FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

SUPPORTING DOCUMENT 4

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

Chemical Safety

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Contents

Glossary

ADI	Acceptable daily intake
ALT	Alanine aminotransferase
AP/ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
BAT	Brown adipose tissue
BMI	Body mass index
Bq	Becquerel. The International Standard (SI) unit of radioactivity
BUN	Blood urea nitrogen
CLA	Conjugated linoleic acid
CRP	C-reactive protein
EKG/ECG	Electrocardiogram
EFSA	European Food Safety Authority
FSANZ	Food Standards Australia New Zealand
HDL	High density lipoprotein
GLP	Good laboratory practice
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvic transaminase
IGF1	Insulin-like growth factor-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IU	International unit
LD_{50}	Median lethal dose. The quantity of chemical that is lethal to 50% of the experimental animals exposed to it.
LDL	Low density lipoprotein
mRNA	messenger RNA
NOAEL	No observed adverse effect level
NOEL	No observed effect level
OECD	Organisation for Economic Co-operation and Development
PPAR	Peroxisome proliferator-activated receptor
TAG/TG	Triacylglycerol
TNFα	Tumour necrosis factor alpha
UL	Upper limit
USEPA	United States Environmental Protection Agency
WAT	White adipose tissue
WBC	White blood cell

Summary and conclusions

This Application seeks permission for the addition of CLA to a range of foods. CLA is a collective term for a mixture of isomers of linoleic acid in which the two double bonds are conjugated (separated by one single bond). For this assessment, CLA refers to a chemically defined mixture of approximately equal amounts of the *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA in the form of triglyceride esters (Tonalin® CLA).

The *cis*-9, *trans*-11 isomer of CLA is a normal constituent of the human diet, present principally in milk and other dairy products. Addition of synthetic CLA to food would result in an increase in dietary exposure to both isomers, but a greater proportional increase in exposure to the *trans*-10, *cis*-12 isomer, which is not normally found in significant quantities in the diet.

The toxicological database consisted of a range of studies in animals and humans. FSANZ has evaluated these studies, with a focus on those studies using a CLA mix containing approximately 1:1 of the two major isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. All of the known physiological effects of CLA were induced by these two main isomers, although in some cases, the observed effects appeared to have been produced by one of the isomers acting alone. In other cases, the two isomers have appeared to act together to produce an effect.

The majority of animal studies with CLA have been conducted in mice and rats, with a smaller number of studies in pigs, hamsters and rabbits also being available. These studies were of variable duration, e.g. ≤8 weeks in mice; 2 to 36 weeks in rats; ≤14 weeks in pigs; and 8 weeks in hamsters, with key data coming from two 90-day studies in rats. Two developmental studies in rats were also conducted, as well as a number of genotoxicity studies.

A number of human studies, while primarily being conducted for the purpose of establishing efficacy, examined certain clinical chemistry and haematology parameters, and therefore were also included as part of this assessment.

No toxicological endpoint relevant to humans was identified from these studies in animals or humans. However, given that the clinical studies were primarily designed to assess efficacy, very little relevant toxicological information could be derived from them. Furthermore, given the nature of these fatty acids, their presence already in the diet and their known metabolic pathways, it was considered that hazards would not have been identified from the measured parameters. Hence with these limitations, it was not considered appropriate to establish a health standard for CLA. The highest intakes of CLA were in the range of 6 – 6.8 g per day in humans. As higher intakes greater than this have not been reported in the data dossier, no further comment can be made regarding the safety of CLA in humans at higher consumption rates.

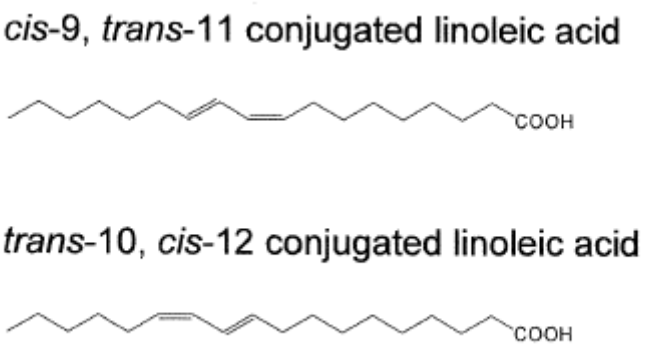
1. Introduction

Linoleic acid is a polyunsaturated fatty acid with 18 carbons and two *cis* double bonds at the 9 and 12 positions (octadecadienoic acid, C18:2). CLA consists of a group of geometric and positional isomers of linoleic acid. All CLA isomers have double bonds with a single carbon bond in between (conjugated double bonds).

Tonalin®TG80 is manufactured from food grade safflower oil .The CLA preparation proposed to be added to food in this Application (Tonalin®TG80) consists of a mixture of *cis*-9, *trans*-11 CLA (this isomer is the predominant form found naturally in ruminant meat and milk products and is also known as rumenic acid), and *trans*-10, *cis*-12 CLA (see Figure 1). In the human diet, CLAs contribute about 0.5 to 2.0% of the fatty acids. The *cis*-9, *trans*-11 isomer contributes >70% of the CLA in these foods (McLeod *et al*., 2004). Various other CLA isomers may also be present in these foods, and it has been estimated that this may include 0 to 2% of the *trans*-10, *cis*-12 isomer (Fritsche *et al*., 1999). The proposed formulation of Tonalin CLA would consist of equivalent quantities of both isomers. It has been estimated that consumption of Tonalin® CLA would result in an increase of 2-fold and >50-fold of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers, respectively.

Tonalin®TG80 contains approximately 40% of each of these two isomers, with a total CLA content of 78-84%. These are present primarily as triglyceride esters (>75%). In addition to the two main CLA isomers, a number of other fatty acids are present in small amounts; oleic acid being the other main fatty acid present.

Figure 1: Structural formula of main isomers of CLA



2. Proposed mode of action

The mode of action by which CLA is believed to alter body composition is thought to include: a reduction in lipid uptake; an increase in energy expenditure; and an increase in fatty acid oxidation. Further detail from the applicant has been reproduced below. It has also been suggested that this mode of action is similar to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Li *et al*., 2008). It is, however, unclear if these actions can adequately explain the variable effects observed with the consumption of CLA in animal and human studies.

2.1 Inhibition of lipid uptake in adipose tissue

'*In vitro* and *in vivo* studies have found that *trans-10*, *cis-12* CLA and mixed CLA isomers reduce lipid uptake by adipocytes, through modulation of lipoprotein lipase (LPL), a key enzyme in lipid metabolism which hydrolyses the circulating triglycerides and releases free fatty acids (Park *et al*., 1997; Park *et al*., 1999; Brown *et al*., 2003; Xu *et al*., 2003; Brown *et al*., 2004; Kang *et al*., 2003; Lin *et al*., 2001).

Studies in mice, human breast cancer cells, human hepatocytes and murine adipocytes suggest that *trans-10*, *cis-12* CLA and mixed CLA isomers reduce the expression or activity of stearoyl-CoA desaturase (SCD-1), an enzyme responsible for the delta-9 desaturation of palmitate and stearate (Choi *et al*., 2000; Choi *et al*., 2001; Choi *et al*., 2002; Ntambi *et al*., 2002; Pariza *et al*., 2001). Loss of SCD-1 function has been shown to protect from obesity, by decreasing the synthesis of long-chain mono-unsaturated fatty acids, which are the preferred substrates for triglyceride synthesis.'

2.2 Increasing energy expenditure in adipose tissue and skeletal muscle

'Accumulating evidence from *in vivo* studies in mice and *in vitro* experiments suggests that uncoupling protein 2 (UCP2), which is abundant in white adipose tissue, is involved in increased energy expenditure (Ryder *et al*., 2001; Tsuboyama-Kasaoka *et al*., 2000; Kang *et al*., 2004; Ealey *et al*., 2002; Takahashi *et al*., 2002). Increased UCP2 expression and mRNA levels have been observed with mixed CLA isomers.'

2.3 Increasing fatty acid oxidation in muscle, liver and adipose tissue

'In murine muscle cells, mixed CLA isomers have been shown to increase the activity of carnitine palmitoyltransferase (CPT), which is the enzyme catalysing the rate-limiting step in β-oxidation, resulting in stimulation of fat oxidation in skeletal muscle (Park *et al*., 1997). Both *cis-9*, *trans-11* and *trans-10*, *cis-12* CLA isomers have been shown to increase CPT activity in the muscle, liver and adipose tissue (Martin *et al*., 2000; Rahman *et al*., 2002).

CLA and predominantly the *trans-10*, *cis-12* isomer modulates enzyme responsible for the breakdown and synthesis of lipids in adipose tissue, resulting in a reduction in adipose tissue mass. At the same time, increases in fat oxidation and energy expenditure primarily in the skeletal muscle, which may explain observed increases in lead body mass. As a result, adipose tissue reduction with lean body mass increase can explain the lack of weight reduction seen in CLA-supplemented subjects.'

3. Absorption, distribution, metabolism, excretion

The general physiological processes for digestion, absorption and metabolism of fatty acids and triacylglycerides are well described in the general scientific literature and are not specific to different fatty acid types. The CLA-triglycerides are therefore expected to be treated by the body in a similar way to other dietary fatty acids and their triglycerides, in particular the closely related linoleic acid. The absorption, distribution, metabolism and excretion of CLA have been investigated mainly in rats, although a number of human studies are also available. A review of relevant studies is provided below.

3.1 Absorption

TAG molecules are present in the small intestine as hydrophobic droplets which cannot be absorbed as such, but are broken down by pancreatic lipase. Lipase mainly attacks the primary ester bonds, resulting in the production of free fatty acids and monoacylglycerol.

These form mixed micelles in the presence of bile acids, in which form they are absorbed by intestinal epithelial cells for re-esterification to triglycerides and / or oxidised as a source of energy.

Upon absorption into the cells that line the intestinal wall, fatty acids of length C>12 (either as part of monoacylglycerol or in free form) are resynthesised into TAG. These TAG are then combined with lipoproteins and other lipid substances to form chylomicrons, which are released into the lymph vessels, from where they enter the bloodstream. Fatty acids of length C<12 are released into the portal vein as monoacylglycerol or free fatty acids bound to albumin, and travel directly to the liver. Fatty acids of lengths C<12 are more water soluble and can be readily placed into the blood, while other fatty acids need to be included in lipoprotein structures before they can be transported.

In an *in vitro,* dynamic, multi-compartmental model of the stomach and small intestine, the digestibility, determined by the recovery of CLA incorporated into micelles in the jejunum and ileum compartments, of CLA triglycerides (Tonalin TG80) and CLA methyl esters was investigated. The CLA-triglyceride was found to be slightly more efficiently digested (90%) compared to the methyl ester (72%) (Venema, 2006). It was hypothesised that either the triglyceride is more efficiently incorporated into mixed micelles, thus giving the lipase better access to the triglyceride molecule or, alternatively, that the lipase may have greater specificity for the triglyceride compared to the methyl ester.

An eight-day study in rats (two groups of six) examined the absorption of free and esterified *cis*-9, *trans*-11 CLA. No CLA was detected in the faeces of rats consuming 330 ± 30 mg/day TAG CLA (mean and standard deviation) or 338 ± 14 mg/day free CLA, leading to the conclusion that both free and esterified *cis*-9, *trans*-11 CLA is entirely absorbed. The analysis of the main fatty acids in the liver, plasma and adipose tissue indicated that *cis*-9, *trans*-11 CLA is deposited mainly in the neutral lipid fraction rather than in phospholipids, and is mainly in esterified form (Plourde *et al*., 2006).

In a study of six healthy overweight (BMI 25-30) men aged 18-45 years, the absorption of CLA in free, triglyceride or ethyl ester forms was investigated. Subjects were fasted overnight and then given 3 g of CLA (1:1 of the two main isomers) in 100 mL yoghurt drink, followed by an oral fat load to assist the formation of chylomicrons. Blood was sampled at t=0 (just before feeding) and at t=1, 2, 4, 6, and 8 hours. Each subject received all of the lipid forms at intervals of two weeks (randomised and double-blinded). Triacylglycerol concentrations were measured in plasma, chylomicrons and triacylglycerol rich lipoproteins at each time point. Variation between the small number of subjects was high, however it was reported that: there was little difference in the incorporation into chylomicrons between the TAG CLA and the free CLA, and no significant difference between the absorption of the *cis*-9*, trans*-11 and the *trans*-10, *cis*-12 isomers; ethyl ester CLA was not incorporated to such an extent as the other forms; and even when free CLA was given, the form found in chylomicrons was the TAG form (Fernie *et al*., 2004).

Daily intake of 2.1 g CLA (equal parts of *cis*-9*, trans*-11 and *trans*-10, *cis*-12 CLA) by 17 healthy, sedentary women (BMI <30) aged 19-24 for 45 days increased the proportion of CLA in total fatty acids in triglycerides and phospholipids in the serum by a factor of two to five. No change in CLA in cholesterol esters was observed. After a washout period of 15 days, increased CLA levels had returned to the levels prior to supplementation (Petridou *et al*., 2003).

3.2 Distribution

In a study in rats fed a CLA preparation (different isomer ratios to Tonalin) or linoleic acid at 1% in the diet for two weeks, significantly more linoleic acid was recovered from the lymph than CLA (78.5% compared to 53.4%). CLA was found in adipose tissue, lungs, kidney, spleen, serum, liver, heart and brain, however no difference was found between the treated and control rats (given linoleic acid). Approximately 80% of the CLA was carried as chylomicrons and the remaining 20% as very low-density lipoproteins. 95% of CLA was present as triacylglycerides and 5% as phospholipids (Sugano *et al*., 1997).

In a published study designed to assess the β-oxidation of CLA, male rats were given (by gavage) 1.5-2.6 MBq of [1-^{14}C]-linoleic acid, *cis*-9*, trans*-11 CLA or *trans*-10, *cis*-12 CLA, and $^{14}CO_2$ production was monitored for 24 hours (Sergiel *et al*., 2001). Animals were then killed and radioactivity in different tissues determined. Approximately 71.8% and 70.3% of the radioactivity was found in expired CO_2 in the rats given *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA, respectively. This was significantly more than for rats given linoleic acid (60.3%, $P < 0.05$). The amount of radioactivity incorporated in a range of tissues was also measured. In most cases levels were similar between groups, however, the incorporation of radioactivity was greater in the group given linoleic acid than the groups given the *cis*-9, *trans*-10 and *trans*-10, *cis*-12 isomers in the brain, heart, adrenals, testes and carcass, indicating that CLA appeared to be oxidised to a greater extent than linoleic acid. No explanation could be given for the slightly variable incorporation of the two isomers (*cis*-9, *trans*-11 & *trans*-10, *cis*-12) in the same tissues.

