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Original article

Liver lipid metabolism disruption in cancer cachexia is aggravated by cla supplementation -induced inflammation

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SUMMARY

Background & aims: The liver is the main organ regulating metabolism. In spite of that, few studies examine liver metabolism in cachexia, a wasting syndrome associated with increased morbidity and mortality in cancer. Cachexia induces major metabolic disruption, inflammation and fat and lean mass loss. We have previously shown impairment of hepatic lipid metabolism in cancer cachexia that contributes to the aggravation of the symptoms. The present study addresses the effects of Conjugated Linoleic Acid supplementation upon liver lipid metabolism in cachectic rats. Methods: Male Wistar rats were randomly assigned to control groups (C) receiving 0.9 NaCl (Placebo –CP); or to groups supplemented with sunflower oil (CSF), supplemented with CLA (CCLA), or still, to tumour bearing animals (T) receiving NaCl (TP), sunflower oil (TSF), or CLA (TCLA). Supplementation (0.5 ml) by gavage was carried out for 14 days. Body weight, dietary intake, glucose, cholesterol and triacylglycerol plasma content, liver glycogen and triacylglycerol content and mRNA expression of liver carnitine palmitoyltransferase I and II (CPT I and II), as well as microsomal triglyceride transfer protein (MTP), liver fatty acid-binding protein (L-FABP), peroxisome proliferator-activated receptor-alpha (PPAR-alpha), and apolipoprotein B (apoB), were assessed.
Results: Liver CPT II activity was reduced in all groups, when compared with CP. Hepatic mRNA expression of MTP, apoB and FABP was reduced in TCLA, when compared with all groups. TCLA also presented increased hepatic and plasma triacylglycerol content, when compared with all T groups. Adipose tissue-derived inflammatory factors were assessed. No differences among the groups were observed in regard to Retro Peritoneal Adipose Tissue cytokine (IL-1β, IL-6, and TNF-α) protein content and expression, with the exception of IL-10 in tumour-bearing animals. In the Epididymal Adipose Tissue, the inflammatory cytokines were augmented in TCLA, compared with all other groups.
Conclusion: CLA supplementation fails to promote the re-establishment of hepatic lipid metabolism in tumour-bearing animals, and therefore is not recommended in cancer-related cachexia.

1. Introduction

Cancer Cachexia is a paraneoplastic syndrome characterised by weight loss, progressive skeletal muscle and adipose tissue wasting, fatigue, and anorexia, and affects around 80% of all hospitalised cancer patients, representing the direct cause of death of up to 40% of the cases. Cachexia associated chronic systemic inflammation induces marked disruption of intermediary metabolism, both in animal models and cancer patients [1–4,62].

The rat Walker 256 carcinosarcoma is the most studied experimental model of cancer cachexia, as it induces similar symptom to those of human cachexia [6]. Previous studies [5,6] have shown that in one such model, the liver, the central organ controlling intermediary metabolism, suffers many morphological and functional

Abbreviations: CLA, conjugated linoleic acid.

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alterations during cachexia. Nevertheless, despite its unequivocal importance, few studies examine cachexia associated - hepatic symptoms. In the Walker 256 cachectic model, liver capacity for oxidation of fatty acids and ketone body production is decreased [5,6], while oleate incorporation in the organ lipid stores increased, and VLDL assembly disrupted [7]. The cachectic animals present higher circulating VLDL levels, a consequence of decreased peripheral tissue uptake and of disrupted hepatocyte metabolism. All these changes markedly contribute to the aggravation of cachexia, as the energy deficit imposed by the Cori cycle between tumour and liver may not be prevented by hepatic fatty acid oxidation derived ATP [7].

Conjugated Linoleic Acid (CLA) is a common nomenclature adopted for a family of positional isomers of linoleic acid (C18:2 n-6) employed in nutritional therapy, which has been associated with beneficial effects on hepatic lipid metabolism, and modulation of circulating lipids [8–12]. A study performed by Purushotam (2007) [9], reported CLA supplementation to attenuate the development of hepatic steatosis and enhance lipid oxidation in the organ, due to activation of transcriptional factors involved in liver lipid metabolism, such as PPAR-α. Nevertheless, other studies [10,11] have found deleterious responses, or no response at all to CLA supplementation, is regard to liver lipid metabolism modulation. This contradiction arises probably due to differences in the isomer of CLA adopted, animal species, patient characteristics, gender, supplementation time, dose and age.

Since many authors report positive results with CLA supplementation in respect to liver lipid metabolism, the aim of the present study was to investigate whether CLA treatment would be able to prevent liver steatosis and hepatic lipid metabolism disruption in cancer cachexia. As CLA has also been associated with decreased adiposity, and lipid profile modulation [12–14], we also examined its effect on three different adipose depots.

2. Material and methods

2.1. Animals

Male adult Wistar rats (160–250 g) were obtained from the Institute of Biomedical Sciences, University of São Paulo. They were maintained under controlled temperature conditions (23 ± 1 °C), in metabolic cages, under a 12 h light/12 h dark cycle (lights on at 7:00 a.m.). The animals received water and food (commercial chow, Nuvilab®, Nuvital, Brazil) *ad libitum*. We assessed weight and food intake daily, always in the afternoon period. All procedures were carried out in accordance with the ethical principles stated by the Brazilian College of Animal Experimentation, (protocol number 041, 2004) and were approved by Biomedical Sciences Institute/USP Ethical Committee for Animal Research.

2.2. Experimental design

Animals were initially divided into two groups (control and cancer). Walker 256 tumour cells (2×10^7 cells) were injected into the right flank of the animals subcutaneously in cancer groups [19]. Control rats received saline (0.9 NaCl) injections on the same day of tumour inoculation (C). The two groups were re-distributed within six groups, according to the type of supplementation (sunflower, CLA or saline), which started on the first day after tumour inoculation and was maintained for 14 days. The groups were divided as described below (Fig. 1):

On the 14th day post tumour cells inoculation, rats from all groups were killed by decapitation, in the interval between 8:00 and 11:00 a.m. The liver, the gastrocnemius muscle, the adipose tissues (EAT, RPAT and MES) and the tumour were excised after euthanasia and weighted. Food intake was measured and the average intake calculated. Caloric and macronutirent intake was estimated from declared values provided by the companies providing chow, sunflower oil and CLA.

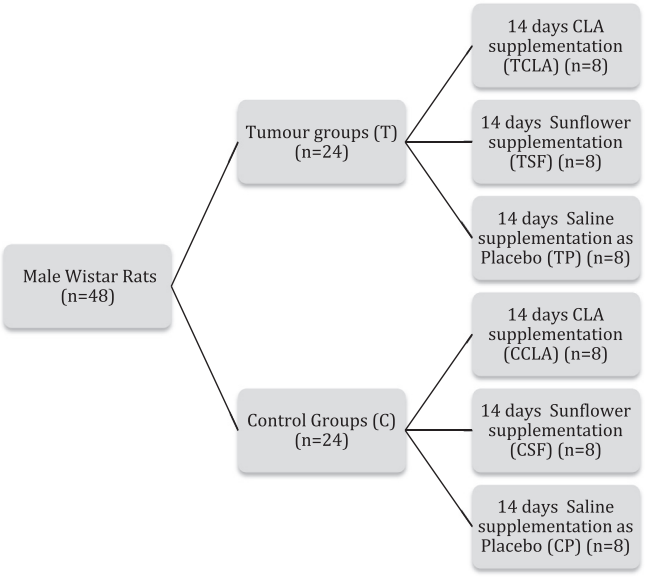


Fig. 1. Animal groups.

2.3. Supplementation protocol

All rats received 0.5 mL intragastrical supplementation (2% w:w of total food intake) of either CLA (tonalin®) (CLA and TCLA groups), commercially available SUNFLOWER oil (SF and TSF groups) or saline solution – 0.9% NaCl, (C and T groups). Supplementation started on the same day of tumour inoculation. Sunflower oil, rich in linoleic acid, was employed as a control for CLA, in order to isolate the effect of increased PUFA ingestion from that of those of specific types of fatty acids. Sunflower oil is the most adopted control for CLA supplementation [20–22].

2.4. Analysis of gene expression

Fragments of 100 mg from the liver of cachectic and control rats were obtained and total RNA was extracted with TRIZOL®, as described previously [23]. RNA concentration was determined by assessing absorbance (wave length), using a spectrophotometer (Beckman DU 640, Fullerton, CA, USA).

