

# Association of liver steatosis with lipid oversecretion and hypotriglyceridaemia in C57BL/6j mice fed *trans*-10,*cis*-12-linoleic acid

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**Abstract** Conjugated linoleic acids (CLA) have recently been recognized to reduce body fat and plasma lipids in some animals. This study demonstrated that the steatosis accompanying the fat loss induced by *trans*-10,*cis*-12-C<sub>18:2</sub> (CLA2) and not *cis*-9,*trans*-11-C<sub>18:2</sub> (CLA1) isomer in C57BL/6j mice was not due to an alteration of the liver lipoprotein production that was even increased. The 3-fold decrease in plasma triacylglycerol contents and the induction of mRNA expression of low-density lipoprotein receptors concomitantly observed in CLA2-fed mice suggested an increase in the lipoprotein clearance at the level of the liver itself. CLA1 feeding produced similar but attenuated effects on triglyceridaemia only.

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**Key words:** Liver steatosis; Conjugated linoleic acid; Lipoprotein; Triacylglycerol; Fatty acid; Low-density lipoprotein receptor

## 1. Introduction

During the last years, considerable attention has been focused on conjugated linoleic acids (CLA), a group of dienoic isomers of linoleic acid, that have been shown to exhibit unique properties such as anti-carcinogenic [1,2], anti-atherogenic [3] and body fat reducing [4,5] effects in animal models. Two CLA isomers, *cis*-9,*trans*-11-C<sub>18:2</sub> (CLA1) that has been found in ruminant meat and dietary products, and *trans*-10,*cis*-12-C<sub>18:2</sub> (CLA2), were used in approximately equal amounts in commercial mixtures administered for most of the physiological studies (for a review see [6]). More recently with the use of purified CLA isomers, evidence has accumulated that CLA2 was the isomer actually altering body fat mass [7], while both CLA1 and CLA2 were found to be responsible for cancer prevention [8,9]. However, in addition to the reduction of the total body fat mass reported with CLA

mixtures in rats, chickens [10] and mice [4] and with CLA2 in mice [5] and even men [11], a dramatic enlargement of liver with steatosis was shown to develop during CLA2 feeding [12,13].

The reduction of body fat mass by CLA mixtures has been associated with a decreased activity of lipoprotein lipase (LPL) of adipose tissues and with an increased activity of total carnitine palmitoyltransferase in periepididymal adipose and muscle tissues [4]. Adipocyte apoptosis has also been evoked [12].

Liver steatosis is often associated with obesity, diabetes, hyperinsulinemia and very low-density lipoprotein (VLDL) overproduction [14]. The hyperinsulinaemia met in CLA2-fed mice [13] would cause the greater level of gene expression of liver enzymes related to fatty acid (FA) uptake and to lipogenesis, which would explain, at least in part, the setup of the steatosis, as it has been shown in the hyperinsulinaemic fa/fa Zucker rats [15]. Besides, an alteration of VLDL secretion rates could also result in liver fat accumulation as described in suncus [16]. However, little is known about the effects of CLA on liver lipoprotein secretion. Recent studies indicate that CLA2 reduced both apoB synthesis and triacylglycerol (TAG) secretion in HepG2 cells [17,18], but the potential lipid-lowering effects of CLA in various species remain confused since decreases or increases in plasma TAG levels in rabbits, rats and hamsters have been observed [19–22].

Owing to the pivotal role of the liver in the lipid partitioning, the aim of the study was to investigate, in C57BL/6j mice, the individual effects of the main two purified CLA isomers on body and lipid parameters, on the capacity of the liver to secrete VLDL, on activity and mRNA expression of proteins involved in VLDL clearance and FA esterification.

