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ORIGINAL ARTICLE

Dietary Supplementation of Calcium may Counteract Obesity in Mice Mediated by Changes in Plasma Fatty Acids

Sarah Laraichi · Pilar Parra · Rocío Zamanillo · Ahmed El Amarti · Andreu Palou · Francisca Serra

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Abstract The scope of this study was to assess the impact of calcium and conjugated linoleic acid (CLA) supplementation on plasma fatty acid profiles and to evaluate potential synergistic effects of both compounds against dietary obesity. Mice separated into five experimental groups were followed: control (C), high-fat diet (HF), HF with calcium (Ca), HF plus CLA and HF with both Ca and CLA. Plasma metabolites and fatty acids were determined by commercial kits and gas chromatography, respectively. Both dietary calcium and CLA supplementation contributed to lower body fat gain under a HF diet. Maximum efficacy was seen with calcium; no additional effect was associated with the combined treatment with CLA. Plasma leptin, adiponectin and HOMA index were in accordance with an altered glucose/insulin homeostasis in the HF and HF + CLA groups, whereas control levels were attained under Ca-enriched diets. Plasma fatty acids showed minor changes associated to CLA treatment, but a high impact on PUFA was observed under Ca-enriched diets. Our results show that the mechanism underlying the anti-obesity effects of calcium supplementation is mediated mainly by changes in PUFA plasma profile. In addition, the lack of synergy on body weight reduction in combination with associated lipid profiles of calcium and CLA suggests that calcium may interfere with absorption and/or bioactivity of CLA, which can be of relevance when using CLA-fortified dairy products against human obesity.

Keywords Conjugated linoleic acid · Calcium supplementation · Obesity · Plasma fatty acids · PUFA · Leptin · Adiponectin

Abbreviations

ANOVA	Analysis of variance
CLA	Conjugated linoleic acid
DHA	Docosahexaenoic (C22:6n-3)
EPA	Eicosapentaenoic (C20:5n-3)
FAME	Fatty acid methyl ester
FFA	Unesterified fatty acids
GC	Gas chromatography
HF	High-fat diet
HOMA	Homeostatic model assessment for insulin resistance
LSD	Least significant difference
NF	Normal-fat diet
WHO	World Health Organisation

Introduction

Obesity is an emerging health crisis in Europe (as exposed by a recent WHO report) where its prevalence has increased three-fold over the last 30 years and continues to rise [1]. More importantly, the prevalence of childhood

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S. Laraichi · P. Parra · R. Zamanillo · A. Palou (✉) · F. Serra
Laboratory of Molecular Biology, Nutrition and Biotechnology, University of the Balearic Islands and CIBER de Fisiopatología de la Obesidad y Nutrición (CIBER-OBN), 07122, Palma de Mallorca, Spain
e-mail: andreu.palou@uib.es

F. Serra
e-mail: francisca.serra@uib.es

S. Laraichi · A. El Amarti
Laboratory of Calorimetry and Materials, Faculty of Sciences, Abdelmalek Essaâdi University, 93030 Tétouan, Morocco

obesity is increasing at an alarming rate [1, 2]. Besides the risks of obesity in development of chronic health problems such as type II diabetes and cardiovascular disease, the economic impact of this rise in obesity is reported to increase health care costs by 2–8 % and to increase obesity-related death by 10–13 % in various parts of Europe [1, 3]. The impact of this increased mortality and morbidity due to obesity in Europe represents more than 1 million deaths and 12 million life-years per year, respectively [1, 4]. Therefore, the increasing prevalence of obesity demands more effective approaches for prevention and treatment. Specific nutrients may be valuable tools in the treatment of this chronic disease that requires life-long intervention [5–11]. Evidence in animal models demonstrates that both calcium and conjugated linoleic acid (CLA) are able to modulate body composition and prevent fat accumulation [12–15]. Epidemiological studies suggest that milk consumption and/or a diet high in dairy products are amongst the few known foods that are inversely related to body mass index (BMI) and that dairy calcium specifically accounts for the body weight-regulating effects of dairy products [16–18]. Using animal models of dietary obesity, we have shown that high calcium intake attenuates the fat and body weight gain promoted by high-fat feeding and favours a higher rate of weight loss when animals are switched to a normal-fat diet [12, 13]. The potential beneficial effects of CLA have been investigated extensively in terms of its ability to induce body-composition changes, such as lowering fat mass and increasing lean body mass [19–24].

CLA is a collective term used for the positional and geometric isomers of linoleic acid. The main isomer occurring naturally in foods is *cis*-9, *trans*-11 CLA (9,11CLA), which comes from fermentation in the rumen and is therefore found in meats and dairy products. *Trans*-10, *cis*-12 CLA (10,12CLA), which is a quantitatively minor isomer in CLA-containing foods, has been identified as the main isomer responsible for reducing fat and body weight (reviewed in [25, 26]). Despite this background, only a few studies have addressed the combined effect of diets enriched in both calcium and CLA, and are focussed mainly on the potential beneficial effects on bone [27, 28].

