

## The effect of dietary supplementation using isomeric blends of conjugated linoleic acid on lipid metabolism in healthy human subjects

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Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid. Studies using animal models have shown that CLA reduces adiposity, improves plasma lipoprotein metabolism and insulin sensitivity and reduces arteriosclerosis. Whilst CLA may have therapeutic potential with regard to coronary artery disease risk factors in human subjects, there has been little investigation into its effects in human subjects. This current study investigated the effects of dietary supplementation using two isomeric blends of CLA on triacylglycerol (TAG)-rich lipoprotein metabolism and reverse cholesterol transport in human subjects and evaluates whether CLA modulated cardiovascular disease risk factors. Fifty-one normo-lipidaemic subjects participated in this randomised double-blind placebo-controlled intervention trial. Subjects were randomly assigned to receive 3 g *cis*-9,*trans*-11–*trans*-10,*cis*-12 isomeric blend (50:50) or a *cis*-9,*trans*-11–*trans*-10,*cis*-12 isomeric blend (80:20) CLA or linoleic acid (control)/d for 8 weeks. The 50:50 CLA isomer blend significantly reduced ( $P \leq 0.005$ ) fasting plasma TAG concentrations. The 80:20 CLA isomer blend significantly reduced ( $P \leq 0.05$ ) VLDL-cholesterol concentrations. CLA supplementation had no significant effect on LDL-cholesterol, HDL-lipid-protein composition or reverse cholesterol transport. CLA supplementation had no effect on body weight, plasma glucose and insulin concentrations. Fatty acid analysis revealed that the *cis*-9,*trans*-11 CLA isomer was incorporated into total plasma lipids following supplementation with both isomeric blends of CLA. The present study demonstrates that CLA supplementation significantly improves plasma TAG and VLDL metabolism in human subjects. The study confirms that some of the cardio-protective effects of CLA that were shown in animal studies are relevant to man.

**Conjugated linoleic acid: Triacylglycerol: Very-low-density lipoprotein: Low-density lipoprotein: High-density lipoprotein**

Conjugated linoleic acid (CLA) is the term used to describe a mixture of positional and geometric isomers of linoleic acid, with conjugated double bonds, which may be of *cis* or *trans* configuration at positions 9 and 11 or 10 and 12 (Ha *et al.* 1987). CLA is a natural food constituent, produced by the intestinal flora of ruminant animals (Kepler *et al.* 1970) and is found in the lipid fraction of meat and dairy products. Chin *et al.* (1992) estimated the daily intake of CLA in human subjects to be 160 mg/d. Several animal studies have established that CLA has profound effects on lipoprotein metabolism and prevents diet-induced atherosclerosis. Lee *et al.* (1994) showed that CLA significantly reduced plasma triacylglycerol (TAG) and LDL-cholesterol concentrations and demonstrated

that cholesterol deposition in the aorta was 30% less in the CLA-fed rabbits. Nicolosi *et al.* (1997) demonstrated that CLA supplementation significantly reduced plasma TAG (–28%) and cholesterol (–26%) concentrations, which were associated with a significant reduction of aortic streak formation (–26%) in hypercholesterolaemic hamsters fed an atherogenic diet. In contrast, Munday *et al.* (1999) showed that CLA promoted fatty streak formation in C57BL/6 mice fed an atherogenic diet. This occurred despite the fact that CLA treatment improved the lipoprotein profile. Kritchevsky *et al.* (2000) completed an intensive investigation into the dose–response effect of CLA on the progression and regression of atherosclerosis in rabbits and demonstrated that CLA supplementation

**Abbreviations:** Apo, apolipoprotein; CETP, cholesteryl ester transfer protein; CLA, conjugated linoleic acid; PPAR, peroxisome proliferator activated receptor; TAG, triacylglycerol.

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caused substantial (–30%) regression of established atherosclerosis. CLA improves insulin sensitivity and glucose tolerance (Houseknecht *et al.* 1998) and reduced adiposity (Park *et al.* 1999) in several animal models. Therefore, CLA could be a therapeutic nutrient capable of reducing the risk of obesity, diabetes and CHD in human subjects. Two recent human studies (Benito *et al.* 2001; Smedman & Vessby, 2001) have shown that CLA supplementation at 3.9 g/d for 63 d and 4.2 g/d for 12 weeks respectively had no significant effect on plasma lipid concentrations.

Reverse cholesterol transport refers to the process whereby cholesterol in peripheral tissues is transferred to the liver via lipoproteins (Tall, 1998). It represents the only form of cholesterol clearance from the circulation, hence it may have a protective effect against atherosclerosis. The cholesteryl ester transfer protein (CETP) is a key enzyme in this process. It catalyses the transfer of cholesteryl ester and TAG between VLDL and HDL. HDL-cholesterol concentrations are inversely related to VLDL-TAG concentrations (Nestel *et al.* 1987) and this relationship is mediated in part by CETP. Several studies have established that CLA is effective in reducing plasma (Nicolosi *et al.* 1997; Munday *et al.* 1999) and VLDL- (Stangl, 2000) TAG concentrations. Therefore, the reductions in plasma TAG concentrations associated with CLA intake would be expected to improve LDL- and HDL-cholesterol metabolism, which may be mediated, in part, through the activities of CETP. Evidence to date from animal experiments demonstrates that CLA supplementation has no effect on HDL-cholesterol concentrations (Lee *et al.* 1994; Nicolosi *et al.* 1997; Stangl *et al.* 1999; Gavino *et al.* 2000). However, it is effective at reducing LDL:HDL ratios (Lee *et al.* 1994). The effect of CLA supplementation on the reverse cholesterol transport pathway has not been investigated in human subjects, but it may underlie the anti-atherogenic effect of CLA, which has been shown in several animal models.

