Selective effect of conjugated linoleic acid isomers on atherosclerotic lesion development in apolipoprotein E knockout mice

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Abstract

Research suggests that conjugated linoleic acid (CLA) may inhibit atherosclerosis, but there are contradictory results in different animal models fed heterogeneous mixtures of CLA isomers. This study addressed the hypothesis that the individual CLA isomers may exert different atherogenic properties. ApoE$^{-/-}$ mice were fed isocaloric, isonitrogenous westernized diets containing 0.15% cholesterol and enriched with 1% (w/w) *cis*-9,*trans*-11-CLA (c9,t11-CLA), *trans*-10,*cis*-12-CLA (t10,c12-CLA) or linoleic acid (control diet) for 12 weeks. At the end of the dietary intervention, the effects of CLA isomers on the development of atherosclerotic vascular lesions, lipid metabolism, inflammation and oxidative stress were assessed. The t10,c12-CLA diet had a profound pro-atherogenic effect, whereas c9,t11-CLA impeded the development of atherosclerosis. *En face* aortic lesion assessment showed more dorsal and lumbar extensions presenting atherosclerotic foci after the t10,c12-CLA diet. Furthermore, animals fed t10,c12-CLA had pronounced hyperlipidemia, higher 8-*iso*-prostaglandin F$_{2\alpha}$ levels, higher vulnerable atherosclerotic plaque with a lower smooth muscle and fibre contents and higher macrophage content and activation, assayed as plasma chitotriosidase compared to the control or c9,t11-CLA dietary groups. Plasma chitotriosidase activity was more closely associated with the extent of the plaque than with MOMA staining or than monocyte chemoattractant protein-1 levels. Our results demonstrate that CLA isomers differentially modulate the development of atherosclerosis, c9,t11-CLA impedes, whereas t10,c12-CLA promotes atherosclerosis. These opposing effects may be ascribed to divergent effects on lipid, oxidative, inflammatory and fibro muscular components of this pathology. Plasma chitotriosidase is a better indicator of dietary fat interventions that alter plaque monocyte activity in this murine model.
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Keywords: CLA; Apolipoprotein; Atherosclerosis; Chitotriosidase; Paraoxonase

1. Introduction

Conjugated linoleic acid (CLA) is the term used to describe a group of positional and geometric isomers of linoleic acid where one or both of the double bonds are in the *cis*- or the *trans*- configuration and separated by a simple carbon–carbon linkage rather than by the normal methylene group, this change produces a diene structure. The most abundant natural form of the CLA isomer is *cis*-9,*trans*-11-CLA (c9,t11-CLA) [1], although synthetic CLA sources provide almost equal amounts of *trans*-10,*cis*-12-

CLA (t10,c12-CLA) [2]. c9,t11-CLA is formed during an incomplete ruminal biohydrogenation of linoleic acid and Δ9 desaturation of *trans* vaccenic acid in animal tissues. Therefore, ruminant food products (meat and dairy products) are the major source of CLA in the human diet, and the daily intake of CLA can varies between 400 and 600 mg in man [3]. Multiple biological effects have been ascribed to CLA, including control of body composition and energy metabolism, modulation of immune responses, insulin resistance and diabetes, lipid metabolism and platelet activity [3–9].

Atherosclerosis, a disease of the large arteries, is the primary cause of heart disease and stroke. In westernized societies, it may be the indirect cause of approximately 50% of all deaths [10]. Inflammatory and oxidative mechanisms, coupled with dyslipidemia, initiate atheroma formation. Leukocyte recruitment and expression of pro-inflammatory cytokines into the sub-endothelial space characterize early atherogenesis. Later, the atheroma progresses into an enlarged and complicated atherosclerotic plaque. Atherosclerosis is a chronic pro-inflammatory condition that can be converted into an acute clinical event by plaque rupture and thrombosis [11,12].

The potential effects of CLA on atherosclerosis are controversial. In vivo studies have reported anti-atherogenic properties of CLA rich diets in rabbits [13–16] and hamsters [17,18]. In contrast, increased fatty streak lesions were found in C57BL/6 mice fed CLA-containing atherogenic diets [19]. Furthermore, the anti-atherogenic mechanisms ascribed to CLA are unclear. Decreased prostanoid synthesis as a result of cyclooxygenases (COX-1 and COX-2) inhibition has been reported [20,21]. However, other studies show these mechanisms are insufficient to explain the complex and multiple effects of CLA on vascular homeostasis [22,23]. While a diet of mixed CLA isomers activate peroxisome proliferator activated receptor (PPAR) α and γ in the aorta [24,25], t10,c12-CLA antagonizes PPARγ activation in adipocytes [26,27] which would in turn modulate key genes involved in atherosclerosis. A key issue lies in the composition of the CLA intervention, studies to date have used heterogeneous blends of CLA isomers. The different CLA isomers have divergent effects on markers of lipid metabolism and insulin resistance [9]. Therefore, this study tested the hypothesis that the main CLA isomers had divergent isomer specific effects on atherosclerotic lesion development, which may explain the variability between studies. ApoE-deficient (ApoE$^{-/-}$) mice were selected because they spontaneous develop atherosclerosis and are a valuable tool to investigate the effect of dietary interventions [28]. Also the progression and histopathology of lesions, including fatty streaks, necrotic cores, fibrous caps and plaque rupture, show similar features to those observed in man [29–33]. Moreover, this experiment was designed to determine the isomer specific effect of CLA on the nature of atherosclerosis, as determined by lipid, oxidative, inflammatory processes and SMC involvement.

