SUPPORTING DOCUMENT 1

APPLICATION A1005
EXCLUSIVE USE OF TONALIN® CLA AS A NOVEL FOOD

Consideration of the Effect of a 1:1 Isomer Mix of Conjugated Linoleic Acid on HDL- and LDL-Cholesterol Levels

December 2010

Contents

Summary

As discussed elsewhere, a key aspect of the consideration by FSANZ of the impact of permitting the addition of CLA to food is the nature of the effect of dietary CLA on the blood lipid profile of consumers, due to the potential for a cis/trans polyunsaturated fat to display adverse effects on blood lipids related to the presence of a trans double bond. To address this concern, FSANZ examined the published literature describing the effect of CLA on HDL-cholesterol and LDL-cholesterol levels for the 1:1 isomer mix proposed for use by the Applicant, and other ratios of the two isomers under consideration. A quantitative meta-analysis was used to summarise the overall effects of the 1:1 isomer on these parameters. Given the known effects of different control fats on blood lipids, trials comparing the 1:1 isomeric ratio of CLA to saturated or cis-unsaturated fats were combined to examine effects on HDL-cholesterol. Given the known effects of different control fats on blood lipids, trials comparing the 1:1 isomeric ratio of CLA to cis-unsaturated control fats were combined to examine the effects on LDL-cholesterol. The effect of adding other ratios of the two CLA isomers on blood lipids was described qualitatively.

The results of these meta-analyses are that consumption of 1.4 - 5.6 g of the 1:1 isomer mix of CLA reduces HDL-cholesterol by 0.036 mmol/L (95% Confidence Interval (CI): -0.069 to -0.002, p=0.04) when compared to saturated and cis-unsaturated fats. There was an elevation, albeit not significant, of LDL-cholesterol when the 1:1 isomer mix was compared to oils rich in cis-unsaturates (increase of 0.049 mmol/L; 95% CI: -0.008 to 0.106; p=0.09).

The Epidemiology Scientific Advisory Group (EpiSAG) took the view that it would be reasonable to combine the results of the 1:1 isomer ratio studies with the results of the isomers individually or when combined in other ratios. Therefore the studies shown in Figures 4 and 5, except for Benito *et al.,* (2001) who used a mix of four different isomers of CLA, were combined with studies shown in Figures 1-3. Specifically this added three arms testing a 4:1 *c-9, t-11:t-10, c-12* ratio, four arms testing the *c-9,t-11* isomer alone and three arms testing the *t-10,c-12* alone to the HDL cholesterol level analysis. For the LDL-cholesterol analysis, all except one of the 4:1 isomer studies had used unsaturated fat controls and were added to the analysis. Combining all studies in the same dose range (1.4-5.6 g) reduces the effect on HDL-cholesterol levels slightly (to -0.031mmol/L). For studies with *cis*-unsaturated controls, including the other trials the 1.4-5.6 g does range increases the effect on LDL-cholesterol level (from 0.049 to 0.057mmol/L).

Owing to the increasing number of studies over time, and the increased dose range when the single isomer and other ratios are added to the results of the 1:1 ratio trials, a dose-response relationship was examined. There was a statistically significant relationship for both lipid outcomes. Compared to any fatty acid, there was a change in HDL of -0.005 mmol/L per gram of CLA (standard error of the mean (SEM): 0.002 mmol/L, p=0.04) for all studies. Compared to cis-unsaturates, there was a change in LDL-cholesterol of 0.012mmol/L per gram of CLA (SEM: 0.001mmol/L, p<0.001) for all studies.

The results of the analysis using other ratios of the two isomers support the results of the trials using the 1:1 ratio.

The randomisation methods or the diets of the subjects used in studies are rarely described. Given the quantities of CLA or control fat used in most studies, it is possible that other small changes in the diet might have occurred that affected lipid levels. When studies have small numbers, randomisation cannot be relied upon to equalise important confounders between groups. Although meta-analysis can combine small studies with inadequate power to examine effects, it cannot overcome any lack of equalisation of confounders that might exist within the studies unless the original data are obtained for each study and pooled. However, these studies are the best available scientific data available at present.

FSANZ concludes that, based on the currently available evidence, CLA lowers HDL-cholesterol and this is inconsistent with action as a *cis*-polyunsaturated fatty acid. Although the primary focus of the current assessment is on the 1:1 isomer ratio, FSANZ regards the results of the analysis of any ratio of the two isomers as supporting the view that the 1:1 isomer ratio probably has an effect on LDL-cholesterol which is an additional concern.

1. Introduction

CLA is a set of polyunsaturated fatty acid isomers with 18 carbon atoms and two double bonds: one in the *trans* configuration and one in the *cis* configuration, and is conjugated (only one single bond between the two double bonds). Thus, chemically, CLA is both a *trans* and a polyunsaturated fatty acid but is classified as a *trans* fat for labeling purposes in the Code. The CLA mixture that is the subject of the Application has two isomers – C18:2, *c-9,t-11* and C18-2, *t-10,c-12* - in a 1:1 ratio.

Linoleic acid (C18:2, *c-9,c-12*) is a polyunsaturated fatty acid which has favourable effects on blood lipids by elevating HDL-cholesterol and lowering LDL-cholesterol levels. Linoleic acid and similar unsaturates such as oleic acid (C18:1, c-9) and linolenic acid (C18:3, c-9,c-12,c-15) are associated with decreases in heart disease incidence, while the *trans* fat, elaidic acid (C18:1 t-9), is associated with unfavourable effects on lipids and heart disease incidence (Mozaffarian *et al,* 2009). Given the similarities and differences between CLA isomers and other *cis* and *trans* unsaturates, it is possible that CLA would affect blood lipids - specifically LDL and HDL cholesterol;

- similarly to other polyunsaturated fats, or
- similarly to *trans* fats, or
- have a unique effect of its own.

It is also well known that the effect of saturated fat on LDL cholesterol levels varies among the saturated fatty acids with stearic acid having little or no effect (Mensink *et al.,* 2003). Given the known variation in function among the various saturated fats, despite similar structure, and in view of the presence of both *cis* and *trans* bonds in CLA, FSANZ examined the functional effect of CLA on lipids rather than relying on its labeling definition. Available studies comparing the effect of the 1:1 isomer mix of CLA to other macronutrients on HDL-cholesterol and LDL-cholesterol levels were examined quantitatively. Studies of other ratios of the two isomers on the same outcomes were also included in a subsidiary analysis.

2. Choice of HDL-cholesterol and LDL-cholesterol levels as primary outcome markers

Serum total, LDL- and HDL-cholesterol levels are recognised predictors of heart disease. The New Zealand Cardiovascular Risk Chart shows total/HDL ratio as one of the primary predictive factors, together with cigarette smoking habit, systolic blood pressure, age, sex and presence/absence of diabetes (NZGG, 2009). This chart has been adopted by the National Heart Foundation of Australia (NHFA, 2009). The US National Heart Lung and Blood Institute recommends that low HDL-cholesterol level is one of several risk factors which should be used to determine the level of LDL-cholesterol that warrants intervention (NHLBI, 2001). The primary mode of action of HDL-cholesterol in protecting against heart disease is thought to be ‘reverse cholesterol transport’ i.e. carriage of cholesterol from macrophages in arterial walls to the liver (Singh *et al.*, 2007; deGoma *et al.*, 2008).

A pooled analysis of 61 cohort studies found that both HDL-cholesterol and non HDL-cholesterol (which is predominantly LDL-cholesterol) levels predicted vascular mortality. “On average, 1 mmol/L lower non HDL-cholesterol, 0.33 mmol/L higher HDL-cholesterol, a 1.33 lower total/HDL cholesterol were each associated with about a third lower" ischaemic mortality although the exact magnitude of the relationship varied by age and blood pressure (Prospective Studies Collaboration, 2007). This study also found that the total/HDL ratio was the most predictive of the various ratios examined. A pooled analysis of 25 cohort studies conducted in the Asia-Pacific region, including studies conducted in Australia, examined fatal and non-fatal vascular events (Woodward *et al.*, 2007). The authors reported that a 0.4mmol/L decrease in HDL-cholesterol levels was associated with an increased relative risk of coronary heart disease of 1.39 (95% CI: 1.22 to1.57) over 6.8 years of follow-up (Woodward *et al*., 2007). These two studies also found associations in the same directions for ischaemic stroke but had variable results for haemorrhagic stroke (Prospective Studies Collaboration, 2007; Woodward *et al.*, 2007).

Both HDL- and LDL-cholesterol can be subdivided into fractions which are thought to have different actions (Singh *et al.*, 2007; deGoma *et al.*, 2008; Ip *et al*., 2009). Reverse cholesterol transport is thought to be the primary action of HDL-cholesterol. It is also postulated that some sub-components of HDL-cholesterol may have anti-oxidant, anti-inflammatory, anti-thrombotic and other actions (Singh *et al.*, 2007; deGoma *et al*., 2008, Briel *et al.*, 2009). Briel *et al.,* (2009) reported that, after taking the reduction in LDL-cholesterol into account, studies of currently available drug treatments did not indicate that observed increases in HDL-cholesterol levels conferred extra protection. Like others, they noted that it is desirable to develop assays for HDL-sub fractions and investigate these for predictive ability (Singh *et al.*, 2007; deGoma *et al.*, 2008; Briel *et al*., 2009). Work on the development of drugs targeting specific enzymes in the HDL-cholesterol particles is also underway (Singh *et al.*, 2007). Until more specific assays for, and drugs targeting HDL-cholesterol sub-components, are developed, low levels of HDL-cholesterol remains one of the major predictive risk factors for heart disease and is used to help determine the goals of therapy with drugs that treat LDL-cholesterol levels (NHLBI, 2010; NZGG, 2009; NHFA, 2009). Similarly, the clinical usefulness of sub fractions of LDL-cholesterol remains to be proven (Ip *et al.*, 2000).

Therefore, this review has focused on LDL-cholesterol and HDL-cholesterol levels, which have proven clinical usefulness as predictors and which were reported in many of the randomised controlled trials that tested CLA in humans. Total cholesterol level was not included in the current analysis because it is affected by both LDL-cholesterol level and HDL-cholesterol level which, in turn, affect heart disease risk in opposite directions. The effect of the CLA isomers on cholesterol ratios, such as LDL/HDL or total/HDL, was not examined because these were generally not reported in the papers identified and so their variances, which are used to weight the summary effect in a quantitative meta-analysis, could not be calculated. Few papers reported cholesterol sub fractions and so it was not possible to do an extensive quantitative analysis of these parameters.

3. Choice of control macronutrient

No macronutrient is inert in relation to serum lipids because increasing the intake of one must result in the intake of another being reduced to keep total energy constant and prevent changes in body weight from confounding the results. These relationships were initially described in terms of the P:S ratio (ratio of polyunsaturated to saturated fats). As further research was done, the role of mono-unsaturates and then *trans* fatty acids was elaborated.

For example, Clarke *et al.,* (1997) described the effect of general classes of fatty acid compared to carbohydrate and also the effect of specific saturated fatty acids.

Mensink *et al*., (2003) updated this work and included *trans* fatty acids as a separate item (Table 1 below). In 2009, the relationship for *trans* fatty acids versus the other classes of fatty acids was reported by Mozaffarian and Clarke (2009) (Table 1). Mozaffarian and Clarke (2009) estimate that replacing 1% energy from cis-monounsaturated fat with trans fat would reduce HDL-cholesterol by 0.010mmol/L and raise LDL-cholesterol by 0.038mmol/L (Table 1).

Table 1: Change in LDL cholesterol and HDL cholesterol levels (mmol/L) predicted in two reviews when 1% energy from one macronutrient is replaced by another macronutrient

Macronutrient exchange; x->y indicates that 1% energy from x is replaced with 1% energy from y	LDL-cholesterol, mean change per 1% energy replacement x->y (mmol/L)		HDL-cholesterol, mean change per 1% energy replacement x->y (mmol/L)	
	Mensink et al (2003)	Mozaffarian & Clarke (2009)	Mensink et al (2003)	Mozaffarian & Clarke (2009)
cho -> SFA	0.032	-	0.010	-
cho -> MUFA	-0.009	-	0.008	-
cho -> PUFA	-0.019	-	0.006	-
cho -> TFA	0.040	-	0.000	-
SFA->TFA	0.008*	0.008	-0.010*	-0.013
MUFA->TFA	0.049*	0.038	-0.008*	-0.010
PUFA->TFA	0.059*	0.051	-0.006*	-0.013

TFA: *trans* fatty acids; SFA: saturated fatty acids; MUFA: *cis*-monounsaturated fatty acids; PUFA: *cis*-polyunsturated fatty acids; cho: carbohydrate; * calculated from the relationship of the various fatty acids versus exchange with carbohydrate

4. Identification of studies and approach to analysis

As part of the Application, the Applicant provided a range of studies on CLA that measured HDL-cholesterol and LDL-cholesterol in humans and FSANZ had also searched the literature several times, the last time being 31st March, 2010. In all, 34 randomised double blind studies were included. The primary analysis examined the effect of the 1:1 isomer ratio. Secondary analyses examined the effect of other ratios of the two isomers under consideration. See the Appendix for further detail, including reasons for exclusion of many trials.

