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SCIENTIFIC OPINION

Scientific Opinion on the safety of "conjugated linoleic acid (CLA)-rich oil" (Tonalin® TG 80) as a Novel Food ingredient[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2], [3]

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ABSTRACT

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to carry out the additional assessment for Tonalin® TG 80, a conjugated linoleic acid (CLA)-rich oil, as a food ingredient in the context of Regulation (EC) No. 258/97. Tonalin® TG 80 consists of approximately 80 % of the two CLA isomers a *c*9,*t*11:*t*10,*c*12 (1:1). Tonalin® TG 80 is intended by the applicant as an ingredient for milk-, yoghurt- or fruit-type products and other – unspecified products for adult consumers. The applicant suggests a daily intake of 4.5 g Tonalin® TG 80. The available data from non-human studies do not indicate a risk for genotoxicity, reproductive toxicity, carcinogenicity or allergenicity. The extent of the effects of CLA on insulin resistance and on markers of cardiovascular risk appears to be species-dependent. Therefore the focus of this safety assessment relies mainly on the large number of available human studies. Based on the assessment of these studies, the Panel considers that CLA consumption does not appear to have adverse effects on insulin sensitivity, blood glucose control or liver function for up to six months, and that observed effects on blood lipids are unlikely to have an impact on cardiovascular risk. Long-term effects of CLA intake on insulin sensitivity and the arterial wall have not been adequately addressed in humans. The Panel concludes that the safety of Tonalin® TG 80 has been established for the proposed uses at intakes of 4.5 g per day (corresponding to 3.5 g CLA), for up to six months. The safety of CLA consumption for periods longer than six months has not been established under the proposed conditions of use. The safety of CLA consumption by type-2 diabetic subjects has not been established.

KEY WORDS

Conjugated Linoleic Acid, insulin sensitivity, blood lipids, novel food ingredient, Cognis.

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SUMMARY

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to carry out the additional assessment for Tonalin® TG 80, a conjugated linoleic acid (CLA)-rich oil as a food ingredient in the context of Regulation (EC) No. 258/97 taking account of the comments/objections of a scientific nature raised by the Member States.

Tonalin® TG 80 which consists of approximately 80 % of the two CLA isomers *c*9,*t*11: *t*10,*c*12 at a ratio of 1:1. Tonalin® TG 80 is manufactured from safflower oil via trans-esterification with ethanol to form fatty acid ethyl esters, isomerisation (conjugation) under alkaline conditions, hydrolysis of the ethyl esters and separation of the CLA from the reaction mixture by distillation, and lipase-catalysed re-esterification of the CLA with glycerol. The single steps of the process are procedures commonly applied in the isolation, refinement and modification of vegetable fats and oils.

Tonalin® TG 80 is intended by the applicant for use as an ingredient in milk-, yoghurt- or fruit-type products and other unspecified products. The intended target consumers are adults. The applicant suggests a daily intake of 4.5 g Tonalin® TG 80, corresponding to 3.5 g CLA.

The average intake of naturally occurring CLA from food is estimated to be about 0.3 g/day in Europe. A supplementation of 3.5 g CLA would therefore lead to a 11.7-fold increase in CLA intake. However the most abundant CLA isomer naturally occurring in foods is the *c*9,*t*11 isomer, accounting for more than 90 % of the dietary CLA intake. Thus an intake of 3.5 g CLA from Tonalin® TG 80 with a *c*9,*t*11: *t*10,*c*12 ratio of 1:1 would increase the intake of the *c*9,*t*11 and the *t*10,*c*12 isomers approximately 6 and 58 fold, respectively.

The applicant provided data from animal studies on absorption, distribution, metabolism and excretion. The metabolism follows standard pathways known for fatty acids consumed as triglycerides. A number of non-human studies have been provided on the acute, subchronic, chronic, geno-, reproductive, and developmental toxicity and carcinogenicity of CLA. The available data from non-human studies do not indicate a risk for genotoxicity, reproductive toxicity, carcinogenicity or allergenicity.

