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The absence of lactation after pregnancy induces long-term lipid accumulation in maternal liver of mice

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ABSTRACT

Aims: The present investigation evaluated whether pregnancy followed by lactation exerts long-term impacts on maternal hepatic lipid metabolism.

Main methods: Female mice were subjected to two pregnancies, after which they were either allowed to breastfeed their pups for 21 days (L21) or had their litter removed (L0). Age-matched virgin mice were used as controls (CTL). Three months after the second delivery, serum was collected for lipid profiling, and fragments of liver were used to assess lipid content and to evaluate the key steps of *de novo* non-esterified fatty acid (NEFA) synthesis, esterification and β-oxidation, very low density lipoprotein (VLDL) assembly and secretion and autophagy.

Key findings: L0 exhibited a significant increase in hepatic TG and reduced apolipoprotein B-100 (ApoB-100) expression. L21 mice had increased ATP citrate lyase (ACLY) activity and reduced acetyl-CoA carboxylase (ACC) phosphorylation but no increased hepatic TG. On the other hand, L21 mice had reduced hepatic sequestosome 1 (SQSTM1/p62) levels. Increased high density lipoprotein (HDL) cholesterol and hepatic apolipoprotein A-1 (ApoA-1) expression were found exclusively in L21.

Significance: The present study reveals that long-term hepatic lipid accumulation is induced by the history of pregnancy without lactation. On the other hand, reduced SQSTM1/p62 levels indicate that increased autophagic flux during life may prevent hepatic fat in dams subjected to lactation. Lactation after pregnancy is also obligatory for a long-term increase in maternal HDL. The present data may contribute to the understanding of the mechanisms leading to elevated cardiometabolic risk in women limited to short periods of lactation.

1. Introduction

The adaptation of maternal metabolism to lactation is an example of integrated regulation that provides proper lipid concentration in the milk. The mammary gland (MG) itself exhibits a high capacity of glucose uptake and *de novo* lipogenesis [1,2]. However, exogenous non-esterified fatty acids (NEFAs) are also esterified within the MG and contribute to triglyceride (TG) milk content. NEFAs can be taken up from the circulating pool or acquired from hydrolysis of TG carried by very low density lipoproteins (VLDLs) and chylomicrons [3].

This anabolic role played by the MG is synchronized to changes in the white adipose tissue (WAT) and liver. The WAT of the lactating mother displays an increased lipolysis rate and reduced lipid incorporation due to reduced lipoprotein lipase (LPL) activity. NEFAs taken up by the liver, instead, are preferentially shunted to re-esterification and then secreted within VLDLs [3]. These integrated changes taking place during lactation are fully dependent on breastfeeding. Premature removal of the litter has immediate impacts on maternal metabolism, such as reduction of insulin-induced glucose clearance, exacerbation of TG storage in the liver and increased lipogenesis in WAT [4,5].

More recently, short or absent lactation has been shown to produce long-lasting consequences for maternal metabolism. Observational studies consistently report an inverse relationship between lactation

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duration and maternal risk for developing Type 2 Diabetes, metabolic syndrome and hypertension later in life [6–11]. Breastfeeding for longer periods is also associated with reduced maternal body weight and visceral adiposity 3 and 7 years after delivery, respectively [12,13]. Not only body composition but also maternal lipid metabolism itself and its cardiovascular impacts are affected by a history of lactation. Women who experience longer lactation periods have a reduced risk for hyperlipidemia and coronary heart disease [14,15].

The long-term consequences of pregnancy and lactation for maternal metabolism have also been explored in rodents. Rats and mice exposed to cycles of pregnancy without lactation have increased adiposity later in life when compared to age-matched females that were allowed to breastfeed their pups after delivery [16,17]. It is not clear, however, whether exposure to lactation after pregnancy also impacts the maternal hepatic lipid metabolism later in life.

The current study was conducted to evaluate multiple aspects related to hepatic lipid metabolism in female mice subjected to repeated cycles of pregnancy with or without lactation. Age-matched virgin females were used as controls, and the key steps that contribute to lipid and cholesterol metabolism and lipoprotein synthesis were assessed.

2. Materials and methods

2.1. Experimental design

Four-week-old male and female C57BL/6J mice were obtained from the Animal Breeding Center at the University of Campinas (CEMIB, Campinas, Sao Paulo, Brazil) and were housed at 22 ± 2 °C under a 12:12 h light:dark cycle (lights on at 7:00 a.m.) with free access to food and water for 5 weeks. All mice received Nuvilab CR-1 standard chow composed by 16.2 g of protein, 1.5 g of fat and 40.5 g of carbohydrates (per 100 g of chow) (Quimtia, Colombo Brazil). This chow contained no added sucrose or glucose. At 9 weeks of age, one group of females was kept virgin to be used as age-matched controls (CTL), and the other group was mated (housing 1 male with two females for 5 days). At this time point, CTL mice were housed as two mice per cage.

After removal of males, mated females were kept in isolated cages until the end of the experiment. At this time point, CTL mice were also isolated. As of the 18th day after the beginning of mating, females were monitored twice a day for the presence of litter. Pregnant mice were either allowed to breastfeed for 21 days (L21) or had the litter removed no longer than 24 h after delivery (L0). The L21 litter was adjusted to 5–6 pups per lactating mother.

A second mating was performed in L0 and L21 mice four weeks after delivery (one week after weaning for the L21 mice). The procedure of litter removal was repeated for L0 mice, and the litter adjustment before a 21-day lactation was repeated for L21 mice. The L0 and L21 mice were used for the experiments 3 months after the second delivery along with age-matched CTL mice.

2.2. Adiposity determination and biochemical analysis

Mice were weighed and euthanized with lethal doses of sodium thiopental ip (80 mg·kg^{-1} body weight). Retroperitoneal and perigonadal fat pads were removed and weighed. The sum of the fat pads weights (perigonadal plus retroperitoneal) was expressed as the percentage of the body weight. Whole body adiposity was measured in anesthetized mice using a dual-energy X-ray absorptiometry (DEXA) system (Discovery Wi QDR Series; Hologic Apex Software, Hologic Inc.).

Trunk blood was removed for serum and cardiac puncture was performed for plasma samples. Both serum and plasma samples were used for colorimetric determination of total cholesterol, triglycerides and HDL with commercial kits (Labtest Diagnóstica S.A., MG, Brazil).