The objective of the current study was to investigate the effects of dietary supplementation using two isomeric blends of CLA on: (1) plasma TAG and VLDL metabolism;

(2) indices of reverse cholesterol metabolism, HDL composition, LDL-cholesterol, CETP mass and activity; (3) plasma glucose, insulin and non-esterified fatty acid concentrations in a large normolipidaemic group of human subjects. Two isomeric blends of CLA were used to determine the relative efficacy of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers on lipoprotein metabolism in human subjects. One group received an 80:20 isomeric blend of the *cis*-9,*trans*-11–*trans*-10,*cis*-12 CLA isomers (80:20 CLA group). Another group received a 50:50 isomeric blend of the *cis*-9,*trans*-11–*trans*-10,*cis*-12 CLA isomers (50:50 CLA group).

## Methods

### Study subjects

The present study was approved by the Ethics Committee of the Federated Dublin Voluntary Hospitals. The present study was conducted on an outpatient basis. Fifty-one healthy, non-smoking volunteers, eighteen males and thirty-three females, with a mean age of 31.6 (SD 10.03) years, a mean weight of 68.53 (SD 11.23) kg and a mean BMI 23.33 (SD 4.5) kg/m<sup>2</sup> were recruited from the personnel of Trinity College and St James's Hospital, Dublin, Republic of Ireland. All volunteers gave a written informed consent to participate in the study and adhered to the following inclusion criteria: BMI < 25 kg/m<sup>2</sup>, fasting plasma cholesterol < 6.5 mmol/l, fasting plasma TAG < 2 mmol/l, fasting  $\gamma$ -glutamyl transferase < 60 IU, haemoglobin > 110 mg/l, < 90 min strenuous exercise per week and were not habitual consumers of any fatty acid nutritional supplements or drugs known to affect lipid metabolism.

This was a randomised double-blind placebo-controlled study. Volunteers were randomly assigned into three groups receiving 3.0 g (three 1 g capsules) of: (1) 50:50 CLA isomer blend/d; or (2) 80:20 CLA isomer blend/d; or (3) linoleic acid/d. Supplements were taken each day in one dose with meals. CLA supplements were supplied by Lodders Crooklaan, B.V., Wormerveer, The Netherlands. Table 1 shows the fatty acid composition of the CLA supplements and the amounts

**Table 1.** Capsule composition of the 50:50 and 80:20 *cis*-9,*trans*-11–*trans*-10,*cis*-12 isomer blends and the control treatment, linoleic acid\*†

Ingredient	50:50 CLA isomer blend	80:20 CLA isomer blend	Control (linoleic acid)
Fatty acid composition (g/100 g fatty acids)			
16:0	ND	ND	3.7
18:0	ND	ND	1.3
18:1	22.7	28.8	24.2
18:2	ND	ND	66.9
SFA	7.0	5.9	ND
Others	ND	ND	3.9
CLA isomers:			
Total CLA	67.2	58.8	ND
<i>cis</i> -9, <i>trans</i> -11	31.0	44.9	ND
<i>trans</i> -10, <i>cis</i> -12	31.5	11.0	ND
<i>trans</i> -10, <i>trans</i> -12	2.4	1.1	ND

CLA, conjugated linoleic acid; SFA, saturated fatty acid; ND, not detected.

\* Supplements given as free fatty acid.

† Materials and analysis details kindly provided by Lodders Crooklaan, B.V., Wormerveer, The Netherlands.

of fatty acids consumed by each group. Study compliance was 96% using a pill count and was also measured by the incorporation of the *cis-9,trans-11* isomer of CLA into total plasma lipids.

Subjects attended the Nutrition Laboratory, at the Trinity Centre for Health Science, St James's Hospital, for blood sampling between 07.30 and 08.00 hours following a 12 h overnight fast at weeks 0 and 8. Subjects were also asked to abstain from alcohol 24 h prior to blood sampling. Subjects were given a supply of capsules following blood sampling at week 0. Blood was taken into 10 ml EDTA vacutainer (Beckton & Dickinson, Plymouth, Devon, UK). Blood samples were centrifuged at 1000g for 10 min at room temperature. Plasma for protein and lipid analysis was stored at  $-70^{\circ}\text{C}$ .