The estimation of the concentration of CPT I, CPT II, FABP, PPAR – α, apoB and MTP mRNA was assessed by real time reverse transcriptase-polymerase chain reaction method. A 33 μl assay mix containing 3 μg RNA, 10 units of placental RNAse inhibitor, 2 μl oligo (dt), 2 μl dNTP (10Nmol), 2 μl DTT, 10 units of Moloney-murine leukaemia virus reverse transcriptase (Invitrogen, USA), and 4 μl 10× reaction buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 150 mM $MgCl_2$ in nuclease-free water) were employed to produce cDNA. This mixture was incubated at 80 °C for 3 min, 21 °C for 10 min, 42 °C for 30 min and then 99 °C for 10 min respectively. A 1% agarose and ethidium bromide gel was employed after loading 2 μl of the final product to verify the quality of the reaction. cDNA was amplified employing real time polymerase chain reaction (PCR) with SYBR® green (Applied Biosystems). For the PCR reaction, primers were designed from sequences published by Genebank, shown in Table 1.

Table 1
List of primers.

Gene	Sequence 5'–3'
CPT I	Fw: CCGAGCTCAGTGAGGACCTA
NM_031,559	Rev: ATCTGTTTGAGGGCTTCGTG
CPT II	Fw: GAGCCCCTAGTAGGCCCTTA
NM_012,930	Rev: AGGCTTCTGTGCATTGAGGT
FABP	Fw: ACCTCATTGCCACCATGAAC
NM_ 0125,561	Rev: CTTCCCTTCATGCACGATTT
PPAR -α	Fw: CCTGCCTTCCCTGTGAACT
NM_ 0131,961	Rev: ATCTGCTTCAAGTGGGGAGA
ApoB	Fw: AGCGCCACCAAGATTAACTG
NM_0192,872	Rev: GCTTGAGCTCGTACCTGGAC
MTP	Fw: GTCACACAACTGGCCTCTCA
NM_ 0011,077,271	Rev: GCCGTTATCGTGACTTGGAT
GAPDH	Fw: ACATCATCCCTGCATCCACT
NM_0170,083	Rev: GGGAGTTGCTGTTGAAGT

2.5. Measurement of maximal carnitine palmitoyltransferases I and II activity

The livers were minced with scissors and manually homogenized in isolation buffer (220 mM Mannitol, 70 mM sucrose, 2 mM Hepes, 0.1 mM EDTA, pH 7.4). The homogenates were filtered and centrifuged twice at 1000 *g* (12 min), the supernatant was taken and then centrifuged twice at 10,000 *g* for 15 min to obtain isolated mitochondrias. The mitochondrias were centrifuged after suspension in buffer (0.15 mM KCl and 5 mM Tris HCl pH 7.5), and again centrifuged (10,000 *g*, 15 min). Then, the isolated mitochondria were resuspended in a second buffer (10 mM Phosphate buffer, pH 7.5) and ultracentrifuged (100,000 g, 1 h). The obtained pellet was resuspended in Phosphate (pH 7.5) buffer to which Tween 20 (1% w/v) was added, and stirred on ice for 30 min, to separate CPT I from CPT II [24]. Another ultracentrifugation was carried out to separate CPT I (pellet) and CPT II (supernatant).

CPT activity was measured as in Seelaender et al. (1998). The assay medium was prepared with: 60 mM KCl, 40 mM Mannitol, 20 mM Hepes, 0.15 mM EGTA, 1.5 mM KCN, fat-free bovine serum albumin (0.5%), 42 μM palmitoyl CoA, 0.35 mM carnitine (0.6 Ci ^{3}H-methylcarnitine). 0.03 mg of the isolated enzyme fraction was added to initiate the reaction. Blanks received water in substitution to the enzyme fraction. The assay mixture final volume was 0.5 ml, and the pH, 7.3. To stop the reaction, 1.5 ml of 7% perchloric acid was added and tbs were transferred to ice. The acylcarnitine formed was extracted with n-Butanol. CPT activity was expressed as nmol/min per mg of protein in the isolated enzyme fraction. Sample protein content was assessed with the method of Lowry et al. [25].

2.6. Serum determinations

Serum was obtained from the collected blood samples, and stored at −80 °C for posterior analysis. Serum glucose, total cholesterol and TAG were quantified with commercial kits (Labtest®, Brazil). Plasma insulin levels were assessed with radioimmunoassay commercial kits (Coat-A-Count (DPC), Los Angeles, CA, USA). Plasma leptin levels were measured using a radioimmunoassay method, with commercial kits (Linco Research Inc., St. Louis, MO, USA).

2.7. Liver TAG content

The method described by Folch et al. (1957), was employed to assess Liver TAG content [26].

2.8. Determination of liver glycogen content

The method described by Hassid, 1957 [27] was employed to determine Liver Glycogen content. To digest liver samples, we employed 30% KOH at 100 °C and ethanol to precipitate glycogen. The samples were centrifuged at 3000 rpm for 15 min between precipitation phases, and then, submitted to acid hydrolysis in the presence of phenol. The values were expressed in mg/100 mg of wet weight.

2.9. Cytokine content measurement

Adipose tissues samples were obtained and carefully rinsed with ice-cold 0.9% NaCl, frozen in liquid nitrogen, and stored at − 80 °C. A fragment of the frozen tissue (0.1–0.3 g) was homogenised in RIPA buffer (0.625% Nonidet P-40, 0.625% Sodium Deoxycholate, 6.25 mM Sodium Phosphate, 1 mM Ethylenediamine tetra acetic acid and 10 μg/ml of protease inhibitor cocktail at pH 7.4) (Sigma–Aldrich, St. Louis, Missouri, USA). To obtain the supernatants, homogenates were centrifuged at 30.000 *g* for 10 min at 4 °C. The protein content was determined by the method of Lowry 1961, with a commercial kit (Bio-Rad, Hercules, CA, USA) [25]. Quantitative assessment of IL-1β, IL-6, TNF-α, and IL-10 protein was carried out by ELISA (DuoSet ELISA, R & D Systems, Minneapolis, MN, USA). The following kits were used: DY510 for TNF-α, DY501 for IL-1β, DY506 for IL-6, and DY522 for IL-10. Assay sensitivity was 5.0 pg/ml in the range of 31.2–2 000 pg/ml. The intra- and inter-assay variability of the TNF-α, IL-1β, and IL-6 kits were 2.7–5.2, and 4.9–9.5%, respectively. Assay sensitivity for IL-10 was 10 pg/ml in the range from 31.2 to 2 000 pg/ml. The intra-assay variability of the IL-10 kit was 2.0–4.2%, and its inter-assay variability was of 3.3–6.4%. All samples were run as duplicates and the mean value was reported.

2.10. Immunodetection of ApoB

Liver samples were homogenised in lysis buffer (RIPA) containing protease inhibitors with a Polytron® and centrifuged for 10 min. The infranatant was collected and stored at − 80 °C. ApoB content in the liver was determined by Western blot. Proteins were separated by polyacrylamide (7%) gel electrophoresis for two hours and transferred to nitrocellulose membranes (Highbond extra, Amersham®, UK). ApoB immunodetection was performed with ApoB primary antisheep antibody (Bristol Myers Squibb) and antigoat secondary antibody (Santa Cruz®). After two washes in TBS, containing 0.5% nonfat dry milk, the membrane was incubated for 30 min with the antigoat antibodies (Santa Cruz®), at room temperature. Membranes were revealed with the ECL kit (GE®), and the bands, quantified with the Scion Image program. Alpha-tubulin was adopted as the internal control.

2.11. Statistical analysis

Data are presented as means ± SEM and Statistical Analysis was performed using two-way ANOVA test (control vs. supplemented SF and CLA groups effects and tumour-bearing vs. control group effects), followed by a Tukey *post hoc* test. The homogeneity of variances was verified and when necessary the data were transformed into the logarithm and the percentages were transformed into sinae arc of the square root. The 0.05 probability level was considered to indicate statistical significance. Statistical analysis was performed by Statistical Department of Biomedical Sciences Institute (University of São Paulo).

Table 2
Relative (%) weight gain after 14 days of supplementation and Relative weight (percentage of total body mass) of the liver, muscle (gastrocnemius), white adipose depots and tumour.