Lipid extraction from liver samples was performed in CHCl3 and methanol (2:1, v/v) using a rotor-stator homogenizer. The homogenates were incubated for 16 h at 4 °C under gentle homogenization in a closed glass tube. After this, a 0.6% NaCl solution was added to the extracts and the mixtures were centrifuged at 2000 ×*g* for 20 min. The organic layer was collected and dried using an Eppendorf Vacuum Concentrator Plus (Eppendorf, Hamburg, Germany). The lipids were solubilized in 200 µl of isopropanol and quantified using commercial kits (Labtest Diagnóstica S.A., MG, Brazil).

Serum samples were not run on all together in the same plate. Serum parameters were, instead, read in two different plates (following samples availability). With the attempt to minimize plate-to-plate variability we systematically use the same standard solutions provided by the kit along the different plates (specifically for these two experimental sets, the same batch was used in the different plates). The absorbance of the standard solution (a 2.26 mM glycerol solution for the triglyceride kit and a 200 mg/dl cholesterol solution for the cholesterol kits) was then used to calculate the serum concentrations.

2.3. Neutral lipid staining by Oil Red O

Liver sections were obtained and processed as previously described [18]. Briefly, liver fragments were frozen in *n*-hexane with liquid nitrogen, and cryostat sections (12 µm) were mounted on glass slides. Glass slides with the sections were incubated with Oil Red O and then rinsed with water. After rinsing, glass slides were coated with water-soluble mounting medium and coverslips. Images were captured under a final magnification of 20× using a bright field Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon FDX-35, Nikon, Tokyo, Japan). Analysis was performed using the free software ImageJ (http://imagej.nih.gov/ij).

Colored images were converted to grayscale, and densitometry was performed on the entire captured field. The section staining and image acquisition were performed in a single experimental round, and analysis was performed assuming an individual background signal for each image. Data are presented as Oil Red O optical density.

2.4. Western blot analysis

Fragments of liver (approximately 100 mg) were removed and processed for western blotting as previously described [19]. The primary antibodies used were anti-phospho-acetyl-CoA carboxylase (ACC) (cat. # 07-303) from Millipore (Temecula, CA, USA), anti-Beclin 1 (BECN1) (cat. # sc-48341) and anti-stearoyl-Coenzyme A desaturase (SCD) (cat. # sc-23016) from St. Cruz Biotechnology (Dallas, TX, USA), anti-fatty acid synthase (FASN) (cat. # 3189) from Cell Signaling Technology (Danvers, MA, USA), anti-sterol regulatory element binding transcription factor 1/2 (SREBP) (cat. # bs-1402R) from Bioss Inc. (Boston, MA, USA) and anti-microtubule associated protein 1 light chain 3 alpha (LC3) (cat. # ab48394) and anti-sequestosome 1 (SQSTM1/p62) (cat. # ab91526) from Abcam (Cambridge, UK). Secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) were used for chemiluminescent detection of the bands through visualization on a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA). Band intensities were quantified by optical densitometry using ImageJ software (http://imagej.nih.gov/ij). The results were normalized to the total protein transferred to the membranes as indicated by Ponceau S staining.

2.5. Enzymatic activity

Liver samples were quickly removed, weighed, washed with ice-cold phosphate-buffered saline (PBS), frozen with liquid nitrogen and ground in a mortar. Powdered samples were maintained at −80 °C until assessment. The activities of L-3-hydroxyacyl-CoA dehydrogenase (β-HAD), carnitine palmitoyltransferase-1 (CPT1) and ATP citrate lyase (ACLY) were measured using spectrophotometric assays following standard methods described elsewhere [20,21].

Table 1
Primers sequences.

Gene	Forward	Reverse
Slc27a5	GCCACACCTCATTTCATCCG	GGTTCCTTCACACACAGCCTG
Dgat2	CAGGTGATCTTTGAGGAGGGTTC	TGATGGGCTTGGAGTAGGGC
Gpam	TCTTCAGAGGCTTCTAGGTCCC	CAGTATGTGGCACTCTCAGCG
Fasn	GAGGGATCTGGTGAAAGC	ACATTTCCTGAAGTTTCCG
Scd2	GTAAACAGCCTGTGCGTTAGC	ACCAAGATGTTCTCCCGAGAG
Srebf1	CAGTCACCAGCTTCAGTCCAGG	CTGCTCAGGTCATGTTGGAAACC
Mttp	GTGGAAAGCAGAGCGGAGAC	CTCGAATTGCCTGAGTGGG
Sec22b	CATTGATAGCCGTGCTCGGAG	CGCATGTTCAAGTACTTCGCATC
Apob100	GAGAGGCTCACCTGGACATC	CCATCAGACTCCTTGTACCTCC
Apoa1	CCTTGAACGAGTACCACACCAGG	GCATGGGCATCAGACTATGGC
Lcat	TCTCTACGGTGTGGGCAGAC	TGGGCAGCAAATGTACGG
Abca1	GGCAATTCGCAAACTGGG	CTCCTTGACAATGCTTAGGGC
Hmgcr	GGGACCAACCTTCTACCTCAGC	CACAGTGCCACATACAATTCGG
Ldlr	GACACTGTACTGACCACCCAG	CTGCTCCTCATTCCCTCTG
Srp14	TGTCTGTTGAGAGCCACGGATG	GCCTGTCACTGTGCTGGTTTGC
Actb	TCAAGATCATTGCTCCTCCTG	GCTCAGTAACAGTCCGCCTAG
Gapdh	AAGGTGGTGAAGCAGGCATC	GAAGGTGGAAGAGTGGGAG

Solute carrier family 27 member 5 (*Slc27a5*), diacylglycerol *O*-acyltransferase 2 (*Dgat2*), glycerol-3-phosphate acyltransferase, mitochondrial (*Gpam*), fatty acid synthase (*Fasn*), stearoyl-Coenzyme A desaturase 2 (*Scd2*), sterol regulatory element binding transcription factor 1 (*Srebf1*), microsomal triglyceride transfer protein (*Mttp*), SEC22 homolog B, vesicle trafficking protein (*Sec22b*) Apolipoprotein B-100 (*Apob100*), apolipoprotein A-I (*Apoa1*), lecithin cholesterol acyltransferase (*Lcat*), ATP-binding cassette, sub-family A (ABC1), member 1 (*Abca1*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*), low density lipoprotein receptor (*Ldlr*), signal recognition particle 14 (*Srp14*), actin beta (*Actb*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Table 2
Zoometric and biochemical characteristics of mice subjected to pregnancy with or without lactation.