In a published study, 17 healthy women were given a low fat diet (30% of energy from fat, 19% from protein and 51% from carbohydrates) for 30 days. After 30 days, women were randomly grouped into two groups and given either 3.9 g CLA per day (n=10) or an equivalent amount of high linoleic sunflower oil (n=7) for a further 63 days. The CLA contained 11.4% *cis*-9, *trans*-11-CLA, 10.8% *trans*-8, *cis*-10-CLA, 15.3% *cis*-11, *trans*-13-CLA and 14.7% *trans*-10, *cis*-12-CLA, and their corresponding cis/cis (6.74% total) and trans/trans (5.99% total) isomers.

Blood was taken after 30, 60 and 93 days. Adipose tissue samples were taken by needle biopsy at 30 and 93 days. There was no change in plasma cholesterol, LDL cholesterol, HDL cholesterol or triglycerides. CLA in plasma increased in the test group (weight percentage from 0.28 to 1.09, p<-.05), however only approximately 4.23% of ingested CLA was in plasma at any one time. CLA in adipose tissue was not affected by CLA ingestion. It was concluded that in humans, dietary CLA is rapidly metabolised to other products (Benito *et al*., 2001).

3.3 Metabolism

The metabolism of CLA has been reviewed by Sébédio *et al*., (2003). Absorbed CLA appears to be metabolised in the same way as linoleic acid, and is subject to either desaturation and chain elongation, or to β-oxidation in peroxisomes and mitochondria. In rats, β-oxidation is the prevailing metabolic pathway for CLA (Sergiel *et al*., 2001). When each isomer (*cis*-9, *trans*-11 & *trans*-10, *cis*-12) was administered separately to rats for 6 weeks (diet consisted of 6% lipids of which 1% was of either isomer), the *trans*-10, *cis*-12 isomer decreased the triacylglycerol content of the liver by 32% and increased the 18:0 content at the expense of the 18:1n-9, indicating an alteration of the Δ9 desaturase activity. This was not observed with the *cis*-9, *trans*-11isomer. The *trans*-10, *cis*-12 isomer also induced an increase in the C22 polyunsaturated fatty acids in the liver lipids. The *trans*-10,*cis*-12 isomer was found to be mainly metabolised into conjugated 16:2 and 18:2 fatty acids, whereas the *cis*-9, *trans*-11isomer was preferentially metabolised into a conjugated 20:3 isomer (Sébédio *et al*., 2001).

In normolipidaemic humans, CLA supplementation (3 g/day, for 8 weeks) as either a 50:50 or 80:20 isomeric blend of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, resulted in low or undetectable levels of the *trans*-10, *cis*-12 isomer, indicated poor incorporation into the plasma lipids compared with the *cis*-9, *trans*-11 isomer (Noone *et al.*, 2002). Belury (2002) similarly concluded that there appeared to be a more rapid metabolism of the *trans*-10, *cis*-12 isomer.

CLA can undergo Δ6 desaturation, elongation and further Δ5 desaturation while maintaining the conjugated diene (CD) double bond where metabolites similar to those of linoleic acid are produced (Banni, 2002). The *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers may thus be metabolised into conjugated isomers of 18:3, 20:3 and 20:4. The type and ratio of metabolites produced exhibit species specific differences and also appears to be influenced by the fat content of the diet.

3.4 Excretion

Less than 0.5% of a gavage dose of radio-labelled CLA was detected in the faeces of rats after 24 hours. About 1.3-2% was excreted in the urine with the majority (72%) in the form of $^{14}CO_2$ being excreted in expired air (Sergiel *et al*., 2001). The remaining CLA was deposited in the organs and carcass; however this is not necessarily CLA, but may be products of CLA, including products of 1-^{14}C-acetyl CoA, a breakdown product of 1-^{14}C-CLA

4. Hazard identification

4.1 Acute toxicity

No acute toxicity studies with CLA have been published or submitted with this Application. However, an unpublished review of the safety of CLA states that an acute toxicity study with CLA methyl ester (unknown purity) 'beadlets' found the LD_{50} in rats to be greater than 2 g/kg (Berven *et al*., 2002).

4.2 Repeat dose toxicity

4.2.1 Mice

(i) Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice (Tsuboyama-Kasaoka *et al*., 2000).

Eight-week old female C57BL/6J mice were fed diets containing 1% CLA (equivalent to 1500 mg/kg bw per day of 34% *cis*-9, *trans*-11/*trans*-9, and *cis*-11; 36% *trans*-10 and *cis*-12; 3% *cis*-9, *cis*-11/*cis*-10, and *cis*-12; 2% *trans*-9, *trans*-11/*trans*-10, and *trans*-12) for up to eight months. The control diet contained 11% safflower oil. In the test diet, 25% of the safflower oil was replaced with CLA to keep the fat intake constant between the two groups.

No differences in body weight or energy intake were observed, although CLA-fed mice exhibited decreased fat mass, manifested as ablated BAT and reduced subcutaneous WAT. There was also little renal and retroperitoneal BAT and WAT in CLA-fed mice. The mean diameter of adipocytes in CLA-fed mice was 41% smaller than control mice. Further analysis indicated that in addition to decreased cell size, cell death also contributed to the observed BAT and WAT mass.

TNFα and uncoupling protein 2 (UCP2) mRNA expression increased 12- and 6-fold respectively in adipocytes after 11 days of CLA supplementation. TNFα induces apoptosis

of adipocytes and up-regulates UCP2. These mRNAs were also upregulated in nonadipocytes but to a lesser extent. Fatty acid synthase and acetyl-CoA carboxylase (enzymes involved in lipogenesis) were reduced in CLA supplemented animals after 5 months (88% and 72% respectively).

The liver was very enlarged in CLA-fed mice, with histological analysis revealing panlobular macrovesicular steatosis but no increased inflammation. No enlargement of kidneys, heart or skeletal muscle was noted.

Although there was no significant alteration in blood glucose concentration after oral glucose tolerance testing, insulin tolerance testing showed marked insulin resistance in CLA-fed mice. CLA supplementation also decreased fasting and feeding blood leptin levels by 49% and 79%, respectively and markedly down-regulated insulin-sensitive glucose transporter (GLUT4) mRNA in parametrial WAT. The levels of plasma leptin in CLA-fed mice could be returned to normal through leptin infusion. The livers of CLA-fed mice also showed massive vacuolisation due to fat deposition, however this was reversed partially by leptin infusion, which also had the effect of decreasing liver weight by 30%.

It was concluded that supplementation of 1 % CLA in female mice results in reduced adipose tissue by apoptosis, lipodystrophy and hyperinsulinemia. The hyperinsulinemia can be normalised/reversed by leptin administration.

Comment: The use of only one dose level in this study makes the interpretation of the results difficult as it is not possible to consider potential dose-response effects. It was speculated that the marked insulin resistance may be partly explained by leptin deficiency and a decrease of GLUT4 in adipocytes.

(ii) Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse (West *et al*., 1998).

Groups of male AKR/J mice[1] (8-10 per group) were fed a high fat (45 kcal%) or low fat (15 kcal%) diet with or without CLA (1.2 and 1.0 % by weight, equivalent to 1800 and 1500 mg/kg bw per day, in high and low fat diets, respectively) for 6 weeks. Control groups were given high or low fat diets without CLA supplementation. The CLA preparation contained *cis*-9,*trans*-11-CLA and *trans*-9, *cis*-11-CLA (39.1%), *trans*-10, *cis*-12-CLA (40.7%), and *cis*-9, *cis*-11-, *cis*-10, *cis*-12-, *trans*-9, *trans*-11-, *trans*-10, *trans*-12-, and *cis*-9, *cis*-12-CLA (6.1 % total) and remainder (14.1 %). Body weights and food intake were measured. At six weeks, mice were placed in metabolic chambers and 24 hour CO_2 production and O_2 consumption measured. After the end of six weeks, mice were killed, and liver, kidney, testes, spleen, fat depots and eviscerated carcasses weighed.

CLA significantly reduced growth rate and body weight in both diet groups compared to controls. CLA intake also significantly reduced energy intake independent of diet group. Most of the effect on energy intake occurred during the first three weeks of the study. Overall, CLA reduced total cumulative energy intake in the high fat diet group by about 14 % and in the low fat diet group by about 9.6 %.

CLA treatment had a significant effect on organ weights, increasing the weight of both liver and spleen independent of diet group and body weight. CLA treatment also significantly reduced adipose depot weights relative to controls for both diet groups, with CLA treatment having the largest effect on the retroperitoneal adipose depot, reducing it by 78.2 and

[1] The AKR/J strain of mice is sensitive to the development of obesity associated with adipocyte sensitivity to insulin and is hyporesponsive to diets containing high levels of fat and cholesterol, http://jaxmice.jax.org/strain/000648.html

87.7 % in the high and low-fat treatment groups, respectively. The effect was less marked on the epididymal adipose depot. CLA also reduced total carcass lipid and protein in both diet groups. CLA also significantly increased metabolic rate and decreased the night-time respiratory quotient (RQ).

Comment: In relation to the increased metabolic rate and decreased night-time RQ, it was speculated the CLA blocks normal diurnal RQ differences by promoting lipolysis during the night, providing more fatty acids for metabolism and thereby lowering the RQ.

(iii) Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake (DeLany *et al*., 1999)

In a study by DeLaney *et al.,* (1999), male AKR/J mice (12/group) were given 0 (control), 0.25, 0.5, 0.75 and 1.0 % CLA by weight (equivalent to 375, 750, 1125 and 1500 mg/kg bw/day) in a high fat diet (45 % of energy from fat) for 39 days. The isomer content of the CLA preparation was: 39.1% *cis*-9, *trans*-11-CLA; 40.7% *trans*-9,*cis*-11-CLA; and 1.8% *cis*-9,*cis*-11-CLA; 1.3% *cis*-10,*cis*-12-CLA; 1.9% *trans*-9,*trans*-11 and *trans*-10,*trans*-12-CLA; 1.1% *cis*-9,*cis*-12 linoleic acid; and 14.1% remainder.

As part of the same study, a time course was also undertaken where male AKR/J mice (40/group) were fed a high fat diet (% fat 45 kcal) containing either 1% CLA or no CLA (control). Eight animals from the control group and the test group were killed after 2, 4 6, 8 and 12 weeks of CLA feeding.

In the first study, a dose-dependant decrease in body weight gain and reduced body fat depot mass was observed. This was statistically significant in the two highest doses. No effect was seen on food consumption. Absolute liver weights increased in a dose dependant manner (4%, 11%, 13% and 28% for the CLA concentrations of 0.25, 0.5, 0.75 and 1%, respectively), as did absolute spleen weights (4%, 7%, 7% and 18% respectively). This was significant ($p < 0.001$) only at the highest dose levels for both organs.

Histopathological examination of the liver showed cytoplasmic vacuolation typical of lipid accumulation in the 1% CLA group. No treatment related effects were seen in the spleen. Fasting plasma leptin was increased non-significantly associated with CLA ingestion. Fasting plasma insulin concentrations were dose-dependently increased by up to 120%. This was statistically significant only in the 1% CLA group.

In the 12-week time course study, the main observations from the first dose-response study were also seen in the time-course study: reduced body weight gain, reduced body fat depot mass, increased liver weight, decreased lipid content and increased protein content in carcasses, and increased plasma insulin levels with time which became statistically significant by eight weeks.

Comment: A reduction in adipose mass is usually associated with improved insulin sensitivity and decreased plasma insulin levels, therefore the increased plasma insulin finding is paradoxical. It was noted that the AKR/J strain has been shown to have higher insulin levels and respond to a high fat diet with higher insulin levels than SWR/J mice[2], therefore the observed effect would need to be verified in other strains. In relation to effects on insulin levels, it has been noted that mice react in a species-specific manner to treatment with CLA (Pariza, 2004) and also appear to be the most sensitive and responsive species in terms of the effects of CLA on lipid metabolism (Park et al., 2002)

[2] A general purpose mouse strain.

It is possible that the reductions in insulin sensitivity that have been observed in mice and occasionally in rats (see Section 3.2.2 below) are due to high fat content of the diets, rather than to CLA.

(iv) Conjugated linoleic acid modulates hepatic lipid composition in mice (Belury and Kempa-Steczko, 1997).

In a published study, groups of 12 female SENCAR mice[3] were fed diets containing 0, 0.5%, 1% or 1.5% CLA for six weeks (equivalent to 0, 750, 1500 and 2250 mg/kg bw per day). The CLA contained 43% *cis*-9, *trans*-11- and *trans*-9, *cis*-11-CLA, 45% *trans*-10, *cis*-12-CLA, and 6% *cis*-9, *cis*-11-, *cis*-10, *cis*-12-, *trans*-9, *trans*-11-, *trans*-10,*trans*-12-CLA, 2% linoleate and 4% unidentified compounds. Mice fed CLA had lower body weights and increased lipids in the liver compared with the control mice. Food intake was similar between the groups. Analysis of lipids in the liver revealed CLA was incorporated into neutral and phospholipids at the expense of linoleate.

4.2.2 Rats

(i) Lymphatic recovery, tissue distribution, and metabolic effects of conjugated linoleic acids in rats (Sugano *et al*., 1997).

Groups of male Sprague Dawley rats (5-6/group) were fed diets containing either 1% CLA or 1% linoleic acid (equivalent to approximately 500 mg/kg bw per day) for 2 weeks.

No differences in food intake and weight gain were observed between the CLA and linoleic acid fed groups. The weights of the liver, heart, lungs, kidneys, spleen, brain and perirenal adipose tissue were also comparable between the two groups.

No statistically significant differences were observed in serum concentrations of total and HDL-cholesterol, triacylglycerol, phospholipid and liver lipids, with the values all being within the ranges reported for rats fed cholesterol-free diets.

(ii) Conjugated linoleic acid modulates tissue levels of chemical mediators and immunoglobulins in rats (Sugano *et al*., 1998).

In a study designed to assess the immune effects of CLA, no difference in food intake and body weight gain was observed in four week-old rats given 0, 0.5 or 1% CLA (equivalent to 0, 250 or 500 mg/kg bw per day) in the diet for three weeks. Linoleic acid was used as a control and to give the same fat intake in each group. There was a tendency to increased liver weight and decreased peritoneal adipose tissue (both significant in the high dose group).

(iii) A sub-chronic 90-day oral rat toxicity study and in vitro genotoxicity studies with a conjugated linoleic acid product (O'Hagan and Menzel, 2003).