	C			T		
	CLA	SF	P	CLA	SF	P
Weight gain[1] (%)	8.0 ± 0.3	16.9 ± 0.5	16.1 ± 1.6	3.0 ± 0.2	10.3 ± 0.4	10.3 ± 0.7
Liver[2] (%)	2.8 ± 0.1	3.4 ± 0.2	3.5 ± 0.2	3.0 ± 0.1	2.9 ± 0.2	2.9 ± 0.1
Muscle[3] (%)	2.01 ± 0.20	1.47 ± 0.08	1.41 ± 0.17	0.64 ± 0.05	0.87 ± 0.03	0.47 ± 0.05
EAT[4] (%)	0.74 ± 0.08	0.91 ± 0.02	0.46 ± 0.07	0.32 ± 0.01	0.36 ± 0.01	0.25 ± 0.01
RPAT[5] (%)	0.43 ± 0.08	0.71 ± 0.04	0.46 ± 0.07	0.38 ± 0.05	0.41 ± 0.06	0.25 ± 0.01
MES[6] (%)	0.09 ± 0.01	0.15 ± 0.01	0.06 ± 0.01	0.11 ± 0.03	0.10 ± 0.01	0.04 ± 0.01
Tumour[7] (%)	–	–	–	2.34 ± 0.39	2.98 ± 0.51	3.26 ± 0.35

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, $p = 0.404$; Group, $p < 0.001$ (T≠C, independent of Supplementation); Supplementation, $p < 0.001$ (Tukey, CLA≠SF and P, independent of Tumor, $p < 0.05$).
[2] Group x Supplementation, $p = 0.025$ (Tukey, CCLA≠CP, $p < 0.05$).
[3] Group x Supplementation, $p = 0.004$ (Tukey, TCLA, TSF and TP≠CCLA, CSF and CP; TSF≠TP; CCLA≠CSF and CP, $p < 0.05$).
[4] Group x Supplementation, $p = 0.008$ (Tukey, all groups are different from CSF and CCLA; TP, CCLA and CSF≠CP, $p < 0.05$.
[5] Group x Supplementation, $p = 0.052$; Group, $p < 0.001$ (T≠C, independent of Supplementation); Supplementation, $p = 0.001$ (Tukey, CLA≠SF and SF≠P, independent of Tumor, $p < 0.05$).
[6] Group x Supplementation, $p = 0.072$; Group, $p = 0.112$; Supplementation, $p < 0.001$ (Tukey, CLA and SF≠P, independent of Tumor, $p < 0.05$).
[7] Supplementation, $p = 0.270$.

3. Results

Table 2 shows the mean body weight gain of the animals and tissues after supplementation. T groups final body weight were lower in relation to that of C groups ($P < 0.001$). Besides that, CLA supplementation groups (CLA and TCLA) gained less weight in relation to the other supplemented groups ($P < 0.05$). Only CCLA showed differences concerning the relative weight of the liver in relation to CP ($P < 0.05$). The relative weight of the muscle showed differences when T groups were compared do C groups ($P < 0.05$). In addition, TSF showed an increase in relation to TP ($P < 0.05$). CCLA relative muscle weight was higher in relation to CSF and CP ($P < 0.05$). Regarding EAT, the tumour bearing state caused a decrease in relative weight, compared to control groups ($P < 0.05$). In control groups, supplementation increased relative weight in comparison with placebo ($P < 0.05$). RPAT showed a decrease in relative weight in T groups, when compared to C groups, regardless of the supplementation ($P = 0.001$). SF supplemented groups demonstrated an increase in RPAT relative weight when compared with CLA supplementation and Placebo, regardless of the presence of the tumour ($P < 0.05$). MES relative weight showed an increase in the supplemented groups in relation to placebo, regardless of the presence of the tumour ($P < 0.05$). No difference concerning tumour weight could be observed among the groups ($P = 0.270$).

As illustrated in Table 3, daily food intake was lower in CLA in comparison with P, regardless of the tumour presence ($P < 0.05$), but there were no differences in relation to ingested calories among groups, therefore, anorexia was not observed in the tumour-bearing groups. Intake of carbohydrate and protein was lower for the CLA groups, regardless of the presence of the tumour ($P < 0.05$). However, the groups supplemented with oil (CLA or Sunflower) presented increased fat intake, as expected, in comparison with the P, regardless of tumour presence ($P < 0.05$).

Table 4 shows the plasma levels of Cholesterol, Triacylglycerol (TAG), Glucose, Insulin and Leptin. Cholesterol levels were higher in tumour groups, in comparison with control, regardless of the supplementation ($P < 0.001$). Cholesterol content in CLA groups were higher in comparison with SF and P groups (control and tumour) ($P < 0.05$). Regarding Triacylglycerol, the tumour-bearing groups showed higher values compared with the control group, while TCLA supplementation further augmented these levels, compared with TSF and TP ($P < 0.05$). Sunflower supplementation also increased TAG levels in comparison with P in the tumour group ($P < 0.05$). All supplemented groups showed enhanced Glucose levels when compared with Control group ($P < 0.05$). TCLA glucose levels were higher when compared with TSF ($P < 0.05$). The Tumour groups showed a decrease in insulin levels compared with control group, regardless of supplementation ($P = 0.010$). Leptin levels was lower in TP in relation to CP ($P < 0.05$). Only in the control group we observed a decrease in leptin levels due to CCLA supplementation, when compared to CSF and control ($P < 0.05$).

TCLA presented the highest liver neutral fat content ($P < 0.05$). Tumour groups showed higher values in relation to control groups

Table 3
Daily food intake (Average ingestion in 14 days), estimated total caloric intake (TCI) and macronutrient intake.

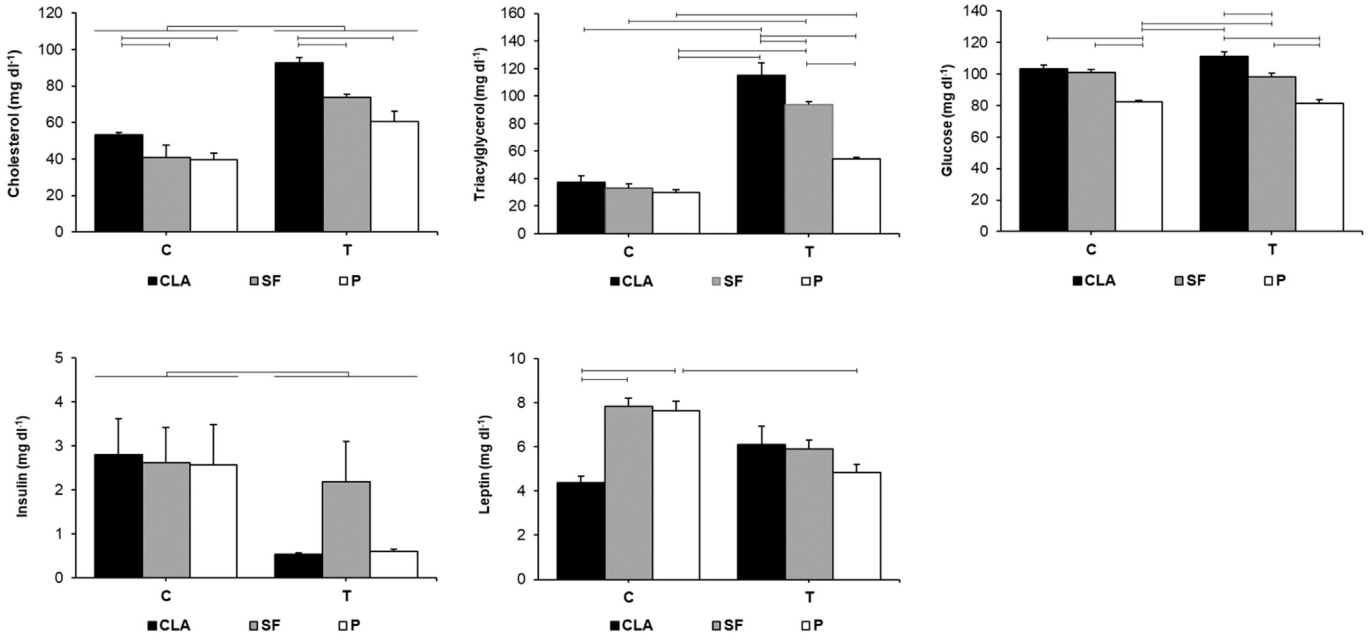
	C			T		
	CLA	SF	P	CLA	SF	P
Food intake[1] (g)	19.5 ± 0.6	19.6 ± 0.5	22.9 ± 1.2	18.6 ± 0.7	20.9 ± 0.7	20.9 ± 0.5
TCI[2] (Kcal)	72.9 ± 2.0	73.0 ± 1.8	80.4 ± 4.3	69.6 ± 2.6	77.9 ± 2.3	73.3 ± 1.7
Carbohydrate[3] (g)	10.7 ± 0.3	10.8 ± 0.3	12.6 ± 0.7	10.2 ± 0.4	11.5 ± 0.4	11.5 ± 0.3
Protein[4] (g)	4.41 ± 0.13	4.40 ± 0.11	5.15 ± 0.28	4.18 ± 0.17	4.71 ± 0.15	4.73 ± 0.10
Total Lipids[5] (g)	1.38 ± 0.03	1.39 ± 0.02	1.03 ± 0.06	1.35 ± 0.04	1.44 ± 0.03	0.95 ± 0.03