	CTL	L0	L21
Body weight (g)	26.0 ± 0.8 (19)	26.2 ± 0.05 (19)	26.8 ± 0.4 (16)
Relative fat pads weight (%)	2.4 ± 0.3 (19)	2.3 ± 0.3 (19)	2.1 ± 0.2 (16)
Whole body adiposity (%)	11.7 ± 0.9 (5)	11.7 ± 1.8 (7)	9.7 ± 0.9 (7)
Triglycerides (mg/dl)	60.2 ± 6.9 (19)	70.8 ± 5.7 (19)	60.9 ± 7.4 (16)
Cholesterol (mg/dl)	74.6 ± 4.0 (19)	86.2 ± 4.6 (19)	91.9 ± 9.4* (16)
HDL (mg/dl)	43.5 ± 2.4 (19)	55.4 ± 3.5 (19)	58.6 ± 4.8* (16)

L0: mice subjected to two pregnancy cycles without subsequent lactations. L21: mice subjected to two pregnancy cycles followed by 21 day lactation periods. CTL: age-matched virgin mice. Data are presented as mean ± SEM (n).
* $P < 0.05$ *vs*. CTL.

2.6. RNA extraction and real-time PCR

Fragments of liver were homogenized in TRIzol (50 mg/ml), and the total RNA was extracted and quantified using a Nanodrop 8000 device (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using 2 μg of total RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time amplifications were performed using KAPA SYBR Fast DNA polymerase (Kapa Biosystems, Boston, MA) in a Step One Plus sequence detection system. Primer sequences are shown in Table 1. Values of mRNA expression were normalized using the geometric mean of three internal control genes (*gapdh*, *actb* and *srp14*). Fold changes were calculated by the $2^{-\Delta\Delta CT}$ method.

The mean variations of the Ct values of *srp14* were 0.24 for L0 and 0.7 for L21 (relative to CTL). The mean variations of the Ct values of *gapdh* were 0.54 for L0 and 0.64 for L21 (relative to CTL). The mean variations of the Ct values of *actb* were 0.83 for L0 and 1.09 for L21 (relative to CTL).

2.7. Statistical analysis

All results are presented as the mean ± SEM. Parametric distribution was checked using the Shapiro-Wilk test. Comparisons of parametric data were performed using one-way ANOVA followed by a Tukey-Kramer *post hoc* test. Non-parametric data were compared using the Kruskal-Wallis test (GraphPad Prism - Graph Pad Software, Inc., San Diego, USA). P values < 0.05 indicated significant differences.

3. Results

3.1. Repeated cycles of pregnancy with lactation lead to long-term increased maternal high-density lipoprotein (HDL) levels

We initially assessed body weight, relative fat pads weight and whole body adiposity in L0 and L21 mice 3 months after the second delivery and in age-matched CTL. None of these parameters were altered among the groups. Lipid profiles were also assessed 3 months after the second delivery. At this time point, L0, L21 and CTL mice exhibited similar levels of serum TG, but L21 had increased levels of total cholesterol ($P = 0.04$) and HDL cholesterol ($P = 0.01$) when compared to CTL (Table 2). Plasma samples from cardiac puncture were also analyzed. The plasma concentrations of TG of CTL ($n = 5$), L0 ($n = 7$) and L21 ($n = 7$) were, respectively, 45.8 ± 3.5, 42.2 ± 3.5 and 53.2 ± 7.5. The plasma concentrations of total cholesterol of CTL ($n = 5$), L0 ($n = 7$) and L21 ($n = 7$) were, respectively, 77.9 ± 2.8, 80.3 ± 3.6 and 89.3 ± 2.5. The plasma concentrations of HDL cholesterol of CTL ($n = 5$), L0 ($n = 7$) and L21 ($n = 7$) were, respectively, 35.3 ± 2.7, 28.6 ± 4.4 and 49.5 ± 5.5.

As observed in serum samples, plasma TG values were not different among the groups. In opposition to what was found in serum samples, plasma total cholesterol values were not increased in L21. On the other hand, plasma levels of HDL from L21 were consistently increased when compared to those from L0 ($P = 0.01$).

3.2. Repeated cycles of pregnancy without lactation result in maternal hepatic lipid accumulation later in life

Lipid staining by Oil Red O was performed on liver samples from L0 and L21 mice 3 months after the second delivery and in age-matched CTL. L0 mice exhibited increased levels of hepatic lipid content when compared to either CTL or L21 (54% higher than in CTL and 60%

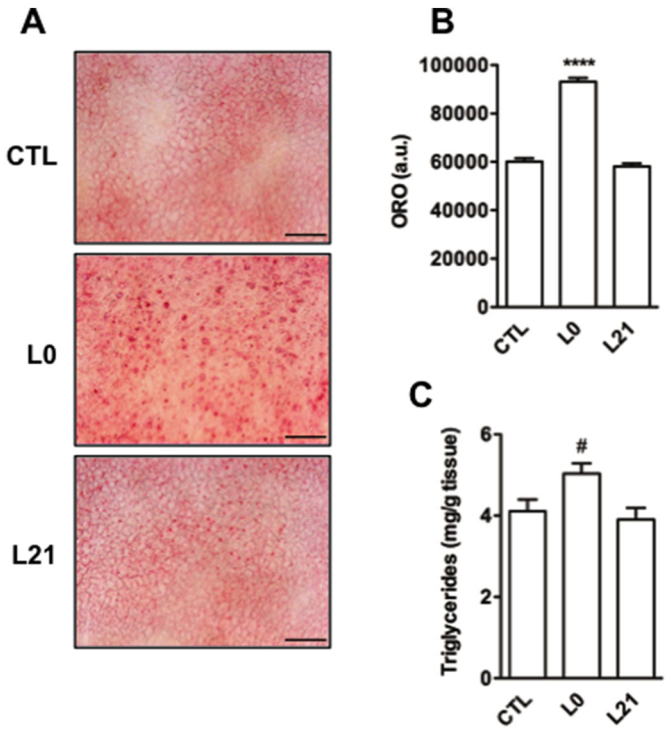

Fig. 1. Hepatic triglyceride (TG) content in CTL, L0 and L21 mice. Liver samples were processed for neutral lipid staining with Oil Red O. Images were acquired under a 20× magnification. Horizontal bars correspond to 100 μm (A). Quantification was performed using ImageJ software (B). Additional samples were subjected to lipid extraction and colorimetric TG determination (C). Data are presented as the mean ± SEM. CTL (n = 8 for Oil Red O and n = 5 for TG), L0 (n = 10 for Oil Red O and n = 7 for TG), L21 (n = 7). ****P < 0.0001 *vs.* CTL and L21; #*P* < 0.05 *vs.* L21.

higher than in L21; P < 0.0001) (Fig. 1B). Colorimetric determinations after lipid extraction also revealed increased TG levels in liver of L0 mice (28% higher than L21; P < 0.05) (Fig. 1C).