Groups of Wistar outbred [Crl:(WI)WU BR] rats (20/sex/group) were fed a modified AIN-93G diet containing different dietary levels of Clarinol™ G80[4], as shown in Table 1, for 90 days.

[3] The acronym SENCAR is derived from SENsitivity to CARcinogenesis. SENCAR mice are commonly used for studies of susceptibility and resistance to the induction of skin tumours. http://jaxmice.jax.org/strain/002748.html

[4] Clarinol™ G80 contains the 9-*cis*, 11-*trans* and 10-*trans*, 12-*cis* CLA isomers in approximately equal proportions, accounting for approximately 75% of the total fatty acid content of the product. Minor amounts of other CLA isomers are also present, accounting for approximately 4.4% of the total fatty acid content.

Standard rat diet (AIN-93G, which contained 7% w/w fat from safflower oil) was used for the low fat control group.

Table 1: Level of safflower oil and Clarinol™ G80 in the diet

Group	Dietary level of safflower oil[5] (% w/w)	Dietary level of Clarinol™ G80 (% w/w)[6]	Number of rats (male/female)	
			Main groups	Recovery groups
Low fat – control	7	0	20/20	10/10
High fat – control	15	0	20/20	10/10
High fat – low dose	14	1	20/20	-
High fat – mid dose	10	5	20/20	-
High fat – high dose	0	15	20/20	10/10

The study also included three recovery groups of 10 rats/sex in the two control groups and the high dose group. These animals were maintained on the study for four weeks at the end of the 90 day period. The control animals continued with the same diet while the high dose animals were changed to the high fat control diet. The high fat diets (test substance and control) had their nutrient levels increased by 10% to take into account the fact that rats will consume food according to their caloric need (i.e. dietary intake of the high fat diets was expected to be less than the control low fat diet).

Food consumption was significantly lower in high dose animals of both sexes compared to the control animals during days 7-14 of the study; this was attributed to decreased palatability of the test diet. Decreased food consumption resulted in significant reduction in body weight in high dose male and female rats at day 7 and high dose females at day 14. This delay in growth in the first weeks of the study resulted in consistently lower body weights in the high dose animals. In week 12, water consumption was significantly lower in the high dose group than either control group (20% and 24% reduction in males and females respectively).

No changes were observed in haematological parameters considered to be an adverse effect related to treatment. Enzyme markers of liver cell damage, ALP and ALT were significantly increased throughout the study in high dose males but were not accompanied by histopathological changes in the liver. Sorbitol dehydrogenase and 5'-nucleotidase (5'-ND) were unaffected in this group. Relative and absolute liver weights were increased in the high dose, and relative liver weight in the mid-dose group males, but these effects disappeared at the end of the recovery period. No fat accumulation was observed in the liver. ALP, ALT and AST were significantly increased in high dose females. Both ALT and AST remained elevated but only in comparison to the low fat control group. SD was also significantly increased in high dose females in week 13. Changes of 5'-ND occurred sporadically in the female dose groups but showed no consistent dose-response relationship. Relative liver weights in high dose females were statistically significantly increased even after the recover period, however no histopathological change other than hepatocellular hypertrophy was apparent, nor was any fat accumulation observed in the liver. In the mid dose group, liver weights were increased at the end of the treatment period, however the increase was not evident at the end of the recovery period, and was not

[5] Safflower oil contains very low levels of CLA isomers, 0.7 mg CLA/g fat

[6] Actual intakes in mg/kg bw/day were calculated at the end of the study. The 5% Clarinol™ G80 in diet is equivalent to 2433 and 2728 mg Clarinol™ G80 /kg body weight per day in male and female rats, respectively. As Clarinol™ G80 contains approximately 80% CLA, this is equivalent to 1946 and 2182 mg CLA/kg body weight per day.

accompanied by changes in clinical chemistry or histopathology and was therefore not considered an adverse treatment related effect.

Plasma cholesterol levels were significantly decreased in high dose males throughout the study. An increase in cholesterol levels was seen in mid dose females throughout the study, but not in the high or low dose groups. Plasma triglyceride levels were increased in high dose females compared with both control groups. In males, plasma triglyceride levels in all treatment groups and the high fat control group were lower than those in the low fat control group. No significant differences in cholesterol or plasma triglycerides were seen following the recovery period.

Blood glucose was significantly decreased in high dose male rats in week 13 compared with both controls. At the end of the recovery period, plasma glucose levels were still decreased in high dose male rats compared to the low fat, but not high fat, controls. Plasma insulin levels were significantly increased in high dose males in weeks 4 and 8 but not in week 13. Insulin levels were increased in high dose females in weeks 8 and 13 compared to both control groups. The effect on insulin levels had reversed by the end of the recovery period.

No treatment related changes in renal function (urinary volume or density) were observed. An increase in urinary crystals in high dose males was observed.

The relative organ weights of kidney and spleen were significantly increased in the high dose animals of both sexes when compared to both the low fat and high fat control diets. In males, absolute spleen weights were also significantly increased. High dose females also had increased relative pancreas weights. In high dose females, the increases in relative organ weights were still evident, albeit reduced, at the end of the recovery period, as was the increase in absolute spleen weight in males. Relative adrenal weight was increased in high dose males, whereas absolute adrenal weight was significantly lower in high dose females.

The effects on adrenal weight were not evident at the end of the recovery period. Data on organ weights are presented in Table 2.

Brown fat was assessed *in situ* at necropsy. Significantly less brown fat was present in mid and high dose males, and females of all treatment groups when compared with both control groups. After the recovery period, males and females in the high dose group had reduced brown fat compared to both control groups.

It was concluded on the basis of effects on the liver in the high dose group, that the NOAEL in this study was 5% Clarinol^{TM} G80 in diet (the mid dose group). Liver hypertrophy and increased plasma insulin levels are the key effects on which the NOAEL is based, with liver hypertrophy considered an adaptive effect that is reversible and not considered adverse, and increases in insulin levels transient in male rats, with no adverse effect on blood glucose or in the pancreas in either sex. The 5% Clarinol^{TM} G80 in diet is equivalent to 2433 and 2728 mg Clarinol^{TM} G80 /kg body weight per day in male and female rats, respectively. As Clarinol^{TM} G80 contains approximately 80% CLA, this is equivalent to a NOAEL of 1946 and 2182 mg CLA/kg body weight per day.

Table 2. Mean organ weights following 90 day CLA treatment and 4 weeks recovery (from (O'Hagan and Menzel, 2003), Table 6)

		End of treatment period					End of recovery period		
		LF control	**HF control**	**1%**	**5%**	**15%**	**LF control**	**HF control**	**15%**
Males									
Pancreas	g	1.23	1.16	1.21	1.23	1.21	1.05	1.23	1.22
	g/kg bw	3.09	2.96	3.00	3.02	3.27	2.64	3.01	2.97
Adrenals	g	0.053	0.051	0.051	0.054	0.056	0.047	0.051	0.050
	g/kg bw	0.133	0.131	0.128	0.134	0.150*@@	0.118	0.122	0.122
Kidneys	g	2.36	2.34	2.42	2.45	2.47	2.25	2.36	2.45
	g/kg bw	5.95	5.95	6.04	6.02	6.65**@@	5.63	5.71	5.92
Spleen	g	0.602	0.584	0.612	0.621	0.683**@@	0.610	0.629	0.693*
	g/kg bw	1.52	1.48	1.52	1.53	1.84**@@	1.52	1.53	1.67
Liver	g	9.58	9.44	9.76	10.28@	10.59**@@	9.05	9.51	9.70
	g/kg bw	24.1	23.9	24.3	25.2*@@	28.5**@@	22.6	23.0	23.3
Females									
Pancreas	g	0.74	0.80	0.70	0.69	0.91	0.76	0.91	1.85
	g/kg bw	3.45	3.81	3.12	3.12	4.37*	3.22	3.76	4.06*
Adrenals	g	0.066	0.064	0.067	0.066	0.058*	0.055	0.059	0.054
	g/kg bw	0.309	0.307	0.298	0.300	0.279	0.230	0.243	0.258
Kidneys	g	1.43	1.36	1.48	1.52	1.57	1.50	1.57	1.51
	g/kg bw	6.70	6.46	6.53	6.87	7.44**@@	6.28	6.43	7.16**@
Spleen	g	0.386	0.376	0.391	1.399	0.418	0.396	0.418	1.392
	g/kg bw	1.81	1.79	1.73	1.80	1.98*@@	1.65	1.71	1.86**
Liver	g	4.88	4.78	5.29	5.45	7.27	4.90	5.25	5.05
	g/kg bw	22.9	22.7	23.3	24.6@	34.5**@@	20.4	21.5	24.0**@@

* P < 0.05 ** P < 0.01 versus LF-control, @ P < 0.05 @@ P < 0.01 versus HF-control

(iv) CLA-methylester-beadlets and CLA-ethylester-beadlets. Subchronic oral toxicity study in Wistar rats. Administration in the diet for 3 months. Unpublished study (Melert *et al*., 2002)

A 90-day study in rats was conducted and the raw data submitted by the Applicant. The study was GLP compliant and met the requirements of OECD test guideline 408.

Groups of Wistar rats (10/sex/group) were administered 'CLA-methylester beadlets' (linoleic acid-methylester; 39% purity) in the diet at concentrations of 0, 0.5, 1.5 and 5% for 3 months (Table 3). Additional groups of 10 male and 10 female Wistar rats received either 'CLA-ethylester beadlets' (linoleic acid-ethylester; 40% purity) or 'CLA-placebo beadlets' at the 5% level only. The isomer composition of the CLA beadlets are not provided, nor is the composition of the beadlets.

Table 3: Doses used in test diets for 90 day study in rats

Test group	Dose
0	0
1	CLA-placebo beadlets 5.0% in diet[7]
2	CLA-methylester beadlets 0.5% in diet
3	CLA-methylester beadlets 1.5% in diet
4	CLA-methylester beadlets 5.0% in diet
5	CLA-ethylester beadlets 5.0% in diet

Food consumption and body weight were recorded weekly. Animals were examined daily for clinical signs or mortality. Detailed clinical examinations were conducted weekly. A functional observational battery, measurement of motor activity, clinical chemistry, haematology, and urinalysis measurements were carried out towards the end of the administration period. Ophthalmological examinations were carried out prior to the start and towards the end of the administration period. At termination of the study, all animals were assessed by gross pathology followed by histopathological examinations. Test group results were compared statistically with the control group (group 0).

No animal died during the study. No treatment related clinical signs were observed during the study. One female in group 4 (from day 91 onwards) and one female in group 5 (from day 42 onwards) had alopecia, however this was considered incidental.

Food consumption was significantly decreased in group 5 males on days 56-77, in group 4 females on day 70, and in group 5 females on day 21. Due to the isolated occurrence and the lack of a dose-response relationship, this was considered incidental.

Body weight was significantly increased in group 4 males on days 28 and 35. Body weight change was significantly increased in group 1 females on days 7 and 35, group 3 females on day 35 and group 5 females on day 35. These findings were isolated and not dose related and therefore were considered incidental. Similar isolated and non-dose related findings were seen in the food efficiency calculations, and were considered incidental.

In the functional observation battery and the motor activity measurements no substance related effects were observed.

Of the measured haematological and clinical chemistry parameters, platelets were significantly increased in females in groups 1, 2 and 3 ($p \leq 0.01$) and group 4 ($p \leq 0.05$).

[7] For males, 5% was calculated to be approximately 3400 mg/kg bw weight per day and for females approximately 4000 mg/kg bw per day. The beadlets were approximately 40% CLA (1360 and 1600 mg/kg bw per day for males and females respectively).

Similar changes were not seen in males. In females in groups 4 and 5 triglycerides were significantly increased ($p \leq 0.05$). These values were also increased compared to group 1, however no statistical analysis was performed. In males, triglycerides were increase non-significantly in group 4, however this was due to a high value obtained in a single animal (animal 50). These changes were not considered to be treatment-related. No significant differences in blood glucose or cholesterol levels were observed. No significant differences in levels of the enzyme markers ALP, ALT or AST were found.

In the high dose CLA-methylester group (group 4) absolute kidney weight was significantly increased in both male ($p \leq 0.05$) and female ($p \leq 0.01$) rats. Absolute kidney weights were also increased in females in the high dose CLA-ethylester group (group 5) ($p \leq 0.05$). In females absolute liver weights were increased in groups 4 and 5 ($p \leq 0.01$) and spleen weight in group 4 ($p \leq 0.05$).

Relative liver weights were increased in males in group 4 and in females in groups 4 and 5 ($p \leq 0.05$) (Table 4).

Table 4: Mean organ weights following 3-month CLA feeding in rats

	Group	**0**	**1**	**2**	**3**	**4**	**5**
Males							
Liver	g	8.302	8.711	8.18	8.685	9.602	8.578
	%	2.308	2.396	2.28	2.343	2.531*	2.388
Kidney	g	2.339	2.408	2.258	2.36	2.753*	2.378
	%	0.651	0.663	0.63	0.637	0.728	0.664
Spleen	g	0.583	0.546	0.556	0.604	0.617	0.609
	%	0.162	0.15	0.155	0.163	0.163	0.17
Females							
Liver	g	5.17	5.223	5.177	5.288	5.871**	5.978**
	%	2.494	2.478	2.443	2.507	2.72*	2.789*
Kidney	g	1.46	1.518	1.487	1.469	1.667**	1.574*
	%	0.704	0.72	0.701	0.694	0.772	0.733
Spleen	g	0.415	0.385	0.396	0.418	0.476*	0.437
	%	0.2	0.182	0.187	0.199	0.22	0.203

In the control animals (group 0), one male had a gross lesion of the thymus (focus) and another had a kidney cyst. In males in groups 2, 4 and 5 gross lesions observed included erosion/ulcer of the glandular stomach (one group 2 animal), focal constriction of the liver (one group 4 animal), granular surface of the kidney (one group 4 animal), thymus focus (two group 4 and five group 5 animals). One group 5 male had reduced testes and epididymides size. One female in test group 2 had pelvic dilation of the kidney, and one female in group 5 had erosion/ulcer of the glandular stomach. One female in each of groups 4 and 5 had sparse hair. With the exception of red foci in the thymus, which were believed to be agonal haemorrhages, all findings were single observations and considered to have developed spontaneously, with no relationship to treatment.

Histopathology findings correlated with the gross lesions. Only the erosion/ulcer of the glandular stomach in two animals lacked a microscopic correlate. There was no histopathological finding related to the increased organ weights in some of the animals in the high dose groups (4 and 5), therefore these were considered to be likely to be transient changes and of no toxicological significance. All microscopic findings were either single observations or recorded at low incidence, or they occurred in control animals only or at comparable incidence and severity in control and high dose groups. No treatment related microscopic findings were detected in any of the organs examined.