Table 1
Composition of the control, c9,t11-CLA and t10,c12-CLA semi-purified diets

	Control	c9,t11-CLA	t10,c12-CLA
Maize starch	60.8 (53.9)	60.8 (53.9)	60.8 (53.9)
Calcium caseinate	15.84 (16.1)	15.84 (16.1)	15.84 (16.1)
Cellulose	5.83	5.83	5.83
Mineral mix	4.07	4.07	4.07
Vitamin mix	1.17	1.17	1.17
Cholesterol	0.15	0.15	0.15
Fat blend	12.3 (30)	12.3 (30)	12.3 (30)
Variable fatty acid composition of the PUFA fraction			
Linoleic acid	1.0	0.03	0.03
c9,t11-CLA	–	0.86	0.12
t10,c12-CLA	–	0.10	0.85
Other CLA isomers	–	0.01	0.01

Dietary components are expressed as % (w/w). The percentage of energy provided by protein, fat and carbohydrate fractions is presented in parentheses.

2. Methods

2.1. Animals

Twenty-nine 3-month-old homozygous apoE-deficient male mice, bred in the Unidad Mixta de Investigación, Zaragoza, were used. Initial plasma cholesterol and triglyceride concentrations were estimated in samples taken from retroorbital plexus in isofluorane-anesthetized animals after overnight fasting, and were used to randomly distribute in three groups of similar concentrations. The mice were housed in sterile filter-top cages in rooms maintained on a 12-h light/12-h dark cycle and had ad libitum access to food and water. Food consumption, which was determined using metabolic cages (Biosys, Barcelona, Spain), and body weights were recorded weekly throughout the experiment. The protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

2.2. Diets

The description of semi-purified diets is shown in Table 1. All of them were isocaloric and isonitrogenous, contained 0.15% (w/w) cholesterol and provided 30% of energy as fat. The diets provided equal proportions of SFA, MUFA and PUFA (40, 33 and 27% of total fat, respectively). The PUFA fraction of the control diet contained linoleic acid as differential fatty acid, and the CLA enriched diets contained an equivalent amounts of either c9,t11-CLA or t10,c12-CLA isomers, as previously described [9]. All diets were prepared by Unilever (Vlaardingen, The Netherlands) and stored in N$_2$ atmosphere at −20 °C. Fresh diet was provided daily. Experimental diets were fed for 12 weeks and all diets were well tolerated.

2.3. Biochemical determinations

After the experimental period, animals were sacrificed by suffocation with CO_2 and blood was drawn from their hearts. Plasma NEFA, triglyceride and total cholesterol concentrations were measured in a microtiter assay, using commercial kits from (Wako, and Sigma Chemical Co., Madrid, Spain). HDL cholesterol was determined in a similar manner after phosphotungstic acid–$MnCl_2$ (Roche, Barcelona, Spain) precipitation of apo B containing particles [34]. Measurement of the total 8-*iso*-prostaglandin $F_{2\alpha}$ was carried out by immunoassay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Paraoxonase was assayed as arylesterase activity following the protocol previously described [35]. Results were expressed as μmol phenylacetate hydrolyzed ($min^{-1} L^{-1}$ (IU/L)).

Chitotriosidase enzyme assay was carried out in a microtiter plate as described [36]. Briefly, chitotriosidase activity was determined by incubating 4 μL of serum with 40 μL of 22 μM fluorogenic, substrate 4-methylumbelliferyl-β-D-N,N',N'-triacetylchitotrioside (Sigma Chemical, Madrid, Spain), in 100 mM citric acid and 200 mM sodium phosphate buffer, pH 5.2 for 15 min at 37 °C. The reaction was stopped with 0.3 M glycine–NaOH buffer pH 10.6 by mixing at room temperature. The chitotriosidase hydrolysis of the substrate produces the fluorescent molecule 4-methylumbelliferone, which was quantified with a fluorometer (SPECTRAfluor Plus, TECAN), using excitation at 366 nm and emission at 446 nm, and compared with a standard 4-methylumbelliferone (Sigma Chemical) calibration standards. Chitotriosidase activity was expressed as nmol of substrate hydrolyzed per minute per litre of serum. Serum chitotriosidase activity was measured in duplicate and the intra-assay coefficient of variation was lower than 5%.