3.3. Biochemical changes leading to increased NEFA synthesis are seen exclusively in the liver of mice subjected to repeated cycles of pregnancy with lactation

Aiming to elucidate the mechanisms leading to long-term increased lipid storage in the liver of L0 mice, we evaluated the hepatic signaling pathways associated with *de novo* lipogenesis. The mRNA expression and the protein content of SREBP 1/2, a key transcription factor that controls the expression of key enzymes involved in the synthesis of NEFAs [22], were not altered in either L0 or L21 (Fig. 2B and E).

We also evaluated the following four enzymes involved in NEFA synthesis: FASN, SCD, ACC and ACLY. Similar levels of *fasn* and *scd2* mRNAs (Fig. 2A and C) and FASN and SCD protein contents (Fig. 2D and F) were found in the liver of L0 and L21 mice 3 months after the second delivery and in age-matched CTL. ACC was evaluated for serine phosphorylation, which is known to inhibit its activity and lead to increased hepatic NEFA synthesis [23]. Our experiments revealed that ACC phosphorylation was reduced in the liver of L21, but not in L0, when compared to CTL (30% lower than in age-matched CTL; P = 0.04) (Fig. 2G). ACLY activity was increased exclusively in the liver of L21 mice (45% higher than in age-matched CTL; P = 0.01) (Fig. 2H).

3.4. The activity of enzymes that mediate NEFA β-oxidation is not reduced in the liver of mice subjected to repeated cycles of pregnancy without lactation

As no changes in the parameters associated with *de novo* lipogenesis were detected to explain the prominent increase in lipid storage observed in the liver of L0 mice, we next assessed potential changes in the activities of β-HAD and CPT1, two limiting steps for NEFA β-oxidation [24,25]. Our experiments revealed, however, that liver samples from CTL, L0 and L21 mice exhibited similar levels of β-HAD and CPT1 activities (Fig. 3A and B).

3.5. The expression of genes associated to NEFA uptake and esterification are not increased in the liver of mice subjected to repeated cycles of pregnancy without lactation

As no evident long-lasting changes in the biochemical machinery controlling NEFA synthesis and oxidation were detected to justify the increased lipid content observed in the liver of L0 mice, we tested the hypothesis that the absence of lactation would interfere with the expression of enzymes that mediate NEFA esterification.

The expression levels of diacylglycerol *O*-acyltransferase 2 (*dgat2*) and mitochondrial glycerol-3-phosphate acyltransferase (*gpam*) were not altered in the liver of L0 and L21 3 months after the second delivery when compared to age-matched CTL (Fig. 4A and B). Similarly, we did not detect differences in the expression of solute carrier family 27 member 5 (*Slc27a5*), the gene that encodes the fatty acid transporter FATP5, in liver samples from CTL, L0 and L21 (Fig. 4C).

3.6. Mice subjected to repeated cycles of pregnancy with lactation have reduced hepatic levels of SQSTM1/p62 later in life

Autophagy has been recently associated to the progression of several metabolic diseases including non-alcoholic fatty liver disease (NAFLD) [26]. We thus evaluated the levels of three proteins that are involved in the autophagic flux, BECN1, LC3 and SQSTM1/p62.

Although we have found no differences in BECN1 and LC3 levels (Fig. 4D and E), hepatic SQSTM1/p62 content was reduced in L21 when compared either to CTL (50% lower) or to L0 (34% lower) (P < 0.05) (Fig. 4F).

3.7. Mice subjected to repeated cycles of pregnancy without lactation display reduced expression of apob100 later in life

Hepatic synthesized TGs are usually packed in VLDL to be secreted. We therefore investigated apolipoprotein B-100 (*apob100*), microsomal triglyceride transfer protein (*mttp*) and SEC22 vesicle-trafficking protein homolog B (*sec22b*) expression to identify possible changes in the cellular machinery that mediates VLDL assembly and secretion.

Although we found no changes in the expression of *mttp* and *sec22b*, *apob100* was reduced in L0 compared to age-matched CTL (39% lower; P = 0.02). Such reduction was not detected in L21 mice (Fig. 5A, B and C).

3.8. Mice subjected to repeated cycles of pregnancy with lactation display increased expression of apoa1 later in life

In order to clarify the mechanism by which L21 mice had increased HDL levels 3 months after the second delivery, we assessed the expression of genes associated with HDL synthesis and the flux of cholesterol.

We found no differences among the three groups in the hepatic expression of lecithin cholesterol acyltransferase (*lcat*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*hmgcr*), low density lipoprotein receptor (*ldlr*), and ATP-binding cassette transporter A-1 (*abca1*) (Fig. 6A, B, C and D). When compared to CTL, the expression of