In addition non-human studies were provided on the effects of CLA on lipid metabolism parameters, hepatic lipid accumulation, liver function, markers of inflammation, insulin sensitivity and glucose metabolism. *In vitro* data suggest that the *t*10,*c*12 CLA isomer is involved in the regulation of fatty acid synthesis and mediating suppression of insulin sensitivity in mature human adipocytes. This isomer has also been reported to be responsible for undesirable effects on fat and glucose metabolism *in vivo*. Mice seem to be particularly sensitive to the effects of CLA on fat and glucose metabolism. However the extent of the effects of CLA on insulin resistance and also on markers of cardiovascular risk appears to be species-dependent. Therefore the focus of the safety assessment relies mainly on human studies.

The administration of the 1:1 isomer mixture of CLA to normal weight, overweight and obese non-diabetic subjects does not appear to have adverse effects on insulin sensitivity, blood glucose control or liver function at the proposed conditions of use for up to six months. Effects of CLA consumption over periods longer than six months on insulin sensitivity and liver steatosis have not been adequately addressed in humans. With respect to type-2 diabetic subjects, the evidence provided does not establish the safety of CLA under the proposed conditions of use, since the CLA 1:1 isomer mixture appears to adversely affect both static (HOMA-IR) and dynamic (ISI, OGIS) surrogate markers of insulin sensitivity as well as fasting blood glucose and no studies on blood glucose control (e.g., HbA1c) are available for periods of consumption beyond eight weeks. Under the proposed conditions of use, CLA has no effect on LDL-cholesterol concentrations or the LDL:HDL-cholesterol ratio, and the magnitude of the changes observed in HDL- and triglyceride concentrations are unlikely

to have an impact on CVD risk. However, the observed increase in plasma and urinary concentrations of isoprostanes, which may indicate an increase in lipid peroxidation, and the increase in some markers of subclinical inflammation (i.e., 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ and possibly C-reactive protein) associated with CLA consumption, together with the limited data available on the effects of CLA on vascular function may indicate a potential for vascular damage (i.e., atherosclerosis) in the longer term. No data on effects of CLA intake on the arterial wall have been provided in humans.

The Panel considers that CLA consumption does not appear to have adverse effects on insulin sensitivity, blood glucose control or liver function for up to six months, and that observed effects on blood lipids are unlikely to have an impact on cardiovascular risk. Long-term effects of CLA intake on insulin sensitivity and the arterial wall have not been adequately addressed in humans. The evidence provided does not establish the safety of CLA consumption by type-2 diabetic subjects under the proposed conditions of use.

The Panel concludes that the safety of Tonalin® TG 80, an oil with approximately 80 % CLA 1:1 mixture of *t*9,*c*11 and *t*10,*c*12 isomers, has been established for the proposed uses at intakes of 4.5 g per day (corresponding to 3.5 g CLA), for up to six months. The safety of CLA consumption for periods longer than six months has not been established under the proposed conditions of use. The safety of CLA consumption by type-2 diabetic subjects has not been established.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

On 27 April 2007, Bioresco, Food Scientific and Regulatory Services, on behalf of Cognis Deutschland GmbH & Co KG submitted a request under Article 4 of the Novel Food Regulation (EC) N° 258/97 to place on the market 'Tonalin® TG 80 conjugated linoleic acid (CLA)-rich oil as a novel food ingredient.

On 1 July 2008, the competent authority of Spain forwarded to the Commission its initial assessment report.

On 31 July 2008, the Commission forwarded the initial assessment report to the other Member States. Several of the Member States submitted additional comments and some Member States raised objections. The concerns of a scientific nature can be summarised as follows:

- The verification and specification data are insufficient. Accreditation credentials of the test laboratories have to be supplied.
- The applicant must confirm which plant oils are used for the production and must show that the production process is under control and that the composition of the product meets the specification.
- Data on the contents of phytosterols and residual amounts of ethanol should be provided.
- The stability of the novel food ingredient in the intended final applications should be demonstrated.
- The efficacy in use is not sufficiently demonstrated.