Apolipoproteins A-I and B were quantified by enzyme-linked immunosorbent assays with specific polyclonal antibodies (Biodesign, Saco, ME) as previously described [37]. All assays were done in triplicate and all samples were processed in the same day. Intra-assay CV's was lower than 4.6%. Orosomucoid concentration was determined by single radial immunodiffusion test using the mouse α1-AG plate kit (Cardiotech Services, Louisville, KY) according to the manufacturer's instructions. MCP-1 was determined by using the ELISA development kit from BioVision (Mountain View, CA).

2.4. Evaluation of atherosclerotic lesions

The heart and the arterial tree were perfused with phosphate-buffered saline under physiological pressure. Hearts and aortas were dissected out and cleaned. For *en face* studies, aortas stored in neutral formaldehyde, were longitudinally opened and stained as described [38]. The aortic base of the hearts was taken, transferred to liquid OCT (Bayer

Diagnostic, Germany) and frozen in liquid N_2-cooled isopentane. Serial cryosections were made. Average lesion sizes were used for morphometric evaluations based on the method of Ref. [39]. Images were captured and digitized using a Nikon microscope equipped with a Cannon digital camera. Morphometric analyses were performed using Scion Image software.

Macrophage staining immunohistochemistry was carried out using a monoclonal rat antibody to the mouse monocyte–macrophage marker MOMA-2 (Serotec, Oxford, UK; 1:25 dilution) followed by detection with a polyclonal goat anti-rat IgG coupled to Alexa fluor 594 (Molecular Probes, Eugene, OR, USA; 1:200 dilution). Sections were counterstained for nuclei with SYTOX green (Molecular Probes). Fluorescent images were captured as described above. Data represent area of MOMA-2 staining from six sections per mouse.

Fibro muscular cap as index of plaque vulnerability was assessed by estimating the presence of smooth muscle cells (SMC) and fibre content of plaques. The former was evaluated by immunohistochemistry of cryosections using an anti mouse α-actin conjugated to alkaline phosphatase (Sigma Chemical; diluted 1:25). Non-specific binding was removed by repeated washes and bound alkaline phosphatase activity was revealed with Fast Red TR/Naphtol AS-MX (Sigma Chemical) as substrate. Visualization of red colour indicated presence of SMC. The staining of fibres was carried out by counterstaining with aniline blue. Images were captured as described above and data are expressed as percent of plaque covered by SMC and fibres.

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. Unless otherwise stated, non parametric Mann–Whitney U-test for comparison between pairs was used for unpaired observations. Only differences in weight were evaluated by repeated-measures ANOVA analysis. Association between variables was assessed by Spearman correlation test (ρ). A covariance analysis (ANCOVA) was performed in order to check whether the observed effects on lesion were biased by cholesterol values. All statistical tests were performed with the use of SPSS Version 11.0 (SPSS Inc., Chicago, IL), and a value of $p \leq 0.05$ was taken to indicate statistical significance.

3. Results

3.1. The isomer specific effects of CLA on body weight and plasma lipid metabolism

Fig. 1 shows the variations in body weight during the dietary experiment for each group. The t10,c12-CLA isomer fed mice showed significant lower weight gain ($p = 0.05$) compared to those fed the control and c9,t11-CLA diets. The lower weight gain was due to less white adipose tissue mass,
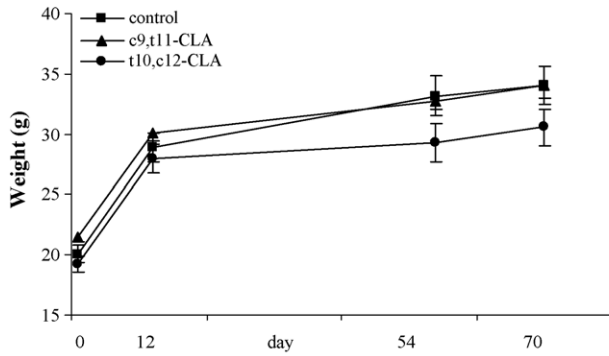
Fig. 1. The effect of control, c9,t11-CLA and t10,c12-CLA diets on body weight in apoE$^{-/-}$ mice. Data are shown as mean ± S.E.M. Statistical analysis was carried out by repeated-measurements ANOVA. Differences within-subjects in weight ($p < 0.001$) and in the interaction weight-group ($p < 0.05$) were observed. Analysis between-subjects gave difference between control and t10,c12 groups ($p < 0.05$).

Fig. 2. Cross-sectional lesion area in the aortic root of apoE$^{-/-}$ mice fed the control, c9,t11-CLA and t10,c12-CLA diets. Each symbol represents one animal; bars represent means. $^*p \leq 0.05$ vs. control; $^{\#}p \leq 0.01$ between isomers (Mann–Whitney).

epididimal adipose tissue deposits were significantly lower in the t10,c12-CLA fed mice, compared to control and c9,t11-CLA fed mice (data not shown).