Therefore, the aim of this work was to assess the impact of calcium and CLA supplementation on plasma fatty acid profiles and to evaluate potential synergistic effects of both compounds against dietary obesity. One of the relevant aspects of this study is the use of equimolar and moderate doses of the two main CLA isomers [14, 15, 29], which replicates human treatments and intends to avoid some of the side-effects that are found with the higher doses regularly used in mice [30–32]. Our results show that the mechanism underlying the anti-obesity effects of calcium supplementation is mediated mainly by changes in PUFA plasma profile. In addition, the lack of synergy on body weight reduction in combination with associated lipid profiles of calcium and CLA suggests that calcium may interfere with absorption and/or bioactivity of CLA, which can be of relevance when using CLA-fortified dairy products against human obesity.

Materials and Methods

Animals, Diets and Experimental Groups

Male C57BL/6J mice from Charles River (Barcelona, Spain) weighing 21 ± 0.1 g (5-weeks-old) were housed in groups of four in plastic cages and kept in a 12-h light:dark cycle at 22 °C with free access to food and water.

We designed an experimental dietary approach for 56 days based on three diets: a standard normal-fat diet (NF), containing 12 % calorie content as fat, was used as control; a high-fat diet (HF), which contained 43 % calorie content as fat; and a HF diet enriched with calcium (3.10 mg Ca/kcal) (HF + Ca). Dietary sources of fat were lard and soybean oil; the standard amount of calcium was supplied in the form of calcium carbonate (1.04 mg Ca/kcal); and the enrichment in the HF + Ca diet was performed by adding non-fat dry milk as previously described [12]. The gross energy density of these formulations was calculated to be 3.9 kcal/g in NF and 4.6 kcal/g in HF diets. All diets were prepared by Research Diets (New Brunswick, NJ), provided the same amount of protein (≅20 %), and were presented as pellets to the animals; fresh food was offered twice a week. Detailed composition of diets is included as ESM (Table S1).

In addition, mice fed with the HF diets were treated orally with CLA (HF + CLA and HF + Ca + CLA groups), receiving a daily amount of Tonalin (kindly provided by Cognis, Monheim am Rhein, Germany). Tonalin® TG 80, derived from safflower oil, is composed of TAG containing approximately 80 % CLA with a 50:50 ratio of the active CLA isomers 9,11CLA and 10,12CLA. In order to reproduce human dosage and administration pattern when taking CLA supplements, we tested a dose that is around five times higher than that usually used in human trials. Therefore, 6 mg CLA/day (240 mg CLA/kg BW) was supplied orally to the animals.

The rest of the animals under HF diets (HF and HF + Ca groups) received an isocaloric load of sunflower oil. A comparable volume used in CLA treated groups was administered as water to mice fed the NF diet, in order to simulate animal manipulation.

In total, we followed five experimental groups ($n = 8$ per group) according to the diet and treatment received: NF, HF, HF + CLA, HF + Ca and HF + Ca + CLA. All

experimental procedures were performed according to both national and institutional guidelines for animal care and use (approval 13 February 2006).

Animal Follow-up

Food intake and body weight were recorded every 3 days throughout the experiment. Calorie intake was measured for each cage and expressed as the daily average of the two cages per group. Body composition analysis (individual body fat and lean mass) was determined by EchoMRI in live mice on days 20 and 40 of treatment.

Sacrifice and Sample Collection

At the end of the treatment, animals were anaesthetised by an intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight), at the beginning of the light cycle and after 10-h of fasting. Fat depots were excised and weighed. Blood samples were collected by cardiac puncture with a heparinised syringe and needle (0.2 % heparin diluted with saline, Sigma, Madrid, Spain). Plasma was obtained by centrifugation at 2,500 rpm for 10 min at 4 °C and stored at −20 °C for further analysis.

Plasma Analysis

Fatty acid metabolites were determined using commercial kits and following the indications of the providers [FFA from Wako Chemicals, (Neuss, Germany) and TAG and glycerol from Sigma Diagnostics (Madrid, Spain)]. Insulin, leptin and adiponectin were measured by enzyme-linked immunosorbent assay (ELISA) kits [insulin mouse ultra-sensitive ELISA kit from DRGInstruments (Marburg, Germany), mouse leptin quantikine from R&D Systems (Minneapolis, MN) and rat/mouse adiponectin ELISA kit from Phoenix Europe (Karlsruhe, Germany)].

For fatty acid analysis, plasma samples were prepared following conditions previously described [33, 34] but adjusted to working with a reduced amount of sample (10 μL) as shown in a previous study [35]. Final hexane extracts containing methylated fatty acids from samples were transferred into an automatic injector vial for gas chromatography (GC) analysis. Analysis were performed on a HP-5890 Series GC System (Hewlett-Packard, Waldbronn, Germany) equipped with a flame ionisation detector and a Hewlett-Packard ChemStation software system. Separation of FAME was carried out on a fused silica capillary column Rtx-2330 (40 m × 0.18 mm, ID, 0.10 μm) coated with a non-bonded stationary phase (poly 90 % biscyanopropyl-10 % cyanopropylphenyl) siloxane from Thames Restek (Saunderton, UK). Operating conditions were as follows: injector port temperature 250 °C; helium as carrier gas with 0.37 mL/min; injection volume of 1 μL; detector temperature 270 °C; H_2 flow 30 mL/min; air flow 360 mL/min. The temperature program was as follows [33, 34]: initial temperature 120 °C (10 min), increased at 20 °C/min to 170 °C and held for 20 min, then increased to 230 °C (10 °C/min) and held for 8 min. Total analysis time was 46.5 min for each sample.