The effect of CLA was not specifically described in the two reviews from which Table 1 is drawn. Based on Table 1, FSANZ grouped trials using any of saturated fat, *cis*-monounsaturated fat and cis-polyunsaturated fat as the control macronutrient together when HDL-cholesterol was considered. This is because these three classes of fatty acids have broadly similar effects on HDL-cholesterol to each other, both in magnitude and direction although a small amount of variability would be introduced as the control fats do not have exactly the same effect. Only one trial used trans fat as the control and has been considered separately. Trials which did not replace CLA with a fat were excluded from both HDL- and LDL-cholesterol analysis. Yonei *et al.,* (2007) used a lactose placebo and Laso *et al.,* (2007), Bonet-Serra *et al.,* (2008) and Lopez-Roman *et al*., (2007) gave CLA in milk or yoghurt drinks but did not replace the CLA in the intervention vehicle with fat in the placebo vehicle and so altered the energy intake as well as the fat intake between the groups.

The situation is different when LDL-cholesterol is the parameter of interest. Trials using saturated fat could be grouped with trials using *trans* fat as the control macronutrient because these have similar effects on LDL-cholesterol levels (Table 1). Trials using *cis*-monounsaturated and *cis*-polyunsaturates could also be grouped together owing to their similar effects on LDL-cholesterol. However, trials using saturated and *cis*-unsaturated fat controls should not be grouped together as the control substances have opposite effects on LDL-cholesterol levels and so averaging the results would be meaningless. Thus FSANZ grouped trials using olive oil, flax and soya oil, linoleic acid, safflower oil and high oleic sunflower oil as the control fat together as "unsaturated controls" and a quantitative analysis of the LDL outcome was done only on this grouping. The results of trials using butter or a mixture of fats designed to resemble the usual diet would have higher levels of saturated fat and are presented separately as "other controls". This is a crude grouping but the limited number of studies did not allow greater discrimination.

The predictive equations presented in Table 1 assume a linear dose-response relationship between fatty acid intake and blood lipid response and describe intake as % energy whereas the trials examining the effects of CLA generally describe their doses in grams. Using the mean intake of *trans* fatty acid in Australian adults (0.6% total energy or 1.5g *trans* fat) and New Zealand (0.7% total energy or 1.7g *trans* fat) (FSANZ, 2007) we have estimated an approximate conversion factor of 1.5g fat = 0.6% energy. Therefore a study that compared 2.5g CLA to 2.5g other fat compared an exchange of approximately 1.0% energy.

Studies were divided into those which tested a 1:1 isomer mix of *c-9,t-11* and t-*10,c-12* and studies which tested other ratios of these two isomers. The quantity of CLA used in all studies of the 1:1 ratio mix was narrow, ranging from 1.4–5.6 g (true dose, after allowing for the quantity of other fatty acids); this is also relatively narrow as it equates to approximately 0.5-2.2% energy from CLA.

Therefore it was appropriate to combine the results in a single meta-analysis rather than examining a dose-response relationship using meta-regression.

5. Results

5.1 1:1 isomer mix

When compared to other fats, a 1:1 isomer mix of CLA reduced HDL-cholesterol by 0.036 mmol/L (95%CI: -0.069 to -0.002, p=0.04) (Table 2, Figure 1)[1]. The 95% confidence interval indicates that the study results are consistent with a range of almost no reduction up to a reduction of 0.069 mmol/L (p=0.04). Although the inconsistency, I^2,was moderate to high, and could be reduced to 0% by removing three studies with extreme values (Zhao *et al.*, 2009, Tholstrup *et al.*, 2008, and Whigham *et al.*, 2004), this did not alter the result (see Appendix for detail).[2]

When compared to *cis*-unsaturated fat controls, the 1:1 isomer mix raised LDL-cholesterol by 0.049 mmol/L (95% CI: -0.008 to 0.106, p=0.09) (Table 2, Figure 2). The 95% confidence interval indicates that the result is consistent with a range from a reduction in LDL-cholesterol of 0.008mmol/L to an increase of 0.106mmol/L. The inconsistency (I^2=0%) suggests that variation between studies might be attributed to chance.

Table 2: Effect of 1:1 CLA isomer mix (1.4 - 5.6g/day) on HDL- and LDL-cholesterol levels.

Description	Difference: intervention – control (95% CI) (mmol/L)	I^2 (95% CI)
Effect on HDL-cholesterol: 1.4-5.6g CLA compared to saturated and cis-unsaturated fat controls (Figure 1)	-0.036 (-0.069 to -0.002) p=0.04	65.1% (46.2% to 75.3%)
Effect on LDL-cholesterol: 1.4 to 5.6g CLA compared to cis-unsaturated fat controls (Figure 2)	0.049 (-0.008 to 0.106) p=0.09	0% (0% to 38%)

Only three studies fell into the group of ‘other controls’ (Figure 3). As these studies used a range of controls from butter to a mix of fats resembling the Chinese diet, no overall summary effect was derived (Figure 3) because it is not clear which studies had comparable control groups that could be reasonably combined. These three studies all found reductions in LDL-cholesterol compared to fats that would be relatively high in saturated fatty acids compared to the studies shown in Figure

[1] The size of the black square for each study in the figure indicates its relative weighting in the overall result
[2] I^2 describes the “percentage of total variation across studies that is due to heterogeneity rather than chance” and 0%, 25%, 50% and 75% could be interpreted as indicating no, low, medium and high heterogeneity respectively (Higgins *et al*, 2003).

2, but the small number of studies makes it difficult to draw conclusions.

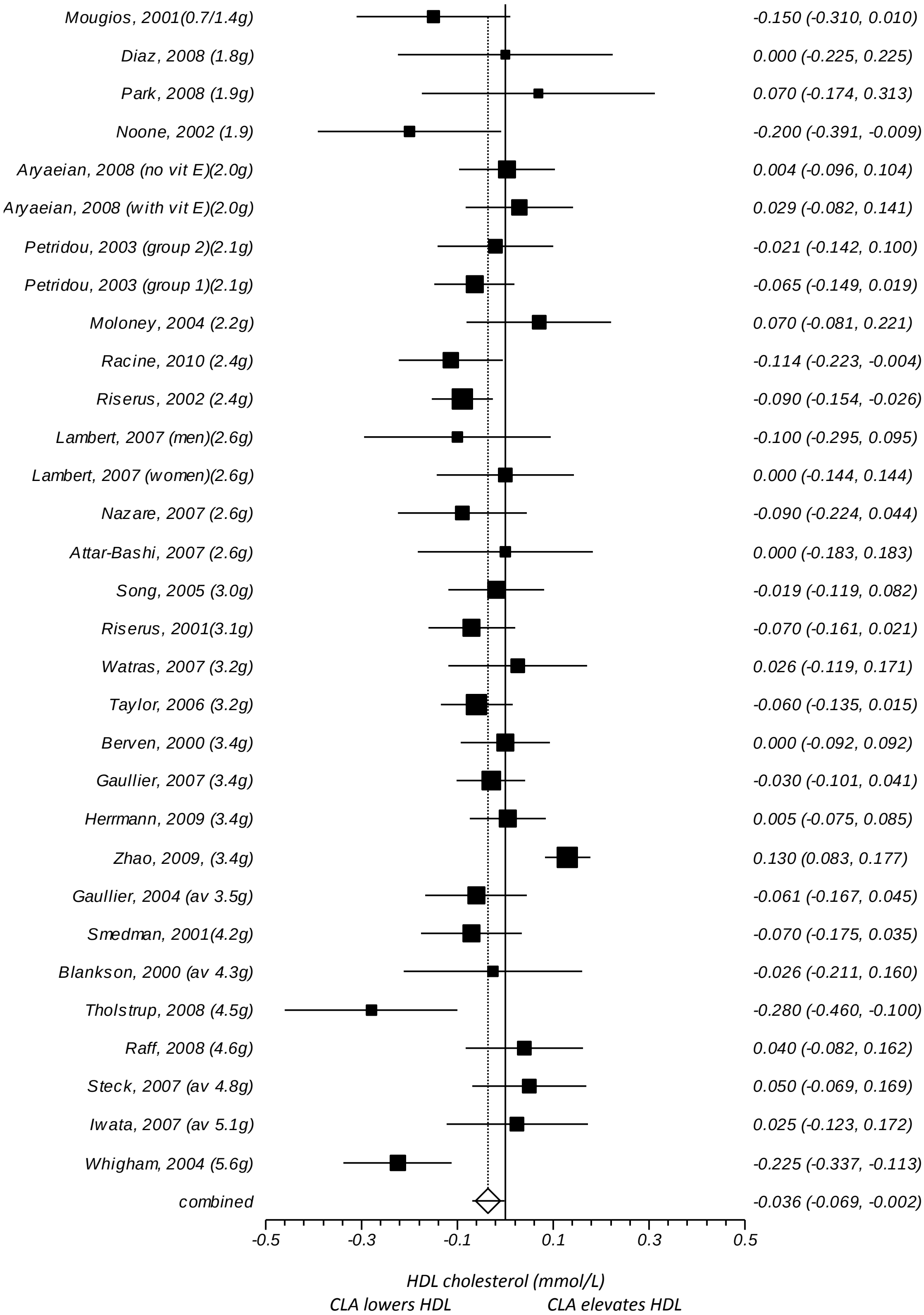

Figure 1: Difference between intervention and control groups (95% confidence interval) in ***HDL-cholesterol*** *levels,* ***1:1 CLA isomer mix*** *and* ***any type of fatty acid control****, ordered from top to bottom by increasing dose of CLA (daily dose of CLA shown next to author's name)*

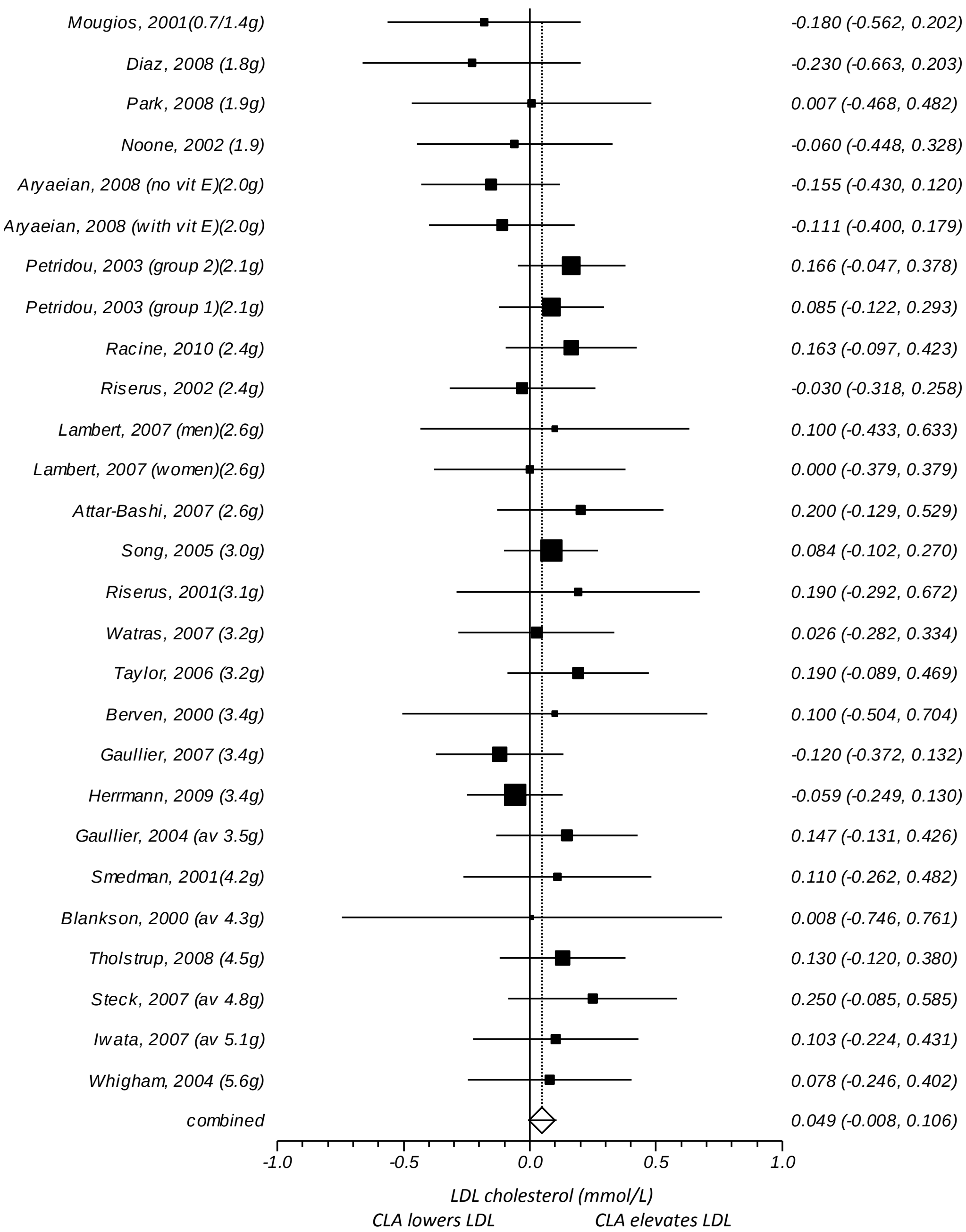

*Figure 2: Difference between intervention and control groups (95% confidence interval) in **LDL-cholesterol** levels, **1:1 CLA isomer mix** and **cis-unsaturated fat controls,** ordered from top to bottom by increasing dose of CLA (daily dose of CLA shown next to author's name)*