Table 2 shows the isomer specific effects of CLA rich diets on plasma lipid and lipoprotein metabolism. Pre-intervention plasma cholesterol concentrations were not significantly different between experimental groups. Following the administration of the diets, a selective behaviour between isomers was observed. The c9,t11-CLA diet significantly reduced plasma cholesterol and NEFA concentrations and increased apo A-I concentrations, compared to the control diet. In contrast, the t10,c12-CLA diet significantly increased plasma total and HDL cholesterol, triglycerides and NEFA concentrations and reduced apo A-I levels compared to control and c9,t11-CLA diets. Plasma apo B concentration were significantly greater after the t10,c12-CLA diet, compared to c9,t11-CLA, but not the control diet. Furthermore, the t10,c12-CLA diet was associated with hyperglycaemia (22.6 ± 2.1 mmol/L versus 17.3 ± 0.9 mmol/L for control group; $p < 0.05$). In contrast, the c9,t11-CLA fed group reduced plasma glucose (14.4 ± 0.7 mmol/L versus 17.3 ± 0.9 mmol/L in control group; $p < 0.05$) and insulin concentrations (217 ± 7.3 pmol/L versus 295 ± 32 mmol/L
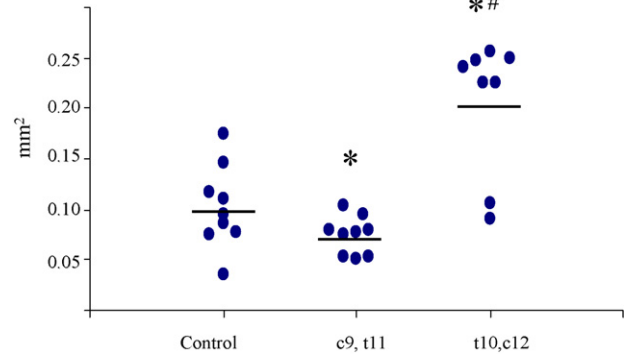
in control group; $p < 0.05$). These data highlight the selective action of the individual CLA isomers on metabolic markers of insulin resistance that are relevant to atherosclerosis.

3.2. Divergent isomer specific effects of CLA on atherosclerotic lesion area

Two quantitative methods are generally used to measure the surface of atherosclerotic lesions. The first, measures cross-sectional area in slices taken at the level of the aortic sinus, and is useful to assess mild or early stage disease. The second *en face* method involves pinning out the whole aorta and quantifying lesion area as a percentage of total surface area, and its purpose is to measure advanced lesions [40]. In the present report, both methods have been used. Fig. 2 shows atherosclerotic lesion expressed as aortic root cross-sectional lesion areas in mice killed at the end of the dietary intervention at 20 weeks of age. The lesion area was significant less after feeding apoE$^{-/-}$ mice the c9,t11-CLA diet compared to the control diet. In contrast, the t10,c12-CLA fed group demonstrated significant greater lesion area compared to both the control and c9,t11-CLA fed mice. These results demonstrate a clear difference in lesion development between CLA isomers.

Table 2

Initial cholesterol and final plasma lipid and apolipoprotein concentrations in apoE knockout mice fed control, c9,t11-CLA and t10,c12-CLA diets for 12 weeks

	Control ($n = 10$)	c9,t11-CLA ($n = 10$)	t10,c12-CLA ($n = 9$)
Initial cholesterol (mg/dL)	459 ± 12	467 ± 24	417 ± 23
Final cholesterol (mg/dL)	2209 ± 125	1590 ± 67*	2596 ± 154$^{*,\#}$
HDL cholesterol (mg/dL)	12 ± 3	8 ± 2	22 ± 3$^{*,\#}$
Triglycerides (mg/dL)	164 ± 8	123 ± 14	428 ± 56$^{*,\#}$
NEFA (mg/dL)	3.2 ± 0.3	2.1 ± 0.4*	8.6 ± 1.5$^{*,\#}$
Apo A-I (mg/dL)	77.5 ± 3.64	84.2 ± 2.8*	59.2 ± 3.7$^{*,\#}$
Apo B (A.U./dL)	63 ± 0.7	60 ± 1	65 ± 1.4$^{\#}$

Results are expressed as means ± S.E.M. Mann-Whitney *U*-test was used to pair-wise comparisons.

* $P \leq 0.05$ vs. control.

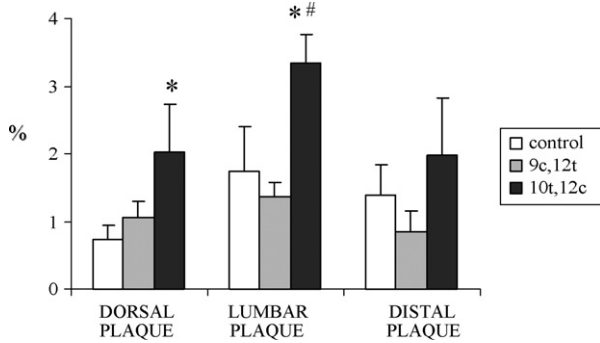$^{\#}$ $P \leq 0.05$ vs. c9,t11-CLA isomer.

Fig. 3. Lesion coverage in the aortas of apoE$^{-/-}$ mice fed the control, c9,t11-CLA and t10,c12-CLA diets. Lesion areas occupied in the different regions are shown. Results are expressed as mean ± S.E.M. Mann–Whitney test between pairs was used as statistical analysis. [*]$p \leq 0.05$ vs. control; [#]$p \leq 0.01$ between isomers.