#### Laboratory methods

Plasma samples were analysed for TAG (PAP; Biomerieux, Lyon, France) cholesterol (PAP; Biomerieux), non-esterified fatty acids (Acyl Co A synthetase–acyl Co A oxidase; Wako Chemicals, GmbH, Neuss, Germany), glucose (Biomerieux), apolipoprotein (ApoA-I) (Biomerieux) and ApoB (Biomerieux) concentrations using an RA-XT clinical chemistry analyser (Technicon Inc., Tarrytown, NY, USA). Plasma insulin concentrations were measured using fluoro-immunoassay (AutoDELFIA Insulin kit; Wallac Oy, Turku, Finland). Total, HDL- and HDL<sub>3</sub>-cholesterol and apoA-I concentrations were measured using a RA-XT clinical chemistry analyser following the precipitation with both Immuno Quantolip total HDL precipitating reagent (Immuno AG, Vienna, Austria) and Immuno Quantolip HDL<sub>3</sub> precipitation reagent (Immuno AG). HDL<sub>2</sub>-cholesterol and HDL<sub>2</sub> ApoA-I concentrations were calculated by subtracting values for total HDL cholesterol and ApoA-I concentrations from values for HDL<sub>3</sub> cholesterol and HDL<sub>3</sub> ApoA-I concentrations. LDL-cholesterol concentrations were measured following the precipitation of LDL using Biomerieux LDL precipitation reagent. VLDL were isolated using ultracentrifugation. Briefly, the density of the plasma was adjusted to 1.019 g/ml, overlaid with saline solution of equal density and then subjected to ultracentrifugation at 250 000g for 3 h at 4°C. VLDL were then isolated and analysed for cholesterol and TAG concentrations.

#### Preparation of radiolabelled high-density lipoprotein 3 for the measurement of cholesteryl ester transfer protein activity

CETP activity was determined using a radiolabel [<sup>3</sup>H]cholesteryl ester in HDL<sub>3</sub> as a donor particle and endogenous ApoB lipoproteins as acceptor particles. Radiolabelled HDL<sub>3</sub> was prepared by a method derived from that of Tollefson *et al.* (1988). HDL<sub>3</sub> was isolated from 20 ml fasting plasma by adjusting its density to 1.13 g/ml using solid KBr. Plasma (2.1 ml) was placed in an ultracentrifugation tube and overlaid with saline solution of  $d$  1.13 g/ml. HDL fraction  $d > 1.13$  g/ml was recovered via tube slicing, and was dialysed for 24 h in Tris-buffered saline pH 7.4. A quantity of 10 nmol [1,2,(n)-<sup>3</sup>H]cholesterol

(specific activity  $1.71 \times 10^6$  MBq/mmol) was evaporated to dryness using a vortex evaporator. This was redissolved in 50  $\mu\text{l}$  ethanol. The plasma fraction of  $d > 1.13$  g/ml was then added to the radiolabelled cholesterol under gentle stirring. The mixture was incubated for 24 h at 37°C in a shaking water-bath to allow esterification and incorporation of [<sup>3</sup>H]cholesterol into HDL<sub>3</sub>. After incubation, the density of the solution was adjusted to 1.13 g/ml and subjected to ultracentrifugation at 250 000g for 6 h at 4°C. The resulting infranatant fraction was adjusted to  $d$  1.21 g/ml and the fraction  $d < 1.21$  g/ml with HDL<sub>3</sub> was recovered. The HDL<sub>3</sub> was subject to another ultracentrifugation at 250 000g for 10 h to ensure purity of HDL<sub>3</sub> subfraction. The HDL<sub>3</sub> preparation was dialysed against Tris-buffered saline pH 7.4 for 24 h. The labelled preparation of HDL<sub>3</sub> had a specific activity of about 717 Bq/nmol cholesterol using TLC. More than 96% radioactivity was associated with the cholesteryl ester fraction.

#### Measurement of cholesteryl ester transfer activity using radiolabelled cholesteryl ester

CETP activity was measured by its ability to promote the transfer of radiolabelled cholesteryl ester from HDL<sub>3</sub> to endogenous ApoB lipoproteins (Abbey & Nestel, 1994). HDL<sub>3</sub> (20  $\mu\text{l}$ ) containing 2.5 nmol cholesterol (667 Bq/30  $\mu\text{l}$ ) was added to 300  $\mu\text{l}$  plasma. 5,5'-Dithiobis(2-nitrobenzoic acid) (1  $\mu\text{l}$ ; 90 mM) was added to this mixture to inhibit lecithin co-acyl transferase activity. Samples were incubated for 3 h at 37°C. After the incubation, ApoB lipoproteins were precipitated using MnCl<sub>2</sub>–heparin using the following procedure. Plasma (250  $\mu\text{l}$ ) was placed in glass centrifuge tube. Heparin solution (10  $\mu\text{l}$ ; 5000 IU/ml) and 12.5  $\mu\text{l}$  1 M-MnCl<sub>2</sub> were added to plasma and mixture vortexed. Samples were allowed to stand in a water-bath for 30 min. Samples were then centrifuged at 1500g for 30 min at 4°C. The precipitant was washed with 1 ml 0.5 M-NaCl. Pellets were then resuspended in NaCl (100 g/l). Radioactivity of the supernatant fraction and pellet was measured using 5 ml scintillation fluid. The % radioactivity transferred to ApoB lipoproteins was determined (Abbey & Nestel, 1994). All assays were carried out in triplicate. The analytical CV for CETP activity using radiolabelled cholesteryl ester was 4.9% ( $n$  15).