It was concluded that the highest doses tested represented the NOAEL for CLA-methyl ester and CLA-ethyl ester. For males this was approximately 3400 mg/kg body weight and for females, approximately 4000 mg/kg body weight. As the CLA compounds comprised only 40% of the 'beadlets', this is equivalent to approximately 1360 mg/kg bw for males and 1600 mg/kg for females.

Comment: The Applicant states that the test article was Tonalin CLA methyl and ethyl esters, produced using the same source and manufacturing processes as for Tonalin TG 80. As Tonalin CLA methyl and ethyl esters are hydrolysed during intestinal passage in the same way as triglycerides to form bioavailable Tonalin CLA free fatty acids, this study is also relevant for the safety assessment of Tonalin TG 80.

(v) Modulation of body fat and serum leptin levels of dietary conjugated linoleic acid in Sprague Dawley rats fed various fat-level diets (Yamasaki *et al*., 2003).

Groups of four-week old male Sprague-Dawley rats (5/group) were fed diets containing 4%, 7% and 10% fats with or without 1.5% CLA (equivalent to approximately 1500 mg/kg bw per day) for three weeks. CLA was replaced with safflower oil in the control groups. The two main isomers of CLA were present in approximately equal proportions in the CLA preparation. Small amounts of *cis*-9 *cis*-11-, *cis*-10 *cis*-12-, *trans*9 *trans*-11, and *trans*-10 *trans*-12-CLA were also present. In all CLA diets, the amount of CLA was the same (approximately 338 mg/day for each rat).

Body weight and food intakes were measured. GOT and GPT activities were measured as indices of hepatic injury. Serum leptin and TNFα were also measured.

No differences in final bodyweight were observed between test and control animals at each dietary fat level. There was no significant difference in the weights of the heart, lung, spleen, kidney, and brown adipose tissue across all dietary groups. Food efficiency was increased with the increase of dietary fat level, but was not affected by CLA. Liver weights of CLA fed animals were higher than controls in the 4% and 7% dietary fat groups (difference not significant), but not in the 10% fat group.

Perirenal white adipose tissue (PWAT) and epididymal white adipose tissue (EWAT) weights were decreased in the CLA groups. In the 4% group PWAT and EWAT were 64.1% and 70.4% of the controls, respectively. In the 7% and 10% fat groups PWAT was 75.3% and 74.5% of the controls, respectively. The difference between test and control groups decreased with increased dietary fat levels. This was also the case for EWAT.

Serum GOT activity was non-detected in the 7% control group, but was detected in the other dietary groups. GOT was significantly increased in CLA groups when assessed using two-way analysis of variance but not pairwise comparison. However, CLA feeding did not affect serum GPT activity in any dietary group.

Serum leptin was reduced significantly in the CLA groups compared to the control groups. Dietary CLA reduced TNF- α significantly. Compared to the controls the 4%, 7% and 10% fat groups had 31%, 16% and 44% of the levels of TNFα.

(vi) Effects of conjugated linoleic acid on long term feeding in Fisher 344 rats (Park *et al*., 2005).

Male Fisher 344 rats were given control diet (10 rats) or diet containing 1% CLA (11 rats, equivalent to 1000 mg/kg bw per day) composed of 88.7% CLA; 41.9% *cis*-9, *trans*-11, 43.5% *trans*-10, *cis*-12, 1.5% *trans*-9, *trans*-11/*trans*-10,*trans*-12, 1.8% other CLA isomers for up to18 months.

Body weight gain, food consumption, behaviour and general signs of toxicity were recorded for all test animals during the treatment period. After 12 weeks, 3 animals selected randomly from each group were killed and body fat measurements made. Water consumption was measured and urine samples taken at 70 weeks. At 72 weeks, blood samples were taken and clinical chemistry and haematological analyses performed. At termination of the study (81 weeks) all surviving animals were killed for gross and microscopic examination.

One control animal died at week 69, due to pituitary tumour and chronic renal disease. Three rats from each group were killed prior to the end of the study due to lethargy, anorexia and/or weight loss greater than 25% of the animal's body weight. All these animals had severe chronic renal disease. Three animals of each group also had pituitary or testicular tumours.

Of the animals that were killed after 12 weeks for body fat measurements, the CLA fed animals had slightly less body fat compared to controls, however this was not statistically significant.

Food intake was significantly lower in the test group; body weights were slightly lower although not statistically significant.

CLA feeding significantly reduced blood glucose concentration in both fasted and fed animals (n=4-6 for control and n=7-8 for CLA). Mean corpuscular volume was increased in CLA fed animals. However, this was not considered to be of concern as it was small and there was no difference in haematocrit.

Necropsy and histopathological examinations indicated no differences in tissue weights (n=3 for control and n=4 for CLA). All animals had chronic renal disease. Pituitary tumours, testicular tumours/masses and/or prostatitis were observed in both groups. One CLA fed animal had an enlarged spleen, which was diagnosed as granular cell lymphoma. Two animals from each group had early stage granular cell lymphoma. Other disorders observed in one animal from each group included gastritis, cardiomyopathy, focal hepatopathy and interstitial pneumonitis.

It was suggested that the renal disease observed in all animals may have been due to the high protein content of the diet compared to conventional rat chow (14-16%). It was concluded that, under the conditions of the study, CLA did not cause any adverse effects.

(vii) Toxicological evaluation of dietary conjugated linoleic acid in male Fisher 344 rats (Scimeca, 1998).

Groups of male rats (20/group) received either a basal diet, or a diet supplemented with 1.5% non-esterified CLA (equivalent to 1500 mg/kg bw per day, comprised of 90% CLA, with *cis*9, *trans*11-, *trans*9, *cis*11- and *trans*10, *cis*12 isomers accounting for 85% of the total) for 36 weeks. This study was part of a larger study of CLA chemoprevention in a chemically induced model of intestinal tumourgenesis, therefore the control group of rats received weekly subcutaneous saline injections from week 2 through to week 4. The authors stated this procedure had no bearing on the use of the animals as controls for this study.

Food disappearance and body weights were determined weekly throughout the course of the study along with physical examinations. Daily observations were made for clinical signs and/or mortality. Animals surviving to study termination were killed for necropsy and microscopic examination.

No differences were observed in mean body weight gain and food consumption between the groups. Organ weights (relative and absolute) were not different between groups except for the adrenals (increased in the test group) and thymus (decreased in test group), however no histomorphological alterations were seen to explain these differences.

Haematological analysis revealed no significant differences between groups. Other standard tests such as urinalysis, ophthalmological examination and clinical chemistry were not performed. Mean intake of CLA ranged from 1970 mg/kg bw per day to 467 mg/kg bw per day over the course of the study (study week 1 to 36).

(viii) Mammary cancer prevention by conjugated dienoic derivative of linoleic acid (Ip *et al*., 1991).

Groups of female rats (30/group) were fed a diet supplemented with 0, 0.5, 1 and 1.5% (2 groups) by weight of CLA (equivalent to 0, 500, 1000, 1500 mg/kg bw per day) for 26 weeks. The basal diet, which contained no added CLA, included 5% corn oil which naturally contains about 0.2 mg CLA/g fat. This amount of CLA is negligible compared to the amount of CLA added to the test diets. Two weeks after commencement of the study, each group received an oral intubation of 10 mg of 7,12-dimethylbenz(*a*)anthracene (DMBA) in corn oil for the induction of mammary tumours. The second group of rats receiving 1.5% CLA in the diet was administered corn oil without any DMBA, and thus served as the negative control.

The total number of mammary adenocarcinomas in the 0.5, 1.0 and 1.5% CLA groups was reduced by 32, 56 and 60%, respectively. The final tumour incidence and cumulative tumour weight were similarly diminished in rats fed the CLA-containing diets. In general, there appeared to be a dose-dependent level of protection at levels of CLA of 1% and below, but no further beneficial effect being apparent at levels above 1.0%.

The feeding of CLA at levels up to 1.5% in the diet of rats for 26 weeks did not appear to be associated with any adverse effects: no affects on growth rate, organ weights (liver, spleen, kidney, uterus), or gross pathology and histopathological examination did not reveal any morphological abnormalities.

(ix) Increase in vitamin A status by the feeding of conjugated linoleic acid (Banni *et al*., 1999)

In a study in female Sprague Dawley rats fed CLA in the diet at 0, 0.5%, 1%, 1.5% or 2% (equivalent to 0, 500, 1000, 1500 or 2000 mg/kg bw per day) for one month, an increase in the amount of retinol in the liver was observed (Table 5). All diets contained a constant level of Vitamin A, as 8 mg retinyl palmitate/kg diet[8] (1mg retinyl palmitate = 1830 IU, EFSA UL 2006, so 8mg = 14,640). The increase was statistically significant in all groups, dose-related and reached a maximum in the 2% CLA group of about five-fold over the control group. Liver retinyl esters were also significantly increased, peaking at about two-fold over the control group between 0.5% and 1% CLA. The plasma retinol concentration was also

[8] Nutrient Requirements of Laboratory Animals, National Research Council (US) recommends 0.7 mg retinol/kg diet (equivalent to 2,300 IU/g (sic, this should be IU/kg), or 1.3 mg β-carotene/kg diet. http://books.google.com.au/books?id=y0_4nhYwRfwC&pg=PA11&lpg=PA11&dq=lab+rat+diet&source=web&ots=84hK1Hq2-u&sig=XaBTuTZN7Xbzdh7ADr7_SlPW23E&hl=en#PPA13,M1
Kliba maintenance diet 14,000 IU/kg http://www.kliba-nafag.ch/neutral/download/3430.pdf vitamin fortified 18,000. breeding vitamin fortified 24,000 IU/kg http://www.kliba-nafag.ch/neutral/download/3804.pdf
Specialty feeds retinol 10,950 IU/kg http://www.specialtyfeeds.com/sp-frameset.html?standiets/labanimals.html

significantly increased in all CLA groups, although no dose-response was evident. Retinyl esters were not detected in plasma.

The mechanism by which CLA might cause this increase is not known, although it was speculated that, as CLA is known to activate peroxisome proliferator-activated receptor-α (which has also been shown to be involved in retinol binding protein expression in intestinal cells), CLA may enhance the level of cellular retinol-binding protein in intestinal cells and thus the sequestration of retinol from the lumen.

Table 5: Vitamin A Content in Livers and Plasma of Rats Fed Increasing Levels of CLAa (Banni et al., 1999)

Dietary CLA %	**Liver Retinol[b] µg/g tissue**	**Liver Retinyl esters[c] µg/g tissue**	**Liver Retinyl esters/ retinol**	**Plasma Retinol[d] µg/ml**
Control (0%)	1.9 ± 0.11	47.2 ± 3.4	23.9	0.23 ± 0.02
0.5	3.3 ± 0.19	70.3 ± 5.9	21.2	0.33 ± 0.02
1.0	5.7 ± 0.50	84.1 ± 6.8	14.6	0.36 ± 0.02
1.5	7.5 ± 0.94	91.3 ± 3.5	12.1	0.37 ± 0.07
2.0	10.7 ± 1.2	85.2 ± 5.0	7.9	0.40 ± 0.03

[a] Values are means ± SE; n = 6.
[b] Retinol concentrations showed a dose-dependent increase as a function of CLA intake ($p < 0.05$ by regression analysis)
[c] Retinyl ester concentrations in all CLA groups are significantly different from control ($p < 0.05$)
[d] Retinol concentrations in all CLA groups are significantly different from control ($p < 0.05$)

Comment: It is not clear from the published report what the fat content of the control diet is, or the exact form of CLA administered.

(x) Dietary conjugated linoleic acids alter serum IGF-I and IGF binding protein concentrations and reduce bone formation in rats fed (n-6) or (n-3) fatty acids (Li *et al*., 1999)

In a study to examine the effects of dietary CLA on serum concentrations of IGF-I and IGF binding proteins (IGFBP) and the relationship of these factors to bone metabolism, male weanling Sprague-Dawley rats were fed CLA in the diet at 0 or 1% (equivalent to 0 or 1000 mg/kg bw per day) for 42 days. A group at each dose received diets rich in (n-6) or (n-3) fatty acids (containing 70 g/kg of soybean oil or menhaden oil plus safflower oil, respectively). The ratio of CLA isomers was not stated.

The serum concentration of IGF-I was significantly lower in rats ingesting CLA (17% and 21% reduced in rats ingesting soybean oil and menhaden plus safflower oil respectively). CLA increased IGFBP level in rats fed soybean oil but reduced it in those fed menhaden plus safflower oil. Rats given CLA had significantly reduced rates of tibial mineral apposition and bone formation.

4.2.3 Pigs

(i) The pathology, haematology, and blood chemistry of pigs fed conjugated linoleic acid synthesized from sunflower oil. (Cook *et al*.,1998).

In a 98-day study in male pigs fed diets containing 0.48% or 0.95% of a CLA product (approximately 60% CLA, isomers not stated, equivalent to approximately 115 or 228 mg CLA/kg bw per day), no effects were seen on organ weight or histomorphology. Blood magnesium and albumin increased slightly. Total white cell count increased due to elevated lymphocyte numbers. These changes were not considered clinically significant.

(ii) Distribution of conjugated linoleic acid (CLA) isomers in tissue lipid classes of pigs fed a commercial CLA mixture determined by gas chromatography and silver ion-high-performance liquid chromatography (Kramer *et al*., 1998)

Two groups of eight pigs (sex not specified) were fed CLA or sunflower oil at 2% of the basal diet from 61.5 to 106 kg liver weight (duration of study not specified). The CLA was approximately 19% *cis*-11, *trans*-13-CLA, 26% *trans*-10, *cis*-12-CLA, 20% *cis*-9, *trans*-11-CLA and 16% *trans*-8, *cis*-10-CLA. No gross pathological changes were reported in this study.

(iii) Conjugated linoleic acid and vitamin A: a nutritional therapy for post-weaning multisystemic wasting syndrome. J. Bassagnaya-Rieri. 2001. Performing Laboratory: Nutritional Immunology & Molecular Nutrition Laboratory, Iowa State University. Project number NPB/IPPA # 01142. (Bassaganya-Riera, 2001).