For the *en face* method, the aortic tree was divided into four zones: aortic arch, dorsal (from the first intercostal until diaphragmatic artery), lumbar (since diaphragmatic until iliac artery) and distal (since iliac artery onward). Fig. 3 presents the percentage lesion coverage in the dorsal, lumbar and distal regions. There were significantly more dorsal and lumbar extensions presenting atherosclerotic foci after the t10,c12-CLA diet. In contrast, the aortic arch did not show changes between groups (data not shown), which suggests that deposit of atherosclerotic lesions in this region was saturated and not sensitive to dietary intervention. These results clearly show selective development of atherosclerosis dependent on the CLA isomer provided.

3.3. Isomer specific effects of CLA on atherosclerotic plaque stability

Fibres and smooth muscle cell content in atherosclerotic plaques are considered indexes of plaque stability, both parameters were characterised and presented in Fig. 4. The presence of smooth muscle cells was evidenced as colour red following the immunohistochemistry with anti α-actin and fibres appeared as blue (Fig. 4I). Quantitative data of these parameters for the experimental groups are displayed in Fig. 4II. There were significantly more smooth muscle cells and fibre content in the atherosclerotic plaques of the control and c9,t11-CLA groups compared to the t10,c12-CLA. These results show that the CLA isomers also selectively modulate plaque stability by modifying fibro muscular characteristics.

3.4. Effect of CLA isomers on oxidative and inflammatory parameters

To study the effect of CLA isomers on anti-oxidative mechanisms, paraoxonase aryl esterase activity, an antiox-
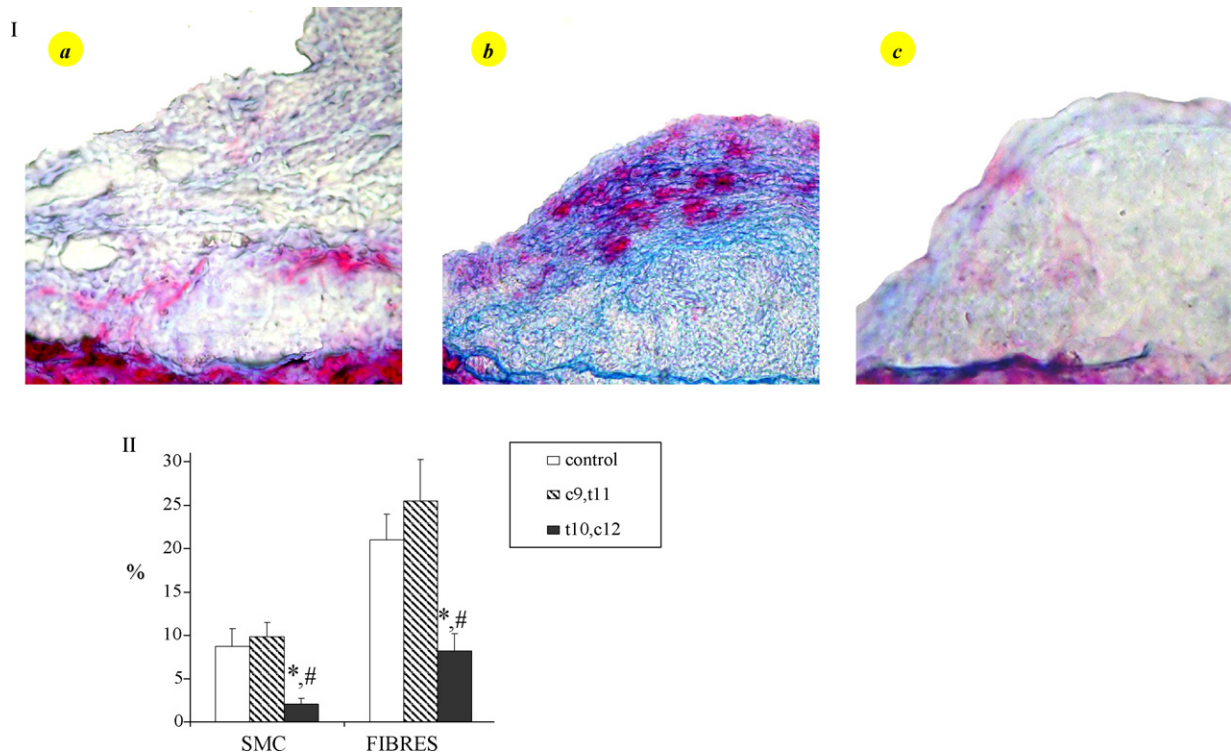

Fig. 4. Fibro muscular characteristics of atherosclerotic plaques in apoE$^{-/-}$ mice fed the control, c9,t11-CLA and t10,c12-CLA diets. (I) Representative images from each group evidencing presence of smooth muscle cells, as red deposits of immunostaining with anti α-actin, and fibres as blue filaments in sub-endothelial space: a, control; b, c9,t11-CLA; c, t10,c12-CLA. Magnification ×400. (II) Morphometric quantification of areas covered by SMC and fibres. Results are expressed as percentage of lesion area stained for each parameter and are mean ± S.E.M. Mann–Whitney test between pairs was used as statistical analysis. [*]$p \leq 0.01$ vs. control; [#]$p \leq 0.01$ between isomers.