#### Measurement of cholesteryl ester transfer protein mass

CETP mass was measured using an ELISA devised by Clarke *et al.* (1995). Capture and detection antibodies for CETP ELISA were obtained from Pfizer Pharmaceuticals (Gorton, CT, USA). Absorbance values of samples were measured using a microtitre ELISA plate reader (Titertek, Huntsville, AL, USA). CETP concentration of each sample was calculated using a CETP standard. Results were expressed as CETP mass in  $\mu\text{g/ml}$ .

#### GC analysis of total plasma lipid fatty acid composition

Total plasma lipids were isolated using the method derived by Folch *et al.* (1957). Lipid present in the organic phase was dried using a vortex evaporator (AGB Scientific,

Dublin, Republic of Ireland). When samples were dried, they were flushed with N<sub>2</sub>, sealed to prevent lipid oxidation and stored at -20°C. Methyl esters of total plasma lipids were prepared by adding 0.5 ml 0.01 M-NaOH in dry methanol. Samples were vortexed and flushed with N<sub>2</sub> and then placed in a heating block at 60°C for 15 min. Boron trifluoride (0.75 ml) was then added to mixture, samples were vortexed and incubated at 60°C for 15 min. Lipids were extracted three times using 0.5 ml hexane. Samples were dried in a vortex evaporator and stored under N<sub>2</sub> at -20°C until analysis.

The fatty acid methyl ester composition of total plasma lipids for CLA analysis was analysed using a Shimadzu GC-14A GLC (Mason Technologies, Dublin, Republic of Ireland) that was fitted with a Shimadzu C-16A integrator. A CP Sil 88 fused silica column (50 m × 0.22 mm, 0.2 file thickness; Chrompack Ltd, Middelburg, The Netherlands) was fitted. N<sub>2</sub> was used as a carrier gas. Conditions for the GC analysis of plasma lipids were as follows. An initial column temperature of 120°C increased at 8°C/min to 180°C. Column temperature was held at 180°C for 40 min. Column temperature was increased at 4°C/min to 220°C and was held at 220°C for 15 min. Peaks were identified using a fatty acid methyl ester standard spiked with known concentrations of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA. Fatty acids were identified by retention times compared with standard and fatty acid compositions and were calculated as % total fatty acids.

#### Data analysis

All statistical analysis was carried out on an Apple Macintosh statistical package Data Desk 4.1 (Data Description Inc., New York, NY, USA). Data for plasma TAG, HDL-cholesterol, HDL and ApoA-I concentrations were transformed to normalise the distribution of the data sets to give data a normal Gaussian distribution. One-way ANOVA investigated differences in baseline variables between the study groups. Repeated-measures ANOVA examining the treatment × time interaction was used to investigate statistical changes in biochemical variables following CLA intervention. *Post-hoc* statistical analysis (least significant difference) was used to ascertain which data points were significantly different when repeated-measures ANOVA showed a significant treatment ×

time interaction. A *P* value <0.05 was considered significant.

## Results

### Characteristics of the study population

Fifty-one subjects completed the dietary intervention. ANOVA demonstrated no significant differences, baseline variables were found between study groups at week 0 and following dietary intervention with regard to body weight, BMI and age as shown in Table 2.

### Effects of conjugated linoleic acid supplementation on plasma lipids, glucose, insulin, cholesteryl ester transfer protein mass and activity

Plasma lipid, glucose and insulin concentrations and CETP mass and activity of the three supplementation groups at week 0 and week 8 are presented in Table 3. One-way ANOVA showed no significant difference in these variables between each of the study groups at week 0. Three-way ANOVA showed that plasma TAG concentrations were significantly (*P* ≤ 0.005) reduced by the 50:50 CLA supplement. Plasma TAG concentrations were not significantly altered in the 80:20 CLA and linoleic-acid-supplementation groups. Plasma cholesterol, non-esterified fatty acids, glucose, insulin, CETP mass and activity remained unchanged in all the supplementation groups throughout the present study. There was no significant change in body weight in either the 50:50 or 80:20 CLA groups or the linoleic acid (control) group as a result of dietary supplementation over 8 weeks.

### Lipoprotein analysis for all groups at week 0 and week 8

Lipid and protein concentrations of lipoproteins of all supplementation groups at week 0 and 8 are presented in Table 4. VLDL-TAG concentrations were reduced by 24.5 and 29.4% in the 80:20 and 50:50 CLA groups respectively, however these changes did not reach significance. When both CLA treatment groups were combined, the reduction in VLDL-TAG concentrations was significant (*P* = 0.05). VLDL-cholesterol concentrations were significantly (*P* ≤ 0.05) reduced by 32.2% in the 80:20 CLA group and non-significantly by 27.5% in the 50:50 CLA

**Table 2.** Comparison of study subjects between each group\*  
(Mean values and standard deviations)

	50:50 CLA				80:20 CLA				Linoleic acid			
	Week 0		Week 8		Week 0		Week 8		Week 0		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)	33.22	11.78	–	–	28.58	6.08	–	–	32.31	10.86	–	–
Weight (kg)	65.56	8.26	67.25	7.61	68.07	12.57	65.98	11.16	71.56	12.79	71.46	15.50
BMI (kg/m <sup>2</sup> )	23.51	3.1	23.59	2.67	24.08	7.08	23.39	3.95	23.35	3.35	24.72	5.36
Male (n)/female (n)	6/10				10/7				7/11			

CLA, conjugated linoleic acid.