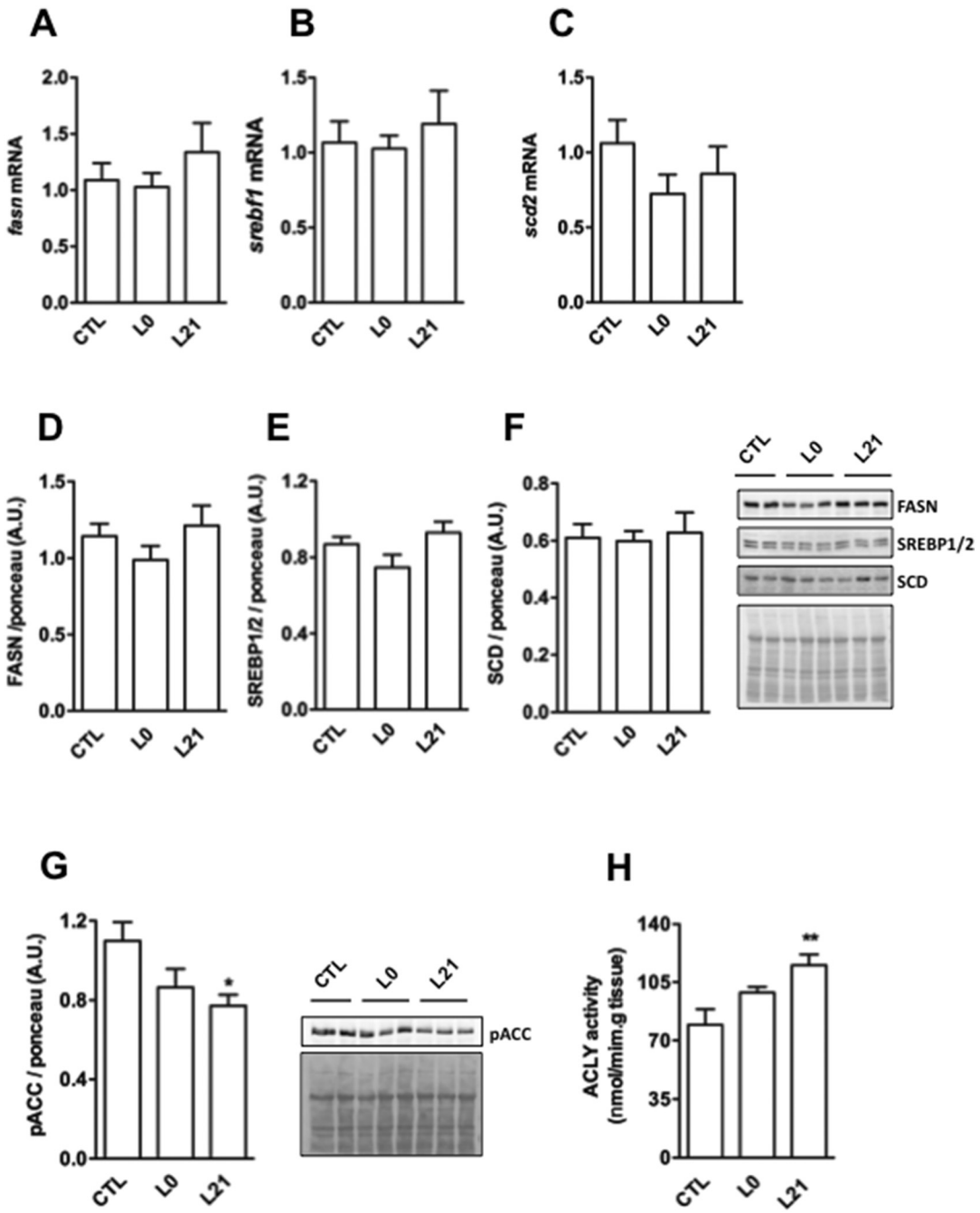

Fig. 2. Enzymatic machinery associated to *de novo* lipogenesis in CTL, L0 and L21 mice. Liver samples were processed for mRNA quantification of *fasn* (A), *srebf1* (B) and *scd2* (C). Western blot was performed to evaluate FASN (D), SREBP1/2 (E), SCD (F) and phosphorylated ACC (pACC) (G). Target proteins were normalized by Ponceau S stained blot. Samples were also processed for measurement of ACLY enzymatic activity (H). Data are presented as the mean ± SEM. CTL (n = 8), L0 (n = 9 for qPCR and n = 10 for western blot and enzymatic activity), L21 (n = 7). *P = 0.04 *vs.* CTL; **P = 0.01 *vs.* CTL.

apolipoprotein A1 (*apoa1*) was increased in the liver of L21 (65% higher; P = 0.04) but not in L0 mice. No differences were found between L0 and L21 (Fig. 6E).

4. Discussion

The present study demonstrates that the history of pregnancy without lactation in mice results in long-term lipid accumulation in the maternal liver. Such outcome was not detected in mice allowed to breastfeed their progeny. This phenomenon was not previously described and adds relevant information to the literature that consistently reports that women who do not breastfeed their babies are prone to develop multiple components of metabolic syndrome later in life [8–15].

NAFLD, which is characterized by excessive lipid accumulation in the liver, is by itself a risk factor for Type 2 Diabetes [27], an outcome already reported to be inversely correlated with the duration of lactation [6,7]. Notably, the increase in the risk for Type 2 Diabetes seen in patients with NAFLD is independent of being overweight/obese [27]. NAFLD *per se* is also a risk factor for nonalcoholic steatohepatitis (NASH), which may progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma [28]. Although the present investigation did not address aspects related to the morphological alterations related to liver inflammation and progression to NASH, it provides detailed

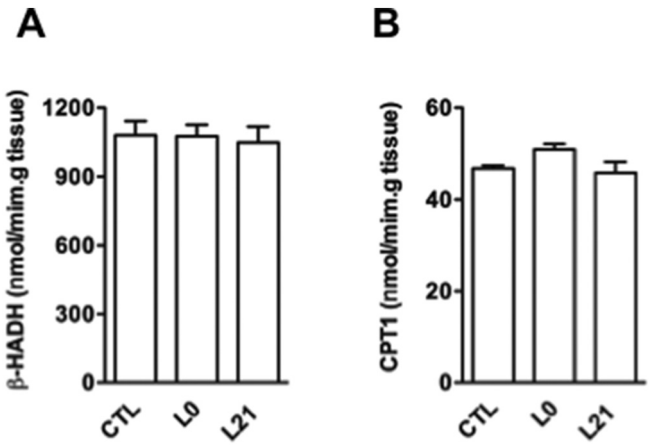

Fig. 3. Activity of enzymes involved in fatty acid β-oxidation in CTL, L0 and L21 mice. Liver samples were frozen and processed for measurement of β-HAD (A) and CPT1 (B) enzymatic activities. Data are presented as the mean ± SEM. CTL (n = 8), L0 (n = 10), L21 (n = 7).

biochemical background to elucidate the mechanism underlying increased lipid content in liver of mice not allowed to lactate their progeny. Whether mice not allowed to breastfeed their progeny are prone to spontaneously develop NASH later in life is an aspect that certainly deserves further investigation.