A total of forty-eight pigs with an initial body weight of 4.3 – 5.1 kg were weaned at 14 days and placed into three dietary groups of 16 animals: (1) soybean oil-supplemented diet (control diet); (2) CLA-supplemented diet (1.33 g/100g diet containing 1.516 mg retinyl acetate/1000 g diet) (Diet 2); and CLA-supplemented diet with additional vitamin A at 10 fold recommended levels (1.33 g CLA/100 g diet containing 15.093 mg retinyl acetate/1000 g diet) (Diet 3). The CLA source was Clarinol^{TM} G80. Pigs were fed the experimental diets for 42 days and then each block of 16 animals was separated into two blocks of eight animals, with one group being challenged with type 2 porcine circovirus (PCV2). Pigs were killed on day 63 of the study.

The feeding of CLA at 1.33 % of the diet containing recommended levels of vitamin A (Diet 2) for 63 days was not associated with any significant increases in the concentration of liver retinyl esters, liver retinol or plasma retinol in the non-infected pigs. In contrast, Diet 3 (which contained 10 fold the amount of recommended vitamin A) was associated with significantly increased levels of liver retinyl esters and retinol but not plasma retinol.

Comment: Only the results for the non-infected pigs are reported in this summary. The results obtained with Diet 3 confirm that plasma retinol levels are under tight homeostatic control.

(iv) The effects of feeding conjugated linoleic acid on pig liver vitamin A and retinol binding protein mRNA (Dugan *et al*., 2008).

Groups of 10 barrows (castrated male pigs) were fed diets containing 0, 0.25 and 0.5% CLA (equivalent to 0, 100 or 200 mg/kg bw per day) in combination with canola oil to make up 2% of total oil. The CLA was made up of 33% *cis*-9, *trans*-11-CLA and 32% *trans*-10, *cis*-12-CLA. Diets were fed from 37 to 116 kg live weight (about 84 days), at which time pigs were slaughtered and liver samples removed and stored at -80 ºC for analysis. The effects on vitamin A levels are summarised in Table 6.

Table 6: Effects of feeding CLA on growth performance, liver weight, liver vitamin A status and relative level of liver retinol binding protein mRNAz

	Dietary CLA (%)			
	0%	**0.25%**	**0.5%**	**SE**
Days on feed (d)	83.4	83.5	84.2	0.76
Avg. daily gain (kg/day)	0.907	0.992	0.947	0.016
Liver weight (kg)	1.94	1.97	1.99	0.03
Retinol (µg/g)	1.56*a*	1.92*a*	2.56*b*	0.11
Retinyl-palmitate (µg/g)	599	628	589	21.6
Retinol to retinyl-palmitate ratio	0.0026*a*	0.0021*a*	0.0045*b*	0.0002
Relative Retinol Binding Protein mRNA	1.30*a*	ND	1.52*b*	0.04

[z] values are means, for each diet, n=10

a, b Means within row with different letters are significantly different (p<0.05).

Feeding pigs CLA at the 0.5% level for up to 84 days was associated with a significant increase in: retinol levels; the retinol to retinyl-palmitate ratio; and the relative level of retinol binding protein (RBP) mRNA in liver. The levels of retinyl-palmitate in the liver were not similarly affected. It was noted that vitamin A in pigs is mainly stored in the liver as retinyl palmitate, and because its level was unaltered, they speculate that feeding CLA is unlikely to change pig vitamin A requirements. However, as the active forms of vitamin A (retinol, retinal and retinoic acid) are derived from retinyl-palmitate via hydrolysis and progressive oxidation, they added that feeding CLA may induce some of its physiological effects by changing the balance between protein bound and esterified retinol. Plasma retinol levels were not determined.

4.2.4 Hamsters

(i) Effects of conjugated linoleic acid (CLA) on lipid levels and peroxisome proliferation in hamster (de Deckere *et al*., 1999).

Male F_1B hamsters were given CLA (single or mixed isomers) in the diet for eight weeks. Diets contained 30% energy as fat and 0.1 g cholesterol/kg. Four groups (32/group) received either a control diet or diets supplemented with either CLA mix (47.3% *cis*-9, *trans*-11, 47.5% *trans*-10, *cis*-12, and 5.2% other CLA isomers), a CLA preparation enriched with *cis*-9, *trans*-11 CLA (89.5%), or a CLA preparation enriched with *trans*-10, *cis*-12 CLA (76.7%). CLA made up 1.5% energy, or 6.6 g/kg diet.

A small but statistically significant (p<0.05%) reduction in food intake was observed in animals fed the CLA mixture or the *trans*-10, *cis*-12 CLA. The *trans*-10, *cis*-12 group had a 20% reduction in growth (statistically significant), but no effects on growth or body weight were seen in the other groups. The CLA mixture and *trans*-10, *cis*-12 CLA groups had significantly increased mean absolute hepatic weight (26% and 25% increased, respectively) and also absolute renal weight (increased by 6% and 7%, respectively). Increase hepatic weight was due to hypertrophy. No further histology was performed.

Markers of peroxisome proliferation (cyanide-insensitive palmitoyl CoA oxidase and carnityl acetyl transferase) were not increased by CLA treatment. No changes were observed in animals given diets containing *cis*-9, *trans*-11 CLA.

4.3 Reproductive toxicity

No studies on reproductive toxicity were available.

4.4 Developmental toxicity

(i) CLA-methylester-beadlets and CLA-ethylester-beadlets – prenatal developmental toxicity study in Sprague-Dawley rats, oral administration (gavage). Study author Jacques Richard. 18 February 2002. Performing laboratory CIT BP 563-27005 Evreux France. BASF project number 30R0746/009048. (Richard J., 2002)

Six groups of pregnant Sprague Dawley rats (25/group) received the test substance by gavage in 'beadlets' from day 6 to day 19 post coitum inclusive. Animals received vehicle only (0.5% carboxymethylcellulose), CLA-placebo beadlets at 1,000 mg/kg/day, CLA-methylester beadlets at 100, 300 or 1000 mg/kg/day, or CLA-ethylester beadlets at 1,000 mg/kg/day (See Table 7). Test 'beadlets' contained approximately 40% CLA.

The study was carried out according to the OECD test guideline No. 414 (Final 22 January 2001) and the USEPA Health effects Test Guidelines OPPTS 870.3700 (August 1998).

Table 7: Dose levels in developmental toxicity study in rats

Group number	Dose
0	0 (vehicle only - 0.5% carboxymethylcellulose in purified
1	CLA-placebo beadlets at 1,000 mg/kg/day
2	CLA-methylester beadlets 100 mg/kg/day
3	CLA-methylester beadlets 300 mg/kg/day
4	CLA-methylester beadlets 1,000 mg/kg/day
5	CLA-ethylester beadlets 1,000 mg/kg/day

Clinical signs and mortality were checked daily. Body weight and food consumption were recorded at designated intervals. Dams were killed on day 20 post coitum and subjected to a macroscopic examination. Foetuses were removed and the following parameters recorded: weight of gravid uterus; number of corpora lutea; implantation sites; early and late resorptions; and dead and live foetuses.

The foetuses were weighed, sexed and examined externally. Soft tissue was examined in half the foetuses and skeletal examination occurred in the other half.

Some statistically significant differences were seen between the control group and the test groups (including the placebo group). These were not considered to be related to the test substance as findings were seen in the placebo group, or only in the low dose groups and there was no dose-relationship.

It was concluded that there were no test-substance related effects on the parameters measured in this study. The NOEL for maternal and prenatal developmental toxicity was the highest dose tested, 1000 mg beadlets/kg bw per day. This is equivalent to approximately 400 mg CLA/kg bw per day.

(ii) Conjugated linoleic acid is a growth factor for rats as shown by enhanced weight gain and improved feed efficiency (Chin *et al*., 1994)

Two experiments were performed on pregnant Fisher rats. In the first experiment, 8-week old female rats were mated and from day one of gestation fed either a control diet or control diet supplemented with 0.5 g CLA/100 g diet (equivalent to approximately 500 mg/kg bw per day). On day 20, ten rats from each group were killed and liver, mammary gland, skeletal muscle and abdominal adipose tissues were removed. Foetuses were removed, weighed and visually examined for abnormalities. Remaining rats (11 in the control group and 13 in the CLA group) were allowed to deliver at term.

In the second experiment, 10-week old female Fisher rats were mated. The same diets were used as in experiment 1, with the addition of a third group, receiving 0.25 g CLA/100 g diet (equivalent to approximately 250 mg/kg bw per day). A fourth group received the control diet during gestation and the 0.5% CLA diet during lactation. Pups were weighed on day 10 and weaned on day 22 of lactation. Pups were maintained on the same diet as their mothers had received for 8 weeks (males) and 10 weeks (females). Body weights and food intakes were determined weekly.

In both experiments, milk was collected on day 10 of lactation and total protein, total milk lipid and net fat mass determined.

In experiment one, food intake and body weight of the dams was not affected by CLA consumption. Liver weights and mammary gland weights were also similar between groups. Litter sizes, foetal body weights at day 20 of gestation were unaffected by CLA. Foetal liver and brain weights were similar. No gross abnormalities were evident.

CLA was elevated in tissues of dams and foetuses in the 0.5% CLA group compared to the control group. CLA was present in milk from dams in the test group (mean value 46.7 µmol/g milk fat compared to 1.68 µmol/g in milk from control dams). Pups receiving CLA during gestation and lactation were significantly heavier than pups from control dams (statistically significant at $p<0.036$, increased by 11%).

In experiment two, similar results were seen. Pup development and survival were not affected by CLA treatment, however at weaning pups in the group fed 0.5% CLA were significantly heavier ($p<0.03$, increased by 9%) than controls and the group fed 0.25% CLA. Pups from dams fed 0.5% CLA during lactation only were intermediate in weight (increased by 5%, not statistically significant).

From weaning to week eight, rats in the 0.5% CLA group were consistently heavier than control and 0.25% CLA group animals. Food intake was similar, and feed efficiency was greater in the CLA groups (significant in the high dose group only).

It was concluded that this work indicates that CLA is not harmful to pups during gestation or lactation. The authors speculated that CLA might promote growth, possibly through modulation of the immune system.

(iii) Dietary conjugated linoleic acid consumption during pregnancy and lactation influences growth and tissue composition in weaned pigs (Bee, 2000).

Mated Swiss Large White sows were fed from the day of mating, throughout gestation and lactation, diets supplemented with either 2 g oil enriched with linoleic acid/100 g diet (n= 4) or a diet supplemented with 2 g of oil enriched with CLA/100 g diet (n=6). The enriched oil contained approximately 59% CLA, and composition of the CLA was approximately 35% each of c9, t11- and t10, c12-, 9% c9, c11- and 4.2% t9, t11/t10, t12. Total CLA in the supplemented diet was 23 g/100 g fatty acid.

After 35 days of rearing, piglets were assigned randomly to one of two groups and fed with either starter diet supplemented with 2% CLA or 2% linoleic acid. Thus there were four groups of piglets: from sows given linoleic acid and starter diets containing linoleic acid or CLA (LL n=8 and LC n=8), and from sows given CLA and starter diets containing linoleic acid or CLA (CL n=12 and CC n=12).

The body weight of each piglet was recorded at birth, weaning and 35 days of the starter period. At the end of the study, pigs were killed, organs removed and carcasses weighed and measured. Back fat and omental fat were collected for fatty acid analysis.

Body weights at birth were not different between the groups, however irrespective of the starter diets, piglets born to and reared on sows fed CLA had significantly greater total feed intake, daily weight gain, terminal body weight and warm carcass weight than pigs of the control group sows. Feed efficiency was similar.

Total lipid content in back and omental fat was unchanged between groups. However, higher deposition of saturated fatty acids and a lower deposition of monounsaturated and polyunsaturated fatty acids in the fat of piglets from sows fed CLA. This was consistent with the observed down-regulation of Δ9-desaturase that was induced by CLA rather than being due to an altered rates of de novo synthesis.

Glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME), two enzymes involved in supplying energy for reductive biosynthesis of fatty acids, were increased in fat tissues of pigs receiving CLA compared to those pigs receiving linoleic acid in the diet. This effect was greater in pigs from sows fed linoleic acid, and the author suggested this might be due to fat metabolism having already been altered in the piglets from sows fed CLA. Fatty acid synthase (FAS) was not altered by the type of fat in the diet.

4.5 Genotoxicity

(i) *Salmonella typhimurium* reverse mutation assay with C-SAT 030031 (Sokolowski *et al*., 2004).

Non-esterified Tonalin FFA 80 (approximately 80% CLA), referred to as C-SAT 030031, batch GR31143161, was assessed for mutagenic activity.

C-SAT 030031 was examined for mutagenic activity in five strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, TA1537). Experiments were performed with or without metabolic activation using liver S9 fraction from chemically pre-treated rats. The study design complied with OECD guideline 471 (adopted 1997) and the study was conducted under GLP guidelines.

A preliminary toxicity test was performed with strains TA98 and TA100 to select the concentrations of the test article to be used in the main assays. The study comprised of negative and positive controls with or without S9 metabolising system. Ethanol, the solvent used in the study, was employed as a negative control in the main study. Experiments for survival determination and estimation of mutant numbers were carried out in triplicates at each test point. Six doses of test substance were applied with 5 mg/plate as the highest dose level. Two experiments were conducted: experiment I – plate incorporation test; and experiment II – pre-incubation test (Table 8). The sensitivity of the individual bacterial strains was confirmed by significant increases in the number of revertant colonies induced by diagnostic mutagens.

In experiment II, the plates of strains TA1537, and TA102 without S9 mix could not be evaluated due to possible toxic effects. This experiment was repeated with lower concentrations of test substance.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with C-SAT 030031 at any concentration level, either in the absence or presence of S9 mix.

Table 8: Reverse mutation assay

Test material	Concentration	Test object	Result
Tonalin FFA 80	**Experiment I and II** (except for strains TA 1537 and TA 102 without S9 mix): 33, 100, 333, 1000, 2500, 5000μg/plate, with and without S9 mix **Experiment II** (repeated with strains TA 102 and TA 1537 without S9 mix): 0.3, 1, 3, 10, 33, 100, 250 and 500μg/plate	*S. typhimurium* TA98, TA100, TA102 TA1535, TA1537	-ve

Based on the results of these tests, it is concluded that Tonalin 80 is not mutagenic in these *Salmonella* strains.

(ii) A subchronic 90-day oral rat toxicity study and in vitro genotoxicity studies with a conjugated linoleic acid product (O'Hagan and Menzel, 2003)

A CLA preparation – Clarinol^{TM} G80, containing approximately 40% of each main CLA isomer – was tested in a bacterial mutation assay with five histidine requiring *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537 and TA 102). The assay was in accordance with OECD Guideline No. 471. Two separate experiments were performed. In the first, Clarinol^{TM} G80 was tested at concentrations ranging from 1.6 to 5000 µg/plate in all strains except for TA100, which due to an error was only tested up to a maximum dose of 3930 µg/plate. In the second, a preincubation step was included where bacteria were exposed to the test material in the presence or absence of S9 for a period of 1 hour before plating. The concentration range tested for this experiment was 51.2 to 5000 µg/plate.