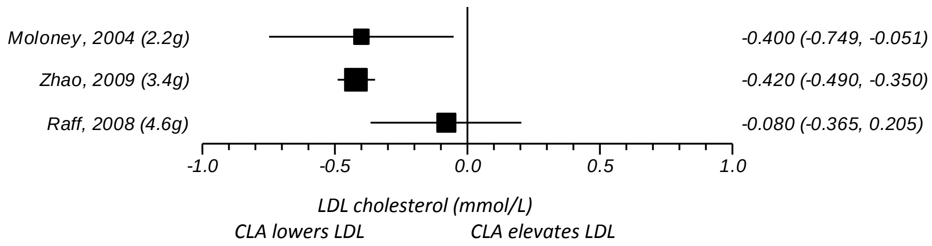

*Figure 3: Difference between intervention and control groups (95% confidence interval) in **LDL-cholesterol levels**, **1:1 CLA isomer mix** and **other fatty acid control groups**, ordered by increasing dose of CLA*

5.2 Conclusions

CLA in the range 1.4-5.6 g (approximately 0.5-2.2% energy) reduces HDL-cholesterol levels by 0.036mmol/L. Trans fats are the only fatty acids which reduce HDL-cholesterol levels when compared to other classes of fatty acids (Table 1). The effect seen for the 1:1 isomeric mixture of CLA (Figure 1) is consistent with that which would be predicted if approximately 3% energy from trans fat were compared to other classes of fatty acids (Table 1).

CLA elevates LDL-cholesterol by 0.049 mmol/L (not statistically significant) when compared to *cis*-unsaturated fats. Because the control fats used are oils which are a mixture of saturated and unsaturated fatty acids, it is not possible to compare the results quantitatively to those shown in Table 1 for different classes of fatty acid separately. However, there is an indication that the average elevation of LDL-cholesterol (when the 1:1 isomeric mixture of CLA is given) may be in the range predicted if a pure *cis*-unsaturated fat were replaced with a *trans* fat.

5.3 Other isomer ratios

Some of the studies included above also tested other ratios of the two relevant isomers (Noone *et al.*, (2002); Riserus *et al.,* (2002a); Tholstrup *et al.*, (2008); Herrmann *et al.*, (2009)) and several additional studies were identified that did not include an arm with the 1:1 isomer ratio. Except for Wanders *et al.,* (2010) who gave 7% energy as CLA (approximately 23 g for those consuming 9270kJ) the range of CLA dose used in these trials was 1.7-4.5 g. Their results are shown below but a quantitative overall summary estimate is not derived owing to the variation in which isomer was used and the much higher dose used by Wanders *et al.,* (2010) compared to the other studies. All of the studies shown in Figures 4 and 5 used *cis*-unsaturated fat controls except Sluijs *et al.,* (2010) who used a mix of fatty acids designed to resemble the Western diet.

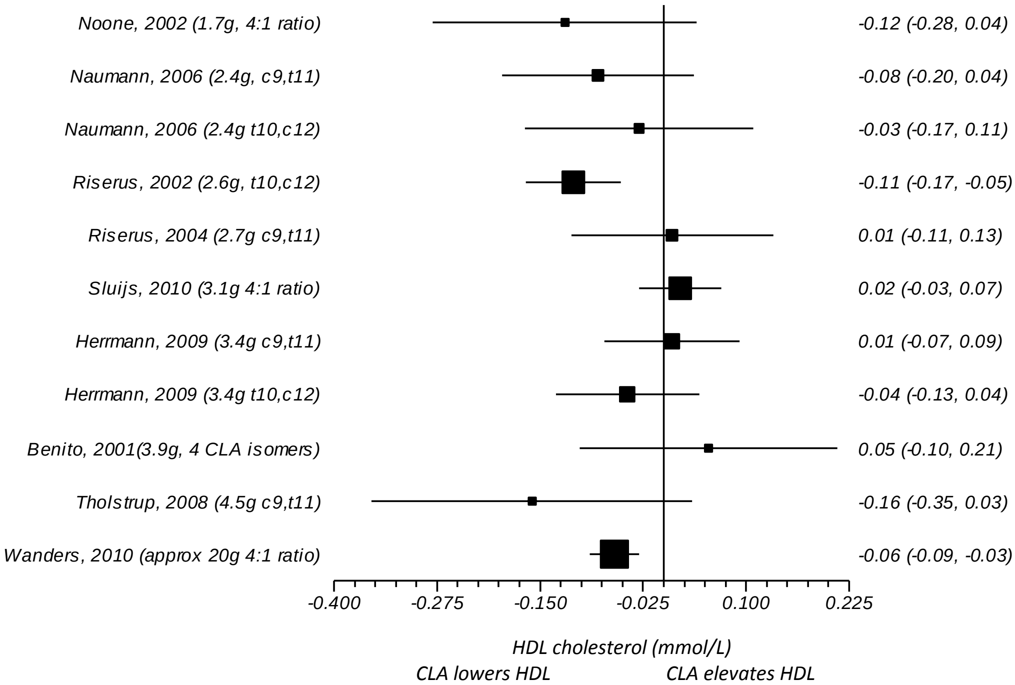

*Figure 4: Difference between intervention and control groups on **HDL-cholesterol** levels, **various ratios** of CLA isomers and **any type of fatty acid control**, ordered by increasing dose of CLA*

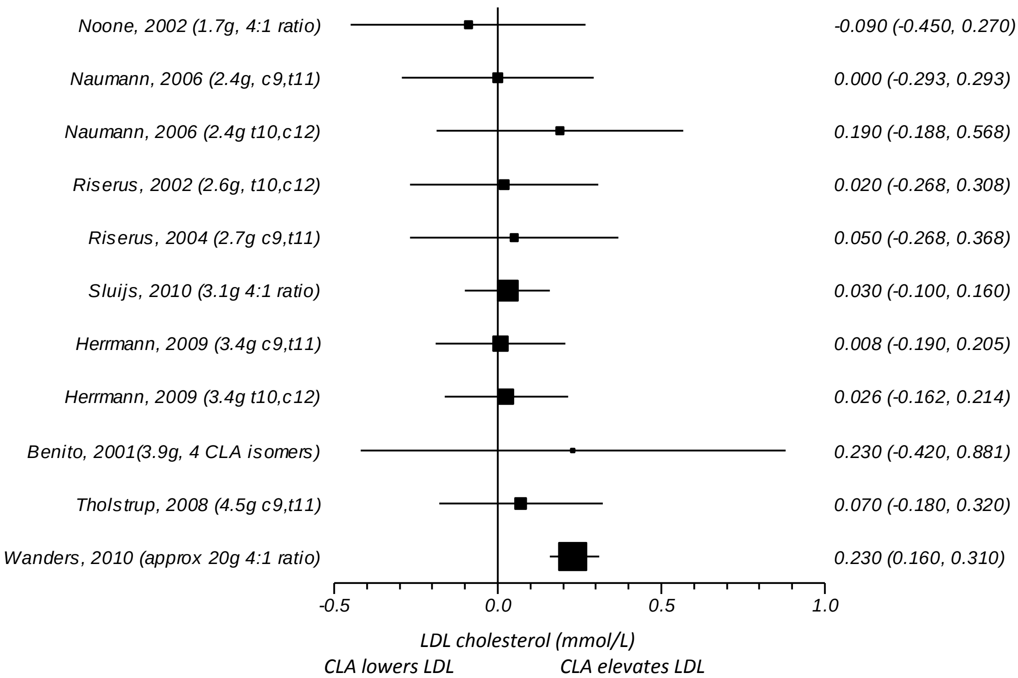

*Figure 5: Difference between intervention and control groups on **LDL-cholesterol** levels, **various ratios** of the CLA isomers and **various types of fatty acid control**, ordered by increasing dose of CLA*

Of the 11 arms, seven found that HDL-cholesterol was reduced when CLA was given compared to the control arm and four reported an increase in HDL-cholesterol levels (Figure 4). Of the 11 arms, nine found that LDL-cholesterol was increased when CLA was given compared to the control arm; one found no difference (0.0mmol/L) and one that LDL-cholesterol levels were reduced (Figure 5).

Only one study compared CLA to more than one control fat and this was also the only trial which has compared any CLA mixture to industrial trans fat (Wanders *et al.*, 2010). This trial used a preparation of CLA containing the same 2 isomers as that proposed for addition to food by the Applicant but in the ratio of 4:1 rather than 1:1. This cross-over trial in 61 subjects also used a much higher quantity of CLA (4:1 *c-9,t-11:t-10,c-12* ratio) than did the other studies, a high oleic acid sunflower oil placebo, and was powered to detect an effect half that predicted for an equivalent quantity of trans fat. The CLA was given in food and this was also the only study to supply 90% of the total dietary intake to the participants. Thus it is the only study to have tight control over the participants' diets. A further difference between this study and the others is that it provided CLA at 7% of each participant's energy need, rather than the same quantity to all participants.

Table 3 shows that this study found that the CLA preparation used lowered HDL-cholesterol to the same extent as industrial trans fat. The CLA preparation used elevated LDL-cholesterol compared to sunflower oil and this elevation was about two-thirds the size of the elevation seen with industrial trans fat (Wanders *et al.,* 2010).

In summary, the direction of the results of the studies using the same 2 isomers but at different ratios is consistent with the studies of the 1:1 isomer ratios in showing that CLA reduces HDL levels and elevates LDL levels. Although these studies used various ratios of the two isomers, rather than the 1:1 mixture, they provide supportive evidence that one or both of the isomers has adverse effects on the levels of these two lipids.

Table 3. Change in HDL- and LDL-cholesterol when three different fatty acid mixes are compared, results from Wanders et al, (2010)

	When high oleic sunflower oil is replaced by industrial *trans* fat	When high oleic sunflower oil is replaced by 4:1 *c-9,t-11:t-10,c-12* CLA	When industrial trans fat is replaced by 4:1 *c-9,t-11:t-10,c-12* CLA
Change in HDL-cholesterol level (mmol/L)	-0.05** (-0.08 to -0.03)	-0.06** (-0.09 to -0.03)	0.0 (-0.03 to 0.03)
Change in LDL-cholesterol level (mmol/L)	0.31** (0.24 to 0.38)	0.23** (0.16 to 0.31)	-0.08* (-0.15 to 0)

* P<0.05; ** P<0.001

5.4 Combined analysis of 1:1 and other isomer ratios

The Epidemiology Scientific Advisory Group (EpiSAG) took the view that it would be reasonable to combine the results of the 1:1 isomer ratio studies with the results of the isomers individually or when combined in other ratios. Therefore the studies shown in Figures 4 and 5, except for Benito *et al.,* (2001) who used a mix of four different isomers of CLA, were combined with studies shown in Figures 1-3. Specifically this added three arms testing a 4:1 *c-9,t-11:t-10,c-12* ratio, four arms testing the *c-9,t-11* isomer alone and three arms testing the *t-10,c-12* alone to the HDL cholesterol level analysis. For the LDL-cholesterol analysis, all except one of the 4:1 isomer studies had used unsaturated fat controls and were added to the studies shown in Figure 2. (The remaining study, Sluijs *et al., (*2010) used a mixed diet control and so was not included in the main LDL-cholesterol analysis but grouped with the other studies shown in Figure 3 and shown in Figure 8. As described above, this group was not combined in a meta-analysis). Many of the additional arms came from studies that also contained a study testing the 1:1 ratio or were multiple arms from the same study. The same procedures were used to average results within studies as for studies that tested different doses of the 1:1 isomer mix (see Appendix). For comparison, Table 4 presents the results for the 1:1 isomer mix from Table 2, followed by the additional analyses.

Combining all studies in the same dose range (1.4-5.6 g) reduces the effect on HDL-cholesterol levels slightly compared to the 1:1 results alone (Table 4). Adding the results of high dose study by Wanders *et al*., 2010 does not alter the result but tightens the confidence interval (Figure 6). For studies with *cis*-unsaturated controls, including the other trials, the 1.4-5.6 g dose range increases the effect on LDL-cholesterol level (from 0.049 to 0.057mmol/L, Table 4, Figure 7). Adding in the results of Wanders *et al.,* (2010) doubles the result, to 0.120mmol/L (p<0.0001, Table 4).