\* One-way ANOVA revealed no significant differences between groups.

**Table 3.** Effect of dietary supplementation using isomeric blends of conjugated linoleic acid (CLA) and linoleic acid (3 g/d) for 8 weeks on plasma lipid, insulin, glucose concentrations and cholesteryl ester transfer protein (CETP) mass and activity\*  
(Mean values and standard deviations)

	50:50 CLA group (n 16)				80:20 CLA group (n 17)				Control: linoleic acid (n 18)			
	Week 0		Week 8		Week 0		Week 8		Week 0		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/l)	4.93	1.31	4.84	0.95	5.01	0.49	4.92	0.70	5.18	1.17	5.28	1.02
Triacylglycerol (mmol/l)	1.20	0.39	0.95†††	0.31	1.08	0.32	1.00	0.40	1.03	0.43	0.94	0.33
NEFA (mmol/l)	0.36	0.26	0.37	0.19	0.33	0.14	0.38	0.19	0.33	0.35	0.33	0.22
Glucose (mmol/l)	4.94	0.31	4.85	0.40	4.97	0.49	4.88	0.41	4.97	0.37	5.21	0.51
Insulin (IU/ml)	10.76	5.08	9.09	6.81	9.14	5.60	9.17	5.58	8.86	3.81	9.27	4.82
CETP activity (cholesteryl ester transfer) (%)	19.26	6.81	20.53	8.01	23.13	6.01	23.51	7.49	22.60	8.46	22.40	9.98
CETP mass (mg/ml)	2.12	0.77	2.45	0.86	2.62	0.58	2.34	0.77	2.27	0.26	1.98	0.70

NEFA, non-esterified fatty acid.

\*For details of supplements, subjects and procedures, see Tables 1 and 2 and p. 244.

Repeated-measures ANOVA showed significant treatment × time interaction: ††† $P \leq 0.005$ .

group. CLA supplementation had no significant effect on the lipid composition of the HDL subfractions. There were non-significant increases in HDL-cholesterol (+11%) and HDL<sub>2</sub>-cholesterol (+29%) concentrations in the control group (linoleic acid). LDL-cholesterol concentrations decreased non-significantly (−8%) in both CLA supplementation groups by week 8 as shown in Table 4.

#### Fatty acid composition of total plasma lipids

Fatty acid composition of total plasma lipids is presented in Table 5. Significant increases of 87 ( $P \leq 0.001$ ) and 90% ( $P \leq 0.0001$ ) in *cis*-9,*trans*-11 isomer of CLA present in total plasma lipids was observed in the 50:50 and 80:20 CLA groups respectively. The *trans*-10,*cis*-12 isomer was undetectable in most samples. This data could not be presented in Table 5. There was a significant ( $P \leq 0.01$ ) reduction in 18:3 *n*-3 in the 50:50 CLA group, while 20:5 *n*-3 decreased significantly ( $P \leq 0.02$ ) in the 80:20 CLA group as a result of dietary supplementation over 8

weeks. The fatty acid composition of the linoleic acid group remained unchanged throughout the 8-week supplementation period of the present investigation.

### Discussion

#### Plasma triacylglycerol and VLDL metabolism

Plasma TAG concentration was significantly reduced by 20% in the 50:50 CLA group. The magnitude of the reduction in plasma TAG concentrations is comparable with that achieved following CLA supplementation in the animal studies (Nicolosi *et al.* 1997; Munday *et al.* 1999). Nicolosi *et al.* (1997) demonstrated that there was a 28 and 36% reduction in plasma TAG concentrations following a CLA diet at 0.06 and 0.11% energy for 11 weeks in hamsters. Benito *et al.* (2001) and Smedman & Vessby (2001) both showed that CLA supplementation had no significant effect on plasma lipid concentrations in human subjects. While Houseknecht *et al.* (1999) demonstrated that CLA has anti-diabetic properties by improving insulin

**Table 4.** Effects of dietary supplementation using isomeric blends of conjugated linoleic acid (CLA) and linoleic acid (3 g/d) for 8 weeks on lipoprotein lipid and protein composition\*  
(Mean values and standard deviations)

	50:50 CLA group (n 16)				80:20 CLA group (n 17)				Control (linoleic acid) (n 18)			
	Week 0		Week 8		Week 0		Week 8		Week 0		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
VLDL-cholesterol (mmol/l)	0.29	0.14	0.21	0.08	0.31	0.16	0.21†	0.13	0.31	0.22	0.26	0.13
VLDL-TAG (mmol/l)	0.68	0.38	0.48	0.22	0.61	0.30	0.46	0.27	0.52	0.36	0.58	0.29
HDL-cholesterol (mmol/l)	1.59	0.57	1.54	0.42	1.44	0.44	1.47	0.37	1.27	0.31	1.42	0.36
HDL-ApoA-I (g/l)	1.27	0.33	1.33	0.27	1.32	0.22	1.21	0.21	1.19	0.24	1.26	0.24
HDL <sub>3</sub> -cholesterol (mmol/l)	1.14	0.23	1.07	0.21	1.06	0.20	1.05	0.19	1.04	0.24	1.11	0.18
HDL <sub>3</sub> -ApoA-I (g/l)	1.11	0.50	1.04	0.15	1.08	0.15	1.14	0.33	1.06	0.15	1.11	0.09
HDL <sub>2</sub> -cholesterol (mmol/l)	0.47	0.26	0.41	0.27	0.39	0.31	0.41	0.28	0.24	0.17	0.31	0.21
HDL <sub>2</sub> -ApoA-I (g/l)	0.19	0.27	0.28	0.15	0.21	0.21	0.16	0.30	0.18	0.19	0.13	0.18
LDL-cholesterol (mmol/l)	1.65	0.83	1.53	0.64	1.69	0.70	1.54	0.59	1.77	1.04	1.71	0.71

TAG, triacylglycerol; Apo, apolipoprotein.