## 2. Materials and methods

### 2.1. Animals and treatment

7 weeks old C57BL/6j male mice (Harlan, Gannat, France) were housed by pools of four animals in plastic cages. After 1 week of adaptation to the control diet (see below), they were randomly allocated to a basal diet containing, in g per kg: casein, 180; corn starch, 460; sucrose, 220; cellulose, 20; mineral mixture, 50; vitamin mixture, 10; sunflower oil, 49; linseed oil, 1 (for the detailed composition of mineral and vitamin mixtures, see [23]). The three experimental diets ( $n = 16$  for each) were obtained by enrichment with 10 g of either of the following FA esterified as TAG: C<sub>18:1</sub> n-9 (oleic acid; control series), *cis*-9,*trans*-11-C<sub>18:2</sub> (CLA1 series) or *trans*-10,*cis*-12-C<sub>18:2</sub> (CLA2 series). After 4 weeks the animals used in the fed state were anesthetized with isoflurane and divided into series of eight mice for each

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**Abbreviations:** CLA, conjugated linoleic acid; DGAT, diacylglycerol acyltransferase; FA, fatty acid; HL, hepatic lipase; LDL-R, low-density lipoprotein receptor; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; SREBP, sterol regulatory element binding protein; TAG, triacylglycerol; VLDL, very low-density lipoprotein

diet. The first series was intended to plasma analysis, then to the determination of VLDL-apoB secretion; the second series was devoted to sampling of liver and periepididymal adipose tissue that were quickly blotted with absorbent paper and frozen in liquid nitrogen for further analysis.

## 2.2. Lipid analysis

Total liver lipids were extracted according to Folch [24] and determined by gravimetry, then TAG contents were quantified by the thin-layer chromatography-flame ionization detector (TLC-FID) method [25]. Plasma TAG concentrations were determined using a commercial kit (Sigma-Diagnostics, Saint-Quentin-Fallavier, France).

## 2.3. Determination of VLDL-apoB secretion by the liver

Shortly after anesthesia, about 0.3 ml of blood was collected from the tail vein as the control sample. Animals were then injected via a jugular vein with 100 µl of 10% Triton WR-1339 in saline solution to block VLDL degradation [26] and blood samplings were performed every 30 min over 90 min. Serum samples were obtained from blood after a clotting time of 30 min at room temperature and a centrifugation of 5 min at 6500×g. ApoB concentrations were determined using a diagnostic kit from Sigma. The control sample (collected before Triton WR-1339 injection) was also used for TAG and apoB basal level measurements.

## 2.4. Determination of heart and liver lipolytic activities

The protocol used was that of Iverius and Ostlund-Lindquist [27]. Heart and liver samples were homogenized in a cold buffer (1/10, w/v) containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA), 12 mM deoxycholate and 10 immunizing units (IU)/ml heparin. The supernatants obtained after centrifugation at 12000×g for 20 min at 4°C were half diluted with the same buffer deprived of deoxycholate and heparin. Lipolytic activities determined on tissue homogenates corresponded to amounts of [<sup>3</sup>H]oleic acid released from radiolabeled triolein in the following procedure: a sample of 100 µl of homogenate supernatant was added to mixtures containing 0.1, 0.5, 1 or 2 mM triolein emulsified with tri(9,10-<sup>3</sup>H)oleoyl)glycerol (Perkin Elmer, Life Sciences Inc., Boston, MA, USA) (0.22 µCi/assay), 5% gum arabic, 0.2 M Tris-HCl (pH 8.6), 10% heat-inactivated chicken serum as the LPL activator and 1 or 0.1 M NaCl (see below), in a final volume of 200 µl. The incubation was carried out at 37°C for 1 h and the amount of [<sup>3</sup>H]oleate released through lipase action was measured by scintillation counting after liquid-liquid extraction [28]. Hepatic and cardiac LPL activities were calculated by subtracting total lipase activity performed in 0.1 M NaCl media from LPL non-specific lipolytic activity performed in 1 M NaCl media [29]. LPL activity was expressed as nmol of oleate released per h per mg of supernatant protein. As the hepatic LPL activity was practically undetectable, lipolytic activities were attributed to hepatic lipase (HL). The protein concentration was determined through the bicinchoninic acid procedure using bovine serum albumin (BSA) as a standard [30].