GC peaks were identified by comparing the retention times of the sample's FAME with those of the standard mixes. Supelco 37 FAME mix and PUFA No2 (Animal Source) mix were purchased from Supelco (Madrid, Spain) and used as standard mixes. Stock standard solutions were prepared by dissolving FAME standards in n-hexane and were stored at −20 °C until usage. Quantification was done by standard normalisation and the results expressed in relative amounts as percentages of total fatty acid analysed in each sample. The areas corresponding to the individual fatty acid peaks were related to the internal standard peak area in the sample. The sum of these relative areas was taken as the 100 % of the fatty acids detected. Finally, the percentage that every individual fatty acid represented of the total identified was calculated. All samples were analysed in duplicate.

Statistical Analysis

Data are presented as mean ± SEM. Repeated-measures analysis of variance (ANOVA) was used to determine differences in body weight gain. One-way ANOVA was used to determine the significance of the differences in body composition, tissue weights, plasma fatty acids and concentrations of metabolites. If there was a significant difference, a least significant difference (LSD) test was used to determine the particular effect that caused that difference. $P < 0.05$ was considered statistically significant and different lower case letters discriminate differences between groups. Linear relationships between key variables were tested using Pearson's correlation coefficients. The analysis was performed using the SPSS program for Windows version 19 (SPSS, Chicago, IL).

Results

Anthropometry

The rate of body weight gain in mice fed with a calcium-enriched diet (HF + Ca and HF + Ca + CLA groups) was similar to those fed a normal-fat diet (NF group), but lower compared with the HF and HF + CLA groups (Fig. 1a). These differences were statistically significant by day 30 and persisted for the duration of the study ($P < 0.05$).

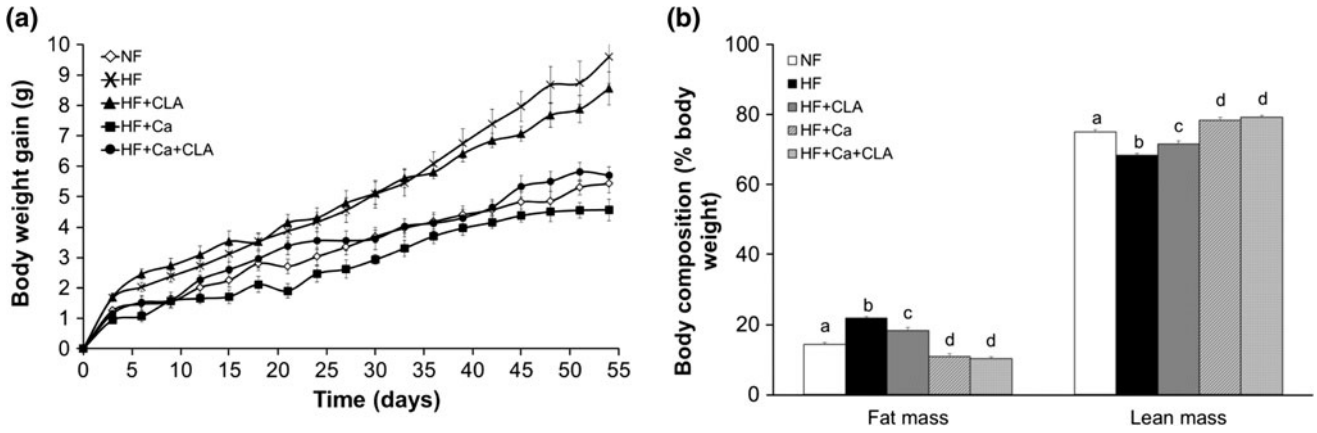

Fig. 1 **a** Cumulative body weight gain during calcium (Ca) and conjugated linoleic acid (CLA) supplementation, and **b** body composition at 40 days of treatment. Each *point* represents the mean ± SEM, $n = 8$. *NF* Normal fat diet, *HF* high fat diet, *Ca* enriched-Ca diet, *CLA* daily oral CLA supplementation (6 mg/day). Body weight gain in NF, HF + Ca and HF + Ca + CLA groups was significantly different from that of HF and HF + CLA from day 30 onwards ($P < 0.05$), determined by repeated measures analyses of variance. Fat and lean content are presented as percentage of the body weight. Mean values with *different lower case letters* are significantly different ($P < 0.05$), ANOVA followed by least significant difference (LSD) test

However, this could not be related to decreased food intake, although HF-fed animals, irrespective of Ca or CLA supplementation, showed a slightly higher calorie intake ($\cong$13 %, $P < 0.05$) than controls over the period studied (supplementary Table S2).

Body composition was improved with CLA and particularly with Ca. In comparison to the NF group, the percentage of body fat in the group fed the calcium-enriched diet was reduced by 18 % in HF + Ca and 21 % in HF + Ca + CLA, while increased by 25 % in HF and 16 % in HF + CLA group at day 20 ($P < 0.001$). In contrast, the percentage of lean mass increased slightly in HF + Ca and HF + Ca + CLA whilst it decreased in the HF and HF + CLA groups when compared to the NF group ($P < 0.001$) (data not shown). After 40 days of treatment, the effects were more robust: the percentage of body fat in HF + Ca and HF + Ca + CLA was lower than in the NF group; body fat was 52 % higher in the HF group in comparison to the NF group and the HF + CLA group presented an intermediate value ($P < 0.001$) (Fig. 1b). Interestingly, the decrease in body fat associated to both Ca and CLA was accompanied by higher lean body mass ($P < 0.01$).