Clarinol^{TM} G80 did not produce an increase in revertant numbers that was considered indicative of mutagenicity.

In a separate study, an in vitro chromosome aberration assay using human peripheral blood lymphocytes was performed. The assay was done in accordance with OECD Guidline No. 473. Two experiments were conducted. In the first, cells were exposed to Clarinol^{TM} G80 for 3 hours, in the presence or absence of S9, followed by a 17 hour recovery period prior to harvesting the cells. The dose levels tested were 128, 160 and 200 µg/ml. In the second, treatment in the absence of S9 was continuous for 20 hours, and treatment in the presence of S9 was for 3 hours followed by a 17 hour recovery period. The dose levels tested were 192, 240 and 300 µg/ml in the absence of S9 and 153, 240 and 300 µg/ml in the presence of S9.

Treatment of human peripheral lymphocytes with Clarinol^{TM} G80 resulted in frequencies of cells with chromosomal aberrations that were similar to those in the concurrent negative control. It was therefore concluded that Clarinol^{TM} G80 did not induce chromosomal aberrations at the concentrations tested.

(iii) Cell mutation assay at the thymidine kinase locus ($TK^{+/-}$) in mouse lymphoma L5178Y cells with C-SAT 030031 (Poth, 2004)

Tonalin FFA 80 (C-SAT 030031, batch number GR31143161) was assessed for its ability to cause mutations in mammalian cells *in vitro*. The assay was done in accordance with OECD Guideline No. 476.

This test aims to determine if a test substance induces mutations in mouse lymphoma L5178Y cells at the thymidine kinase locus; cells with the loss of a functional thymidine kinase enzyme are able to grow in the presence of triflurothymidine. In unchanged cells,

triflurothymidine is converted to its cytostatic and cytotoxic trifluorothymidine-monophosphate derivative, and cell death occurs. Two experiments were conducted, using two parallel cultures each. The first experiment used a treatment period of 4 hours, with and without liver microsomal activation. The second experiment used a treatment period of 24 hours without metabolic activation.

Reference mutagens were run in parallel to establish the sensitivity of the test system. Concentrations of C-SAT 030031 are shown below in Table 9.

Table 9: Concentrations of CLA (C-SAT 030031) in cell mutation assay

	Concentrations in μg/mL							
	Experiment 1							
With S9 mix	**1.25**	2.5	5	10	15	20	25	30
Without S9 mix	**1.25**	**2.5**	5	10	15	20	**25**	**30**
	Experiment 2							
Without S9 mix	10.0	20.0	40.0	60.0	80.0	**100.0**		

Those cultures of concentrations printed in bold were discontinued at day four. In experiment 1 without metabolic activation at 25 and 30 μg/mL and in experiment 2 at 100 μg/L severe toxic effects were seen, hence these doses were discontinued. As only four concentrations are required by the OECD Guideline, the lower doses in experiment 1 were also discontinued.

No precipitation of the test item was observed at any of the doses used. In experiment 1 without S9 mix, no increase in mutant colony numbers was observed up to the highest investigated dose (20.0 μg/mL). In the presence of S9 mix, an isolated and moderate increase was observed in the frequency of mutant colonies reaching the threshold (twice the number of mutant colonies) at 20.0 μg/mL, however this exceeded the historical data range by only 3 colonies (186 versus 189 mutant colonies per 10^6 cells), and was not considered biologically relevant.

In experiment 2 (without metabolic activation) in one of the two parallel cultures, the mutant colony numbers obtained at 10, 20, 40 and 60 μg/mL exceeded the historical controls. However the threshold of twice the frequency of mutant colonies was only exceeded in the second culture at 60 μg/mL. Since no increase was found in the parallel culture, the observed effects were considered not to be biologically relevant.

Under the conditions of the study, it was concluded that C-SAT 030031 did not induce mutations in mouse lymphoma cells.

(iv) Cytogenic study in vivo with linoleic acid-methylester in the mouse micronucleus test after two intraperitoneal administrations (Engelhardt and Hoffman, 2001).

Linoleic acid-methylester (test substance number 00/0526-1, batch number CP 256-2000-02) was tested for clastogenicity and for the ability to induce spindle poison effects (aneugenic activity) in NMRI mice using the micronucleus test method. The test substance was described as a yellow-amber oil. The Applicant states that this is Tonalin CLA methyl ester. The study was conducted in accordance with OECD Guideline No. 474.

The test article was administered twice intraperitoneally, with a 24 hour interval between administrations to male mice (5/group) at dose levels of 500, 1000, and 2000 mg/kg body weight. Olive oil (the vehicle for the test substance) was used as a negative control. Cyclophosphamide and vincristine were used as positive controls for clastogenic effects and aneugenic activity respectively.

Twenty-four hours after the second administration of the test substance, animals were killed and bone marrow from the femora prepared. After staining, 2000 polychromatic erythrocytes were evaluated per animal and investigated for micronuclei. The normocytes with and without micronuclei occurring per 2000 polychromatic erythrocytes were also registered.

Animals given the negative and positive control substances showed no clinical sign of toxicity. Animals given the test substance showed toxic effects (piloerection and squatting posture) at the highest dose level.

No inhibition of erythropoiesis, determined from the ratio of polychromatic to normochromatic erythrocytes, was detected.

Under the conditions of the study, the test substance does not have any clastogenic effect and there was no indication of impairment of chromosome distribution in the course of mitosis in bone marrow cells *in vivo*.

Comment: Although this study uses CLA methyl ester, it is relevant to the safety of Tonalin TG80 because CLA methyl ester is hydrolysed during intestinal passage in the same way as triglycerides to form bioavailable free CLA fatty acids.

4.6 Human studies

A number of studies with CLA have been undertaken using human volunteers. While the primary intent of these studies has been to study the potential benefits of CLA consumption, observations regarding tolerance to CLA, its effects on markers of liver function, inflammatory processes or lipid peroxidation, were often included. The human studies that are considered relevant with respect to the safety of CLA are summarised below.

(i) Conjugated linoleic acid supplementation for 1yr reduces body fat mass in healthy overweight humans (Gaullier *et al*., 2004)

Male and female volunteers (n=180) with body mass indices (in kg/m^2) of 25-30 were included in a double-blind, placebo-controlled study to determine the effect of consuming CLA (a 50:50 mixture of c9, t11 and t10, c12 isomers) for a period of one year on body composition. Subjects were randomly assigned to three groups: CLA-free fatty acid (FFA), CLA-triacylglycerol (TG), or placebo (olive oil) and received daily doses (via soft gel capsules) of either 4.5 g 80% CLA-FFA (3.6 g active CLA isomers, n=61), 4.5 g 76% CLA-TG (3.4 g active CLA isomers, n=60), or 4.5 g olive oil (n=59). No restrictions on lifestyle or caloric intake were imposed on the study subjects, although general dietary advice and exercise recommendations were provided (not specified by study authors).

Body weight, BMI, vital signs and the occurrence of any adverse events were recorded every 3 months, with any serious adverse events monitored continuously throughout the study. Body composition was analysed at 0, 6, 9 and 12 months. Blood samples were obtained from fasting subjects at 0, 3 and 12 months for clinical chemistry and haematology analyses. Compliance with the dosing regime was monitored every 3 months, with a subject being considered compliant if they took ≥75% of the supplement.

Mean body fat mass in both CLA groups was significantly reduced compared to the placebo control group. Subjects receiving CLA-FFA has significantly increased lean body mass compared to the placebo group. These changes were not associated with diet or exercise.

A high compliance and low dropout rate was reported (157 participants completed the study). Only 11.4% of the reported adverse events, which were mainly gastrointestinal in

nature (e.g., abdominal discomfort, loose stools, dyspepsia), were considered to be supplement related. A similar frequency of adverse events were reported for all three groups (placebo included), suggesting they relate to the daily digestion of oil, rather than CLA itself. The CLA supplement was at least as well tolerated as olive oil.

No effect on total cholesterol or TG concentrations were observed although the CLA-TG group had lower HDL concentrations and the CLA-FFA group had higher LDL than at the start of the study. These changes were however small and not significantly different from the placebo group. At the end of the study, both CLA groups had higher lipoprotein concentrations than at the beginning of the study and also in comparison to the placebo group. In addition, the CLA-FFA group had higher leukocytes and thrombocytes, whereas the CLA-TG group only had higher leukocytes.

Fasting serum glucose concentrations were not affected by CLA supplementation, with all subjects having fasting serum glucose concentrations within the normal range throughout the study. A slight increase in glycosylated haemoglobin concentrations was observed in all three groups.

Comment: Elevated lipoprotein concentration is thought to be a risk factor for cardiovascular disease. It was suggested that this combined with higher leukocyte and thrombocyte numbers suggest that CLA may promote an inflammatory response. However other studies have shown an anti-inflammatory role for CLA in animals as well as an enhancement of immune response in animals and humans.

(ii) Supplementation with conjugated linoleic acid for 24 months is well tolerated by and reduces body fat mass in healthy, overweight humans (Gaullier *et al*., 2005)

This study was a follow on study from Gaullier et al., (2004), in which 134 of the 157 participants completing the original study received 3.4 g CLA/day in the triglyceride form (unblinded) for a further 12 months. No placebo control was included in the study therefore only within-group comparisons were possible. The TG form of CLA was chosen because it represents the natural form of lipids. There was no gap in treatment between this present unblinded study and the original placebo controlled study. The study participants remained in their original groups: Group CLA-FFA consisted of 46 subjects previously supplemented with CLA-FFA; Group CLA-TG consisted of 47 subjects previously supplemented with CLA-TG; and Group placebo consisted of 41 subjects previously supplemented with olive oil. To record changes within each group during the 12-24 month period results were compared with month 0 as baseline for the CLA-FFA and CLA-TG groups and month 12 as baseline for the placebo group.

A total of 125 (93%) subjects completed the study, with withdrawal rates being similar across the three groups. Adverse events were reported at similar frequencies for all three groups, with only seven reports being considered treatment related, and all were rated as “mild”. Gastrointestinal complaints were the commonly reported treatment related adverse event.

No changes were observed for the two CLA groups from month 0 or the placebo group from month 12 in haemoglobin, bilirubin, chloride, creatinine phosphokinase, creatinine, erythrocytes, γ-glutamyl transferase, potassium, sodium, thyroid stimulating hormone, thyroxine, and IGF-1.

Circulating lipoproteins were significantly increased in all groups supplemented with CLA. There were also significant increases in leukocyte and thrombocyte counts, although the cell counts remained within the normal range. Serum AST increased significantly in the two CLA groups but not ALT. Plasma total cholesterol and LDL cholesterol were reduced slightly whereas HDL cholesterol and triglycerides were unchanged. Insulin levels were unchanged

in the CLA-FFA and placebo groups but were slightly increased in the CLA-TG group. There was no change in fasting blood glucose. Body weight and body fat mass were reduced in placebo group (i.e. those that had not received CLA during the initial 12 months). No fat or body weight changes occurred in the two groups given CLA during the initial 12 months.

Comment: The clinical relevance of the changes in lipoprotein levels is unclear given the absence of any effect on serum triglycerides and only very small changes to total, HDL and LDL cholesterol. While the observed increases in leukocyte and thrombocyte counts were within the normal range, they were consistent over time and suggest the presence of an inflammatory or immunological response to CLA supplementation. However the clinical relevance of the observed increases is unclear and raise the possibility that their increase may be related to the loss of body fat mass.

(iii) Safety profile of conjugated linoleic acid in a 12-month trial in obese humans (Whigham *et al*., 2004)

The safety of CLA over a 12 month period in obese humans was evaluated in a randomised, double-blind study consisting of three phases in which subjects were given 6 g/day of CLA (equivalent to 7.5 g/day of Clarinol™, composed of 37.3% *cis*-9, *trans*-11 and 37.6% *trans*-10, *cis*-12 isomers) or placebo (high oleic sunflower oil). There were three phases to the treatment protocol. Phase I consisted of a low calorie diet supplied as a liquid providing 13 kcal/kg bodyweight over 12 weeks or until they achieved a 10 – 20% reduction in body weight. Phase II was a weight maintenance/weight-regain phase lasting an additional 16 weeks in which subjects were prescribed a calorie intake of about 25-30 kcal/kg bodyweight. For the Phase I and Phase II, subjects and investigators were blinded to treatment group. Phase III was an open label study for 5 months to continue to assess long-term safety. Subjects were instructed to continue to follow the maintenance diet during this phase. All subjects were given 6 g CLA/day or placebo during the three different phases. Fifty subjects completed Phase I, 48 subjects completed Phase II and 46 subjects completed Phase III.

Subjects were scheduled for clinic visits every 2 weeks where vital signs, weight and body composition were recorded as well as an adverse event questionnaire being completed. Resting metabolic rate (RMR), respiratory quotient (RQ), and EKG were done at baseline and weeks 12, 28 and 52. Limited lab tests were done at weeks 2, 6, 16, 22, 36 and 44 and consisted of: (i) a standard chemistry panel of electrolytes, BUN, creatinine, liver function tests (ALT, AST and AP), serum total protein and albumin, uric acid and magnesium; (ii) a complete blood count consisting of haematocrit, haemoglobin, red blood cell indices, and differential; (iii) a serum lipid panel consisting of total cholesterol, triglycerides, HDL and a calculated LDL; and (iv) fasting serum glucose and insulin.

There were no significant differences between groups in insulin levels at any of the time points. CLA subjects had significantly higher serum glucose levels compared to placebo subjects at week 2 but differences were not significant at any of the other time points. There were no differences between groups throughout the study in insulin resistance.

AP levels were similar between groups through the study. Placebo subjects had significantly higher AST levels at week 12 and higher ALT levels at weeks 6 and 12 but not at any other time points.

At week 28, CLA subjects had significantly higher serum triglycerides and white blood cell (WBC) counts and lower HDL cholesterol. As the CLA subjects started with higher triglyceride and WBC levels at baseline, and the pattern of change with diet were approximately similar between CLA and placebo groups, these differences were not considered to be clinically meaningful. At week 52, CLA subjects had higher triglycerides and pulse. All other measures at weeks 28 and 52 were not significantly different.

When the data were compared as an analysis of change in measures between major time points, the CLA group had a significantly smaller increase in cholesterol levels from weeks 12 to 28. The placebo group had a significantly greater rise in HDL cholesterol from weeks 12 to 28 but then had a decrease from weeks 28 to 52, while the CLA group increased HDL cholesterol levels during that same time. All other changes between major time points were not significantly different. No adverse event was significantly greater in the CLA group compared to the placebo group, and in fact, adverse events were significantly lower in the CLA group compared to placebo group. No significant changes were observed in body weight or body fat between the CLA group and placebo.