Owing to the increasing number of studies over time, and the increased dose range when the single isomer and other ratios are added to the results of the 1:1 ratio trials, a dose-response relationship was examined because this would be more appropriate than averaging the effect over the wide dose range created when the trial by Wanders *et al.,* (2010) was included (Table 4). There was a statistically significant relationship for both lipid outcomes. Compared to any fatty acid, there was a change in HDL-cholesterol of -0.005 mmol/L per gram of CLA (SEM: 0.002 mmol/L, p=0.04) for all studies and of -0.009 mmol/L per gram of CLA (SEM: 0.004mmol/L, p=0.03) when Wanders *et al.,* (2010) was excluded. Compared to cis-unsaturates, there was a change in LDL-cholesterol of 0.012mmol/L per gram of CLA (SEM: 0.001mmol/L, p<0.001) for all studies and of 0.021 mmol/L per gram of CLA (SEM: 0.007mmol/L, p=0.003) when Wanders *et al.,* (2010) was excluded.

Table 4: Effect of the c-9,t-11 and t-10,c-12 isomers of CLA either alone or together on HDL- and LDL-cholesterol levels.

Description	Difference: intervention – control (95% CI) (mmol/L)	I^2 (95% CI)
Effect on HDL cholesterol level		
1.4-5.6g CLA in a 1:1 isomer ratio compared to saturated and cis-unsaturated fat controls (Figure 1)	-0.036 (-0.069 to -0.002) p=0.04	65.1% (46.2% to 75.3%)
1.4-5.6g CLA either alone or in any ratio compared to saturated and cis-unsaturated fat controls (Figure 6)	-0.031 (-0.06 to -0.001) p=0.04	61.4% (40.7% to 72.6%)
Any dose CLA either alone or in any ratio compared to saturated and cis-unsaturated fat controls (i.e. including Wanders *et al.,* (2010))	-0.032 (-0.06 to -0.004) p = 0.02	63.1% (44.1% to 73.5%)
Effect on LDL-cholesterol level		
1.4 to 5.6g CLA in a 1:1 isomer ratio compared to cis-unsaturated fat controls (Figure 2)	0.049 (-0.008 to 0.106) p=0.09	0% (0% to 38%)
1.4 to 5.6g CLA either alone or in any ratio compared to cis-unsaturated fat controls (Figure 7)	0.057 (-0.0002 to 0.113) p = 0.051	0% (0% to 37%)
Any dose CLA either alone or in any ratio compared to cis-unsaturated fat controls (i.e. including Wanders *et al.,* (2010))	0.120 (0.074 to 0.165) p < 0.0001	0% (0% to 36.6%)

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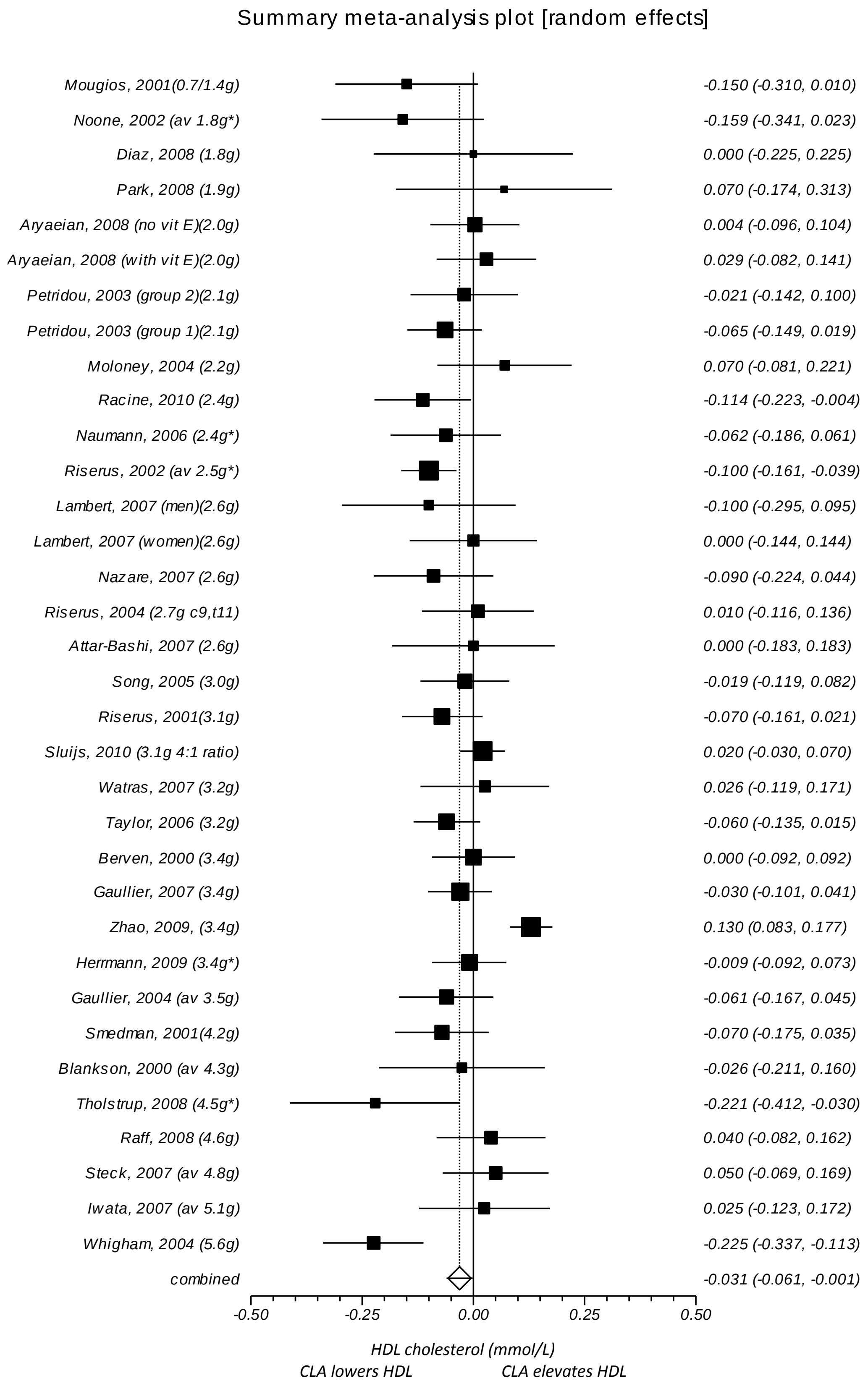

*Figure 6: Difference between intervention and control groups (95% confidence interval) in **HDL-cholesterol** levels, **either or both CLA isomers** and **any type of fatty acid control,** doses from 1.4-5.6g (i.e. excluding Wanders et al., (2010)), ordered from top to bottom by increasing dose of CLA (daily dose of CLA shown next to author's name, studies marked * contain at least one arm that is not in the 1:1 ratio)*

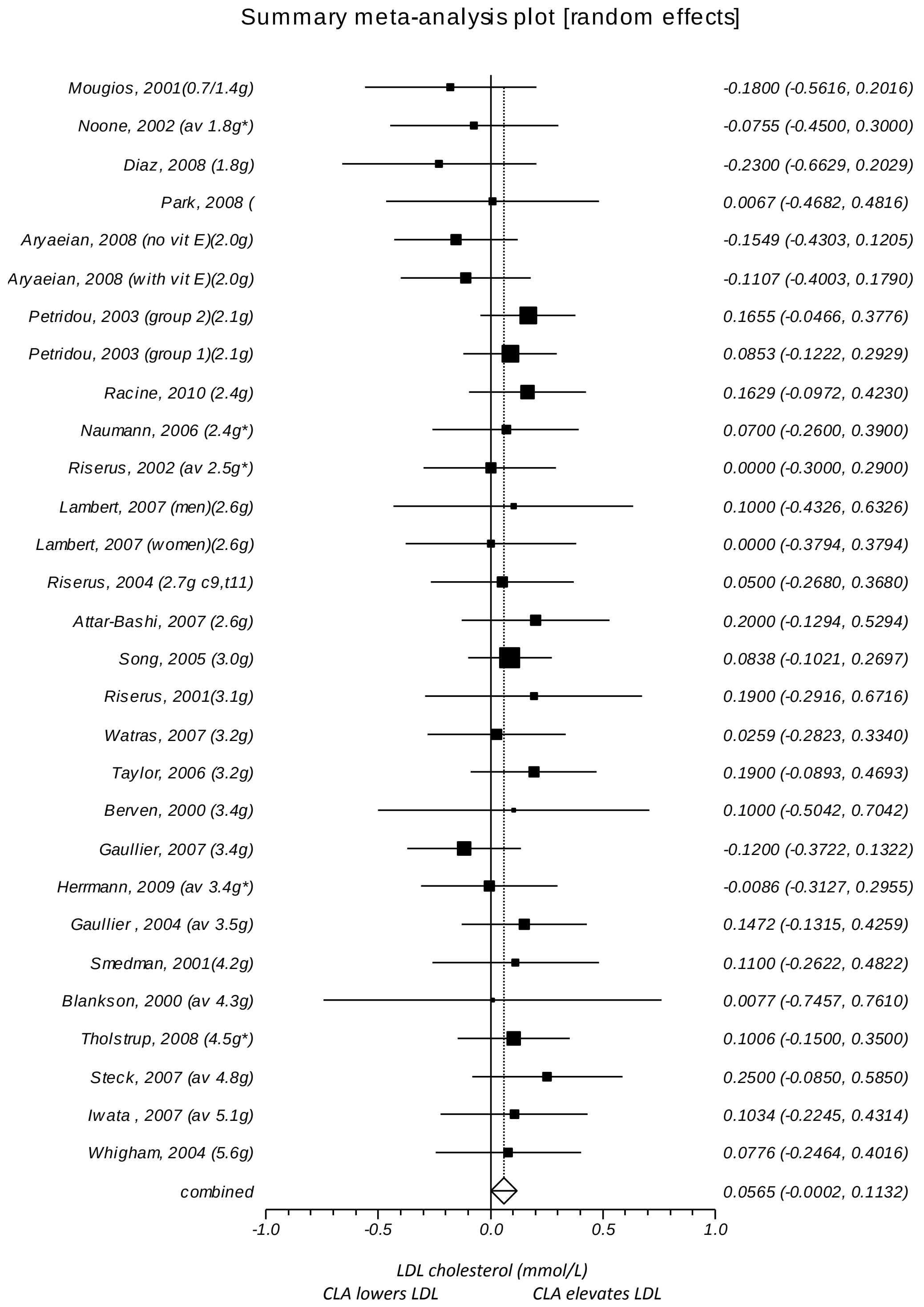

*Figure 7: Difference between intervention and control groups (95% confidence interval) in **LDL-cholesterol** levels, **either or both CLA isomers** and **cis-unsaturated fat controls,** doses from 1.4-5.6g (i.e. excluding Wanders et al., (2010)), ordered from top to bottom by increasing dose of CLA (daily dose of CLA shown next to author's name, studies marked * contain at least one arm that is not in the 1:1 ratio)*

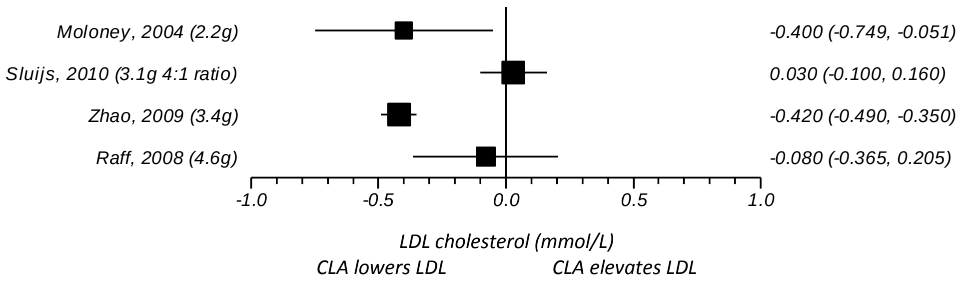

*Figure 8: Difference between intervention and control groups (95% confidence interval) in **LDL-cholesterol** levels, **either or both CLA isomers** and **other fatty acid control groups**, ordered by increasing dose of CLA*

6. Conclusion

The above results indicate that the 1:1 isomer mix of CLA in the range <6 g reduces HDL-cholesterol when compared to saturated and *cis*-unsaturated fats. There was a trend in the FSANZ meta-analysis, albeit not significant, towards an elevation of LDL-cholesterol when the 1:1 isomer mix (<6 g) was compared to oils rich in *cis*-unsaturates.