\*For details of supplements and subjects and procedures, see Tables 1 and 2 and p. 244.

Repeated measures ANOVA showed significant treatment × time interaction: †  $P \leq 0.05$ .

**Table 5.** Fatty acid composition of total plasma lipids (g/100 g total plasma lipids) as a result of dietary supplementation using isomeric blends of conjugated linoleic acid (CLA) and linoleic acid (3g/d) for 8 weeks‡  
(Mean values and standard deviations)

Fatty acid§	50:50 CLA group (n 16)				80:20 CLA group (n 17)				Control (linoleic acid) (n 18)			
	Week 0		Week 8		Week 0		Week 8		Week 0		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
16:0	25.38	2.15	24.80	2.13	25.10	1.98	24.61	1.63	24.63	2.15	24.98	1.48
16:1	2.25	0.72	2.14	0.64	2.76	0.86	3.16	2.14	2.39	2.24	1.84	0.72
18:0	9.15	2.03	8.62	1.07	9.01	1.18	9.33	1.22	9.35	0.87	9.23	2.57
18:1	21.40	3.82	22.84	2.55	21.14	2.28	20.96	1.90	21.51	4.05	20.16	3.77
18:2 n-6	28.49	4.21	27.25	3.39	28.83	3.42	29.44	3.32	29.31	4.54	29.44	2.83
18:3 n-6	0.27	0.12	0.19	0.10	0.25	0.08	0.29	0.12	0.40	0.28	0.29	0.08
18:3 n-3	0.74	0.29	0.50**	0.35	0.68	0.29	0.58	0.27	0.61	0.26	0.62	0.23
<i>cis</i> -9, <i>trans</i> -11 CLA	0.33	0.20	0.62***	0.26	0.42	0.23	0.80****	0.29	0.40	0.16	0.28	0.12
20:2	0.28	0.08	0.19	0.07	0.29	0.12	0.27	0.07	0.26	0.06	0.27	0.06
20:3 n-6	1.54	0.67	1.24	0.53	1.77	0.36	1.79	0.29	1.66	0.54	1.88	0.32
20:4 n-6	7.32	1.17	6.53	1.78	7.13	1.61	6.94	1.74	7.17	1.35	7.42	1.31
20:5 n-3	1.04	0.48	1.02	0.59	1.19	0.52	0.90†	0.47	0.94	0.33	1.05	0.55
22:4 n-6	0.31	0.09	0.27	0.13	0.41	0.33	0.32	0.17	0.41	0.33	0.37	0.12
22:6 n-3	2.32	0.86	2.27	0.73	2.17	0.65	2.13	0.61	2.11	0.66	2.03	0.84

Repeated measures ANOVA shows significance treatment × time interaction † $P \leq 0.02$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

‡ For details of supplements, subjects and procedures, see Tables 1 and 2 and p. 244.

§ *trans*-10, *cis*-12 CLA isomer undetectable in most samples, results not presented.

sensitivity in Zucker diabetic rats. Recent *in vitro* and animal studies suggest that the individual CLA isomers may have different effects on lipid metabolism. We used two isomeric blends of CLA to determine whether providing different proportions of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers had different effects on lipoprotein metabolism in human subjects. The 50:50 CLA supplement significantly reduced plasma TAG concentrations, but the 80:20 CLA supplement did not. These results suggest the *trans*-10,*cis*-12 CLA isomer may be the effective hypotriglycerolaemic isomer. These findings are in agreement with Lin *et al.* (2001), who found that the pure *trans*-10,*cis*-12 was more effective at inhibiting TAG secretion from Hep G2 cells compared with the *cis*-9,*trans*-11 CLA isomer. Yotsumoto *et al.* (1999) demonstrated that the *trans*-10,*cis*-12 CLA isomer inhibited ApoB and TAG synthesis in Hep G2 cells. The hypotriacylglycerolaemic properties associated with the *trans*-10,*cis*-12 CLA isomer were confirmed by Gavino *et al.* (2000), who demonstrated that a CLA isomeric mix containing *trans*-10,*cis*-12 CLA reduced plasma TAG concentrations, but *cis*-9,*trans*-11 CLA had no effect in Golden Syrian hamsters. In contrast to our present study, Benito *et al.* (2001) showed that CLA supplementation had no significant effect on lipid metabolism in human subjects. However, the sample size of that study may not have had sufficient statistical power. A heterogeneous blend of CLA was also used, which may explain the lack of effect. Smedman & Vessby (2001) also showed that CLA supplementation (4.2 g/d for 10 weeks) had no effect on lipid or TAG concentrations, however their cohort showed much greater variability in TAG concentrations compared with our present study.