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, $p = 0.075$, Group, $p = 0.395$; Supplementation, $p = 0.002$ (Tukey, CLA≠P, independent of Tumour, $p < 0.05$).
[2] Group x Supplementation, $p = 0.075$; Group, $p = 0.393$; Supplementation, $p = 0.091$.
[3] Group x Supplementation, $p = 0.079$; Group, $p = 0.407$; Supplementation, $p = 0.002$ (Tukey, CLA≠P, independent of Tumour, $p < 0.05$).
[4] Group x Supplementation, $p = 0.085$; Group, $p = 0.399$; Supplementation, $p = 0.002$ (Tukey, CLA≠P, independent of Tumour, $p < 0.05$).
[5] Group x Supplementation, $p = 0.260$; Group, $p = 0.591$; Supplementation, $p < 0.001$ (Tukey, CLA and SF≠P, independent of Tumour, $p < 0.05$).

Table 4
Plasma Cholesterol, triacylglycerol, glucose, insulin and leptin concentration.

	C			T		
	CLA	SF	P	CLA	SF	P
Cholesterol[1] (mg/dl)	53.2 ± 1.4	40.7 ± 7.2	39.6 ± 3.8	92.7 ± 3.0	73.7 ± 1.6	60.6 ± 5.5
Triacylglycerol[2] (mg/dl)	37.2 ± 5.1	32.8 ± 3.3	30.1 ± 1.7	114.8 ± 9.2	93.7 ± 2.1	54.3 ± 0.9
Glucose[3] (mg/dl)	103.5 ± 2.3	101.1 ± 1.6	82.1 ± 1.0	111.3 ± 2.6	98.0 ± 2.6	81.2 ± 2.3
Insulin[4] (ng/ml)	2.80 ± 0.82	2.63 ± 0.80	2.57 ± 0.91	0.54 ± 0.03	2.19 ± 0.90	0.62 ± 0.05
Leptin[5] (mg/dl)	4.39 ± 0.29	7.83 ± 0.37	7.63 ± 0.46	6.12 ± 0.84	5.91 ± 0.39	4.85 ± 0.36

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, $p = 0.102$, Group, $p < 0.001$ (T≠C, independent of Supplementation); Supplementation, $p < 0.001$ (Tukey, CLA≠P and SF, independent of Tumor, $p < 0.05$).
[2] Group x Supplementation, $p < 0.001$, (Tukey, TCLA, TSF and TP are different from all groups and each other, $p < 0.05$).
[3] Group x Supplementation, $p = 0.036$ (Tukey, all supplemented groups are different from P groups, TCLA≠TSF, $p < 0.05$.).
[4] Group x Supplementation, $p = 0.384$; Group, $p = 0.010$ (T≠C, independent of Supplementation); Supplementation, $p = 0.445$.
[5] Group x Supplementation, $p < 0.001$, (Tukey, TP≠CP, CCLA≠CP and CSF, $p < 0.05$).



(P < 0.05). Supplementation increased liver glycogen in control groups, when compared with Placebo (P < 0.05). Tumour groups presented lower content in relation to control groups (P < 0.05). TCLA and TP showed lower liver glycogen content in comparison with TSF, but not when compared between themselves (P < 0.05). CLA supplementation augmented the protein content in relation to SF and P, regardless of the presence of the tumour (P < 0.05) (Table 5).

Table 6 illustrates Liver CPT I and II maximal activity. The CLA group presented higher CPT I maximal activity when compared with the Placebo groups, regardless of the presence of the tumour (P < 0.05). Regarding CPT 2, all groups showed a decrease in maximum activity in relation to CP, with the exception of TSF (P < 0.05).

CPT I mRNA expression showed a decrease in SF groups when compared to other groups, regardless the tumour (P < 0.05), while CPT II mRNA expression was lower in Tumour groups in relation to Control groups (P < 0.05). Liver FABP mRNA expression was decreased in all T groups in relation to C groups (P < 0.05). Supplementation decreased in relation to Placebo in the C group. Liver PPAR-α mRNA expression was diminished in all groups when compared with CP. TCLA presented the lowest expression when compared with other tumour groups (P < 0.05). TSF showed augmented PPAR-α mRNA when compared with CSF (P < 0.05) (Table 7).

Liver ApoB mRNA expression (Table 8) was lower in TCLA in relation to all groups (P < 0.05). TSF showed increase in ApoB gene expression in relation to CSF (P < 0.05). CSF also was lower than CCLA and CP (P < 0.05). Liver MTP mRNA expression was lower in TCLA in relation to all other groups (P < 0.05). All groups presented diminished ApoB relative protein expression when compared to CP (P < 0.05) (Table 7).

Table 9 illustrates Retroperitoneal adipose tissue related parameters. There were no statistical differences between groups regarding the triacylglycerol, total protein and inflammatory cytokine concentration, with the exception of IL-6 and IL-10. ForIL-6, a decrease was observed in the TP group in relation to CP (P < 0.05). IL-10 content was lower in TP when compared with CP, TCLA and TSF (P < 0.05).

In Table 10, Triacylglycerol concentration was lower in TCLA compared to all other groups (P < 0.05). The concentration of IL-1β was higher in TCLA, when compared with TP, CCLA and CP (P < 0.05). TSF showed increase in IL-1β concentration when

Table 5
Liver TAG, protein and glycogen content.

	C			T		
	CLA	SF	P	CLA	SF	P
TAG[1] (mg/100 g liver weight)	36.0 ± 2.6	39.4 ± 4.4	39.6 ± 3.8	82.0 ± 2.5	58.7 ± 5.5	60.6 ± 5.5
Glycogen[2] (mg/100 g liver weight)	0.606 ± 0.008	0.623 ± 0.022	0.255 ± 0.008	0.195 ± 0.015	0.283 ± 0.011	0.198 ± 0.005
Protein[3] (μg/μL)	895.0 ± 45.1	798.0 ± 35.4	773.0 ± 10.4	893.0 ± 14.4	819.0 ± 25.0	789.0 ± 9.6

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, p = 0.004, (Tukey, TCLA is different from all groups; TSF≠CSF and CP; TP≠CP, p < 0.05).
[2] Group x Supplementation, p < 0.001 (Tukey, TCLA and TP are different from all groups, but not among each other, TSF≠CSF; CCLA and CSF≠CP, p < 0.05).
[3] Group x Supplementation, p = 0.878; Group, p = 0.345; Supplementation, p = 0.001 (Tukey, CLA≠SF and P, independent of Tumour, p < 0.05).