There were a number of other studies that used the isomers singly or in a different ratio. One of these used a 4:1 *c-9,t-11:t-10,c-12* ratio of CLA found a significant reduction in HDL-cholesterol and a significant increase in LDL-cholesterol compared to a control of high oleic sunflower oil (Wanders *et al.*, 2010).

The EpiSAG advised that they thought it was reasonable to combine the studies that used either or both of the isomers only to examine the effect on lipids. There was a statistically significant dose response relationship showing a decrease in HDL and an increase in LDL as dose of CLA increased when only studies using <6g were included and also when the high dose study of Wanders *et al.*, (2010) was included. Although the primary focus of the current assessment is on the 1:1 isomer ratio, FSANZ regards the results of the analysis of any ratio of the two isomers as supporting the view that the 1:1 isomer ratio probably has an effect on LDL-cholesterol.

These effects on both lipids are clearly different from the effects expected of a *cis*-polyunsaturated fatty acid (Mozaffarian and Clarke, 2009). FSANZ concludes that the 1:1 isomer CLA mixture has a different effect on these lipids from that of a *cis*-polyunsaturated fat. Based on currently available evidence, the effect of the 1:1 isomer mix of CLA on lipids is consistent with that of industrial trans fats. As noted above, the New Zealand and Australian heart disease risk charts use the total/HDL ratio as the predictor, with increasing values indicating increasing risk. The decrease in HDL-cholesterol has an unfavourable effect on the total/HDL ratio and this is exacerbated by the likely increase in LDL-cholesterol level.

In summary, FSANZ concludes that the 1:1 isomer mix of CLA decreases HDL-cholesterol levels. The trend towards an increased LDL-cholesterol level in the 1:1 studies and the significant dose-response relationship seen when the 1:1 and other studies were combined leads to the conclusion that the 1:1 ratio probably has an adverse effect on LDL-cholesterol, which is an additional concern.

7. Limitations of the studies examined

The randomisation methods or the diets of the subjects used in studies are rarely described. Given the quantities of CLA or control fat used in the 1:1 ratio studies, it is possible that other small changes in the diet might have occurred that affected lipid levels. Most studies gave the CLA in capsules rather than food and so the possibility of variation in effect relating to the use of different food vehicles was not examined. Most studies have used mixtures of fats (e.g. olive oil or high oleic sunflower oil) as the control substance. As saturated, *cis*-monounsaturated and polyunsaturated fatty acids have different effects on both HDL- and LDL-cholesterol it would be easier to assess the effect of CLA if all trials had used the same control substances.

When studies have small numbers, randomisation cannot be relied upon to equalise important confounders between groups. Lack of statistical significance between the groups in important factors such as body weight, age or sex does not mean that there are no influential differences when numbers are small. Tholstrup *et al*., (2008), Raff *et al*., (2008), Moloney *et al*., (2004) and Aryaeian *et al*., (2008) adjusted for baseline differences in the groups. There was variable reporting of the number of dropouts in the studies with some studies only reporting the number of subjects analysed. Although meta-analysis can combine small studies with inadequate power to examine effects, it cannot overcome any lack of equalisation of confounders that might exist within the studies unless the original data are obtained for each study and pooled.

The possibility of publication bias cannot be assessed properly. FSANZ notes that several studies described elsewhere in this Assessment drew blood from subjects to investigate outcomes such as glucose levels but did not describe any results relating to lipid levels.

7.1 Comparison to meta-analysis provided by the Applicant

In the middle of 2009, the Applicant provided an in-confidence meta-analysis that had similar but not identical results to those described above. This meta-analysis included the studies described above except Attar-Bashi *et al*., (2007); Aryaeian *et al.,* (2008), Park *et al.,* (2008), Zhao *et al*., (2009); Herrmann *et al*., (2009), Sluijs *et al*., (2010) and Racine *et al*., (2010). Some of these differences are due to the date of the Applicant's meta-analysis. It also included intervention-control results from Fielitz *et al.,* (2007) and LDL-cholesterol results from Nazarre *et al*., (2007), neither of which are available to FSANZ. It also included Tricon *et al*., (2006) and Desroches *et al*., (2005) who used a ruminant-derived mix containing *trans*-vaccenic acid and the *c-9,t-11* isomer of CLA but did not include other studies using similar ruminant-derived products (e.g. Chardigny *et al.,* (2008), Motard-Belanger *et al*., (2008). They further included Yonei *et al*,. (2007), Laso *et al*., (2007) and Lopez-Roman *et al*., (2007) who were excluded from the FSANZ analysis because the former used a lactose placebo and the later did not replace the fat despite giving control subjects the food vehicle used to deliver CLA to the intervention group. They also used a fixed-effects model whereas FSANZ has used a random-effects model. The results of these two model types will be different if heterogeneity is present but the same if there is no heterogeneity across the trials.

Despite these differences, the Applicant's meta-analysis found a statistically significant reduction in HDL-cholesterol levels in the studies that used 1:1 isomer mixes ($p<0.001$). The Applicant used standardised mean differences to derive an overall effect and estimated that it approximated a reduction of 0.07mmol/L. This is double the effect that FSANZ found. They also found a significant effect on HDL-cholesterol when the other studies were combined. They did not combine the 1:1 and other studies together.

The effect in the 1:1 ratio trials was dismissed by the Applicant because there was no dose-response relationship and because there was no association with duration of the trials. FSANZ has noted above that the range of CLA doses used is very limited and ranges from approximately 0.5-2.2% energy. Very large sample sizes would be needed to detect the predicted difference in effect across this range (Table 1).

Furthermore, changing the macronutrient content of the diet alters lipid levels within several weeks of the dietary change, but there is no further alteration in lipid levels after a new steady state is achieved. Therefore no association with duration of the trials is the expected result. It is further postulated by the Applicant that the reduction in HDL-cholesterol is an artifact and truly due to the effect of the control fat, oleic acid, on HDL-cholesterol. As shown in Table 1, all types of fatty acid (including saturated fat) except trans fat increase HDL-cholesterol when they replace carbohydrate on an iso-energetic basis. If CLA has the same effect as *cis*-polyunsaturated fatty acids then it, too, should elevate HDL-cholesterol and there would be no difference in HDL-cholesterol levels when CLA is compared to *cis*-unsaturated fats such as oleic acid.

The Applicant's meta-analysis of the effects of CLA on LDL-cholesterol levels did not allow for the different effects that would be predicted if any fatty acid were compared to saturated fat controls versus unsaturated fat controls, but grouped studies with all types of control substances together. Despite this, a non-significant increase in LDL-cholesterol was noted, which may be related to the number of trials using unsaturated fat controls. This is consistent with the results of the FSANZ meta-analysis.

There are differences in the studies included in each meta-analysis. The Applicant's meta-analysis includes the results of a study showing a substantial reduction in both HDL- and LDL-cholesterol (standardized mean difference -0.641; 95% CI: -1.088 to -0.194, $p=0.005$ for both; Fieltz *et al.,* (2007)). However, these data were not supplied to FSANZ and the abstract only describes the change in LDL-cholesterol in the CLA group but does not give the difference between the CLA and control groups. Inclusion of this study would have increased the size of the reduction in HDL-cholesterol levels compared to FSANZ's analysis, and also reduced the size of the increase in LDL-cholesterol. The paper by Nazare *et al.,* (2007) reports only HDL-cholesterol levels but the Applicant's meta-analysis includes LDL-cholesterol results which are not available to FSANZ. There are also several differences in interpretation of the reported results of particular studies ((Moloney *et al.*, (2004), Noone *et al.*, (2002); Watras *et al.*, (2007)) between the two meta-analyses.

7.1.1 Dose-response relationship

The Applicant noted that the meta-analysis that they commissioned did not find a dose-response relationship for HDL-cholesterol or LDL-cholesterol within the 1:1 isomer trials. When testing the dose-response relationship, they included the multiple arms from various studies separately and calculated dose in separate groupings to avoid over-reporting the control groups. They do not say if or how they formally examined the dose-response across the groupings. Dose-response was not tested in the studies using other ratios but an overall average was calculated. In some analyses, the results of the high dose trial of Wanders were corrected to estimate the result if a 5 g dose had been used instead. By contrast, where multiple arms were reported within a trial, FSANZ has used the averaged result of all analyses, including the dose-response assessment.

7.1.2 Dose-response analysis of Brouwer et al, (2010)

Brouwer *et al*., (2010) also examined the dose-response relationship of the two isomers used in any ratio. Their analysis differs from FSANZ's in several respects. Firstly they had different inclusion and exclusion criteria: they included the term "LDL" as part of their search strategy and they restricted the analysis to studies with published values for both HDL- and LDL-cholesterol and in which subjects had stable body weight. This probably accounts for the smaller number of CLA studies in their review. They also recalculated the study result by using the Mensink equations (Mensink *et al.,* 2003) to correct for the different fatty acids used in the control groups and estimated the dose as a percent of energy intake using average energy requirements for men and women (in contrast to the simpler classification of control fats used by FSANZ). The focus of their review was on examining whether there was a difference in dose-response in the studies that had examined industrial trans fat, CLA derived from ruminant sources and the synthetic CLA isomers being examined in the current Application.

They found no difference in the slope of the unweighted regression lines for industrial trans fat, ruminant trans fat and synthetic CLA, although the slope for CLA and HDL-cholesterol alone was not statistically significant (decrease of 0.008mmol/L (95% CI: −0.023 to 0.007mmol/L) for each percent of energy exchanging CLA for *cis*-monounsaturates). There was also no difference between the three lines for LDL-cholesterol, but the increase in LDL-cholesterol with CLA was statistically significant (increase of 0.038mmol/L (95% CI: 0.005 to 0.071mmol/L) for each percent of energy exchanging CLA for *cis*-monounsaturates). They also reported an adverse effect on the LDL:HDL cholesterol ratio (Brouwer *et al.,* 2010). Figures 9 and 10 show that until publication of the study by Wanders *et al.,* 2010 which gave 7% energy as CLA, the range of doses tested for industrial trans fats was much wider than the range tested in the synthetic CLA studies.

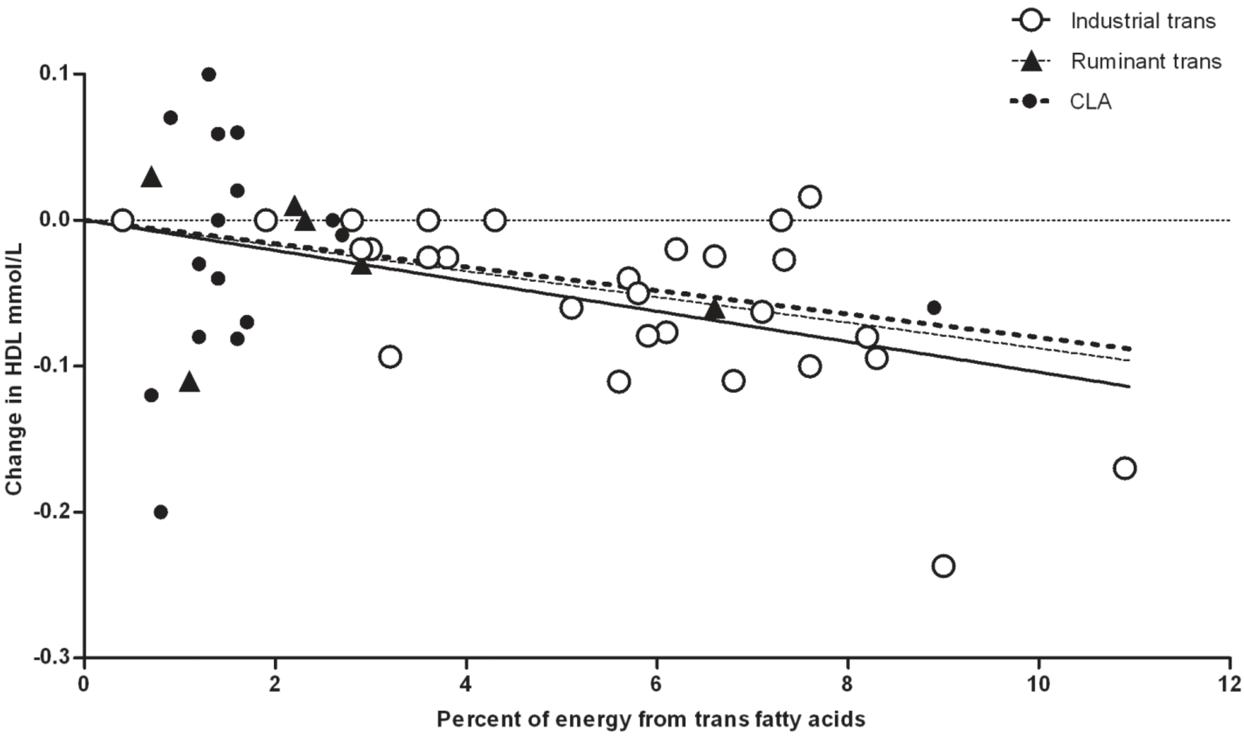

Figure 9: Change in HDL with increasing levels of three types of trans fat in the diet. The horizontal line at zero shows the effect of cis-monounsaturated fatty acids. (Brouwer et al, PLoS ONE, 2010) (Ruminant trans indicates studies using dairy fat from animals fed safflower oil which increases trans-vaccenic acid and c9,t11 CLA content of the fat)