In the fasted state, VLDL are the principal contributors to plasma TAG concentrations. The 80:20 CLA supplement significantly lowered VLDL-cholesterol concentrations.

When the compositional analysis of the VLDL fraction for both CLA groups was pooled, it was demonstrated that decreased VLDL-TAG and -cholesterol concentrations were significant following supplementation. Yotsumoto *et al.* (1999) demonstrated that both the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers are equally effective in reducing cholesteryl ester synthesis in Hep G2 cells, however findings from the present study suggest that the *cis*-9,*trans*-11 isomer is more potent at reducing cholesteryl ester synthesis *in vivo*. Clearly, a reduction in VLDL-TAG and -cholesterol concentrations may be attributed to a decrease in the rate of lipid synthesis. It has demonstrated that CLA isomer blends reduce ApoB secretion, ultimately leading to a reduction in the number of VLDL particles (Pariza *et al.* 2001). Nicolosi *et al.* (1997) demonstrated that CLA supplementation in hamsters reduced non-HDL-cholesterol concentrations, although whether these decreases were attributed to VLDL or LDL fractions was not specified, while Stangl (2000) demonstrated that CLA supplementation in Sprague Dawley rats reduced VLDL-TAG and -cholesterol concentrations when compared with control group. The findings of our study with regard to lipid metabolism were less profound than those observed in animal studies. This probably reflects differences in CLA blends, increased doses of CLA (relative to body weight) and longer supplementation period (relative to life-span).

The ultimate goal of the present study was to determine whether CLA was an effective hypotriacylglycerolaemic agent in human subjects. Whilst investigating the molecular basis of the effects of CLA on TAG and VLDL metabolism was beyond the limits of this current study, animal studies provide several molecular mechanisms to explain the hypotriacylglycerolaemic effect of CLA. Moya-Camarena *et al.* (1999) demonstrated that the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomers of CLA

are potent peroxisome proliferator activated receptor (PPAR)- $\alpha$  agonists. PPAR $\alpha$  is a key transcription factor that regulates hepatic lipid metabolism. Pharmacological PPAR $\alpha$  agonists (e.g. fibrates) improve plasma lipid metabolism by up-regulating PPAR $\alpha$  gene expression (Fruchart *et al.* 1999). Feeding CLA-enriched diets increases the expression of PPAR $\alpha$ -responsive genes in animals (Moya-Camarena & Belury, 1999). Therefore, the TAG-lowering effect of CLA may be partly attributed to the effect of CLA on PPAR $\alpha$ . CLA is also a PPAR $\gamma$  ligand. In adipose tissue, PPAR $\gamma$  regulates the expression of the genes that determine adipogenesis, lipid metabolism and insulin sensitivity (Lowell, 1999). Feeding a CLA-rich diet increased adipocyte PPAR $\gamma$  responsive gene expression (aP2) and improved plasma fatty acid metabolism in diabetic Zucker rats (Houseknecht *et al.* 1998). In the knowledge that pharmacological PPAR agonists improve TAG metabolism, we propose that CLA works in a similar fashion.

#### *Conjugated linoleic acid and cholesterol metabolism*

Reverse cholesterol transport is the process whereby cholesterol is returned from peripheral cells to the liver for excretion. CETP is a key enzyme which regulates reverse cholesterol transport, catalysing the reciprocal transfer of TAG and cholesteryl ester between HDL and VLDL. VLDL-TAG is an important determinant of CETP activity (Mann *et al.* 1991). Based on the anti-atherogenic and hypotriacylglycerolaemic effect of CLA in animals, we assessed the effects of CLA supplementation on VLDL-, HDL- and LDL-cholesterol concentrations and CETP metabolism to determine whether CLA affected reverse cholesterol transport. CLA supplementation had no significant effect on CETP mass or activity. This result was surprising considering the significant reduction in VLDL-TAG associated with CLA supplementation, and the importance of VLDL-TAG concentrations on CETP activity (Mann *et al.* 1991). CLA supplementation had no significant effect on fasting HDL composition or concentrations. Other studies have also shown that CLA supplementation had no effect on HDL lipid composition (Lee *et al.* 1994; Nicolosi *et al.* 1997; Stangl *et al.* 1999; Gavino *et al.* 2000).

CLA supplementation reduced LDL-cholesterol ( $-8\%$ ) concentrations, but this decrease was not significant. Nicolosi *et al.* (1997) also showed that non-HDL-cholesterol concentrations were reduced by (9–13%) in response to a CLA-enriched diet (0.06 and 1.1% energy). The LDL:HDL-cholesterol ratio was reduced in the 80:20 CLA group ( $-7.2\%$ ) and the 50:50 CLA group ( $-2.4\%$ ), however neither reached significance. Lee *et al.* (1994) showed that LDL:HDL-cholesterol ratio was significantly reduced in rabbits fed an atherogenic diet supplemented with 0.5 g CLA/d for 12 weeks. In our present investigation, linoleic acid was more effective than the isomeric blends of CLA at reducing ( $-11\%$ ) LDL:HDL-cholesterol ratios. This reduction in LDL:HDL-cholesterol ratios was accounted for by decreases in LDL-cholesterol concentrations with HDL-cholesterol concentrations remaining unchanged. Several other studies have shown