Individual adipose depots showed the effects of both CLA supplementation and calcium-enriched diet (Table 1). HF diet increased the weight of all depots (compared to NF). CLA supplementation was associated with a reduction in total adipose weight, which resulted from minor effects on the different depots, the strongest impact being on the retroperitoneal depot. A calcium-enriched diet, irrespective of CLA supplementation, was associated to a decrease of the different depots that reached levels comparable to control animals (fed with NF diet). Regarding brown adipose tissue, there were no major differences associated to the treatments.

Plasma Determinations

Plasma leptin levels were in accordance with body fat content (Table 2). In comparison to the NF group, leptin

Table 1 Fat pad weights in mice at the end of the treatment

	NF	HF	HF + CLA	HF + Ca	HF + Ca + CLA
White adipose tissues (g)					
Inguinal	0.171 ± 0.021^{ac}	0.411 ± 0.043^{b}	0.287 ± 0.057^{bc}	0.088 ± 0.021^{d}	0.096 ± 0.012^{ad}
Mesenteric	0.278 ± 0.023^{a}	0.512 ± 0.054^{b}	0.454 ± 0.036^{b}	0.215 ± 0.020^{a}	0.258 ± 0.024^{a}
Epididymal	0.498 ± 0.029^{a}	1.259 ± 0.103^{b}	1.047 ± 0.093^{b}	0.400 ± 0.059^{c}	0.417 ± 0.031^{ac}
Retroperitoneal	0.161 ± 0.015^{a}	0.531 ± 0.053^{b}	0.317 ± 0.035^{c}	0.122 ± 0.03^{a}	0.087 ± 0.012^{a}
Sum	1.108 ± 0.079^{a}	2.712 ± 0.229^{b}	2.105 ± 0.209^{c}	0.836 ± 0.135^{a}	0.858 ± 0.064^{a}
Brown adipose tissue (g)	0.072 ± 0.007^{ab}	0.084 ± 0.007^{b}	0.072 ± 0.004^{ab}	0.058 ± 0.006^{a}	0.058 ± 0.002^{a}

[a] *NF* Normal fat diet, *HF* high fat diet, *CLA* daily oral CLA supplementation (6 mg/day), *Ca* enriched-Ca diet

[b] Data are expressed in grams and are mean ± SEM of eight mice

[c] Means in a row without a common letter are statistically different, $P < 0.05$ [ANOVA followed by least significant difference (LSD) test]

Table 2 Fasting plasma concentration of metabolites in mice at the end of dietary treatment

	NF	HF	HF + CLA	HF + Ca	HF + Ca + CLA
Leptin (ng/mL)	1.03 ± 0.15[a]	5.31 ± 0.86[b]	3.77 ± 0.68[b]	0.56 ± 0.13[c]	0.62 ± 0.20[c]
Adiponectin (μg/mL)	20.27 ± 0.94[a]	17.83 ± 0.81[ab]	16.92 ± 1.11[bc]	14.70 ± 0.83[c]	14.60 ± 1.19[c]
Leptin/adiponectin ratio	0.05 ± 0.01[a]	0.26 ± 0.03[b]	0.19 ± 0.02[b]	0.04 ± 0.01[a]	0.04 ± 0.01[a]
FFA (mg/dL)	26.29 ± 1.79	34.13 ± 3.72	25.63 ± 0.78	23.56 ± 1.03	22.50 ± 2.17
Glycerol (mg/mL)	0.26 ± 0.01	0.25 ± 0.03	0.23 ± 0.02	0.20 ± 0.02	0.19 ± 0.02
TAG (mg/mL)	0.47 ± 0.03[a]	0.58 ± 0.03[b]	0.68 ± 0.04[b]	0.53 ± 0.04[a]	0.48 ± 0.06[a]
Glucose (mg/dL)	121 ± 8[a]	169 ± 8[b]	201 ± 15[c]	107 ± 3[a]	111 ± 7[a]
Insulin (pmol/l)	19.9 ± 0.8	21.3 ± 1.3	20.6 ± 0.6	18.4 ± 0.8	19.4 ± 0.9
HOMA	0.817 ± 0.06[a]	1.23 ± 0.1[b]	1.41 ± 0.1[b]	0.668 ± 0.04[a]	0.730 ± 0.05[a]
QUICKI	0.399 ± 0.006[a]	0.373 ± 0.006[b]	0.364 ± 0.005[b]	0.412 ± 0.004[a]	0.406 ± 0.005[a]

[a] *NF* Normal fat diet, *HF* high fat diet, *Ca* enriched-Ca diet, *CLA* daily oral CLA supplementation (6 mg/day), *FFA* unesterified fatty acids, *TAG* triacylglycerides, *HOMA* homeostatic model assessment for insulin resistance, *QUICKI* quantitative insulin sensitivity check index

[b] Data are the mean ± SEM of 7–8 mice

[c] Means in a row without a common letter are statistically different, $P < 0.05$ (ANOVA followed by LSD test)

was 5.2 and 3.7 times higher in the HF and HF + CLA groups respectively ($P < 0.001$) and decreased in the HF groups with the calcium-enriched diet [by 54 % in HF + Ca and 62 % in HF + Ca + CLA ($P < 0.05$)], reaching control values. In contrast, adiponectin decreased under the HF diet ($P < 0.05$) and did not recover control values with calcium supplementation, despite the lower body fat content associated with the treatment. However, the leptin/adiponectin ratio, which is a good indicator of disturbances associated to the homeostasis of the energy balance modulated by these two signalling molecules [14, 15], indicated that the Ca-enriched diet contributed to normalise the values, whereas CLA on its own was not able to revert the increased leptin/adiponectin ratio induced by HF diet.