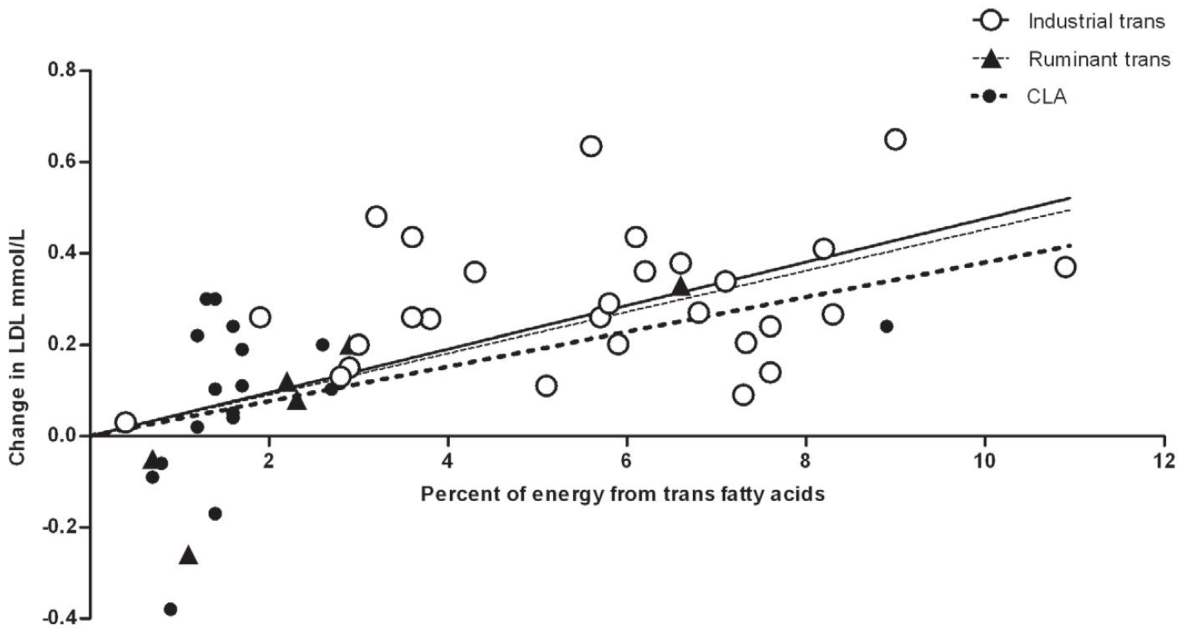

Figure 10: Change in LDL with increasing levels of three types of trans fat in the diet. The horizontal line at zero shows the effect of cis-monounsaturated fatty acids. (Brouwer et al, PLoS ONE, 2010) (Ruminant trans indicates studies using dairy fat from animals fed safflower oil which increases trans-vaccenic acid and c9,t11 CLA content of the fat)

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Appendix

Identification of studies testing CLA

The bulk of the scientific literature reviewed was provided by the Applicant as published peer reviewed papers. The reference lists of the papers provided were searched for further relevant work. PubMed was also searched using the terms: conjugated linoleic acid OR CLA. The following limits were applied to the search: humans, controlled clinical trial. Many of the papers describing human trials provided by the Applicant and identified by other means focused on aspects of weight or body size/shape or measures related to glucose metabolism. Results for lipids were often not mentioned in the abstract and reported only in the tables. Consequently, identification of studies to examine the effects of lipids used the results of the searches for studies of weight- and diabetes-related outcomes (see SD2 and SD3). It is possible that there may be missing studies if the study title and abstract did not use either of the search terms. The search was last run on 31 March 2010.

The following inclusion criteria were applied to the studies:

- Studies in humans
- Statement by authors that the trial was randomised
- A double-blind design, either cross-over or parallel
- Comparing either *c9,t11* and/or *t*10,*c*12 to a control group, (unlike SD2, trials which used ratios other than the 1:1 mixture were included in a subsidiary analysis because the focus was on safety)
- Lasting three weeks or longer
- Reporting results for LDL-cholesterol and/or HDL-cholesterol
- Sufficient numerical data had to be present in the reports to allow the difference between the intervention and control groups and its 95% confidence interval to be calculated
- Trials which gave concurrent treatments that were not expected to affect either lipid level were permitted; for example Aryaeian *et al.,* (2008) conducted a 2x2 factorial trial using vitamin E as the second intervention substance. Zhao *et al*., (2009) gave Rampiril to both intervention and placebo groups. However, trials using treatments which might affect lipid levels or energy intake were excluded. Trials which did not use a fatty acid as the control were excluded, including trials that gave the food vehicle to the control subjects but did not appear to have added an equal amount of fat to it.

Two studies which used a high CLA dairy fat produced by feeding unsaturated oils to cows were excluded because this feeding regime also increased the concentration of *trans*-vaccenic acid (C18:1, *t*11) in the intervention food (Desroches *et al.*, 2005; Tricon *et al.*, 2006). (Other studies which used this approach, such as Motard-Belanger *et al.,* (2008) and Chardigny *et al.,* (2008), are described as tests of the effect of ruminant *trans* fatty acids by their respective authors). By contrast, Raff *et al.,* (2008) added manufactured CLA of a 1:1 isomeric ratio to low CLA butter as the intervention and gave low CLA butter to the control group.

FSANZ abstracted the data relating to effects on LDL-cholesterol and HDL-cholesterol. Abstracted information was checked by a second person and authors were emailed where relevant details were not clear, although many authors did not reply. Figure 1 shows the flow diagram and why various studies were excluded from further consideration. Tables A1a and A1b outlines the features of the studies included and Table A2 notes decisions made during data abstraction and analysis, including when additional information was not received from authors.

Potentially relevant papers identified (**n=70**)

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Papers excluded (**n=36)**

Adams *et al* (2006), Eyjolfson *et al* (2004), Kreider *et al* (2002), **Larsen *et al*, (2006); Medina *et al* (2000), Malpuech-Brugère *et al* (2004), Norris *et al*, (2009), Pinkosky *et al*, (2006), Ramakers *et al*, (2005), Thom *et al*, (2001); Turpeinen *et al* (2006), Zambell *et al*, (2000)** did not contain either HDL or LDL-cholesterol results
Fielitz et al (2007), an abstract, reported results for the intervention group but not the placebo group
Atkinson *et al* (1999) and **Belury *et al*.(2003)** – studies not published in full.
Moya *et al*. (2008) and Thrush *et al* (2007) – no randomisation and no control.
Tricon et al (2004) did not have a control arm given no CLA
Colakoglu *et al*. (2006) – only single-blind with control group not given a placebo, and CLA preparation not adequately described to establish composition or purity.
Gaullier *et al*. (2005) – Data for wk 0-52 captured in Gaullier *et al*. (2004); open-label from week 52-104, i.e. no randomisation and no control.
Tarnapolsky *et al* (2007), Yonei *et al* (2007), Sneddon *et al* (2008), Ahrén *et al* (2009) and **Cornish *et al*. (2009)** gave CLA in conjunction with other potentially active ingredients without a CLA only group for comparison.
Risérus *et al* (2002b), Riserus *et al* (2004b) and **Ingelsson and Riserus (2008)** are additional reports of the study of Riserus et al (2002a)
Chouinard *et al* (2007) and **Close *et al* (2007)** are additional reports from the study of Watras *et al* (2006)
Syvertsen *et al* (2006) is an additional report of Gaullier *et al*. (2007),
Desroches *et al* (2005) and **Tricon *et al* (2006)** examined preparations containing trans-vaccenic acid in addition to CLA

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34 Studies included in the assessment report (n=28 gave 1:1 isomer mixture and 9 gave other ratios of the two isomers including 3 studies which gave both types).

Figure 1: Flow of study consideration and reasons for exclusions in the systematic review

Analysis

Study results were reported in a variety of ways. The difference in change in lipids between the intervention and control group was used when this was reported and calculated when it was not. Some studies reported the difference between baseline and follow-up separately for the intervention and control group and this allowed the test-retest correlation for HDL-cholesterol and LDL-cholesterol to be calculated. The correlations ranged between 0.5 and 1.0 but most were close to 0.8 and so a value of 0.8 was used to calculate the standard deviation of the difference between baseline and follow-up in the intervention and control groups for those studies where this was necessary (Higgins and Green, 2008). The results of studies reporting in mg/dL were converted to mmol/L by dividing by 38.67 after the intervention-control group difference had been calculated.

StatsDirect was used for analysis (StatsDirect Ltd, 2008). The results from the random effects model and I^2 (Higgins *et al.*, 2003) and Cochran's Q for the models are reported for the 1:1 isomers. I^2 describes the "percentage of total variation across studies that is due to heterogeneity rather than chance" and 0%, 25%, 50% and 75% could be interpreted as indicating no, low, medium and high heterogeneity respectively (Higgins *et al.*, 2003). The more familiar Cochran's Q is also shown but it is a less useful descriptor of heterogeneity when study numbers are small (Higgins *et al.*, 2003). Sensitivity analyses were performed to examine the effects of excluding groups of studies (Table A3).

Studies were divided into those which tested a 1:1 mix of *c*-9, *t*-11 and *t*-10, *c*-12 and studies which tested other ratios of these two isomers. Most studies provided enough information to allow the quantity of CLA consumed by subjects to be calculated from the daily quantity of CLA-rich oil and the percentage of the oil that was CLA. Where stated, the percentage ranged from 63% to 81% in those which tested a 1:1 mix, and from 56% to 92% in those which tested other ratios of the two isomers. The quantity of CLA isomer used in all studies of the 1:1 ratio mix was narrow, ranging from 1.4 – 5.6 g, or expressed another way, 0.5 - 2% energy from CLA. Some studies had more than one intervention arm and where they tested isomers in the same ratio but at different doses these were combined to derive an average for all intervention arms versus the control arm.

FSANZ's focus was on the 1:1 isomer ratio although results from studies with other ratios provided supplementary information. The EpiSAG advised that it thought it would be reasonable to combine all studies that used one or both of the isomers together. This increased the available dose range owing to the inclusion of the high dose study that used a 4:1 *c-9,t-11:t-10,c-12* ratio of the isomers (Wanders *et al.,* 2010). To examine the dose-response, a linear weighed regression was calculated for each of the two lipid outcomes in SPSS. The weighings were produced in StatsDirect using the inverse of the variance of the difference of the means of the intervention and control groups. The regression line was forced through the origin because it was assumed that a zero dose would lead to a zero response.

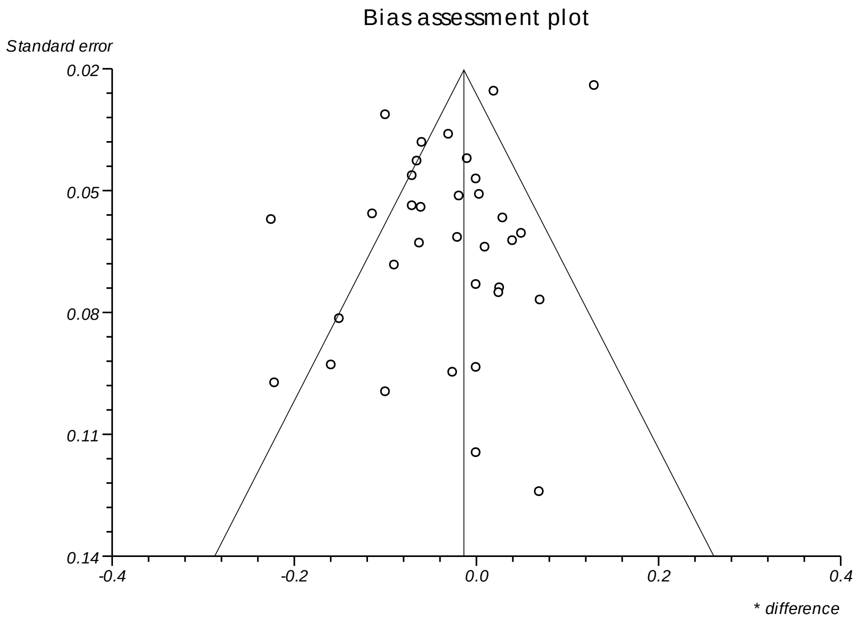
Funnel Plots

Funnel plots are used as a visual tool to assess whether publication bias might be likely. Each dot represents a study and a symmetrical plot suggests there is little likelihood of publication bias.