It was concluded that CLA as Clarinol™ appears safe for use in obese humans for periods of up to one year.

Comment: It was noted that the results obtained for this study with humans are different to those obtained in previous animal studies, particularly in relation to insulin resistance and effects on the liver.

(iv) Conjugated linoleic acid supplementation for 1 yr does not prevent weight or body fat regain ((Larsen *et al*., 2006)).

One hundred and twenty-two obese healthy subjects with a body mass index >28 underwent an 8 week dietary run-in with energy restriction (3300 – 4200 kJ/day). Subjects who lost >8% of their initial body weight (n=101) were subsequently randomly assigned to a 1 year double-blind CLA (4.5 g/day TONALIN consisting of 3.4 g CLA/day consisting of 39% *cis*-9, *trans*-11 and 41% *trans*-10, *cis*-12 as triacylglycerols, n=51) or placebo (4.5 g/day olive oil, n=50) supplementation regime in combination with a modest hypocaloric diet of -1250 kJ/day.

Body weights and adverse events were recorded 14 times during the 52 week period. Physical measurements, blood samples, urine samples, ECGs, body scans, and measurements of blood pressure, waist circumference, hip circumference and pulse were taken at week -8, week 0, and after ~26 and 52 weeks of treatment. Fasting blood samples were also taken at each visit. Blood was analysed for: haemoglobin, erythrocytes, leukocytes, platelets, ALT, AST, γ-glutamyltransferase, creatinine, IGF-1, growth hormone, thyroid stimulating hormone, glucose, insulin, and total testosterone. An index of insulin resistance was calculated from fasting values for glucose and insulin. Urine was analysed for blood, glucose and protein content, as well as for pregnancy testing.

Energy intake was lower in both groups at week 25 and week 52 than before the low calorie diet, with no significant difference being observed between the two groups. During the 1 year of supplementation, both groups regained between 3 and 4 kg of weight, with no significant differences observed between the two groups.

No significant difference in the reporting of adverse events was observed between the two groups. In terms of blood variables, only the change in leukocyte count from week 0 to week 52 was significantly different between groups, with a greater increase in the CLA group than in the placebo group. This difference only became evident after week 25. Urine analyses and vital signs did not reveal any abnormal findings in either study group. CLA did not affect fasting values of plasma glucose and insulin and insulin resistance was also not affected. CLA treatment also did not affect any of the measured hormones.

Comment: In terms of the increased leukocyte count, similar results have been observed in other long term studies. While the actual increase is generally small, the finding may be of concern as it may be an indicator of inflammation. However it is difficult to determine

because only total leukocyte count was done, not differential counts. As the actual leukocyte count remains within reference values, the clinical relevance of this finding remains unclear.

(v) Effect of supplementation with conjugated linoleic acid on human serum lipids and body fat (Mougios *et al*., 2001)

In a double-blind study, 22 volunteers (14 males, 10 females) were divided into a study group and placebo group and received either 0.7 g CLA (49% *cis*-9, *trans*-11 and 51% *trans*-10, *cis*-12) for 4 weeks followed by 1.4 g CLA for a further 4 weeks or placebo (soybean oil). Blood samples were taken at baseline, immediately after the end of the low dosage (week 4) and immediately after the end of the high dosage (week 8) and were analysed for total triacylglyerols, total cholesterol, HDL cholesterol, creatine kinase and cortisol.

The only significant within-group change detected was a decrease in HDL cholesterol during the low CLA intake period, which was maintained after switching to the high dose of CLA. There was also a slight trend towards decreased TAGs and total cholesterol during the first 4 weeks. No significant differences were found within the placebo group, or between groups.

(vi) Conjugated linoleic acid reduces body fat mass in overweight and obese humans (Blankson *et al*., 2000).

A randomised double blind study was conducted using with 60 overweight or obese volunteers. Subjects were divided into 5 groups receiving placebo (olive oil), 1.7, 3.4, 5.1 or 6.8 g CLA per day (as Tonalin) for 12 weeks. Clinical assessment was done at week 0, 6 and 12 and blood sampling was done at week 0 and week 12. Measurements included body fat mass, lean body mass, body weight, blood pressure and heart rate. Blood variables included haemoglobin, erythrocytes, WBCs, platelets, serum creatinine, calcium, sodium, chloride, potassium, serum creatine phosphokinase, lactate dehydrogenase, ALT, AST, serum ferritin, γ-glutamyl transferase, bilirubin, glycosylated haemoglobin A, serum lipase (activity), triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol and lipoprotein (a). Adverse events were monitored throughout the study.

A total of 47 subjects completed the study. Eight subjects withdrew because of adverse events; however the rates did not differ between groups. The most frequent adverse events were gastrointestinal symptoms.

A significantly higher reduction in body fat mass was found in the CLA groups compared to the placebo group, the reduction in body fat within the groups being significant for the 3.4 g and 6.8 g CLA groups.

In all CLA-treated groups, significant reductions in blood lipids (total cholesterol, HDL cholesterol or LDL cholesterol) were observed, although no dose response was evident. There was also a significant increase in potassium, and a decrease in serum creatinine and bilirubin. A significant reduction in creatine phosphokinase was also observed in the 6.8 g CLA group. These changes were not considered to be clinically significant.

(vii) Safety of conjugated linoleic acid (CLA) in overweight or obese human volunteers (Berven *et al*., 2000)

A randomised, double blind placebo controlled study using 60 overweight or obese volunteers was undertaken to investigate the safety of CLA. Subjects were divided into two groups and received either 3.4 g CLA (Tonalin™) or placebo (4.5 g olive oil) daily for 12 weeks. Safety was evaluated by analysis of blood parameters (blood lipids, haematology and chemistry) and by clinical examinations at baseline and week 12. Vital signs and

adverse events were recorded at baseline, week 6 and week 12. Body composition measurements were also made.

Fifty-five subjects completed the study. No differences in blood parameters were observed between the two groups at baseline or study termination. In terms of within group changes, the CLA group exhibited a significant reduction in mean bilirubin level from baseline to week 12. In the absence of changes to other liver parameters, this reduction was not considered to be clinically relevant.

Blood lipids, haematological parameters and blood electrolytes remained unchanged during the study. Both groups exhibited a significant reduction in serum creatinine during the study, however no differences between the groups were observed and the serum creatinine levels were within reference values. These reductions were therefore not regarded as clinically significant.

No differences in clinical signs between groups were observed during the study. The most frequent adverse events recorded, which were all classed as either mild or moderate, were gastrointestinal in nature. The frequency of adverse events was similar between treatment groups.

(viii) Conjugated linoleic acid induces lipid peroxidation in men with abdominal obesity (Basu *et al*., 2000a)

A randomised double blind placebo controlled trial was undertaken to investigate the short term effect of CLA on lipid peroxidation in obese male subjects. A total of 24 subjects were enrolled in the study and were given either 4.2 g CLA (equal amounts of the *c*9, *t*11 and *t*10, *c*12 isomers) or placebo (olive oil) daily for 4 weeks. Urine and blood samples were taken at baseline and week 4, and at 2 and 4 weeks following cessation of CLA intake. Lipid peroxidation was assessed by the measurement of one of the major F_2-isoprostanes, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso- $PGF_{2\alpha}$) and 15-oxo-dihydro- $PGF_{2\alpha}$, a major metabolite of $PGF_{2\alpha}$, which are indicators of non enzymic (free radical induced) and enzymic (cyclo-oxygenase catalysed) arachidonic acid oxidation, respectively.

No significant effects were observed on blood pressure, blood lipids or glucose levels. Significant increases in urinary levels of 8-iso- $PGF_{2\alpha}$ (about 4 times the mean basal level) and 15-oxo-dihydro-$PGF_{2\alpha}$ (about 2 times the mean basal level) were observed in the CLA group. No such effects were observed in the placebo group. Levels of both decreased back to basal levels 2 weeks after treatment ceased and remained at the same level until the end of the study. CLA had no effect on serum α-tocopherol and γ-tocopherol levels or on urinary levels of 2,3-dinorthromboxane B_2.

(ix) Conjugated linoleic acid induces lipid peroxidation in humans (Basu *et al*., 2000b)

A randomised, double blind placebo controlled study was undertaken to investigate the effects of CLA on lipid peroxidation in healthy humans following supplementation for 3 months. A total of 53 adult male and female subjects were enrolled in the study and were given the placebo control (olive oil) daily for an initial 2 weeks and were then divided into two groups and received either 4.2 g CLA (equal amounts of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers) or placebo daily for an additional 12 weeks. Blood and urine samples were collected during the initial 2 weeks and again during the final week of the study and analysed for 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ (urine only). Blood plasma was also analysed for α- and γ-tocopherols. Malondialdehyde (MDA), another product of lipid peroxidation, was also measured in blood plasma as an additional parameter for comparison.

None of the study subjects reported experiencing any side effects during the course of the study. Ingestion of CLA for 3 months was associated with a significant increase in the urine levels of 8-iso-$PGF_{2\alpha}$(3 times basal level for the 24 hour urinary measures). No such increase was observed in the control group. A similar increase was also observed in the plasma 8-iso-$PGF_{2\alpha}$ levels. The urinary levels of 15-keto-dihydro-$PGF_{2\alpha}$ also increased significantly from basal levels (approximately double the basal levels) in the CLA group but not in the placebo group. The individual changes in the urinary levels of 15-keto-dihydro-$PGF_{2\alpha}$ were significantly correlated with those of urinary 8-iso-$PGF_{2\alpha}$. The magnitude of the increase of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ is not comparable to described inflammatory response-related or oxidative injury-induced formation of these compounds.

No significant changes were observed in the serum levels of MDA or α-tocopherol, whereas the levels of γ-tocopherol were significantly increased from the basal level (by 40%) in the CLA group.

Comment: An increase in markers of lipid peroxidation following CLA intake in humans has also been observed in a number of other human studies e.g. (Riserus *et al*., 2002; Smedman *et al*., 2004; Taylor *et al*., 2006; Tholstrup *et al*., 2008)[9]. The Applicant states that products of lipid peroxidation, such as the urinary isoprostanes, have been detected in most diseases involving inflammation and oxidative stress (e.g. cardiovascular disease). While agreeing that significant increases in isoprostanes following CLA supplementation have been observed, the Applicant states that when compared to the reported daily variation in isoprostanes, the reported increases in at least half of the studies are within the normal variance for isoprostanes in general. They also state there are serious flaws in the analysis and methodology of the Basu *et al* (2000a & 2000b) studies[10] – both of which showed the strongest increase in isoprostane levels of all human studies – which makes them unsuitable for safety evaluation. They also note that none of the human studies show a correlation of isoprostane plasma levels with other markers of oxidation or (pro)inflammation, which they claim further supports their view that the published data on isoprostanes should be interpreted with caution and in any case does not point to an increase in oxidative stress due to CLA supplementation.

In a recent article by Basu *et al*., (2006) discussing the role of inflammation in atherogenesis and the potential for dietary intervention to ameliorate the course of this disease, the authors noted some unfavourable outcomes with CLA and especially the *t*10, *c*12 isomer and concluded that: 'Human supplementation with CLA is thus not recommended until further information from studies on the mechanisms of CLA and specific CLA isomers at the molecular level are conducted.'

(x) Six months supplementation with conjugated linoleic acid induces regional-specific fat mass decreases in overweight and obese (Gaullier *et al*., 2007)

A total of 118 healthy overweight and obese adults were included in a randomised double blind placebo controlled trial to examine the effects of CLA on the localisation of body fat mass reduction and also to determine its safety. The subjects were randomised into two

[9] Studies not summarised but included in data package or supplementary data supplied by Applicant.
[10] (Basu *et al*., 2000a) & (Basu *et al*., 2000b) used radioimmunoassay (RIA) to analyse free isoprostanes only. The Applicant claims the RIA method used to be only semi-quantiative at best and also states that the majority of isoprostanes are esterfied in phospholipids rather than in free form, therefore it is necessary to measure both free and esterified levels to adequately address whether CLA increases their formation. The Applicant has also questioned the urine sampling methodology used by the authors, stating that urinary isoprostane output levels vary considerably between days, therefore isoprostanes should ideally be measured over several consecutive days. The Applicant has also pointed to inadequacies with the statistical analyses done by the authors.

groups of 59 individuals and received either 3.4 g CLA (4.5 g Clarinol™) or placebo (4.5 g olive oil) daily for 6 months. Weight, vital signs and BMI were recorded every 3 months. Fasting blood samples were taken at baseline and at 6 months. Adverse events were recorded every 3 months.

A total of 93 subjects completed the study. CLA significantly decreased body fat mass after month 3 and month 6 compared with placebo. CLA was well tolerated and only a very low percentage of adverse events were classed as being related to treatment. These adverse events were mostly gastrointestinal in nature. None of the blood lipids, blood markers of inflammation were affected except HDL cholesterol and CRP. A small reduction in HDL cholesterol and increase in CRP were observed within the CLA group, however both changes were within normal ranges. Lipoprotein (a) levels, cytokines (IL-6 and IL-8), TNFα and leukocytes were not altered in comparison to placebo. With the exception of a small increase in insulin c-peptide within the normal range, all indices of glucose metabolism including fasting glucose and insulin were reduced over the 6 months.

(xi) Effect of dietary supplementation with conjugated linoleic acid on markers of calcium and bone metabolism in healthy adult men (Doyle *et al*., 2005).

In a double-blind placebo-controlled randomised trial on the effect of CLA supplementation on biochemical markers of calcium and bone metabolism, 60 healthy adult men were given either 0 or 3 g CLA daily for 56 days. The CLA was Clarinol^{TM} (50:50 of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA).

Urine and blood samples were collected at 0 and 8 weeks and analysed for biomarkers of calcium and bone metabolism. There was no significant difference in serum calcium, 25-hydroxyvitamin-D, C-terminal telopeptides of type-1 collagen (CTX-1, a marker of bone resorption), osteocalcin and bone-specific alkaline phosphatase levels (markers of bone formation) or urinary calcium, creatinine, urinary type I collagen cross-linked N-telopeptide (NTx/Cr), acetylated pyridinoline(PYD)/Cr and PYD-deoxypyridinoline (DPD) /Cr (markers of bone resorption) between the study groups at week 0.

Serum CTX-1, calcium, 25()H)D osteocalcin and bone-specific alkaline phosphatase, and urinary calcium, Cr, NTx/Cr, PYD/Cr and DPD/Cr levels remained unchanged in both groups over the 8 weeks.