Increased flux of NEFAs from adipose tissue is one common cause of hepatic lipid accumulation. The greater adiposity of obese individuals leads to an elevation of NEFA flux to non-adipose tissues such as the liver. Visceral adiposity is particularly relevant for this adaptation because visceral fat depots display low rates of NEFA re-esterification in response to insulin [29].

The present results reveal that mice exposed to two repeated cycles of pregnancy with or without lactation do not develop increased whole-body adiposity (as revealed by DEXA) or increased relative weights of fat pads (retroperitoneal plus perigonadal). In comparison to the already published data, our results do not substantially differ of those obtained from carcass composition of Osborne-Mendel rats subjected to 3 cycles of pregnancy without lactation [16]. On the other hand, CD-1 mice subjected to one cycle of pregnancy without lactation and analyzed by computed tomography were described as having increased visceral, but not subcutaneous, adiposity two months after delivery when compared to mice that breastfed their pups for 3 weeks after delivery [17]. Although our data were obtained in a different strain of mice (C57BL/6J), it is important to emphasize that the methods presently used were different and, therefore, data are not directly comparable. Apart from the differences among different animal models, our data suggest that long-term hepatic lipid accumulation in C57BL/6J mice with a history of pregnancy without lactation is unlikely to result from exacerbated flux of NEFAs from increased fat depots to the liver.

De novo synthesis of NEFAs is an additional biochemical route that can lead to hepatic lipid accumulation. Liver cells are specialized in converting pyruvate derived from glycolysis into NEFAs in response to the nutritional status. ACLY, FASN, ACC and SCD2 comprise limiting steps for this biochemical route that originates NEFAs to be esterified and packed into VLDL [30]. The genes that encode these enzymes are under the transcriptional control of SREBP1 [22]. ACC activity is also under post-transcriptional control by inhibitory serine phosphorylation [23].

The *de novo* NEFA synthesis contributes to approximately 26% of the amount of TG stored in the liver of obese patients with NAFLD [31]. Unexpectedly, we found increased ACLY activity and reduced ACC phosphorylation in liver of L21, but not in L0 mice. These data show that pregnancy followed by lactation induces an upregulation of biochemical pathways leading to *de novo* NEFA synthesis that does not result in hepatic TG accumulation later in life.

With the attempt to elucidate the mechanism protecting liver of L21 mice from a long-term accumulation of lipids, we next assessed crucial steps related to autophagy. Defective autophagic flux has been suggested to play a role in several metabolic diseases, including NAFLD [26]. Degradation through the autophagic process depends on the initial formation and nucleation of autophagosomes through a BECN1-dependent process. The elongation of its membrane with subsequent closure of the autophagosomes can be mediated my multiple pathways, including those dependent on LC3 [32]. The polyubiquitin-binding protein SQSTM1/p62 is delivered to autophagosomes after its sequestration by LC3 [33].

Although we have found no differences in BECN1 and LC3 protein content, SQSTM1/p62 levels were reduced in liver of L21 mice. Notably, it has been previously described that autophagic dysfunction characterized by suppression of autophagic proteolysis and long-lasting accumulation of SQSTM1/p62 occurs in liver of patients with NAFLD [34]. Experimental studies have also shown that pharmacological activation of autophagy in hepatocytes treated with palmitate resulted in reduced SQSTM1/p62 content and triglycerides accumulation [35]. Accumulation of SQSTM1/p62 and impaired autophagic flux have also been described in liver of mice fed with high fat diet [36]. On the other hand, autophagy was reported to mediate hepatic lipid droplets degradation during starvation [37]. Thus, reduced SQSTM1/p62 levels in liver of L21 mice suggest that mice allowed to breastfeed after pregnancies may experience increased hepatic autophagy during life. Such hypothesis may explain why L21 mice, although displaying slight increased ACLY activity and reduced ACC phosphorylation, fail to develop steatosis later in life.

Interestingly, mice subjected to pregnancy not followed by lactation exhibited an evident hepatic TG accumulation later in life but no conciliating upregulation of *de novo* NEFA steps or impaired autophagic flux that could explain such adaptation.

In addition to *de novo* NEFA synthesis, modulation of the biochemical pathways that control NEFA esterification or β-oxidation can potentially affect hepatic lipid content [38,39]. However, we found no changes either in the mRNA levels of *gpam* and *dgat2* or in the activity of CPT1 and β-HAD that could explain the increase in TG content in the liver of L0 mice. Similarly, L0 mice do not exhibit increased expression of the fatty acid transporter *slc27a5*.

We than evaluated whether changes in the mRNA expression of genes related to VLDL assembly and secretion would possibly explain the increased hepatic lipid content in L0. Nascent VLDLs containing ApoB-100 are formed within the ER lumen through a process dependent on MTTP. Before secretion, nascent VLDLs are transferred towards the Golgi apparatus by the snare protein SEC22b [40]. Although we found no differences in *mttp* and *sec22b* expression, L0 mice exhibited reduced levels of *apob100* expression in parallel with increased hepatic lipid content.

Notably, it has already been described that siRNA-mediated knockdown of *apob100* mRNA leads to hepatic steatosis in mice. Importantly, these authors have also shown that there is an inverse correlation between *apob100* mRNA expression and liver TG content [41]. Other studies have consistently demonstrated that *in vivo apob100* inhibition with siRNA in mice led to increased TG accumulation in the liver [42,43]. Increased hepatic TG was also found in *apob100* knockout mice [44]. Reduced *apob100* mRNA expression was also suggested as a mechanism to explain hepatic steatosis in ATF6 KO mice [45]. Clinical trials also revealed that chronic pharmacological inhibition of hepatic *apob100* also leads to hepatic steatosis in humans [46–48]. We thus propose the suggestion that reduced *apob100* expression in the liver of mice that did not breastfeed their pups may impair the liver's ability to secrete VLDL, resulting in increased TG accumulation.