## 2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was extracted from liver by the Tri-Reagent method adapted from the procedure of Chomczynski and Sacchi [31]. Tri-Reagent was provided by Euromedex (Souffelweyersheim, France). Gene expression was evaluated by semi-quantitative RT-PCR from 1 µg of total mRNA using Access RT-PCR System from Promega (Madison, WI, USA). Reverse transcription and cDNA fragment am-

plification were performed in a Hybaid thermocycler (Omnigene Bio-products Inc., MA, USA). The sequences of the sense and antisense primers designed using 'Primers!' software and synthesized by MWG-Biotech AG (Ebersberg, Germany) were for LPL: 5'-CTAAG-GACCCCTGAAGACACA-3' and 5'-TCTCATACATTCCTCGT-TACCGT-3', for HL: 5'-GTGAATGTGGGGTTAGTGGAC-3' and 5'-ACTTCGCAGATTCCTCCAGC-3', for low-density lipoprotein receptor (LDL-R): 5'-CACCTCCGCTGCAAATCATC-3' and 5'-GTGAATGCAGGAGCCATCTG-3', for diacylglycerol acyltransferase (DGAT): 5'-GTATCCTGAATTGGTGTGTGGT-3' and 5'-TAGGGGTCCTTCAAAAACAGAG-3', and for β-actin: 5'-AAT-CGTGCGTGACATCAAAG-3' and 5'-GAAAAGAGCCTCAGGG-CAT-3'. The number of amplification cycles was determined according to a kinetic profile. Control tubes containing no RNA template were used for contamination checking. RT-PCR products were resolved in a 1.7% agarose gel with ethidium bromide and band intensities were measured by densitometric analysis with a Gel Doc 2000 ultraviolet gel documentation system equipped with 'Quantity One' software (Bio-Rad S.A., Ivry-sur-Seine, France). For every gene studied, β-actin was concomitantly amplified and used for normalization.

## 2.6. Statistics

Differences in mean values between groups were tested by one-way analysis of variance (ANOVA). Significant differences between means were tested by Student's *t*-test for an independent variable. When variances and number of rats were unequal, means were tested by a Kruskal-Wallis non-parametric test.

## 3. Results

### 3.1. Body and lipid parameters

As already reported [5], the body weights of CLA2-fed mice were significantly lower than those of controls and CLA1-fed ones at the end of the experimental period (Table 1). As regards the relative weights of some organs, there was no difference between the hearts of mice fed any of the three diets assayed. CLA2-fed mice were however shown to exhibit a marked regression of the periepididymal adipose tissue and a concomitant enlargement of liver. The increase in the total weight of the liver was in fact associated with a severe fat accumulation, total lipids containing 90 and 60% of TAG in the CLA2 and control series, respectively. The above parameters appeared to be similar in control and CLA1-fed mice. Table 1 also shows that plasma TAG concentrations were reduced after CLA1 feeding and still more after the CLA2 diet (3-fold less) by reference to the control diet. Plasma apoB content underlined the fact that lipoprotein concentration was reduced in CLA2-fed mice, but not in CLA1-fed ones.

### 3.2. Hepatic VLDL secretion

In order to determine whether the hepatic steatosis of CLA2-fed mice resulted from a reduced liver lipoprotein out-

Table 1  
Effects of purified CLA isomers on body, relative tissue weights and lipid parameters in mice

	Control	CLA1	CLA2
Body weight (g)	25.7 ± 0.9	24.7 ± 0.7	23.3 ± 0.6*
Liver			
Mass (% of body weight)	4.7 ± 0.1	4.9 ± 0.1	7.5 ± 0.3***
TAG (mg/g of wet tissue)	8.6 ± 1.6	9.4 ± 2.4	64.9 ± 6.7***
Periepididymal adipose tissue			
Mass (% of body weight)	1.2 ± 0.1	1.1 ± 0.1	0.2 ± 0.1***
Plasma			
TAG (g/l)	1.04 ± 0.15	0.64 ± 0.03*	0.34 ± 0.03***
ApoB (g/l)	0.081 ± 0.012	0.103 ± 0.019	0.038 ± 0.005**

Data are means ± S.E.M. (*n* = 6). Versus control, \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