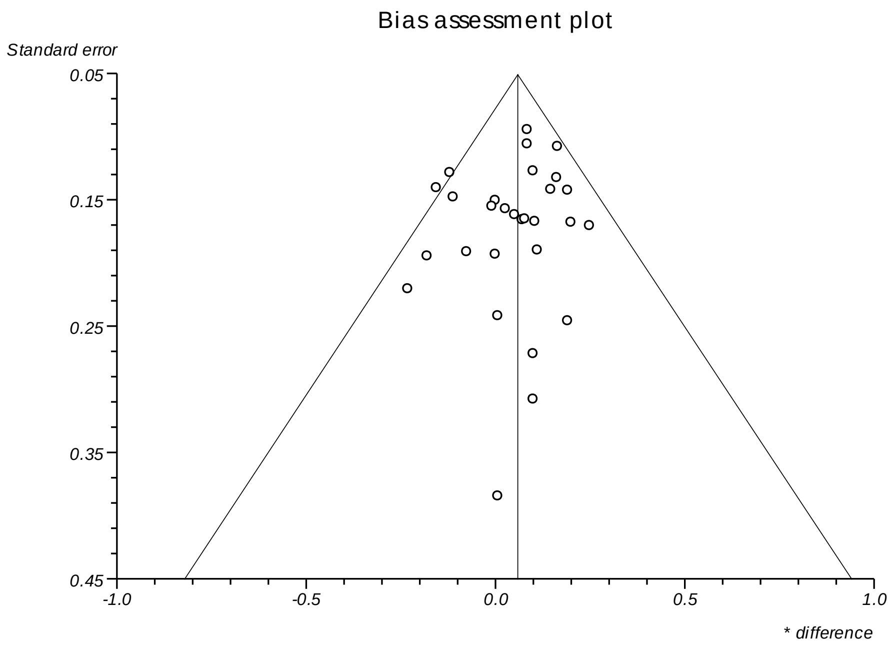
Funnel plot that corresponds with analysis shown in Figure 1 (1:1 isomer ratio and HDL-cholesterol)

Funnel plot that corresponds with analysis shown in Figure 2 (1:1 isomer ratio and LDL-cholesterol)

Funnel plot that corresponds with analysis shown in Figure 6 (all ratios of the two isomers and HDL-cholesterol)



Funnel plot that corresponds with analysis shown in Figure 7 (all ratios of the two isomers and LDL-cholesterol)



There was an a priori decision to subdivide trials reporting LDL-cholesterol into saturated fat versus unsaturated fat controls as these have different effects on LDL-cholesterol. This decision was implemented by grouping trials using olive oil, flax and soya oil, linoleic acid, safflower oil and high oleic sunflower oil as “unsaturated controls” and trials using other fats such as a mixture of fats designed to resemble the usual diet as “other controls”. This is a crude grouping but the limited number of studies did not allow greater discrimination.

Only one trial used two control arms (Wanders *et al.*, 2010). The comparison of the CLA mix against high oleic sunflower oil is included in the main set of results and the other results of this trial are described separately.

The majority of blinded studies lasted for one to three months, with one study being conducted for 12 months. Unlike some other biological parameters (such as weight loss on an energy-restricted diet), lipid levels do not continue to change once the new steady state has been reached and therefore trial duration was not examined as a source of heterogeneity among the trials.

All except three studies used a modified intention-to-treat analysis in that all subjects who returned for the follow-up blood test were included in the group to which they had been randomised. Berven *et al.,* (2000), Diaz *et al.,* (2008) and Racine *et al.,* (2010) stated that they excluded subjects with poor compliance from their analysis, measured as < 70% supplement use and via plasma CLA profile respectively. Some studies also excluded subjects with low compliance from other analyses such as body fat assessment and so the numbers reported here might not match numbers described elsewhere in this Assessment Report. Most studies did not adjust their results for baseline differences between the groups despite the small numbers in the studies, which could mean that randomisation would not ensure that all important differences were equally distributed between the groups.

Sensitivity analyses were done excluding the three studies that had excluded low compliers. For HDL-cholesterol the effect of removing the three studies contributing to the high inconsistency was examined. Three studies had also given an additional substance to both intervention and control although it was thought that these would not affect lipid levels. However an analysis was done with these studies excluded. (The study of Attar-Bashi *et al.,* 2007 who gave flaxseed oil to both groups was not excluded because giving flaxseed oil to both groups is conceptually identical with giving CLA in an olive oil or other base and giving the base to the control group). These analyses caused small changes in the difference between the intervention and control group compared to the primary analysis. In some instances, the HDL-cholesterol results were no longer significant while in others, the LDL-cholesterol results became statistically significant (Table A3).

Table A1a: Summary of Participants and Protocols in Studies with c-9,t-11 and t-10,c-12 CLA isomers given in a 1:1 ratio (ordered alphabetically by first author)

First Author, Year	Initial total n	Final total n	Gender (m/f)	BMI (kg/m^2)	Physical State	Age (years)	Duration (days)	CLA True Dose (g/d)	Co-interventions	Placebo, Dose (g/d)	Dietary/Physical activity management	Inter-group differences at baseline
Aryaeian, 2008	87	87	15/72	~27	Overweight, active rheumatoid arthritis	19-69	84	2	Yes[1]	High oleic sunflower oil, amount not specified	Asked to follow usual diet and activity	Groups were similar w.r.t sex, age, BMI and daily intake of vitamin E at baseline. There were no sig changes in BMI, physical activity or dietary intake during the study period.
Attar-Bashi, 2007	16	16	12/4	~25	Healthy	20-50	56	2.6[a]	Yes[2]	2.0 g soybean oil	Not described	NS difference at baseline between groups in reported measures.
Berven, 2000	60	47	30/17	27.5-39	Overweight or obese	≥18	84	3.4	No	4.5 g olive oil	Diet & physical activity were not reported	NS difference at baseline between groups in reported measures.
Blankson, 2000	60	47	17/30	25-35	Sedentary, light (no sweat) or intense (sweat) exercise, overweight or obese	≥18	84	1.7, 3.4, 5.1, or 6.8	No	9 g olive oil	Diet was not assessed. Participants could join a light or intense exercise program	NS difference at baseline between groups in reported measures. No adjustments for demographic data were done in the statistical analysis.
Diaz, 2008	59	35	0/35	25-34	Overweight or obese	21-50	84	1.8	Yes[3]	2.4 g canola oil	Subjects received dietary counselling to achieve a 500Kcal/day energy deficit. Food records were kept.	NS difference at baseline between groups in reported measures.
Gaullier, 2004	133	119	21/98	25-30	Overweight	18-65	365	3.6 or 3.4	No	4.5 g olive oil	*ad libitum* diet. No restrictions in lifestyle or in caloric intake were implemented.	All groups reduced their energy intake from month 0 to month 12, but the relative change between CLA & control was NS. NS difference in exercise in CLA vs. control

Gaullier, 2007	118	83 (99 for lipid data)	21/84[b]	28-32	Overweight	18-65	182	3.4	No	4.5 g olive oil	*ad libitum* diet and no restrictions in lifestyle or caloric intake were implemented.	NS difference at baseline between groups in reported measures.
Herrmann, 2009[‡]	**38**	**34**	38/0	26.1	No metabolic or gastrointestinal diseases, not diabetic	45-68	4X28	3.4		3.23 linoleic acid	Not described	Not applicable because cross-over study
Iwata, 2007	60	60	60/0	25-35	Overweight or obese	25-60	84	3.4 or 6.8	No	10.8 g high linoleic safflower oil	*ad libitum* diet with no restrictions on caloric intake.	No data
Lambert, 2007	64	62	25/37	<25	Healthy, had been regularly exercising (3 or more time per week) for more than 6 months	21-45	84	2.6	No	3.9 g high oleic acid sunflower oil	Subjects were instructed to keep their training and diet constant throughout the trial and any changes were recorded.	NS difference at baseline between groups in reported measures.
Moloney, 2004	ND	32	ND	~30	Stable, diet controlled Type II diabetics, overweight	~60	56	2.2	No	Palm and soya bean oil. Dose not described	Subjects had diet controlled diabetes and were following healthy eating guidelines and had stable body weight. Diet was monitored by 4-day food records.	No data
Mougios, 2001	24	22	13/9	<30	Healthy	19-24	2 x 28	0.7 x 4 weeks then 1.4 x 4 weeks	No	1 g / 2 g soybean oil	Diet was controlled by giving subjects a balanced isoenergetic weekly dietary plan based on estimated BMR and physical activity. Subjects were asked not to modify their usual way of life, including physical activity, in any other respect. Diet records were collected.	NS difference at baseline between groups in reported measures.
Nazare, 2007	44	44	22/22	~25	Healthy	~29	98	2.6 in yoghurt	No	3.8 g cream (in yoghurt)	*Ad libitum* diet. Physical activity was checked through daily records	NS difference at baseline between groups in reported measures.
Noone, 2002	51	51	18/33	<25	Sedentary	32±10	56	1.9	No	Linoleic acid	No attempts at assessing or controlling diet were reported. To be eligible, subjects had to do < 90 minutes strenuous exercise/week.	NS difference at baseline between groups in reported measures.

Park, 2008	30	30	3/27	≥23	BMI≥23kg/m^2	19-65	56	1.8 (or possibly 2.4)	Yes	2.4g olive oil	Ad libitum diet and participated in a standard physical training program 3 days/week	NS difference at baseline between groups in reported measures.
Petridou, 2003[‡]	17	16	0/16	<30	Sedentary, overweight	19-24	45x2	2.1	No	3.0 g soybean oil	Subjects kept diet records throughout study & were asked not to change physical activity patterns.	Used crossover design. NS difference between the CLA-Con group and the Con-CLA group in values being assessed.
Racine, 2010	62	53	31/22	Not applicable	>85th centile for age-sex-specific BMI	6-10	7 months	2.6		3g sunflower oil	Not described	Some differences in baseline height
Raff, 2008	47	38	38/0	19-27	Healthy	19-35	35	4.6 added to low CLA butter	No	115g low CLA butter	Subjects replaced part of their diet with test foods (butter) incorporated into bread rolls, cake and chocolate milk during the intervention period. Both groups were to obtain similar amounts of fat. Subjects were instructed how to change their diet to consume the test foods without increasing the total fat content of their diet.	NS difference at baseline between groups in reported measures.
Riserus, 2001	25	24	24/0	27-39	Obese with signs of the metabolic syndrome, stable body weight preceeding 3 months	39-64	28	3.1	No	4.2 g olive oil	All men were encouraged to maintain their usual diet and exercise habits throughout the course of the study.	NS difference at baseline between groups in reported measures.
Riserus, 2002 a	66	57	57/0	27-39	Metabolic syndrome, overweight or obese	35-65	84	2.4	No	3.4 g olive oil	All men were encouraged to maintain their usual diet and exercise habits during the study. 3-day weighed food record kept at week 0 & 8	NS difference at baseline between groups in reported measures.

Smedman, 2001	53	50	25/25	19-35	Healthy, wide ranging body weights	23-63	84	4.2	No	4.2 g olive oil	Subjects requested not to change their diet & physical activity habits and to abstain from any vitamin, mineral or fatty acid supplementation prior to and during the study. 3 day weighted diet record kept at baseline, middle & end of study.	NS difference at baseline between groups in reported measures.
Song, 2005	ND	28	8/20	~24	Healthy	~30	84	3	No	3 g high oleic sunflower oil	Subjects were asked not to alter their usual diet and physical activity over the study period.	NS difference at baseline between groups in reported measures.
Steck, 2007	55	48	13/35	30-35	Obese	18-50	84	3.2 or 6.4	No	8 g safflower oil	Participants instructed to maintain their current diet and exercise routines throughout the study period. Five 24-hour recalls collected over study's duration. Brief physical activity questionnaire at baseline, 6 & 12 weeks.	NS difference at baseline between groups in reported measures.
Taylor, 2006	40	40	40/0	33 ± 3	Healthy, obese	35-60	84	3.2	No	4.5 g olive oil	No detail about diet or physical activity protocol provided. No measures of diet or physical activity reported.	NS difference at baseline between groups in reported measures.
Tholstrup, 2008	81	75	0/75	≤35	Healthy, overweight or obese postmenopausal women. Smokers evenly distributed across groups.	~60	112	4.5	No	5.5 olive oil	No dietary restrictions. Weighted food records at baseline and week 8. Physical activity management was not reported.	NS difference at baseline between groups in reported measures.

Watras, 2006	48	40	8/32	25-30	Overweight	18-44	182	3.2	No	4 g safflower oil	No detail about diet or physical activity protocol provided. 7-day physical activity & 3 day diet records kept at baseline & study end. Measures of energy and/or macronutrient intake not provided.	NS difference at baseline between groups in reported measures. Compared to baseline, physical activity decreased by 33% and 40% at 6 months in the placebo and CLA group respectively. Both groups reduced reported energy intake, the placebo group significantly (p=0.02)
Whigham, 2004	63	48[c]	15/35	25-37	Overweight & obese	18-50	365	5.6	Yes[4]	7.5 g high oleic sunflower oil	Diet & physical activity diaries submitted monthly. Initial VLCD followed by maintenance diet that many subjects found difficult to adhere to.	Authors did not specifically report results from baseline comparisons.
Zhao, 2009	80	80	44/36	>30	Obese with stage 1 uncontrolled essential hypertension	~60	56	3.4	Yes[6]	4.5 g oil blend (~60% saturated fat)	Conducted on a free-living, outpatient basis. All subjects given Ramipril. All subjects asked to maintain their usual lifestyle habits. 4-day food records at baseline & study end.	NS difference at baseline between groups in reported measures.

