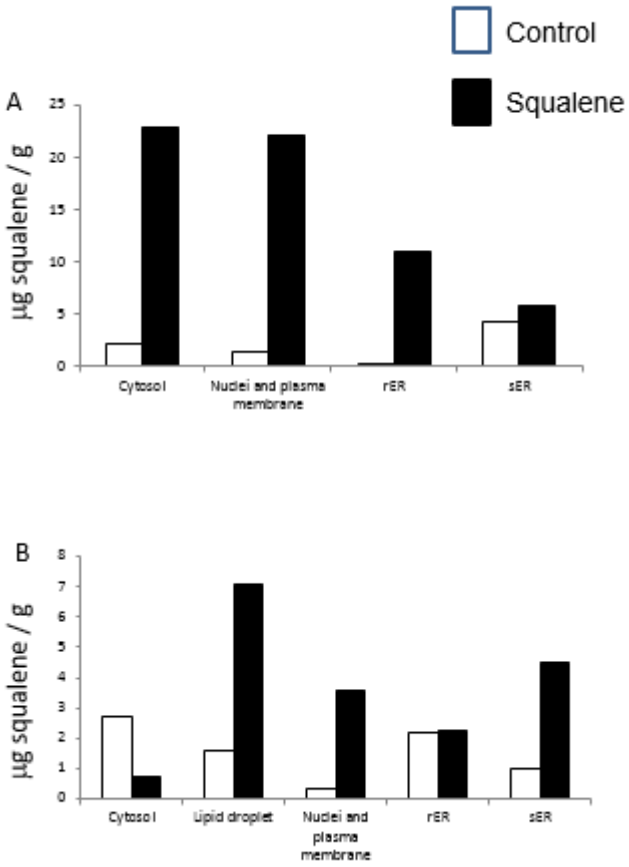
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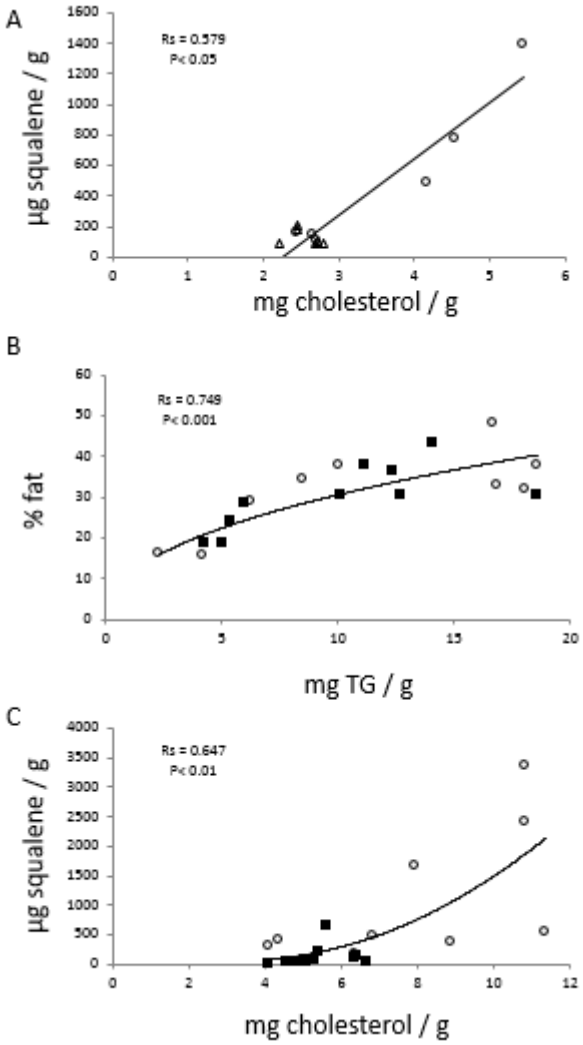
Fig. 4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Rs = 0.579
P< 0.05
µg squalene / g
mg cholesterol / g
B
Rs = 0.749
P< 0.001
% fat
mg TG / g
C
Rs = 0.647
P< 0.01
µg squalene / g
mg cholesterol / g