Table 3
Markers of oxidative stress and inflammation in apoE$^{-/-}$ mice fed a control, c9,t11-CLA and t10,c12-CLA diets for 12 weeks

	Control ($n = 10$)	c9,t11-CLA ($n = 10$)	t10,c12-CLA ($n = 9$)
Arylesterase activity (IU/L)	89890 ± 5340	88670 ± 6420	77960 ± 6230
8-*iso*-Prostaglandin F$_{2\alpha}$ (pg/mL)	157 ± 13	232 ± 25	293 ± 15[*]
MOMA area (mm^2)	0.084 ± 0.010	0.076 ± 0.008	0.108 ± 0.017[#]
Chitotriosidase (nmol/min/L)	1.4 ± 0.1	1.6 ± 0.1	2.0 ± 0.1[*,#]
ORM (µg/mL)	240 ± 5.7	415 ± 15[*]	378 ± 16[*]
MCP-1 (ng/mL)	1.2 ± 0.03	1.7 ± 0.1[*]	1.2 ± 0.04

Results are expressed as means ± S.E.M. Mann–Whitney *U*-test was used to pair-wise comparisons.

[*] $P \leq 0.05$ vs. control.

[#] $P \leq 0.05$ vs. c9,t11-CLA isomer.

idant enzyme present in HDL, and 8-*iso*-prostaglandin F$_{2\alpha}$ levels were measured and presented in Table 3. The t10,c12-CLA diet significantly increased plasma 8-*iso*-prostaglandin F$_{2\alpha}$ levels, compared to the control and c9,t11-CLA enriched diets. Neither isomer induced changes on the catalytic activity of paraoxonase aryl esterase. In order to quantify the inflammatory response, macrophage involvement at two different levels was assessed. At atherosclerotic foci, MOMA-2 antibody staining allowed estimation of monocyte recruitment; and at a systemic level, chitotriosidase activity and MCP-1 levels were used as biochemical markers of macrophage activation, MOMA-2 stained area was significantly greater in mice fed the t10,c12-CLA diet compared to mice fed the c9,t11-CLA enriched diet (Table 3). Similarly, plasma chitotriosidase activity was significantly increased after the t10,c12-CLA diet compared to the control and c9,t11-CLA fed experimental groups (Table 3). Plasma MCP-1 levels increased in the c9,t11-CLA compared with both control and t10,c12-CLA groups. Atherosclerosis is associated with elevated acute phase proteins, including orosomucoid (ORM) [41]. Plasma ORM levels were significantly increased in animals consuming both CLA isomer enriched diets, compared to the control group (Table 3).

4. Discussion

The present work clearly demonstrates pronounced divergent effects of two CLA isomers on the development of atherosclerosis in apoE$^{-/-}$ mice. Two different quantification methods ensured comprehensive plaque size measurement, followed by a histological analysis of the lesion characteristics, showed important differences between CLA isomers on the development and properties of atherosclerotic lesions. In addition, plasma lipid analysis and markers of the oxidative and inflammatory components of atherosclerosis support the hypothesis that CLA isomers have different effects on cellular and molecular components involved in aortic lesion development and stability.

This is the first report comparing the effect of c9,t11-CLA and t10,c12-CLA enriched diets on the development of atherosclerosis in apoE$^{-/-}$ mice. These results may explain some of the discrepancies in the literature in relation to the effect of CLA on atherosclerosis, particularly in those studies

that used a mixture of CLA isomers [13,15,17,19], a summary of which is presented in Table 4. Recent studies determined the isomer specific effects of CLA on the development of atherosclerosis in rabbits [16] and hamsters [42,43]. As in apoE$^{-/-}$ mice, the c9,t11-CLA isomer inhibits atherosclerosis. Despite similar dietary conditions, the t10,c12-CLA did not promote atherosclerosis in rabbits. The different response between species was not related to the hypercholesterolemic state of the t10,c12-CLA fed apoE$^{-/-}$ mice. ANCOVA, with cholesterol as covariable, demonstrated that the significantly different effect between CLA isomers on atherosclerosis remained. Therefore, other pathological processes also affected the progression of atherosclerotic plaques in this experiment. A potential flaw of recent works [42,43] and our study is that the level of the individual CLA isomers (1% of diet) is four times higher than the required amount needed for maximal physiological response in terms of body fat control or the inhibition of tumorigenesis [44]. However, no signs of toxicity of these high doses were reported [42,43]; and even in our study, the diet enriched in c9,t11-CLA was particularly favourable for controlling both the atherosclerotic process-present report- and the hepatic steatosis of the apoE$^{-/-}$ mouse model [45]. Likewise, NZB/W F1 mice, as a model of autoimmune disease, fed a 0.5% CLA-containing diet (0.25% c9,t11; 0.25% t10,c12) showed an extended lifespan [46]. These results suggest that a higher supply of CLA isomers might be required to obtain a benefit in pathological conditions.