Table A1a includes studies where the treatment involved a form of CLA that contained the isomers c9,t11 and t10,c12 in the ratio 1:1. The true dose of CLA is the grams of c9, t11 and t10,c12 given to subjects. This was not always reported, but did range from approximately 60% to 80% of the weight of CLA oil given to subjects. The true dose of CLA isomers of interest rather than the reported dose of CLA (e.g. weight of test capsule) has been reported here.

Six of the thirty studies involved concurrent treatments. [1]Aryaeian *et al* (2008) used CLA and vitamin E in one of four arms of their study. [3]Diaz *et al* (2008) tested CLA along with chromium picolinate. [4]Whigham *et al* (2004) tested liquid low calorie diet for phase 1 followed by attempt at weight maintenance for phase 2. [6]Zhao *et al* (2009) tested CLA and Ramipril (37.5mg/day), an anti-hypertensive medication.

[a]In Attar-Bashi *et al* (2007) dose was reported in mL and had to be converted to a gram weight by FSANZ using a specific gravity of 0.92. Both groups received flaxseed oil ..

[b]In Gaullier *et al* (2007), three months into the study 105 of the original 118 subjects remained. At six months, dropouts reduced the final value of n to 93 and authors then discounted a further 10 subjects due to non compliance, leaving a final value of n=83 reported. In tabulated results of measures of blood lipids, the authors reported the value of n at 6 months as n=50 (CLA group) and n=49 (placebo group). For the purpose of analysis of lipid data, FSANZ used the final n of 99.

[c]In Whigham *et al* (2004), at baseline there were 63 subjects. At week 12 (end phase 1, a VLCD) there were 50 subjects remaining. At week 28 (end phase 2, weight maintenance) there were 48 subjects remaining. For consideration of the effect of CLA on blood lipids, FSANZ used data reported for week 28.

‡ Crossover study design

Acronyms: BMI – body mass index; BMR – basal metabolic rate; CLA – conjugated linoleic acid; f – females; m-male; n – number of participants; NS – no significant; VLCD – very low calorie diet; w.r.t.- with respect to.

Table A1b: Summary of Participants and Protocols in Studies of Mixed Isomers of CLA, ordered alphabetically by first author

First Author, Year	Initial total n	Final total n	Gender (m/f)	BMI (kg/m^2)	Physical State	Age (years)	Duration (days)	CLA *True Dose (g/d)	Co-interventions	Placebo, Dose (g/d)	Dietary/Physical activity management	Inter-group differences at baseline
Benito, 2001	17	17	0/17	~22	Healthy	~28	96	1.6g of the CLA isomers of interest but 3.9g total CLA isomers	No[1]	Equal quantity of high oleic sunflower oil	Each subject's energy requirements were determined, and meals prepared to ensure no wt gain or loss. Dietary intake was strictly monitored. Physical activity management was not reported.	No data
Herrmann, 2009[‡]	38	34	38/0	26.1	No metabolic or gastrointestinal diseases, not diabetic	45-68	4X28	3.4		3.23 linoleic acid	Not described	Not applicable because cross-over study
Naumann, 2006	92	87	48/39	~29	Healthy, with LDL phenotype B, overweight	~53	91	2.4	No	3 g high oleic sunflower oil (in yoghurt)	Subjects were asked not to alter their usual diet and physical activity over the study period.	No data
Noone, 2002	51	51	18/33	<25	Sedentary	32±10	56	1.7	No	Linoleic acid	No attempts at assessing or controlling diet were reported. To be eligible, subjects had to do < 90 minutes strenuous exercise/week.	NS difference at baseline between groups in reported measures.
Riserus, 2002a	66	57	57/0	27-39	Metabolic syndrome, overweight or obese	35-65	84	2.6	No	3.4 g olive oil	Subjects were asked not to alter their usual diet and physical activity over the study period.	NS difference at baseline between groups in reported measures.
Riserus, 2004a	25	25	25/0	27-35	Overweight or obese	35-65	84	2.7	No	3 g olive oil	Subjects were asked not to alter their usual diet and physical activity over the study period.	NS difference at baseline between groups in reported measures.

Sluijs, 2010	401	346	167/179		Overweight or obese; not hypertensive or with total cholesterol≥8 mmol/L	58.5	6 months	3.1		4g palm and soybean oil to resemble fatty acid profile of western diet	Subjects asked not to change their diets	Baseline table presented, not statistics described
Tholstrup, 2008	81	75	0/75	≤35	Healthy, overweight or obese post-menopausal women. Smokers evenly distributed across groups.	~60	112	4.5	No	5.5 g olive oil	Subjects were asked not to alter their usual diet and physical activity over the study period. Food records kept at baseline and week 8.	NS difference at baseline between groups in reported measures.
Wanders, 2010‡	63	61	25/36	22.8	Healthy	30.9	3 x 21	7% of energy	No	7% energy as high oleic sunflower oils	Test fats administered in margarine and yoghurt drinks; 90% energy supplied as foods and subjects allowed to choose remaining food from a list of low-fat foods. One meal eaten under supervision on weekdays; Duplicate diets collected daily and analysed. Subjects seen twice weekly for weighing; diet adjusted to keep weight constant	Not applicable because cross-over study

Table A1b includes studies where the treatment involved a form of CLA that contained c9,t11 and t10,c12 or other isomers of CLA in ratios other than the more commonly examined ratio of c9t11:t10,c12 as 1:1. The *true dose of CLA is the grams of CLA isomers of interest (i.e. c9, t11 and t10,c12) given to subjects. This was not always reported, but did range from 56% (Noone *et al*., 2002) to 92% (Tholstrup *et al*., 2008) of the weight of CLA oil given to subjects. Therefore, the true dose of CLA isomers of interest rather than the reported dose of CLA (e.g. weight of test capsule) has been reported here. Consequently the amount of control fat reported is greater.
[1]Benito *et al* (2001) gave subjects 100mg vitamin E every five days to ensure adequate antioxidant levels. ‡ Crossover study design.
Acronyms: BMI – body mass index; CLA – conjugated linoleic acid; f – females; m-male; n – number of participants;.

Table A2: Decisions made during data abstraction and analysis

Aryaeian *et al*, (2008)	2x2 factorial design entered as two separate studies – one without vitamin E in both groups and one with vitamin E in both groups
Attar-Bashi *et al*, (2007)	Quantity of CLA given in ml and converted to grams using a specific gravity of 0.92
Benito *et al*, (2001)	Although the CLA product used had an approximately equal amount of the two isomers of interest (c9, t11 and t10,c12), it also had approximately the same quantities of two other CLA isomers (t8,c10 and c11,t13) and smaller amounts of 6 additional isomers. Therefore this study was classified as a mixed ratio study rather than a 1:1 ratio study.
Gaullier *et al*,(2007)	Authors included all subjects with data in the lipid results in the analysis even though they excluded 10 non-compliers from some analyses of other outcomes reported elsewhere in this Assessment Report (refer footnote to Table 1a)
Herrmann *et al* (2009)	The author confirmed that the LDL-cholesterol and HDL-cholesterol results had been switched with each other (J Herrmann, personal communication,2010)
Laso *et al*, (2007)	Author clarified that the number of overweight was 10 and obese: 13 (A Lafuente, personal communication, 2009) This study reported that LDL-cholesterol values had standard errors of the mean (SEM) of 29-37mg/dl; other studies reported standard deviations (SD) for LDL-cholesterol in the range of 22-33 mg/dl. Therefore the SEM reported in this study were treated as SD; this decision makes one of the elevations in LDL-cholesterol reported in this study statistically significant but it does not affect any summary estimate because this study used an "other" control (Figure 3).
Lambert *et al*, (2007)	Author clarified that there were 25 men and 37 women (K Charlton, personal communication, 2009) but did not describe further therefore FSANZ assumed 12 treated and 13 control men and 18 treated and 19 control women
Mougios *et al*, (2001)	LDL-cholesterol data supplied by author (V Mougios, personal communication, July 2009)
Petridou *et al*, (2003)	LDL-cholesterol data supplied by author (V Mougios, personal communication, July 2009)
Raff *et al* (2008)	SD for baseline value for HDL-cholesterol in the control group reported as 1.33 mmol/L but assumed to be a misprint for 0.33mmol/L, given the SD in the intervention group
Smedman & Vessby, (2001)	Neither SD nor SEM reported for follow up values so the SDs reported for baseline in intervention and control group were used to calculate SD for difference in each of intervention and control groups; authors note that LDL was normally distributed, but HDL was not
Sluijs *et al*,	Values reported as SD for baseline and follow-up data are likely to be SEMs,

(2010)	but were not used in calculations because the final intervention-control difference and confidence interval was reported
Song *et al*, (2005)	Reported baseline mean total cholesterol of 5.0 and 4.9 mmol/L but no other baseline lipid values. Therefore, based on the two arms of Steck *et al* (2007) which had mean total cholesterol of 4.8mmol/L, values of 1.35mmol/L for HDL-cholesterol and 2.8mmol/L for LDL-cholesterol were used to convert the reported % change from baseline in HDL- and LDL-cholesterol from baseline to mmol/L
Tholstrup *et al*, (2008)	Excluded milk arm as it has additional protein etc which would affect macronutrient % and consequently blood lipid levels; used the least squares results adjusted for baseline presented separately for each group
Whigham *et al*, (2004)	Used results to 28 weeks used as the trial was no longer randomised after this point
Yonei *et al*, 2007	Lactose placebo used (Y Yonei, personal communication, 2009)

Table A3: Examination of altering the assumptions underlying the meta-analysis (sensitivity analysis) for the comparison of 1:1 isomers of CLA versus control fats

Model	Description	Difference (mmol/L) intervention – control (95% CI)	I^2 (95% CI)	Cochran's Q (p)
Results for HDL-cholesterol levels				
0	Figure 1: trials with saturated and *cis*-unsaturated fat controls	-0.036 (-0.069 to -0.002) p = 0.04	65.1% (46.2% to 75.3%)	< 0.0001
1	Figure 1 excluding studies which excluded subjects with low compliance (Berven *et al,* 2000), Diaz *et al*, 2008 & Racine *et al* , 2010)	-0.035 (-0.072 to 0.002) p = 0.06	67.4% (49% to 77.1%)	< 0.0001
2	Figure 1 excluding studies which had a concurrent substance (Diaz *et al*, 2008 (chromium picolinate) Zhao *et al*, 2009 (Rampiril) and Aryaeian *et al* (one arm received vitamin E))	-0.048 (-0.074 to -0.022) p = 0.0003	31.4% (0% to 56.1%)	0.06
Studies with high influence on the inconsistency and possible reason for the inconsistency				
3a	Figure 1 excluding Zhao *et al,* 2009)(subjects given Rampiril)	-0.044 (-0.070 to -0.019) p = 0.0006	29.8% (0% to 54.6%)	0.06
3b	Figure 1 excluding Whigham *et al,* 2004 (subjects placed on weight loss diet prior to study and subjects regained weight during the study)	-0.028 (-0.060 to 0.004) p = 0.09	60.1% (36.2% to 72.4%)	< 0.0001
3c	Figure 1 excluding Tholstrup *et al,* 2008 (analysis adjusted for baseline differences by regression)	-0.030 (-0.063 to 0.003) p = 0.08	62.7% (41.2% to 74%)	< 0.0001
3d	Figure 1 excluding Zhao *et al,* 2009; Whigham *et al,* 2004 & Tholstrup *et al,* 2008	-0.036 (-0.057 to -0.015) p = 0.0006	0% (0% to 37.5%)	0.6

Model	Description	Difference (mmol/L) intervention – control (95% CI)	I^2 (95% CI)	Cochran's Q (p)
Results for LDL-cholesterol levels				
0	Figure 2 Trials with *cis*-unsaturated fat controls	0.049 (-0.008 to 0.106) p = 0.092	0% (0% to 38%)	0.9
1	Figure 1 excluding studies which excluded subjects with low compliance (Berven *et al,* 2000, Diaz *et al,* 2008 & Racine *et al* 2010)	0.048 (-0.011 to 0.107) p = 0.1136	0% (0% to 39.6%)	0.9
2	Figure 2 excluding studies which had a concurrent substance (Diaz *et al*, 2008 (chromium picolinate) and Aryaeian *et al* (one arm received vitamin E))	0.061 (0.002 to 0.119) p=0.04	0% (0% to 39%)	0.95