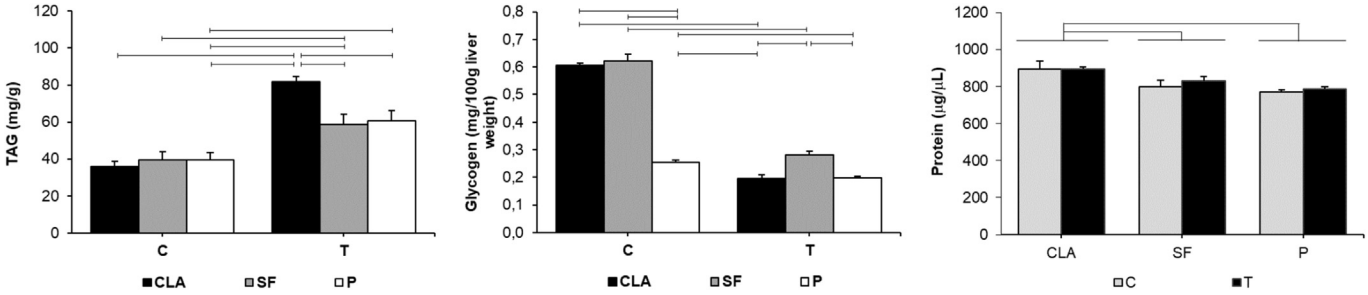
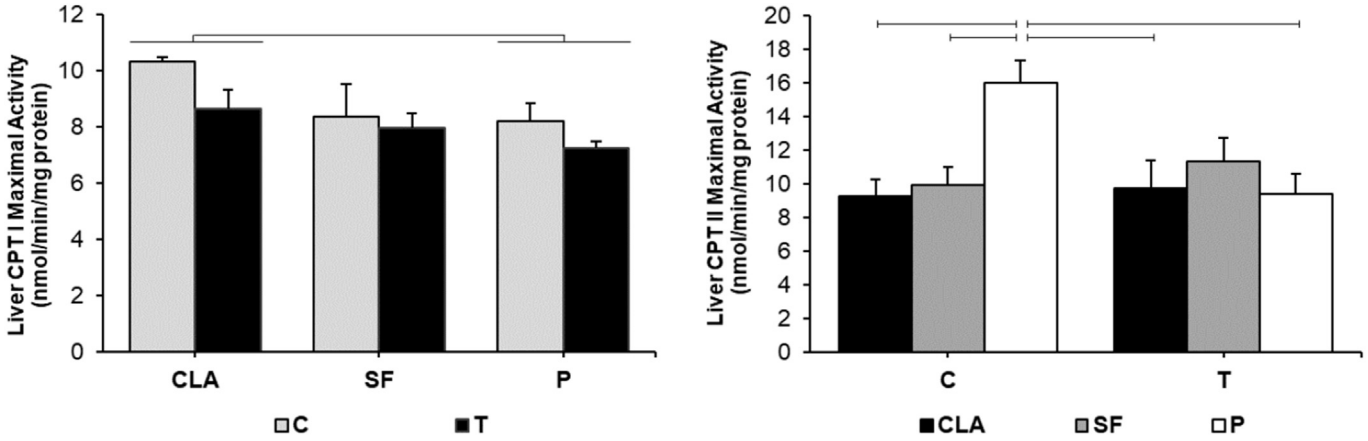


Table 6
Liver CPT I and II maximal activities (nmol/min/mg protein).

	C			T		
	CLA	SF	P	CLA	SF	P
CPT I[1] (nmol/min/mg protein)	10.32 ± 0.18	8.37 ± 1.17	8.20 ± 0.63	8.63 ± 0.68	7.98 ± 0.50	7.24 ± 0.24
CPT II[2] (nmol/min/mg protein)	9.25 ± 1.05	9.96 ± 1.08	15.99 ± 1.32	9.78 ± 1.64	11.34 ± 1.43	9.44 ± 1.20

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, p = 0.617; Group, p = 0.070; Supplementation, p = 0.035 (Tukey, CLA≠P, independent of Tumour, p < 0.05).
[2] Group x Supplementation, p = 0.010 (Tukey, TCLA, TP, CCLA and CSF≠CP, p < 0.05).



compared to TP (P < 0.05). TCLA showed higher concentration of IL-6 when compared with TP and CCLA (P < 0.05). TSF also had higher IL-6 when compared to TP (P < 0.05); and CCLA showed lower IL-6 concentration when compared to CSF (P < 0.05). TCLA showed the highest concentration of TNF-alpha in relation to the other groups (P < 0.05). TCLA showed higher IL-10 concentration in relation to TP and CCLA (P < 0.05), unlike CCLA, which presented a decrease in concentration in relation to CSF (P < 0.05). No statistical differences were observed among groups regarding protein concentration and IL-10/TNF-alpha ratio in the epididymal adipose tissue.

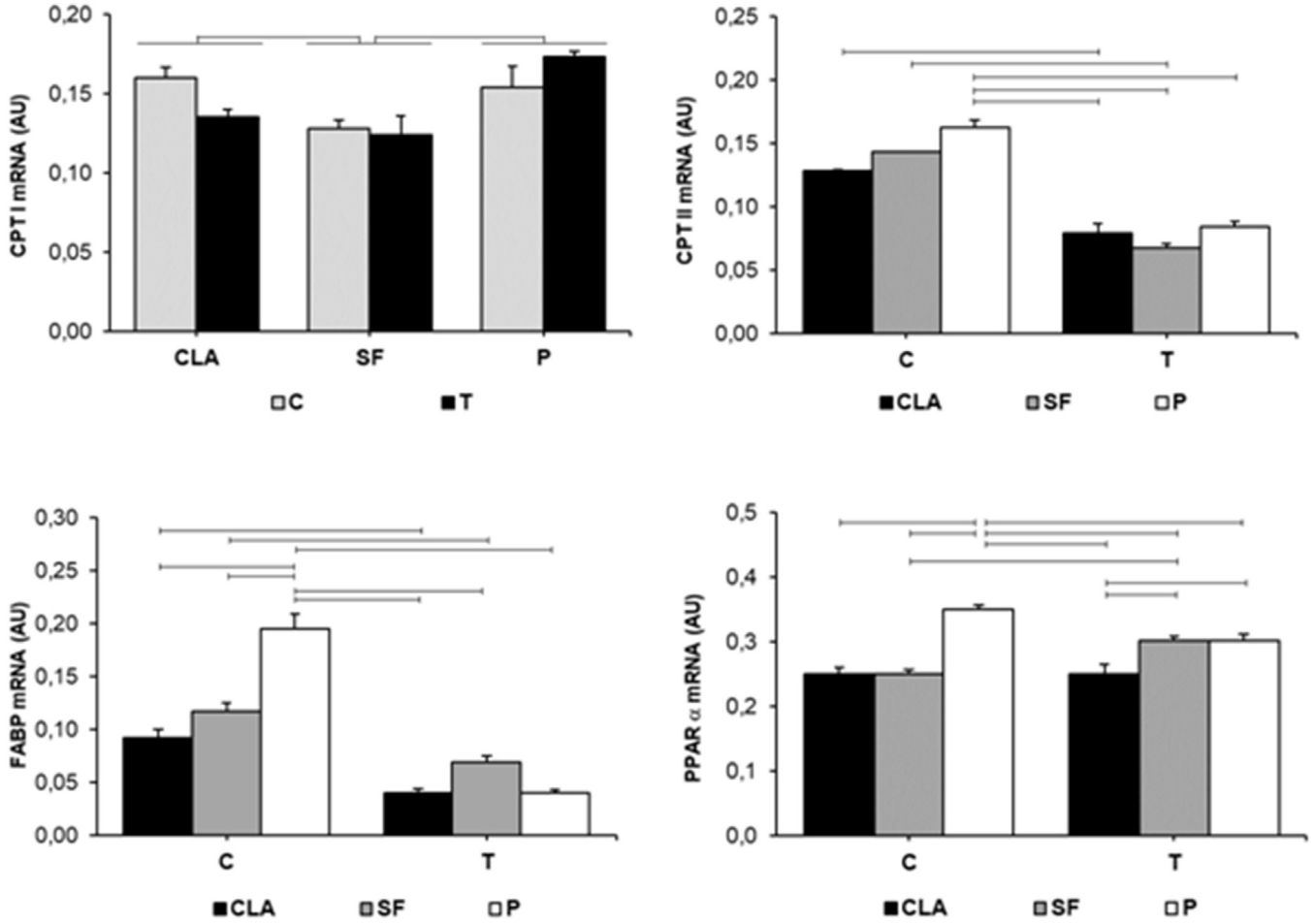
4. Discussion

This study was the first, to our knowledge, to demonstrate the deleterious effect of CLA supplementation on liver lipid metabolism in an experimental model of cancer-related cachexia. Lipid

Table 7
Liver CPT I, II, FABP and PPAR-α mRNA expression (arbitrary units).