The diverse metabolic phenotype induced by the different CLA isomers provided a unique model to determine cellular and molecular effect of CLA isomers on atherosclerosis. The t10,c12-CLA isomer acted as a catabolic agent, impeding weight gain as previously reported [9,26,47]. Of particular interest was the divergent effect on the presence and activation of macrophages in lesions, as well as the fibro muscular characteristics of atherosclerotic plaques. The t10,c12-CLA isomer induced a pro-atherogenic, hyperlipidemic, pro-oxidative state as reported in man [48–51]. In contrast c9,t11-CLA fed animal showed retarded lesion development, associated with lower total cholesterol and NEFA levels. This data agrees with complimentary analysis wherein our group showed that a diet predominant in c9,t11-CLA promotes the regression of established atherosclerosis in apoE$^{-/-}$ mice [52]. Collectively, our data indicate that

Table 4
Effects of CLA on atherosclerotic progression in several animal models

Refs.	Species	Parameter measured	Basal diet	Duration	Control group	CLA type	Outcomes	
Lee et al. [13]	New Zealand male/female rabbits	Aortic cholesterol deposition Percentage of total aortic surface covered by fatty lesions	12% Coconut oil 2% Corn oil 0.1% Cholesterol	22 Weeks	Atherogenic diet, no PUFA added	Isomers mixture 0.5 g/day (0.85 % aprox)	NS in any measured parameter	
Nicolosi et al. [17]	Syrian Golden male hamsters (F₁B strain)	*En face* staining of the aortic lesion for lipid accumulation	10% Coconut oil 1% Safflower oil 0.12% Cholesterol	11 Weeks	Atherogenic diet, no PUFA added	Isomers mixture 0.025% 0.05% 0.5%	Fatty streak NS NS NS	
Munday et al. [19]	C57BL/6 female mice	Morphometric quantification of cross-sectional area of fatty streak	1% Cholesterol 0.5% Cholate	15 weeks	0.5% LA	Isomers mixture 0.25% 0.5%	Fatty streak ↑ 60% ↑ 52%	
Wilson et al. [18]	Syrian Golden male hamsters (F₁B strain)	Morphometric quantification of lipid stained area in aortic sections	20% Coconut oil 2% Safflawer oil 0.12% Cholesterol	12 weeks	1% LA	Isomers mixture 1%	Fatty streak ↓ 40 %	
Kritchevsky et al. [14]	New Zealand male/female rabbits	Visually grading of atherosclerotic lesion	12% Coconut oil 2% Corn oil 0.2% Cholesterol	90 days	Atherogenic diet, no PUFA added CLA was added at the expense of sucrose	Isomers mixture 0.1% 0.5% 1% 1%	Aortic arch NS ↓62% ↓57% NS	Thoracic aorta NS ↓66% ↓57% NS
Kritchevsky et al. [15]	New Zealand male rabbits	Visually grading of atherosclerotic lesion	12% Coconut oil 2% Corn oil 0.2% Cholesterol	12 weeks	Atherogenic diet, no PUFA added CLA was added at the expense of sucrose	Isomers mixture 0.05% 0.075% 0.1% 0.5%	Aortic arch NS NS ↓40% ↓60%	Thoracic aorta NS NS NS ↓50%
Kritchevsky et al. [16]	New Zealand male rabbits	Visually grading of atherosclerotic lesion	13% Coconut oil 1 % Corn oil 0.2% Cholesterol	12 weeks	Atherogenic diet, no PUFA added CLA was added at the expense of sucrose	Isolated isomers 0.5% c9,t11-CLA 0.5% t10,c12-CLA 0.5% isomers mixture	Aortic arch ↓45% ↓54% ↓54%	Thoracic aorta ↓54% NS ↓79%
Valeille et al. [43]	Syrian Golden male hamsters (Janvier strain)	Aortic lipidic deposition	20% Butter fat 0.12% Cholesterol	12 weeks	1% Fish oil	Isolated isomer 1% c9,11t-CLA	↓ 43% EC ↓ 19% FC ↓ 24% TC	
Mitchell et al. [42]	Syrian Golden male hamsters (CR strain)	Morphometric quantification of lipid stained area in aortic sections	20% Coconut oil 2% Safflawer oil 0.12% Cholesterol	12 weeks	1% LA	Isolated isomers 1% c9,t11-CLA 1% t10,c12-CLA	NS NS	

Unless otherwise stated percentages of food are weight/weight. NS, no statistical difference between CLA fed group and control. PUFA, polyunsaturated fatty acid; LA, linoleic acid; EC, esterified cholesterol; FC, free cholesterol; TC, total cholesterol; CR, Charles River.

the different isomers display a different capacity to repress or accelerate the progress of atherosclerosis in this animal model.