Baseline 25(OH)D levels showed that none of the men had severe vitamin D deficiency, although 20% of subjects were marginally deficient.

It was concluded that under the conditions of this study, CLA (Clarinol) had no impact on calcium status or bone metabolism.

(xii) Additional information provided as part of the response to a request for further information

The effects of CLA on endothelial function in healthy subjects as measured by flow mediated vasodilatation or arterial elasticity were at best equivocal (De Roos *et al*., 2003; Raff *et al*., 2006) due to dietary composition and the limited duration of treatment (≤ 5 weeks). Claims that a 50:50 isomeric blend of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 have been found to slow or reverse the progression of atherosclerosis, were not supported after a careful analysis of the data from several non-clinical studies (Lee *et al*., 1994; Nicolosi *et al*., 1997; Kritchevsky *et al*., 2000).Overall, there was no consistent anti- or pro-atherogenic effect of CLA when administered as either one of the individual isomers or the proposed 50:50 isomeric formulation (Rudel, 1999; Arbonés-Mainar *et al*., 2006; Mitchell *et al*., 2005; Mitchell and

McLeod, 2008). A comprehensive mode of action that is consistent with these data is at best speculative at this stage (Li *et al*, 2008).

5. Discussion and conclusion

CLA does not refer to a single substance, but rather is a collective term for a class of conjugated dieonic isomers of linoleic acid. All of the known physiological effects of CLA are induced by two main isomers: *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA (Pariza, 2004). In some cases, the observed effects appear to be produced by one of these isomers acting alone (e.g., *trans*-10, *cis*-12 CLA is believed to be solely responsible for reduction in body fat gain), whereas in other cases, the two isomers appear to act together to produce an effect (e.g., both isomers appear to be equally effective in inhibiting chemically induced mammary carcinogenesis in rodent models). As a consequence, this hazard assessment has focussed on those studies using a CLA mix of isomers that approximates or is identical to the formulation (Tonalin®TG80) that is proposed for approval as a novel food. This formulation contains approximately equal amounts of the *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA in the form of triglyceride esters.

5.1 Scope of assessment

While a variety of treatment-related changes are observed in both animal and human studies, only those of potential toxicological significance are considered as part of this assessment. Effects considered to potentially influence the risk profile for conditions such as obesity, diabetes, and cardiovascular disease, but which themselves do not have direct toxicological relevance, are discussed in Supporting Documents 1, 2 & 3, and in the body of the report.

5.2 Results from toxicity studies

The majority of animal studies with CLA have been conducted in mice and rats, with a smaller number of studies in pigs, hamsters and rabbits also being available. While the majority of the animal studies have been of short duration, two well conducted sub-chronic studies in rats are available, as well as a small number of other studies of longer duration. In addition two developmental studies in rats have been conducted, as well as a number of genotoxicity studies.

A number of human studies, while primarily being conducted for the purpose of establishing efficacy, have also examined certain clinical chemistry and haematology parameters, and therefore were also included as part of this assessment. A limitation of the human studies and some of the animal studies however is that typically only single dose levels of CLA were used, therefore where treatment-related changes were seen it was not possible to determine if any dose-response is present.

The effects observed in the animal studies are not always consistent across studies nor are they consistent between species. Mice, in particular, appear to be the most sensitive and responsive species to the effects of CLA, exhibiting effects such as increased fasting insulin levels, decreased insulin sensitivity, at much lower doses than in rats and also exhibiting effects that are absent in rats at much higher dose levels of CLA, e.g. accumulation of fat in the liver (steatosis). In addition, many of the mouse studies were conducted in inbred strains chosen as models for obesity, and thus may not reflect normal responses. The studies in mice are thus considered less useful for risk assessment purposes therefore greater emphasis has been placed on the studies using rats.

No treatment related adverse effects were observed in two developmental studies where CLA was given to pregnant rats. CLA was also found to be negative in *in vitro* tests for point mutations in bacterial and mammalian cells, and was also negative in an *in vitro* test for chromosome aberrations in human lymphocytes, as well as an *in vivo* mouse micronucleus test. Some treatment related changes were however observed in a number of the other studies that are considered noteworthy from a toxicological perspective. These were: liver effects (animals); increased liver vitamin A levels (animals); increases in markers of lipid peroxidation (humans); increases in markers of inflammation (animals and humans). These are discussed below.

5.2.1 Liver effects

A number of liver effects have been observed in studies with rodents. Increased liver weights have been seen in mice, rats and hamsters but it is only in mice that this is associated with steatosis. In rats, while certain enzyme markers for liver cell damage were also found to increase in the high dose groups in one of the sub-chronic studies, this was not accompanied by any histopathological changes of toxicological relevance. Such effects were also reversible following cessation of treatment. These liver changes are therefore most likely an adaptive response to a high fat diet and in the absence of any adverse histopathological changes, are not considered to be toxicologically significant. Results from human studies also do not indicate any significant effect on liver enzymes as a result of consumption of CLA.

On the basis of a sub-chronic study in rats (O'Hagan and Menzel, 2003), a NOEL of approximately 2000 mg CLA/kg bodyweight/day has been established based on liver changes at the highest dose level. This dose level would be equivalent to 140 g CLA/day for a 70 kg human, which is far in excess of the expected level of daily intake for humans.

5.2.2 Vitamin A status

While the effects of CLA on vitamin A status has not been the focus of extensive research, one short term study in rats does show that the administration of CLA at dietary levels of 0.5 to 2.0% to be associated with a dose-dependent increase in liver retinol levels, with significant (but not dose-dependent) increases also being observed in liver retinyl esters and plasma retinol (Belury *et al*., 2003). No increases in plasma retinyl esters were detected. The dietary levels tested in rats are about 8 fold higher than the expected levels of intake in humans. Significantly increased levels of liver retinol have also been observed in one study with pigs fed CLA at similar dietary levels for a slightly longer duration, although unlike rats, levels of liver retinyl esters were unaffected (plasma retinol levels were not determined).

These observations have raised the question as to whether the consumption of CLA in the long term might increase the absorption of dietary vitamin A in humans, leading to an increased risk of hypervitaminosis A.

A plausible mode of action for the effect of CLA on retinol levels was hypothesised by Banni et al (2003) and is supported by more recent data. Banni *et al.,* (2003) note that CLA is known to activate peroxisome proliferator-activated receptor-alpha (PPARα) (Belury and Kempa-Steczko, 1997); Choi *et al*., 2007), which in turn is known to regulate expression of cellular retinol-binding protein (CRBP) mRNA (Suzuki *et al*., 1998; Takase *et al*., 2000). CRBP has a pivotal role in the intestinal absorption and metabolism of retinol, as retinol absorbed from the intestinal lumen by mucosal cells is bound to CRBP. Thus Banni *et al*., (2003) suggested CLA may enhance CRBP levels in intestinal cells and thus the sequestration of retinol from the lumen (Belury *et al*., 2003). Indeed, CRBPII gene expression may be regulated predominantly by dietary fatty acids (Takase *et al*., 2000) and linoleic acid (note, not CLA) force-fed to rats has been shown to produce an elevation of

CRBP II mRNA levels in a dose-dependent and time-dependent manner (Takayanagi and Suruga, 1994). The CLA isomer *t*10, *c*12 is also known to decrease PPARgamma expression (Kang *et al*., 2003).

While no lifetime studies are available that specifically address the affects of CLA on vitamin A status, one long term study where rats were fed CLA at a dietary level of 1% for 18 months does not indicate any signs of hypervitaminosis A. In addition, the absence of a detectable increase in plasma retinyl ester levels in the short term study indicates that liver storage capacity has not been exceeded. Also, as comparison treatments with different types of added oils were not included in the studies, it is also uncertain if the observed effect is specific to CLA or whether it is a general phenomenon associated with increased levels of fat in the diet. At least one study indicates that liver vitamin A concentrations may be influenced by different types of added oil (Alam *et al*., 1990). Given that adipose tissue harbours about 14% of the rat's total vitamin A store (Penniston and Tanumihardjo, 2006), and it is known that CLA mediates effects on lipid storage and turnover, it is possible that the perturbations to liver vitamin A levels merely reflects CLA-mediated faster turnover of liver lipids and adipose tissue, rather than an increase in vitamin A absorption *per se*.

Overall, the effects on liver vitamin A levels in rats and pigs do not appear to have any toxicological significance and do not, of themselves, point to an increased risk of hypervitaminosis A in humans.

5.2.3 Lipid peroxidation

Some studies in humans showed an increase in markers of lipid peroxidation following CLA intake. For example, Basu *et al*., (2000a, 2000b) showed urinary and plasma isoprostanes were elevated in humans given 4.2 g CLA/day for four weeks or three months, however two weeks after treatment ceased levels had returned to basal levels.

Products of lipid peroxidation, such as the urinary isoprostanes, have been detected in most diseases involving inflammation and oxidative stress (e.g. cardiovascular disease). However, the changes observed following CLA supplementation were mostly within the reported daily variation for isoprostanes. It is also noted that none of the human studies show a correlation of isoprostane plasma levels with other markers of oxidation or (pro)inflammation.

Therefore the observed increase in isoprostanes should be interpreted with caution as its relevance to a safety assessment is not clear, and does not necessarily indicate an increase in oxidative stress due to CLA supplementation.

5.2.4 Markers of inflammation

Some studies in both animals and humans have shown that consumption of CLA may be associated with increases in certain markers of inflammation (e.g., TNFα, CRP, IL-6 and IL-8, leukocytes), although such effects are not consistent across all studies or between species, with other studies demonstrating no such increases. A consistent finding in human studies however is an increase in leukocyte count associated with consumption of CLA. The observed increase in leukocyte count is however generally small and typically remains within reference values, therefore in the absence of other changes, is unlikely to be of toxicological significance.

Adipose tissue consists not only of adipocytes but also a number of other cells types such as leukocytes and macrophages (Tilg and Moschen, 2006); (Fantuzzi, 2005). The adipocytes are sources of bioactive proteins and peptides, such as TNFα, CRP, and IL-6. The observed increase in some of these markers in some studies may therefore simply reflect

the mobilisation of fat deposits followed by a reduction in adipose tissue mass in association with CLA intake, rather than be indicative of a true toxicological effect.

5.2.5 Effects on bone

Evidence for effects on bone metabolism in animals due to CLA is mixed. Li et al (1999) found reduced bone mineralisation in rats fed 1% CLA in the diet. Other animal studies found no effect or an increase in bone formation (e.g. in chicks (Cook *et al*., 1997).

A study in humans (Doyle *et al*., 2005) found no effect on calcium status (urinary or serum) or markers of bone formation and bone resorption at levels of 3 g CLA (Clarinol) per day. Overall, the effects on bone seen in some animal studies do not appear to have any toxicological significance in humans.

5.3 Conclusion

This safety assessment has focussed on those studies using a CLA mix containing approximately 1:1 of the two major isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA.

The *cis*-9, *trans*-11 isomer of CLA is a normal constituent of the human diet, present principally in milk and other dairy products. In contrast, commercial production of CLA by chemical isomerisation of linoleic acid produces a mixture containing predominantly the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in equal amounts. Addition of synthetic CLA to food would result in an increase in dietary exposure to both isomers, but a greater fold increase in exposure to the *trans*-10, *cis*-12 isomer, which is not normally found in significant quantities in the diet.

The potential for other significant changes observed in animal studies, such as changes in insulin, blood glucose and plasma triglyceride levels to impact on human health are discussed in Supporting Documents 1 and 3.

No toxicological endpoint relevant to humans was identified from these studies in animals or humans. However, given that the clinical studies were primarily designed to assess efficacy, very little relevant toxicological information could be derived from them. Furthermore, given the nature of these fatty acids, their presence already in the diet and their known metabolic pathways, it was considered that hazards would not have been identified from the measured parameters. Hence with these limitations, it was not considered appropriate to establish a health standard, such as an ADI, for CLA.

Key animal studies

Type	Authors	Test substance	Dose	Control	Effect	NOAEL
Mouse, 6 weeks	West et al, 1998	CLA (39.1% c9,t11 and t0,c11-CLA and 40.7% t10,c12-CLA)	1.2% (in high fat diet) and 1% (low fat)	High fat (45% energy from fat) and low fat (15% energy)	Reduced energy intake, growth rate, adipose depot weight and carcass lipid and protein content, increased weight of liver and spleen, all independent of diet group	
Mouse, 12 weeks	DeLany et al, 1999	CLA (39.1% c9,t11 and t0,c11-CLA and 40.7% t10,c12-CLA)	1% in high fat diet	High fat diet	Reduced body weight gain, body fat depot mass, increased liver weight, decreased lipid content, increased protein content, increased plasma insulin levels	Not reported
Rat, 90 day plus 4 week recovery (#408)	O'Hagan and Menzel, 2003	Clarinol G80 (37.3% 9c,11t- and 37.6% 10t,12c-CLA)	1, 5 and 15%	Low fat (7%) and high fat (15%)	Insulin increased, organ weight increased, reversed in males, partially in females, liver hypertrophy	5% = 1946 and 2182 mg CLA/kg bw/day
Rat, 90 day (#408)	Mellert et al, 2002	CLA-methylester and ethylester beadlets (report suggests comparable to Tonalin TG 80)	0.5, 1.5 and 5% beadlets, 40% CLA	Placebo beadlets (composition not reported)	Plasma triglycerides increased in high dose females, kidney weight increased in high dose male and female, liver weights increased in high dose females, no histopathological finding related to increased organ weights	Highest dose = 1360 and 1600 mg/kg bw/day
Rat, 18 month	Park et al, 2005	CLA (41.9% c9,t11- and 43.5% t10,c12-CLA)	1%	Equivalent corn oil	Reduced blood glucose in fasted and fed rats, no differences in tissue weights. All test and control animals had chronic renal disease. Small sample size (n=3 and 4)	Not reported, although food intake recorded.
Rat, 36 week	Scimeca, 1998	CLA (42.5% c9,t11- and/or t9,c11- and 43% t10,c12-CLA)	1.5%	Basal diet	Increase in adrenal weight and decrease in thymus weight, no histomorphological or haematological changes	Dose ranged from 1970 to 467 mg/kg bw/day over 36 weeks
Rat, one month	Banni et al, 1999	CLA, composition not specified	0.5, 1, 1.5 and 2%	Basal diet AIN-76A (8 mg retinyl palmitate/kg diet	Dose dependent increase in liver retinol concentration. Also increase in liver retinyl ester and plasma retinol concentration	Liver retinol increased at lowest dose tested – 0.5%

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