Obesity induced by HF diet was accompanied by higher plasma glucose levels (39 % in HF and 66 % in HF + CLA animals) and attained control values when calcium was incorporated to HF diet (Table 2). Improvement of glucose–insulin homeostasis under calcium supplementation was also confirmed by homeostatic model assessment for insulin resistance (HOMA) index.

Concerning lipid related metabolites, no differences in FFA and glycerol concentrations in plasma were found among groups (Table 2). However, plasma TAG increased in the HF + CLA group compared to controls (NF group) and calcium-enriched groups ($P < 0.05$).

Individual plasma fatty acids showed an interesting profile (Table 3), particularly induced by the calcium-enriched diet, whereas CLA treatment was associated with minor changes, except those concerning levels of plasma CLA. CLA supplementation induced an increase ranging between 2- and 2.5-fold in plasma levels of the 9,11CLA isomer. Concerning the 10,12CLA isomer, levels tended to be higher in the HF + CLA group (1.6 times) but with the calcium-enriched diet the profile was closer to those of the non-supplemented diets (NF or HF diets), maybe indicating that the 10,12CLA is not absorbed at the same rate as the 9,11CLA isomer or that it is metabolised at a higher rate. However, 10,12CLA was eluted together with C20:1n-9 (gondoic acid) under the chromatographic conditions used, thus some uncertainty on the precise impact of dietary calcium on 10,12CLA plasma levels remains and would need further study.

In addition, CLA treatment promoted increased levels of C18:1n-7 (vaccenic acid) and C20:2 under HF diet, which were not observed when Ca was added (Table 3). This profile contributes to the suggestion that some fatty acids (including CLA) were not efficiently absorbed when calcium was incorporated to the diet.

The highest impact on plasma fatty acid profile was seen in animals fed with the Ca-enriched diet. Total levels of saturated fatty acids were not different between groups, but this was associated with decreased myristic acid (C14:0) and increased pentadecanoic (C15:0), margaric (C17:0) and stearic (C18:0) acids in Ca-supplemented animals in comparison with controls. Levels of monounsaturated fatty acids decreased in the HF diet, and remained lower when the diet contained either calcium or CLA. Palmitoleic (C16:1n-7) and nervonic (C24:1) further decreased under Ca-enriched diets.

Total PUFA increased in the HF diet and recovered control values under the calcium-enriched diet. However, significant differences were observed in specific PUFA, particularly by the calcium-enriched diet. The high levels seen in HF diet of linoleic acid (C18:2n-6) were also observed when calcium was present, and α-linolenic (C18:3n-3) reached a value in between HF and NF animals with calcium diet, whereas docosahexaenoic (C22:6n-3) (DHA) recovered control values. In addition,