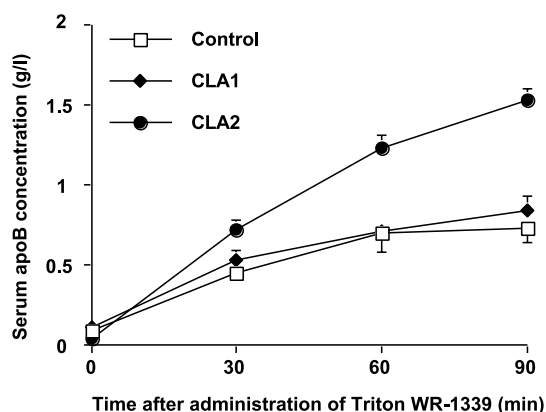


Fig. 1. Effect of CLA on liver VLDL-apoB secretion rate. Plasma ApoB concentrations were determined after intravenous injection of Triton WR-1339. Data are means ( $n=8$ ) and T-bars indicate the S.E.M. All values obtained with CLA2-fed mice were significantly different from those with control mice at  $P<0.01$ .

put, the secretion rate of VLDL was evaluated *in vivo* through the inactivation of LPL [26]: Triton WR-1339 was reported to inhibit LPL activity and to impair consequently the VLDL particle clearance. Under these conditions, as VLDL were no more taken up by cells, their secretion rates were shown to be about proportional to the increase in plasma VLDL-apoB concentration [32].

The data obtained using this procedure (Fig. 1) show that the ability of the total liver to secrete VLDL was practically two times greater in CLA2-fed mice than in controls. However, as the liver of CLA2-fed mice was itself 1.6 times as heavy as in controls, the VLDL secretion rate appeared to be finally about 20% greater per g tissue than in the other two series. Collectively, these results indicate that the liver TAG accumulation in CLA2-fed mice could not be attributed to a decreased VLDL secretion and that the VLDL overproduction from the enlarged liver was however insufficient to eliminate the flux of FA entering the whole esterification and lipoprotein assembling/secretion pathway.

### 3.3. Heart and liver lipolytic activities

VLDL oversecretion and lower plasma TAG content should imply elevated rates of TAG-rich lipoprotein removal essentially performed through LPL activities. In adipocytes cultured in the presence of CLA2, the LPL activity was however demonstrated to be inhibited [7] and we show that the LPL activity in a energy-demanding organ as the heart was unaltered in mice fed any of the CLA isomers checked (Fig. 2a). In the liver, the LPL activity was extremely weak and of about comparable extent in the three series (data not shown). Meanwhile, the HL activity was found to be insensitive to either CLA treatment when expressed per mg protein (Fig. 2b), but in CLA2 series the activity was practically of doubled extent when taking into account the total liver weights by reference to those of control mice.

### 3.4. mRNA expression

It has been shown that, in control mice, some genes, such as FA translocase or adipocyte lipid binding protein, poorly represented in liver and highly expressed in white adipose tissues, were markedly induced in the liver after CLA2 treatment [13]. This could also be the case for the LPL gene that was ex-

pressed to a far greater extent in adipose tissues than in the liver, all the more because LPL might be involved in the clearance of VLDL at the hepatic level in CLA2-fed mice. However unexpectedly, no alteration of the liver LPL mRNA expression was noticeable in either CLA isomer-fed mice, as compared to controls (Fig. 3). The same possibility was investigated with HL whose activity was shown above to be unchanged in the three series. The expression of HL mRNA was found to be comparatively of the same extent for all the diets (Fig. 3). However, as specific mRNA levels were normalized by reference to those of  $\beta$ -actin and not per total liver, LPL and HL mRNA expressions and activities were suggested to be greater in the total liver of CLA2-fed mice.

As compared to the expression of LPL and HL mRNA, the expression of hepatic LDL-R was demonstrated to be signifi-