The present data also show that a history of pregnancy followed by lactation has a long-term effect hallmarked by an increase in maternal HDL cholesterol levels in serum and plasma samples. Serum HDL levels

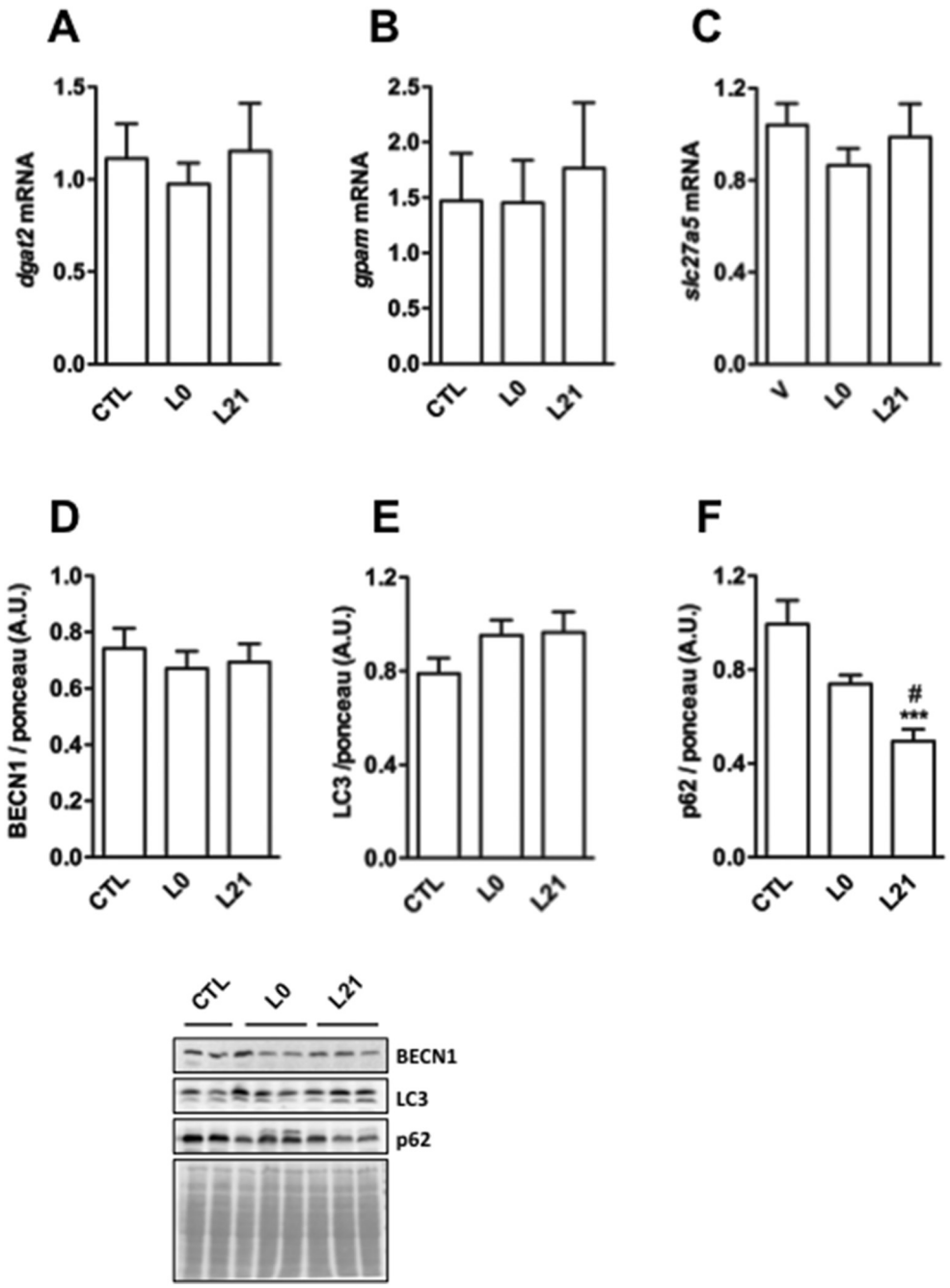

Fig. 4. Evaluation of enzymes involved in fatty esterification and uptake and proteins involved in autophagy in CTL, L0 and L21 mice. Liver samples were processed for mRNA quantification of *dgat2* (A), *gpam* (B) and *scl27a5* (C). Western blot was performed to evaluate BECN1 (D), LC3 (E) and SQSTM1/p62 (p62) (F). Target proteins were normalized by Ponceau S stained blot Data are presented as the mean ± SEM. CTL (n = 8), L0 (n = 9 for qPCR and n = 10 for western blot), L21 (n = 7). ***P < 0.001 *vs.* CTL; #P < 0.05 *vs.* L0.

of L21 mice were higher than those of CTL while plasma HDL levels of L21 mice were higher than those of L0. It is possible that differences between plasma and serum results might have been caused by the presence of tissue fluids in serum samples as these were obtained from trunk blood.

The changes in HDL were not accompanied by an increase in the expression of *abca1*, a transporter that mediates the transport of cholesterol to nascent HDL, or *lcat*, an enzyme responsible for cholesterol esterification within the HDLs. The expression of *apoa1*, instead, was increased in the liver of L21 mice. It is possible, therefore, that increased *apoa1* mRNA in L21 mice plays a role in their higher HDL levels. ApoA1 itself is the major protein component of HDL [49]. Changes in *apoa1* mRNA were already reported to correlate with changes in its protein content [50,51] and mice knockout for this gene were also described to have reduced HDL levels in parallel to reduced *apoa1* mRNA in the liver [52]. ApoA1 also play a crucial role by activating LCAT and thus increasing the amount of cholesteryl esters within HDL, what contributes to stimulate cholesterol efflux from the tissues [53].

Accordingly, it was already reported that changes LCAT activity are not obligatory followed by changes in its mRNA. Hyperhomocysteinemia caused by deletion of cystathionine β-synthase in mice was described to reduce plasma ApoA1 and LCAT activity without changing hepatic LCAT mRNA [54]. In addition, the CAST strain of mice, known to spontaneously develop low HDL levels, has

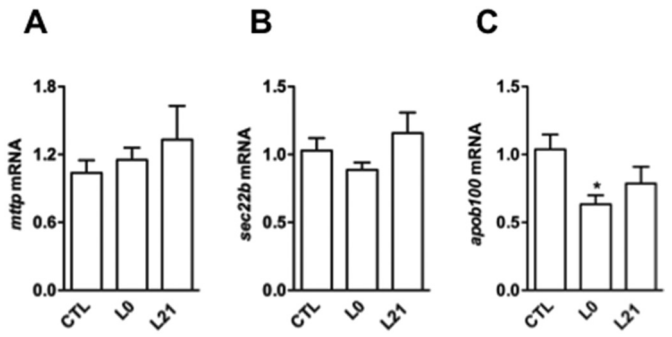

Fig. 5. Evaluation of genes involved in VLDL production in CTL, L0 and L21 mice. Liver samples were processed for mRNA quantification of *mttp* (A), *sec22b* (B) and *apob100* (C). Data are presented as the mean ± SEM. CTL (n = 8), L0 (n = 9), L21 (n = 7). *P = 0.02 *vs.* CTL.