Table 3 Fatty acid composition in plasma at the end of treatment

	NF	HF	HF + CLA	HF + Ca	HF + Ca + CLA
C14:0	0.564 ± 0.11[a]	0.281 ± 0.06[b]	0.326 ± 0.06[b]	0.348 ± 0.03[b]	0.421 ± 0.05[b]
C15:0	0.145 ± 0.01[a]	0.124 ± 0.01[b]	0.145 ± 0.01[a]	0.174 ± 0.01[b]	0.187 ± 0.01[b]
C16:0	25.0 ± 1.4	20.2 ± 1.5	19.2 ± 1.6	21.6 ± 0.5	21.7 ± 0.8
C17:0	0.223 ± 0.02[a]	0.278 ± 0.02[a]	0.271 ± 0.02[a]	0.332 ± 0.01[b]	0.314 ± 0.01[b]
C18:0	6.22 ± 1.0[ab]	8.42 ± 0.3[a]	8.56 ± 0.6[ab]	10.6 ± 0.2[b]	9.85 ± 1.4[ab]
C20:0	0.056 ± 0.01	0.083 ± 0.01	0.109 ± 0.01	0.084 ± 0.01	0.096 ± 0.02
C23:0	0.023 ± 0.01	0.018 ± 0.01	0.039 ± 0.01	0.050 ± 0.01	0.044 ± 0.01
C24:0	0.168 ± 0.01	0.164 ± 0.01	0.185 ± 0.01	0.158 ± 0.01	0.167 ± 0.01
Saturated[d]	32.41 ± 2.22	29.6 ± 1.5	28.8 ± 2.1	33.3 ± 0.6	32.8 ± 2.0
C16:1n-9	0.480 ± 0.04[a]	0.565 ± 0.02[a]	0.656 ± 0.03[b]	0.601 ± 0.03[ab]	0.556 ± 0.05[a]
C16:1n-7	5.03 ± 0.3[a]	1.91 ± 0.1[b]	2.16 ± 0.1[b]	1.28 ± 0.1[c]	1.74 ± 0.2[bc]
C17:1	0.214 ± 0.01	0.174 ± 0.02	0.202 ± 0.01	0.166 ± 0.01	0.169 ± 0.02
C18:1n-9	15.3 ± 0.4[a]	11.9 ± 0.4[b]	12.8 ± 0.4[b]	12.7 ± 0.3[b]	13.2 ± 0.4[b]
C18:1n-7	2.01 ± 0.1[a]	1.09 ± 0.1[b]	1.21 ± 0.1[c]	1.00 ± 0.1[b]	1.04 ± 0.1[b]
C24:1	0.240 ± 0.02[a]	0.153 ± 0.01[b]	0.174 ± 0.01[b]	0.099 ± 0.01[c]	0.120 ± 0.02[bc]
Monounsaturated[e]	23.3 ± 0.6[a]	15.8 ± 0.4[b]	17.1 ± 0.5[b]	15.9 ± 0.4[b]	16.9 ± 0.6[b]
C18:2n-6	24.2 ± 1.6[a]	30.1 ± 0.8[ab]	30.5 ± 1.2[ab]	32.9 ± 0.4[b]	32.7 ± 1.7[b]
C18:3n-6	0.798 ± 0.04[ab]	0.896 ± 0.03[a]	0.823 ± 0.04[ab]	0.727 ± 0.02[b]	0.742 ± 0.05[b]
C18:3n-3	0.510 ± 0.02[a]	0.715 ± 0.04[b]	0.739 ± 0.03[b]	0.625 ± 0.03[c]	0.629 ± 0.06[c]
C20:2n-6	0.163 ± 0.01[a]	0.191 ± 0.01[b]	0.222 ± 0.01[c]	0.191 ± 0.01[b]	0.199 ± 0.01[b]
C20:3n-6	0.772 ± 0.05[a]	0.905 ± 0.05[b]	0.948 ± 0.04[b]	0.500 ± 0.02[c]	0.501 ± 0.02[c]
C20:4n-6	16.2 ± 0.5[a]	20.1 ± 0.7[b]	18.8 ± 0.6[b]	14.4 ± 0.2[c]	14.1 ± 0.5[c]
C20:3n-3	0.200 ± 0.02	0.205 ± 0.01	0.193 ± 0.02	0.178 ± 0.01	0.174 ± 0.02
C20:5n-3	0.413 ± 0.03[a]	0.653 ± 0.02[b]	0.600 ± 0.03[b]	0.295 ± 0.02[c]	0.272 ± 0.02[c]
C22:6n-3	0.172 ± 0.01[a]	0.277 ± 0.03[b]	0.277 ± 0.02[b]	0.200 ± 0.02[a]	0.195 ± 0.02[a]
PUFA[f]	43.4 ± 1.9[a]	53.8 ± 1.6[b]	53.1 ± 1.6[b]	50.0 ± 0.4[ab]	49.4 ± 2.0[ab]
9,11CLA	0.088 ± 0.02[a]	0.060 ± 0.01[a]	0.123 ± 0.01[b]	0.075 ± 0.01[a]	0.190 ± 0.02[b]
10,12CLA	0.291 ± 0.0[a]	0.279 ± 0.03[a]	0.459 ± 0.06[b]	0.344 ± 0.03[b]	0.295 ± 0.02[b]

[a] *NF* Normal fat diet, *HF* high fat diet, *Ca* enriched-Ca diet, *CLA* daily oral CLA supplementation (6 mg/day)

[b] Data are the mean ± SEM of 4–8 mice

[c] Means in a row without a common letter are statistically different, $P < 0.05$ (ANOVA followed by LSD test)

[d] Saturated = C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C23:0 + C24:0

[e] Monounsaturated = C16:1n-9 + C16:1n-7 + C17:1 + C18:1n-9c + C18:1n-7 + C24:1

[f] PUFA = C18:2n-6c + C18:3n-6 + C18:3n-3 + C20:2 + C20:3n-6 + C20:4n-6 + C20:3n-3 + C20:5n-3 + C22:6n-3

[g] 10,12CLA was eluted together with C20:1n-9 (gondoic acid) under the conditions used

the calcium-enriched diet was associated with a decrease in the levels of dihomo-γ-linolenic (C20:3n-) (DGLA), arachidonic (C20:4n-6) (ARA) and eicosapentaenoic (C20:5n-3) (EPA), which were lower than in the NF group.

Correlations between Leptin, Adiponectin and Fatty Acids

Representation of the statistically significant correlations found for leptin, adiponectin and leptin/adiponectin ratio and plasma fatty acids is shown in Fig. 2. Leptin levels showed a positive association with the PUFA α-linolenic ($r = 0.612$, $P = 0.001$), C20:3n-6 ($r = 0.797$, $P = 0.000$), ARA ($r = 0.734$, $P = 0.000$), EPA ($r = 0.863$, $P = 0.000$), DHA ($r = 0.554$, $P = 0.003$), ϖ3 ($r = 0.690$, $P = 0.000$), and an inverse correlation with pentadecanoic ($r = -0.726$, $P = 0.000$) and oleic acids ($r = -0.518$, $P = 0.005$).