	C			T		
	CLA	SF	P	CLA	SF	P
CPT I[1] (AU)	0.160 ± 0.007	0.128 ± 0.006	0.154 ± 0.013	0.136 ± 0.004	0.124 ± 0.011	0.173 ± 0.003
CPT II[2] (AU)	0.1285 ± 0.0005	0.1432 ± 0.0004	0.1624 ± 0.0063	0.0796 ± 0.0075	0.0675 ± 0.0032	0.0843 ± 0.0044
FABP[3] (AU)	0.093 ± 0.008	0.117 ± 0.009	0.196 ± 0.014	0.040 ± 0.004	0.069 ± 0.006	0.040 ± 0.003
PPAR α [4] (AU)	0.251 ± 0.010	0.251 ± 0.007	0.350 ± 0.007	0.251 ± 0.015	0.302 ± 0.007	0.0303 ± 0.010

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, $p = 0.051$; Group, $p = 0.670$; Supplementation, $p = 0.001$ (Tukey, CLA≠SF and SF≠P, independent of Tumour, $p < 0.05$).
[2] Group x Supplementation, $p = 0.006$ (Tukey, TCLA≠ CCLA; TSF≠CSF; TP≠CP; TCLA, TSF and CCLA≠CP, $p < 0.05$).
[3] Group x Supplementation, $p < 0.001$ (Tukey, TCLA≠ CCLA; TSF≠CSF; all groups are different from CP, $p < 0.05$).
[4] Group x Supplementation, $p < 0.001$ (Tukey, all groups are different from CP; TCLA≠TP and TSF; TSF≠CSF, $p < 0.05$).



supplementation *per se* may induce metabolic changes directly related with increased fat consumption, disregard of the type of fat [30,31,53,54]. Therefore, to isolate the specific effect of CLA, we employed sunflower oil as a control, as its fatty acid composition is similar to CLA in relation to n-6 PUFA linoleic acid, yet consisting mainly of Cis-9 linoleic acid (64%), while CLA is composed by a mix of isomers (c-9, T-11 and T-10, C-12).

Liver steatosis is usually present in cachexia, and is related with its aggravation [32,64]. While some studies show a deleterious effect of CLA supplementation, leading to steatosis [33,34], other claim that CLA augments fatty acid oxidation by the liver [8,9]. This contradiction may be explained by differences in the adopted protocols, such as in the time, type of supplemented isomers, and dose of supplementation. In cachectic animals, steatosis was exacerbated by CLA, but not by SF supplementation. Therefore CLA seems to have induced this change as an effect of its specific fatty acid composition.

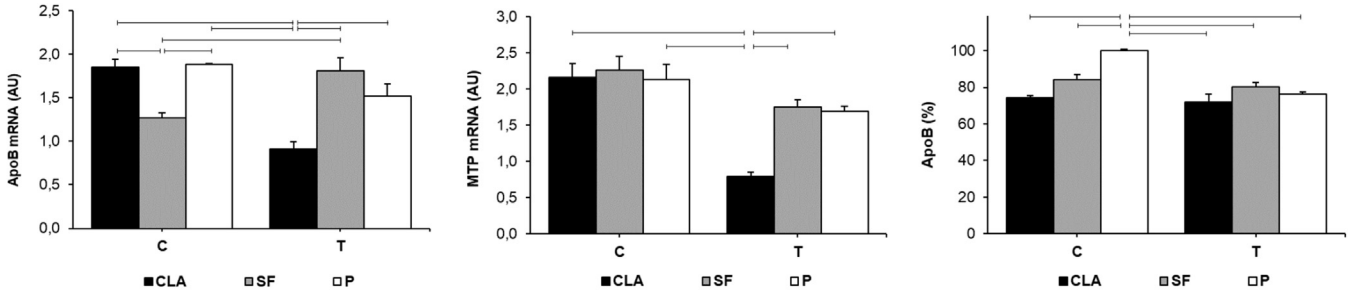
The possible mechanisms involved in the development of steatosis would, in our model of cachexia, potentially derive from the diminished capacity of the liver to oxidise fat and to export lipids as VLDL; as well as from the increased fatty acid uptake by the organ; enhanced liver lipogenesis and TAG esterification, all of which previously reported[18].

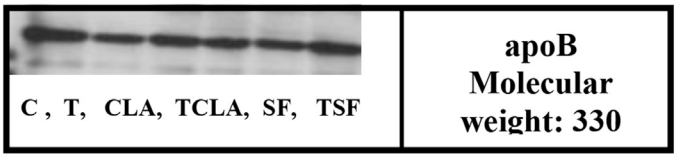
The capacity of the liver to oxidise fatty acids depends on the activity of the enzymatic carnitine palmitoyl transferase (CPT) complex [35,37,38,41]. CPT 1 mRNA expression as well as its maximal activity were measured and we detected an increase of this parameter in CLA when compared with P. CPT II mRNA expression and its maximal activity decreased in T groups,

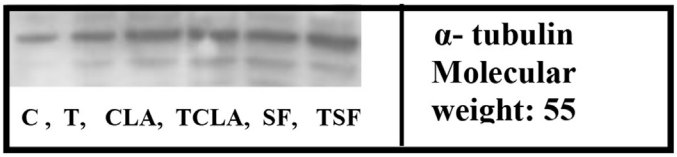
Table 8
ApoB and MTP mRNA expression (arbitrary units) and ApoB protein expression (% in relation to control).

	C			T		
	CLA	SF	P	CLA	SF	P
ApoB mRNA[1]	1.85 ± 0.09	1.27 ± 0.06	1.88 ± 0.01	0.91 ± 0.09	1.81 ± 0.15	1.52 ± 0.15
MTP mRNA[2]	2.16 ± 0.19	2.26 ± 0.20	2.13 ± 0.21	0.79 ± 0.06	1.75 ± 0.10	1.69 ± 0.07
ApoB protein[3]	74.4 ± 1.1	84.3 ± 2.8	100.0 ± 1.0	71.7 ± 4.5	80.3 ± 2.4	76.4 ± 1.0

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, $p < 0.001$ (Tukey, TCLA≠TP, TSF, CCLA and CP; TSF≠CSF; CCLA≠CSF and CSF≠CP, $p < 0.05$).
[2] Group x Supplementation, $p = 0.009$ (Tukey TCLA is different from all groups, $p < 0.05$).
[3] Group x Supplementation, $p < 0.001$ (Tukey, all groups are different from CP, $p < 0.05$).







compared with C, corroborating our previous findings; but CLA supplementation could not reverse this situation [36]. CLA supplementation studies are still controversial, since many show that supplementation does not alter CPT I and CPT II mRNA expression [39,40], while, in contradiction, other authors claim that there is increased CPT I and II gene expression, since long term fatty acid supplementation affects lipid metabolism[8,9,10]. These studies nevertheless, examine a specific CLA isomer, T-10 C-12 [39], and are therefore, not comparable to the presently reported.

We investigated PPAR-α mRNA expression in cachexia and CLA supplementation, as PUFAs (polyunsaturated fatty acids) are known to activate these transcription factors. However, the affinity for n-3 and n-6 of PPAR- α varies [42,50]. Many studies have demonstrated an enhancement in PPAR- α mRNA expression as promoted by CLA intake [43–45], which we presently failed to find, probably due to the short period of supplementation, as others report changes to happen after 1 month of treatment.

Another important mechanism regulating fat content in the liver is VLDL production and secretion [49,61,63]. In Walker 256-induced cachexia, VLDL production and secretion are reduced, as previously reported by Tavares et al. [47] and Carreño et al. [48]. To access whether the supplementation protocol would interfere with this process, apoB, which is the main apolipoprotein present in the particle, and MTP, the protein responsible for the transfer of TAG to nascent VLDL [46] were studied.