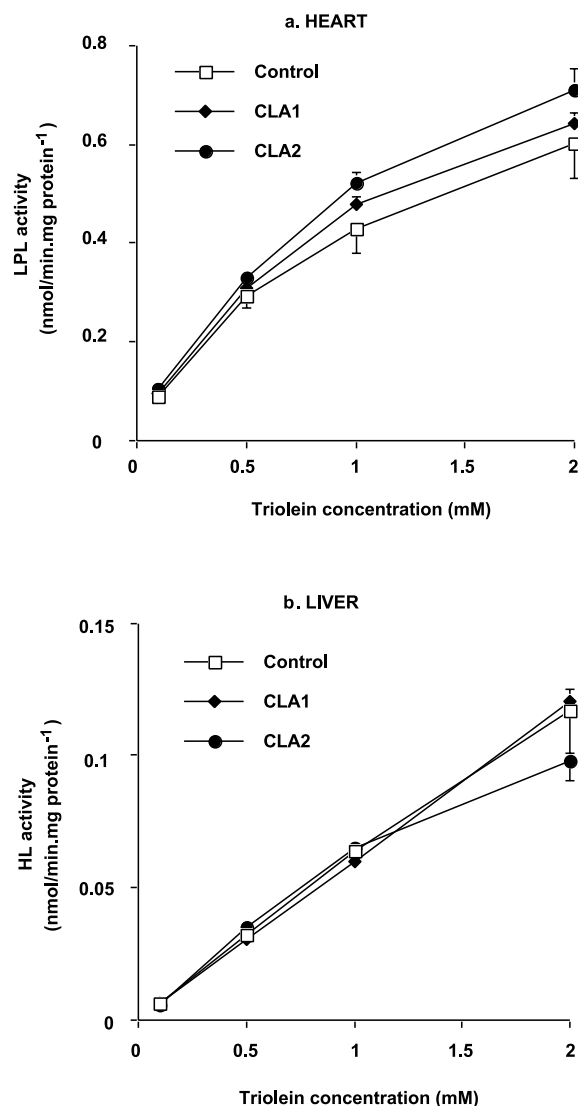


Fig. 2. Effects of triolein concentration on heart and liver lipolytic activities in control and either CLA-fed mice. LPL and HL activities were determined using heart and liver homogenate supernatants, respectively (see Section 2). Results are means ( $n=6$ ) and are expressed as nmol of free FA released from [ $^3$ H]triolein per mg protein; T-bars indicate the S.E.M. For all the range of concentrations tested, no significant difference in enzyme activity was observed between control and CLA-fed mice at  $P<0.05$ .

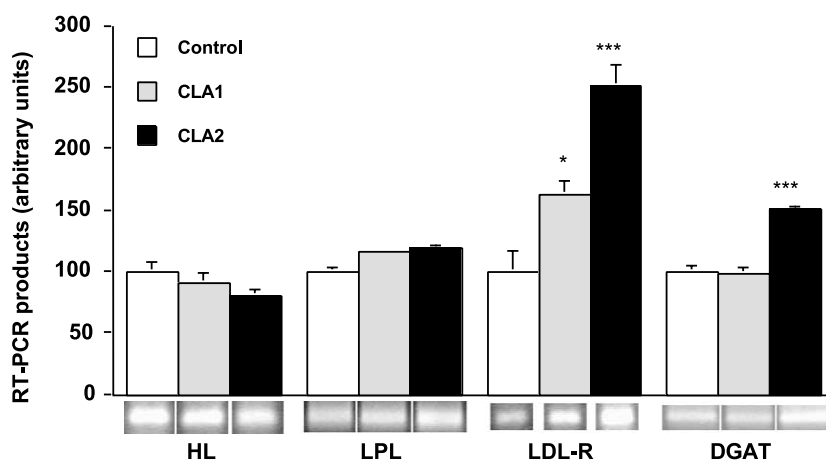


Fig. 3. RT-PCR analysis of gene expression in the liver of control and either CLA-fed mice. Each reaction was performed in triplicate. Results are expressed as RT-PCR product relative abundance in CLA-fed mice as compared to controls (=100) after normalization to the levels of  $\beta$ -actin mRNA. Data are means ( $n=3$ ) and T-bars indicate the S.E.M. Versus controls, \* $P<0.05$ ; \*\*\* $P<0.001$ .

cantly induced in mice fed either CLA isomer. Concomitantly, mRNA level of DGAT, that is the last enzyme of the TAG biosynthesis, was increased in CLA2-fed mice only (Fig. 3).

#### 4. Discussion

The explanations regarding the relations between the setup of the liver steatosis and blood lipids in mice fed diets containing mixtures of CLA or purified CLA2 are very scarce and still confused. For instance, CLA mixtures have been reported to reduce plasma lipids in rabbits [19], in hamsters [3] and more particularly the plasma VLDL fraction in rats [20]. Meanwhile, purified CLA2 was shown to increase plasma VLDL-TAG in hamsters [22]. In HepG2 cells, CLA2 was recently demonstrated to suppress TAG secretion and to increase cell TAG contents [17].