The principal feature of plaque vulnerability, firstly observed in clinically symptomatic patients [53] and validated in apoE⁻/⁻ mice [30] is a thin fibrous cap, mainly comprising collagen, proteoglycans and VSMC which is actively weakened both by a lytic process and a lack of repair [54]. Thus, the thinner the fibrous cap, the higher the risk of rupture [55]. In this sense, stabilized plaques appeared in both control and c9,t11-CLA fed groups with higher content of SMC and collagen than the t10,c12-CLA fed group. In the latter group, the fibrous cap was replaced by a huge a-

cellular mass and underneath the plaques displayed higher macrophage content and activation, as estimated by MOMA staining and raised chitotriosidase activity. Despite the anti-inflammatory properties described for CLA isolated isomers in vitro [24,56,57] and CLA mixtures in vivo [46], our data point out to a selective different way of action between both isomers in vivo and as consequence depending of the proportions in mixtures different results could be observed. All these results indicate that CLA isomers greatly modify plaque characteristics and that the t10,c12-CLA isomer induces features compatible with those present in vulnerable plaques.

The t10,c12-CLA isomer induced a pro-atherogenic lipid profile, with elevated plasma TG, NEFA, total cholesterol and

apo B concentrations. Surprisingly, the t10,c12-CLA group showed elevated HDL cholesterol levels, an effect previously described in mice [19] and humans [50], despite the pro-atherosclerotic state. Apo A-I concentrations were reduced in the t10,c12-CLA group. Correlation analysis failed to show an association between aortic lesion and HDLc, but there was a correlation between aortic lesion and plasma apo A-I concentration ($\rho = -0.542$; $p < 0.01$). This supports the idea that HDL protein composition determines their atherogenic properties [58]. The notion of inefficient HDL particles in the t10,c12-CLA group [59,60] is also supported by no correlation between HDL cholesterol and paraoxonase activity. These findings suggest important modifications of HDL by t10,c12-CLA. Overall, the t10,c12-CLA isomer is modifying dramatically the lipid parameters towards a more atherogenic profile.

Activated macrophages play an important role in the evolution of the vascular plaque. Chitotriosidase, a member of the glycosyl hydrolase family, is one of the proteins exclusively secreted by activated macrophages [61], its activity is elevated up to 55-fold in human atherosclerotic tissue [62]. Serum chitotriosidase activity is elevated in patients with atherosclerosis and it is associated with the severity of atherosclerotic lesions [36]. This study is the first assessment of chitotiosidase activity in murine atherosclerosis and its activity can be modulated by dietary intervention. Also chitotriosidase activity was more associated with the extent of the plaque ($\rho = 0.40$; $p < 0.05$) than the MOMA staining ($\rho = 0.33$; $p < 0.05$). Thus chitotriosidase could represent a marker of plaque activity. MCP-1 mediates monocyte recruitment and systemic administration of antibodies against this molecule reduces atherosclerosis in apoE$^{-/-}$ mice [63]. Plasma MCP-1 levels were determined to investigate whether it could be used as a marker of atherosclerosis and to understand its involvement in lesion progression. Unexpectedly, MCP-1 levels were elevated in mice with less atherosclerosis and it showed an inverse correlation with aortic lesion area ($\rho = -0.48$; $p < 0.02$). However, plasma MCP-1 levels might be influenced by other tissues. MCP-1 is also secreted by adipose tissue [64] and reduced adipose mass in the t10,c12-CLA group fed may have distorted the relationship between plasma MCP-1 and plaque monocyte activation. Taking these results together, plasma levels of chitotriosidase provide a plaque monocyte activity not biased by changes in adipose mass tissue.

The astonishing body fat-lowering effect of CLA in mice, maybe overestimated due to their great metabolic rate [44], has led to the idea that these compounds could be used as a tool in body weight management in humans, but trials on weight lose in humans have shown that the results of CLA are considerably less than that anticipated from mice studies [65]. Regardless, many CLA-containing dietetic supplements have been commercialized and are widespread used in U.S. and Europe without monitoring of the necessary long term side effects, especially for the t10,c12-CLA isomer as we have evidenced. Fortunately, many CLA supplements are a

mix of isomers where prevails the c9,t11-CLA, but they may also contain a variable amount of the t10,c12-CLA isomer.

In conclusion, CLA isomers have divergent effects on the progression of atherosclerosis associated with a Western-type diet. The natural dietary c9,t11-CLA isomeric form had beneficial effects decreasing lipid values, oxidative stress, macrophage infiltration and activation, plaque stability and reducing plaque development. However, the t10,c12-CLA isomer raised oxidative stress, disimproved the plasma lipid profile and transformed HDL into pro-atherogenic particles thereby enhancing lesion development with activated macrophages and plaque instability. The fact that a single isomer has its own properties indicates that varying its proportion in mixtures may radically influence the outcome. Consequently, CLA should be regarded as a heterogeneous substance and composition of the isomers should be clearly indicated to avoid conflicting results, which could create confusion in the field nutrition and for the consumer.

Acknowledgments

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