that linoleic acid reduces the LDL:HDL-cholesterol ratio (Dupont *et al.* 1990; Singer *et al.* 1990; Nydahl *et al.* 1994). The authors gave careful consideration when choosing an appropriate control fatty acid for the present study. We chose linoleic acid because it represented the non-conjugated form of CLA. Linoleic acid is one of the principle fatty acids in the human diet, therefore we felt that an intake of 3 g/d would have little effect on lipoprotein metabolism. Clearly the linoleic acid had some, albeit a minor, effect on HDL- and LDL-cholesterol levels. The findings of the present study show that although CLA is a potential hypocholesterolaemic agent in animal models this is not the case for normolipidaemic human subjects.

#### *Body weight, glucose and insulin*

CLA supplementation had no effect on body weight. Several studies in a variety of rodent models showed that CLA reduced body weight, reduced adiposity and increased lean mass. Body composition was not measured in the present investigation, therefore we cannot conclude whether CLA has an anti-obesity effect. These findings are in agreement with Zambell *et al.* (2000), who found no changes in body weight or body composition attributed to CLA supplementation in human subjects, however Smedman & Vessby (2001) demonstrated that CLA supplementation (4 g/d), although having no effect on body weight, did significantly reduce body fat. In the present study, CLA supplementation had no effect on fasting glucose and insulin concentrations. Results on the effect of CLA on glucose and insulin metabolism from animal studies are controversial. Houseknecht *et al.* (1998) showed that a CLA supplementation normalised impaired glucose tolerance and hyperinsulinaemia in Zucker diabetic fa/fa rats. Stangl *et al.* (1999) showed that a CLA-rich diet (10 g/kg diet) significantly increased (+37%) insulin concentrations in swine. Tsuboyama-Kasoke *et al.* (2000) showed that a CLA-rich diet induced insulin resistance and glucose intolerance in C57B16 mice. Our present study demonstrates that in healthy subjects CLA supplementation has neither pro- nor anti-diabetic effects.

#### *Fatty acid composition of total plasma lipids*

The mean fatty acid concentration of *cis-9,trans-11* isomer of 0.38 g/100 g total plasma lipids at week 0 was comparable with concentrations of this isomer of CLA found in total plasma lipid in human subjects by Jiang *et al.* (1999). Following CLA supplementation the level of *cis-9,trans-11* CLA isomer was significantly ( $P \leq 0.001$ ) increased by 90% in the 50:50 CLA group, and by 87% in the 80:20 CLA group. Britton *et al.* (1992) have shown comparable increases in the incorporation of *cis-9,trans-11* isomer of CLA into plasma phospholipids of human subjects as a result of increased consumption of food products rich in CLA for 3 weeks. Huang *et al.* (1994) demonstrated that the *cis-9,trans-11* isomer of CLA increased by 19–27% as a result of increased intake of dairy products in human subjects. There was a significant reduction in eicosapentaenoic acid (20:5) of total fatty acid composition of plasma lipids in the 80:20

CLA group, suggesting that increased incorporation of *cis*-9,*trans*-11 CLA may be at the expense of *n*-3 polyunsaturated fatty acids. The *trans*-10,*cis*-12 isomer was not detected in the week 0 total plasma lipids in the present study, while it was only detected in some of the week 8 samples, which indicates that the *trans*-10,*cis*-12 isomer of CLA is not incorporated efficiently into plasma lipids. Sebedio *et al.* (1997) have demonstrated that the *trans*-10,*cis*-12 isomer is metabolised into 20:4  $\Delta$  5,8,12,14 and 20:3  $\Delta$  8,12,14 via desaturation and elongation pathways. Martin *et al.* (2000) hypothesised that the *trans*-10,*cis*-12 isomer of CLA is more easily oxidised due to its structure allowing it to bypass a number of rate limiting steps in the peroxisomal  $\beta$ -oxidation pathway. Both these findings offer an explanation as to the difficulties in detecting the *trans*-10,*cis*-12 isomer of CLA in plasma lipid.

### Conclusions

The present study showed that CLA supplementation in human subjects has beneficial effects on some coronary artery disease risk factors. The significant reduction in plasma TAG concentrations was accounted for by reduced VLDL-TAG concentrations. The decrease in plasma TAG concentrations noted in the present study has been estimated to be equivalent to a reduction of 12.5% in coronary artery disease risk according to the findings of Stampfer *et al.* (1996). Significant reductions in VLDL-cholesterol concentrations were observed in the 80:20 CLA group. HDL-cholesterol concentrations are unaffected by CLA supplementation, while LDL-cholesterol concentrations are reduced non-significantly. Animal studies showed that CLA affected insulin and glucose metabolism, our study showed that this was not the case in human subjects. The present study indicates that CLA has positive clinical effects by conserving the cardio-protective HDL-cholesterol concentrations and reducing the potentially atherogenic VLDL-cholesterol concentrations. Given the low level of CLA in food, CLA supplements or CLA-enriched functional foods would be required to achieve comparable doses. A dose-response study would be required to determine the minimum effective dose of CLA in human subjects.

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