Plasma adiponectin showed a positive association with the fatty acids myristic ($r = 0.423$, $P = 0.025$), palmitoleic ($r = 0.587$, $P = 0.001$), heptadecenoic ($r = 0.412$, $P = 0.026$), *cis*-vaccenic ($r = 0.549$, $P = 0.002$), C20:3n-3 ($r = 0.371$, $P = 0.047$), C20:3n-6 ($r = 0.382$, $P = 0.041$), nervonic ($r = 0.719$, $P = 0.000$) and MUFA ($r = 0.457$, $P = 0.000$). Negative associations between adiponectin and fatty acids were confirmed for the

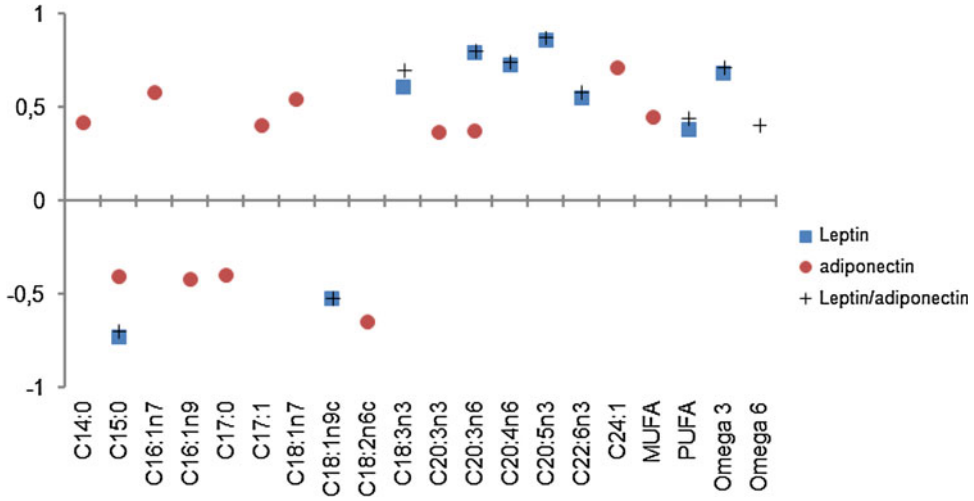

Fig. 2 Correlations of leptin, adiponectin and leptin/adiponectin ratio with plasma fatty acids in mice. Point estimates of Spearman's correlation coefficient is presented for leptin (*filled squares*), adiponectin (*filled circles*) and leptin/adiponectin ratio (*plus signs*) and plasma fatty acids. The analysis was performed using data belonging to all groups ($n = 27$–29) and only statistically significant correlations are represented ($P < 0.05$)

saturated pentadecanoic ($r = -0.398$, $P = 0.033$) and margaric acid ($r = -0.394$, $P = 0.035$), as well as for unsaturated hexadecenoic ($r = -0.416$, $P = 0.025$) and linoleic acid ($r = -0.644$, $P = 0.000$).

The leptin/adiponectin ratio showed associations with the same fatty acids that correlated with leptin (maintaining the respective trend, either positive or negative), plus with ϖ6 ($r = 0.411$, $P = 0.030$).

Discussion

Consistent with previous observations [12–15], our results showed the potential of calcium and CLA supplementation in the prevention of obesity promoted by a high-fat diet. However, the present study demonstrated that dietary supplementation with both calcium and CLA did not have additive effects in modulating body composition. Feeding a HF diet (HF group) elicited an increase of 77 % in body weight gain (Fig. 1a) and of 52 % in the percentage of fat mass (Fig. 1b) compared to mice fed the NF diet. The increase was less pronounced when the group received a moderate daily dose of CLA (increase of 58 % in body weight gain and of 28 % in fat mass, HF + CLA group). Interestingly, when the diet was enriched with calcium, irrespective of the CLA supplementation (HF + Ca and HF + Ca + CLA groups), animals managed to keep body weight at the same level as the controls and to decrease fat accumulation in the different adipose depots. Moreover, fat mass was nearly 50 % and 26 % lower in groups that were fed the calcium-enriched HF diet compared to the HF and NF groups, respectively (Fig. 1b). Furthermore, body composition analysis at 20 (data not shown) and 40 days of treatment (Fig. 1b) confirmed the early effect of calcium on reducing fat accumulation compared to both HF and NF groups, whereas CLA required a longer period to exert a decrease in the percentage of fat mass compared to the HF group. Thus, these results demonstrated that the effects of calcium supplementation against fat accumulation are seen earlier than with CLA and, moreover, that calcium is more efficient than CLA, at least in the conditions assayed.

Interestingly, groups receiving a calcium-enriched diet resembled control animals (NF group), particularly regarding anthropometric and associated parameters (e.g. glucose, insulin). Some mechanisms may be active to avoid a further decrease once body weight and/or adiposity have reached "control" levels and, as a consequence, the potential additional effect of CLA would not be observed under the calcium-enriched diets. Furthermore, plasma metabolites of animals fed calcium-enriched diets are suggestive of a lack of activity and/or deficient absorption of CLA when both compounds are included in diet.

Leptin is a cytokine-like hormone produced mainly by adipocytes and is considered an index of body fat content. Accordingly, the percentage of fat mass in all experimental groups corresponded well with the weights of the adipose tissues collected ($r = 0.907$, $P < 0.001$) and correlated positively with leptin plasma concentration ($r = 0.887$, $P < 0.001$). By contrast, adiponectin decreased under HF diet ($P < 0.05$) as expected [36–39] but did not recover control values with calcium supplementation despite the lower body fat content. Other groups have also reported reduced levels of plasma adiponectin in CLA treated mice [31, 32, 40–43] and different mechanisms have been proposed, such as a reduction in adipocyte number [42] and in the adiponectin gene expression in fat depots [32, 40]. Since adiponectin is primarily released from adipocytes, the reduction observed in plasma adiponectin could be consistent with the lower fat depot sizes, at least in the HF + Ca and HF + Ca + CLA groups. However, we have analysed adiponectin mRNA levels in epididymal adipose tissue and no differences were observed between the CLA supplemented groups and the NF group (data not shown).