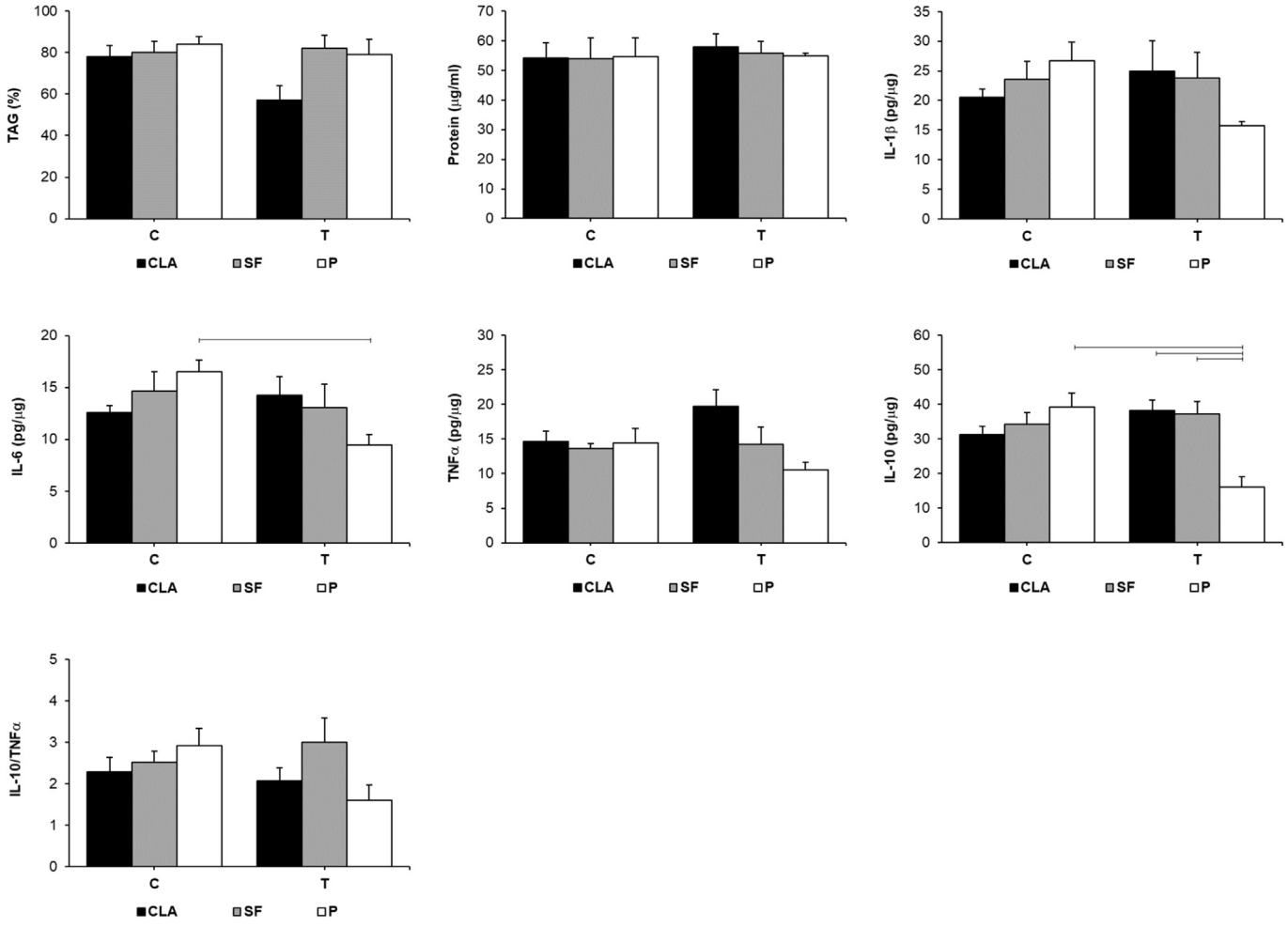
The data show reduced liver mRNA expression of MTP in all supplemented groups in relation to control, with or without tumour, indicating that higher PUFA consumption *per se* changes apoB regulation, even in healthy animals. No studies known to us, have addressed MTP mRNA expression in animals supplemented with CLA, but we consider that the mechanisms are similar to those present after sunflower oil supplementation. TCLA showed significantly lower expression in relation to all groups. The factors inducing a reduction in MTP and apoB in cachectic liver are not fully understood, but we speculate that they could be related with inflammatory factors secreted or uptaken by the liver, such as cytokines and prostanoids, as demonstrated in previous studies [28,51]. CLA supplementation might contribute to this inflammatory profile by inducing even greater secretion of inflammatory cytokines [52].

In relation to apoB mRNA expression, T values were lower, when compared to C, as previously demonstrated [52,53]. TCLA showed diminished ApoB mRNA expression in relation to TP, confirming CLA capacity to reduce ApoB expression, an effect commonly associated with the T-10 C-12 isomer [55]. In the cachectic group, this reduction turned out to be more evident after CLA supplementation. Together, these results demonstrate a lower capacity of

Table 9
Adipose Tissue Parameters (Triacylglycerol and protein concentration, IL-1β, IL-6, TNF −α, IL-10 content, IL-10/TNF−α ratio in the Retro Peritoneal adipose Tissue).

	C			T		
	CLA	SF	P	CLA	SF	P
TAG[1] (%)	78.0 ± 5.5	80.0 ± 5.4	84.0 ± 3.8	57.0 ± 7.1	82.0 ± 6.4	79.0 ± 7.6
Protein[2] (μg/ml)	54.2 ± 5.0	54.0 ± 6.9	54.6 ± 6.4	57.9 ± 4.3	55.7 ± 4.0	54.9 ± 1.0
IL-1β[3] (pg/μg)	20.5 ± 1.4	23.5 ± 3.1	26.7 ± 3.1	24.9 ± 5.1	23.7 ± 4.3±	15.8 ± 0.7
Il-6 [4] (pg/μg)	12.6 ± 0.7	14.6 ± 1.8	16.5 ± 1.2	14.2 ± 1.8	13.0 ± 2.2	9.4 ± 1.0
TNF−α[5] (pg/μg)	14.6 ± 1.6	13.7 ± 0.7	14.5 ± 2.1	19.7 ± 2.4	14.2 ± 2.6	10.6 ± 1.1
IL-10[6] (pg/μg)	31.2 ± 2.3	34.2 ± 3.4	39.3 ± 3.9	38.2 ± 3.0	37.1 ± 3.6	16.0 ± 3.0
IL-10/TNF−α[7] (pg/μg)	2.3 ± 0.4	2.5 ± 0.3	2.9 ± 0.4	2.1 ± 0.3	3.0 ± 0.6	1.6 ± 0.4

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, p = 0.174, Group, p = 0.158; Supplementation, p = 0.047 (no statistical differences were detected by Tukey).
[2] Group x Supplementation, p = 0.943; Group, p = 0.643; Supplementation, p = 0.956.
[3] Group x Supplementation, p = 0.059; Group, p = 0.237; Supplementation, p = 0.683.
[4] Group x Supplementation, p = 0.026 (Tukey, TP≠CP, p < 0.05).
[5] Group x Supplementation, p = 0.073; Group, p = 0.693; Supplementation, p = 0.055.
[6] Group x Supplementation, p < 0.001 (Tukey, TCLA and TSF≠TP; TP≠CP, p < 0.05).
[7] Group x Supplementation, p = 0.094; Group, p = 0.283; Supplementation, p = 0.302.



the liver of TCLA to produce VLDL, contributing to the increase in TAG content in the liver, further aggravating steatosis in cachexia.