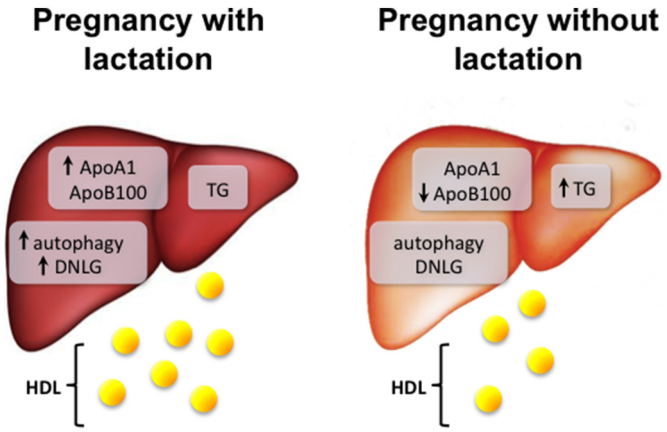

Fig. 7. Molecular changes associated to triglyceride and cholesterol metabolism in liver of mice exposed to lactation or not allowed to lactate after delivery. Arrows indicate upregulated or downregulated steps. Circles represent HDL particles. Apolipoprotein B-100 (ApoB100), apolipoprotein A-I (ApoA1), triglycerides (TG), *de novo* lipogenesis (DNGL).

reduced LCAT activity with unaltered LCAT mRNA [55]. Thus, although *lcat* expression was unchanged in L21 mice, we can also hypothesize that increased *apoa1* can putatively upregulate LCAT activity in these mice, further contributing to their higher HDL cholesterol levels.

5. Conclusion

The present study reveals that long-term hepatic TG accumulation is induced by the history of pregnancy exclusively in mice not allowed to breastfed their progeny. Concordantly, mice that did not breastfeed their progeny exhibited reduced *apob100* expression. Mice that were allowed to lactate showed increased *de novo* NEFA synthesis but also reduced levels of SQSTM1/p62, suggesting that increased autophagic flux during life may have prevented hepatic fat accumulation. Our data also suggest that the history of pregnancy is able to upregulate hepatic *apoa1* expression and HDL levels exclusively in mice that underwent lactation. A summary of the findings is shown Fig. 7. Altogether, the present study may contribute to understanding the mechanisms leading to elevated cardiometabolic risk in women exposed to short periods of lactation.

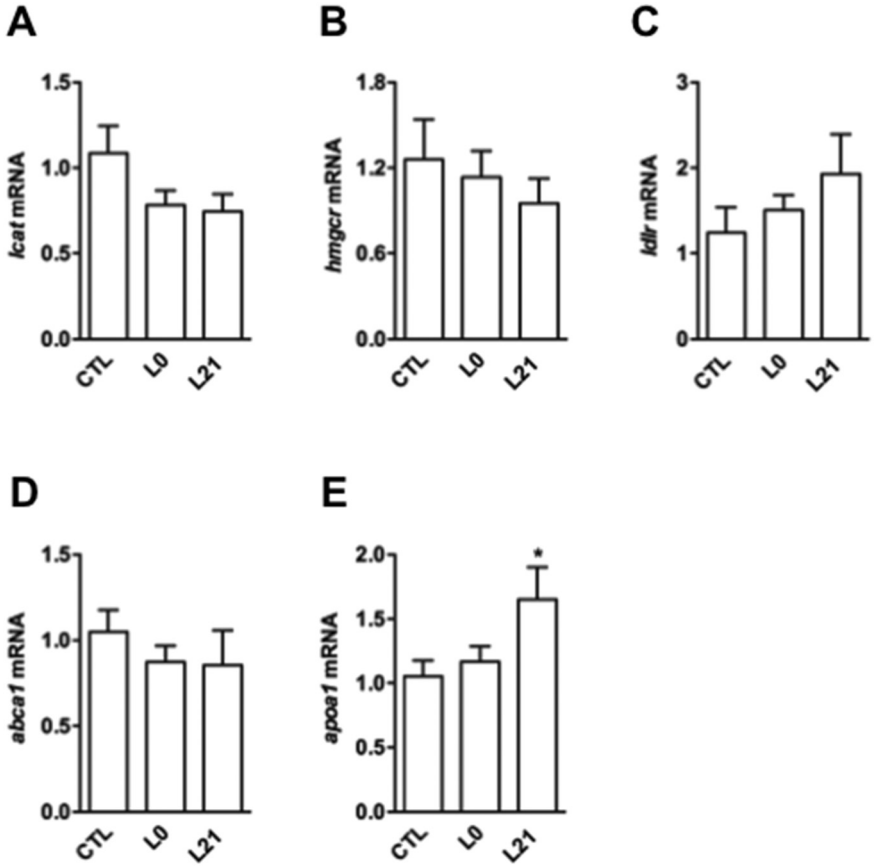

Fig. 6. Evaluation of genes involved in HDL production in CTL, L0 and L21 mice. Liver samples were processed for mRNA quantification of *lcat* (A), *hmgcr* (B), *ldlr* (C), *abca1* (D) and *apoa1* (E). Data are presented as the mean ± SEM. CTL (n = 8), L0 (n = 9), L21 (n = 7). *P = 0.04 *vs.* CTL.

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Conflict of interest

The authors declare no conflicts of interest.

Authors' contributions

J.M.V. and G.F.A. designed the experimental protocols. J.M.V., C.J.T., D.N. de S., J.C.S-S., F.S.F., I.G.A., F.S.S. and G.M. performed the experiments. J.M.V., C.J.T., D.N. de S., J.C.S-S., F.S.F., I.G.A., F.S.S., G.M and G.F.A. analyzed the data. S.B. and G.F.A. wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval

The experimental procedures were approved by the State University of Campinas Committee for Ethics in Animal Experimentation (protocols No. 4496-1, No. 3711-1 and No. 4863-1) and were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation.

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