Interestingly, despite adiponectin reduction, the leptin/adiponectin ratio in the HF + Ca and HF + Ca + CLA groups was maintained close to NF values, but was altered in the HF and HF + CLA groups (Table 2). Deregulation in the production of these two adipocytokines has been observed in both obese and lipodystrophy states [44] and has been proposed to contribute to the impairment of insulin sensitivity [45]. This pattern would fit with the increased levels of glucose found in HF animals and even higher ones with CLA treatment, thus contributing to the altered HOMA index in HF and HF + CLA groups. However, HOMA index was restored to normal values under Ca supplementation (Table 2). Altogether, these data contribute to confirm that the relative amount of the two adipocytokines leptin and adiponectin is likely to be more important than their absolute concentrations, as observed in previous studies using moderate doses of CLA [14, 15], and this relative concentration could be playing a role in the maintenance of glucose/insulin homeostasis, as seen particularly in the groups receiving the calcium-enriched diet. In addition, the strong association found between plasma leptin, adiponectin, its ratio and specific fatty acids, particularly PUFA, contribute to confirm the close relationship between these two hormones and PUFA as well as the impact of calcium enriched diet in the PUFA profile (Fig. 2).

An interesting finding of our study was the different composition of plasma fatty acids, particularly under the calcium-enriched diet (Table 3). Concerning lipid-related metabolites, no differences in FFA and glycerol concentrations in plasma were found among groups, and mobilisation of TAG was seen with CLA (Table 2).There are early reports underlying the hypolipidemic effects of calcium during both saturated and polyunsaturated fat supplementation, and the effects are more pronounced in the presence of saturated fat [46]. However, most attention has focussed on TAG, cholesterol and lipoproteins [47]. To our knowledge, this is the first report regarding the impact of a high-calcium diet on plasma fatty acid profile.

Specifically, a set of fatty acids were decreased with the HF diet (with respect to NF) and remained at lower levels with the calcium-enriched diet; this was the case for saturated myristic (C14:0) and the unsaturated palmitoleic (C16:1n7) and nervonic (C24:1) acids. Plasma palmitoleic acid is considered a marker of endogenous lipogenesis and in human obesity it correlates well with adiposity indexes and insulin [48–50]. HF diet feeding is also associated with increased endogenous lipogenesis in animal models [51, 52]. However, this association may not be so simple because the levels of palmitoleic and oleic acid—the main products of the considered lipogenic enzyme SCD1—were decreased in the HF group (which was obese), and remained at this level in the calcium-enriched groups (which showed reversed obesity). The most striking result concerned plasma PUFA in calcium-enriched diets. Linoleic acid (C18:2 n6) increased in the HF diet and calcium-enriched diets maintained these high levels (by 35 % with respect to NF diet). Somehow, this resulted in significant decreases of the subsequent derived fatty acids, affecting both the ϖ6 and ϖ3 series. We do not know whether the increased levels of linoleic acid were the cause or a consequence, but the essential fatty acids ARA and EPA were decreased by 12 and 46 % respectively in Ca-fed animals, whereas the levels of DHA were not affected. In the present study we do not have precise information about a mechanism that could explain why specific fatty acids were altered in plasma. Although we did not measure fat in faeces, earlier evidence supports increased faecal fat excretion in calcium-enriched diets, which is associated with decreased fat absorption [53, 54]. A potential mechanism suggested by a number of studies is that dietary calcium interferes with fat absorption in the intestine by forming insoluble soaps with fatty acids and/or binding of bile acids (for meta-analysis see [55]); whether this would affect the profile of plasma fatty acids remains to be elucidated. Given the activity of microflora influencing intestinal fermentation and producing fatty acids (mainly short chain) from the digestion products, another tentative mechanism could involve metabolic activity of intestinal microbiota and/or changes in the bacterial community when calcium is incorporated into the diet [56]. This novel hypothesis is under further investigation in our group.

The implication of the results of this study may be significant for two main reasons. Data corroborate that dietary calcium is of importance in the regulation of body weight, although co-supplementation with CLA did not show additive effects in ameliorating fat accumulation under a high-fat diet in mice. Therefore, results suggest that calcium supplementation may interfere with absorption and/or bioactivity of CLA. Since CLA is commercially available in CLA-fortified dairy products, additional studies would be necessary to confirm better efficiency of its anti-obesity properties using other food matrices. Furthermore, the results outline the impact of calcium supplementation particularly on plasma PUFA, linoleic acid and its derived ϖ3 and ϖ6 series. This highlights the importance of analysing plasma fatty acids when dietary calcium supplementation is considered, particularly in the context of bone health and osteoporosis prevention. Further research is needed to replicate these results in other animal models and to assess, in particular, whether this may be the case in dietary calcium-supplemented humans, and to define the mechanism underlying the high impact of dietary calcium supplementation on fatty acid profile and particularly on essential fatty acids.

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Conflict of interest The authors declare that they have no conflict of interest.

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