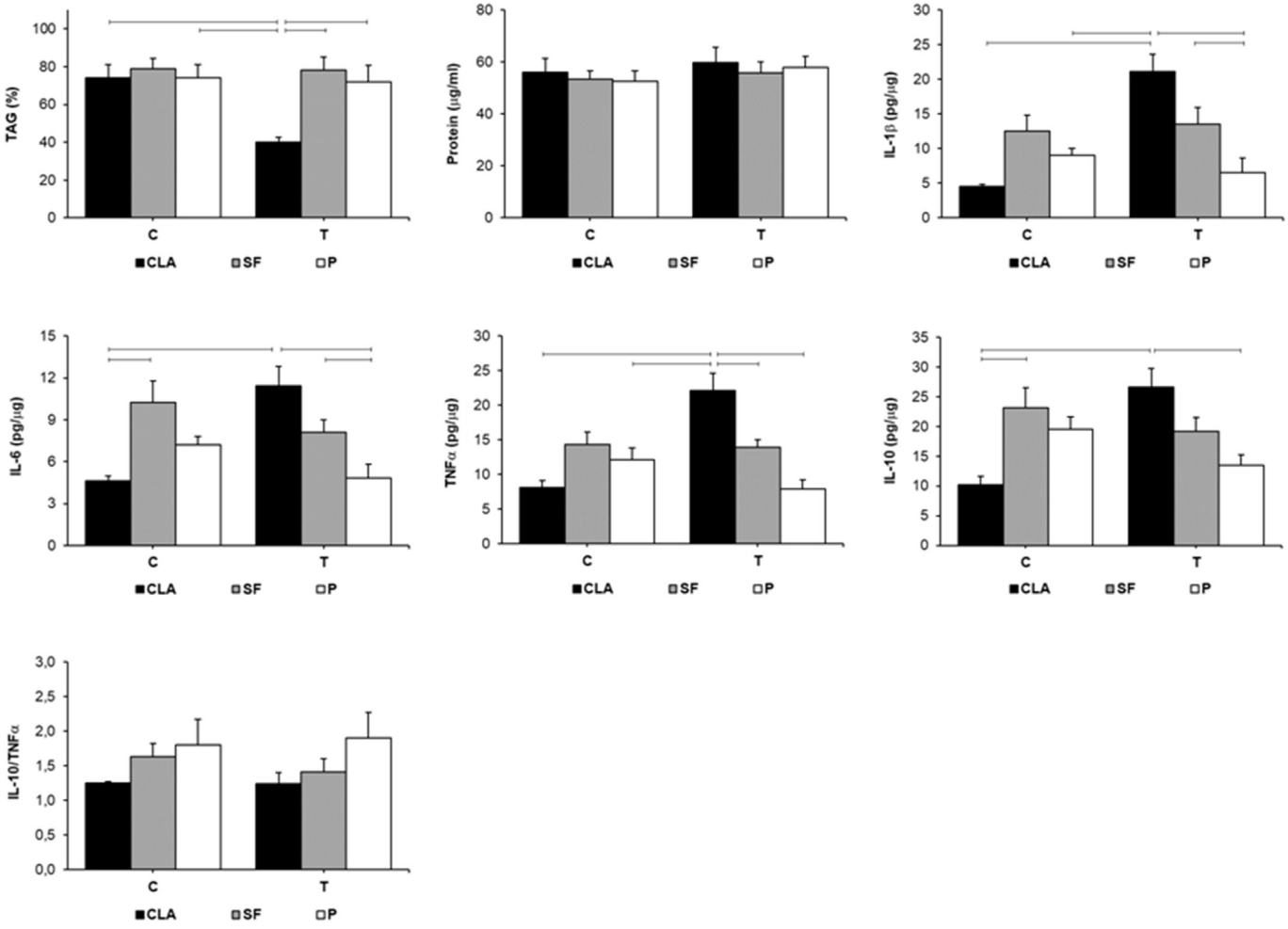
Regardless of the decrease in liver VLDL production, it was found that the supplementation of CLA and SF in cachectic rats increased plasma VLDL TAG in tumour groups. In tumour supplemented groups, increased plasma TAG was expected, as a result of daily high fat content in diet associated with cachectic state, but the opposite was expected in TCLA, as many studies [56–58] have demonstrated hypolipidemic properties of CLA. Nevertheless, other authors report a clear atherogenic property of CLA supplementation in animals [59] and humans [60]. Various factors could be associated with the conflicting results: isomer proportion, animal age and species, supplementation time, fat content in diet, previous lipid profile, and other.

Plasma cholesterol was increased in TCLA in comparison with C, as well as in TCLA in regard to T, suggesting an atherogenic potential of the supplementation protocol. Studies are also controversial in relation to CLA supplementation and plasma

Table 10
Adipose Tissue Parameters (Triacylglycerol and protein concentration, IL-1β, IL-6, TNF −α, IL-10 content, IL-10/TNF−α ratio in the Epididymal adipose Tissue).

	C			T		
	CLA	SF	P	CLA	SF	P
TAG[1] (%)	74.0 ± 7.1	79.0 ± 5.3	74.0 ± 7.2	40.0 ± 2.7	78.0 ± 7.1	72.0 ± 8.9
Protein[2] (μg/ml)	56.1 ± 5.3	53.5 ± 3.1	52.6 ± 4.0	59.7 ± 5.9	55.9 ± 4.2	57.9 ± 4.3
IL-1β[3] (pg/μg)	4.5 ± 0.3	12.6 ± 2.2	9.0 ± 1.1	21.1 ± 2.5	13.5 ± 2.4	6.5 ± 2.1
Il-6 [4] (pg/μg)	4.6 ± 0,3	10.3 ± 1.5	7.2 ± 0.6	11.4 ± 1.4	8.1 ± 0.9	4.8 ± 1.0
TNF−α[5] (pg/μg)	8.1 ± 1,0	14.4 ± 1.7	12.2 ± 1.6	22.1 ± 2.4	13.9 ± 1.2	7.9 ± 1.3
IL-10[6] (pg/μg)	10.2 ± 1,3	23.1 ± 3.3	19.5 ± 2.2	26.7 ± 3.1	19.2 ± 2.3	13.5 ± 1.7
IL-10/TNF−α[7] (pg/μg)	1.25 ± 0,02	1.63 ± 0.18	1.80 ± 0.37	1.24 ± 0.17	1.42 ± 0.19	1.90 ± 0.37

Data are mean ± SEM of 8 rats/group.
ANOVA.
[1] Group x Supplementation, p = 0.049 (Tukey, TCLA is different from all groups, p < 0.05).
[2] Group x Supplementation, p = 0.948; Group, p = 0.319; Supplementation, p = 0.751.
[3] Group x Supplementation, p < 0,001 (Tukey, TCLA≠TP, CCLA, CP; TSF≠TP, p < 0,05).
[4] Group x Supplementation, p < 0,001 (Tukey, TCLA≠CCLA and TP; TSF≠TP; CSF≠CCLA, p < 0.05).
[5] Group x Supplementation, p < 0,001 (Tukey, TCLA is different from all groups, p < 0,05).
[6] Group x Supplementation, p < 0.001 (Tukey, TCLA≠TP and CCLA; CCLA≠CSF, p < 0.05).
[7] Group x Supplementation, p = 0.810; Group, p = 0.836; Supplementation, p = 0.070.



cholesterol content modulation, due to the same factors mentioned in concern to TAG.

The alterations promoted by CLA supplementation on liver metabolism could be also related with the delipidation of adipose tissue, leading to increase in fatty acid uptake by the liver [17,18,65,66]; as induced by local inflammation in fat depots.

WAT consists of the largest endocrine organ in the organism and is responsible for production and release of over 1000 molecules, among which several cytokines and adipokines [15,16,29,67]. The production of these cytokines may be further increased by macrophage infiltration under pathological conditions, such as obesity and cancer cachexia [68,69]. In cancer, there is macrophage infiltration in the adipose tissue [69], diminished plasma leptin [69], and augmented pro inflammatory cytokine production by WAT in rats [15,70,71] and patients [72]. CLA supplementation aggravated WAT inflammation caused by cachexia in rats, increasing proinflammatory cytokine production, as presently reported (IL-1β, Il-6 and TNF- α).

Adipose tissue delipidation due to CLA supplementation is usually associated with the Trans −10, Cis-12 isomer and occurs owing to an enhancement of adipokine production in the tissue [65,66]. This fatty acid isomer activates stromal vascular cells membrane proteins and may enter the cell by diffusion, being converted to metabolites [65]. Both forms of action are responsible for NFκB activation, leading to cytokine production (TNF-α, IL-6 and IL-1β) [65,66].

We hypothesise that CLA supplementation in cachectic animals decreased glucose and fatty acid uptake by the adipose tissue, with reduced TAG synthesis, leading to adipocyte delipidation, as caused by inflammatory cytokines. The compromised capacity of the adipocytes to uptake glucose and fatty acids may contribute to the observed hyperglycemia and higher plasmatic triacylglycerol levels in TCLA, when compared with all groups. Furthermore, adipokines and hyperglycemia are positively correlated with insulin resistance, which has been demonstrated in this study and by others, after CLA supplementation [73,74].

Another interesting result is the effect of CLA supplementation upon WAT IL-10 levels, an anti inflammatory cytokine that has been recently correlated with tumour mass, and with augmented morbidity and mortality, wasting and, poorer outcome [75,76]. TCLA presented higher IL-10 levels in both (EAT and RPAT) fat depots in relation to T.

The present study shows, for the first time, that CLA supplementation does not ameliorate hepatic lipid metabolism in cancer cachexia. Actually, supplementation aggravates cachexia symptoms, including further steatosis and hyperlipidaemia. The mechanisms related with these effects are still unclear, but we suggest that they may be associated with an increased inflammatory status and WAT derived inflammatory factors. Taken together, the results suggest that CLA supplementation is inappropriate for the treatment of cancer cachexia.

Conflict of interest

None declared.

Acknowledgments

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