##### 4.1. VLDL secretion

In the present study, the underlying issue was addressed for the first time *in vivo* by monitoring the secretion rate of hepatic VLDL in mice fed the two CLA isomers used in most studies. The major finding of the investigation was that the liver VLDL output was increased after CLA2 treatment without concomitant hypertriglyceridaemia, the plasma TAG concentrations being even markedly lowered. The estimation of liver VLDL secretion rate was performed using Triton WR-1339 which inhibited LPL activities and therefore the blood clearance of circulating lipoproteins [26] as well as various TAG-rich particle emulsions [33]. The first conclusion of the present work was that the liver steatosis did not result from an impairment of VLDL assembling/secretion.

##### 4.2. Blood lipid clearance

The lowering of plasma TAG concentrations was unexpected in CLA2-fed mice as regards the oversecretion of hepatic VLDL, which suggested that the VLDL clearance should be greatly increased in these mice as compared to controls. LPL activities of extrahepatic organs were factors capable of improving this clearance. However, this possibility was unlikely because LPL activity was found to be low in cultured 3T3-L1 adipocytes treated with CLA2 [7] and owing to the absence of increased LPL activity in the heart (our

study) that represents a very efficient organ using preferentially FA as oxidative substrates. The non-involvement of organs known to use very actively FA led to suggest that the liver was itself mostly responsible for the blood lipid clearance in CLA2-fed mice. However, the activity (see Section 3) and the level of mRNA expression (Fig. 3) of the hepatic LPL were found to be very low and unmodified by any of the CLA studied. No change was also observed for the activity and expression of the HL (Figs. 2 and 3) involved in LDL-R-related protein pathways (for a review see [34]). In CLA2-fed mice, the 1.6-fold greater liver mass has to be taken into account, which should increase about proportionally LPL and HL activities of the whole liver. The pathway mediating the direct uptake of VLDL and lipoprotein remnants deserves much more consideration because of the marked elevation of the LDL-R mRNA expression (Fig. 3). Indeed, the level of expression of the LDL-R gene has been shown to depend on the sterol regulatory element binding protein-1a (SREBP-1a) [35] that was reported to activate genes encoding enzymes of cholesterol and FA synthesis [35] and to be induced in the liver of CLA2-fed mice [13]. Taken collectively, the facts indicate that CLA2-fed mice resembled transgenic mice overexpressing the nuclear form of SREBP-1a since both metabolic situations resulted in enlargement of liver due, at least in part, to lipid accumulation and marked decrease in periepididymal adipose tissue mass [35]. The role of the LDL-R in the VLDL clearance was conversely demonstrated by the disruption of the LDL-R gene in the transgenic mice, which caused an increase in plasma cholesterol and TAG concentrations [36].

##### 4.3. Liver steatosis

Liver fat accumulation and increased blood lipid clearance likely resulted from the imbalance between the slightly greater VLDL secretion and the very active internalization of lipoprotein remnants. Following the lipolysis of remnants in hepatocytes, FA released must be therefore directed towards the esterification pathway. On short term perfusion experiments, normal livers were shown to be capable of esterifying large amounts of FA [37], suggesting that the increase in DGAT mRNA expression in the liver of CLA2-fed mice (Fig. 3) corresponded to an actual metabolic requirement for meeting



the excessive flux of FA. The inhibition of fat deposit into peripheral adipose tissues [7] compelled blood lipids to circulate more actively from liver and intestine to liver and FA user organs. The flux of lipids permanently cleared from blood and taken up more abundantly by a heavier liver was possibly the starting point of the observed inductions.

As CLA1 and CLA2 have been reported to be potent ligands for peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) [38], the underlying regulations might imply the activation of PPAR $\alpha$ , which was shown to repress apoptosis [39] and therefore to favor liver enlargement. The effects of CLA2 were however preserved in PPAR $\alpha$  null mice [40], indicating a complex effect of this CLA isomer on the liver lipid metabolism. Interestingly, CLA1 was shown to share with CLA2 the plasma lipid-lowering effect (Table 1) and the increase in LDL-R gene expression (Fig. 3). The absence of lipoatrophy in CLA1-fed mice strongly suggests that the CLA2 effect primarily affected adipose tissues with further consequences disturbing liver lipid metabolism.

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