  A list of foods to which CLA may be added is required. Portion size should be defined.
- The recommended dose should be 3 g CLA/day rather than 3.5 g/day.
- If used in high-sugar foods, one portion should contain the total recommended daily amount to prevent intake of too much sugar.
- Metabolic effects of the novel food ingredient should be considered.
- Contradictory study findings and low subject numbers make it necessary to further investigate the impact of CLA preparations on parameters such as HDL cholesterol level, lipid and glucose metabolism, insulin sensitivity and inflammation values, particularly in respect of overweight individuals (who constitute the target-group of CLA-enriched foods).
- Additional scientific data are required to demonstrate the safety in use of the novel food ingredient in all kinds of foods and beverages rather than only in food supplements.
- Additional safety data from human studies are required.
- The safety concerns for children need to be further elaborated.

In consequence, EFSA is asked to carry out the additional assessment and to consider the elements of scientific nature in the comments raised by the other Member States in accordance with Article 7, paragraph 1 of Regulation (EC) No 258/97.

TERMS OF REFERENCE AS PROVIDED BY THE COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Food Safety Authority is asked to carry out the additional assessment for 'CLA-rich Oil' in the context of Regulation (EC) N° 258/97.

EFSA is asked to carry out the additional assessment and to consider the elements of scientific nature in the comments raised by the Member States.

ASSESSMENT

The novel food ingredient has been allocated to class 2.1 (complex non-GM novel food ingredient, the source having a history of food use in the Community), as defined in the SCF recommendations concerning the assessment of novel foods (EC, 1997). It is noted that the novel food ingredient Tonalin® TG 80 is intended to be added to foods for consumption by adult subjects as part of weight control programs. This assessment concerns only the risk that might be associated with consumption of the novel food ingredient Tonalin® TG 80, and is not an assessment of the efficacy with regard to any claimed benefit.

1. Specification of the Novel Food

Tonalin® TG 80 is an oil rich in conjugated linoleic acid (CLA) manufactured from safflower oil via (i) trans-esterification with ethanol to form fatty acid ethyl esters, (ii) isomerisation (conjugation) under alkaline conditions, (iii) hydrolysis of the ethyl esters and separation of the CLA from the reaction mixture by distillation, and (iv) lipase-catalysed re-esterification of the CLA with glycerol.

The chemical structures of the two principal CLA isomers contained in Tonalin® TG 80 are depicted in Figure 1.

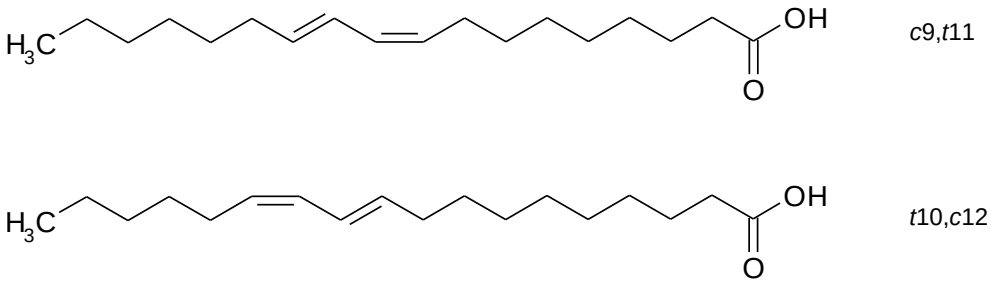

Figure 1: Principal CLA isomers contained in Tonalin® TG 80

Table 1 shows the specifications of Tonalin® TG 80 proposed by the applicant. Data presented for five batches of Tonalin® TG 80 were in agreement with these specifications.

Table 1: Specifications of Tonalin® TG 80 proposed by the applicant.

Parameter	Specification
CLA (mg/g)	≥ 700
Monoglycerides (%)	≤ 1
Diglycerides (%)	≤ 20
Triglycerides (%)	80 -100
Fatty acid composition	
CLA (C18 :2) (%)[a)]	≥ 78
CLA *c*9,*t*11 isomer (%)[a)]	≥ 37.5
CLA *t*10,*c*12 isomer (%)[a)]	≥ 37.5
Sum CLA *t*,*t* isomers	≤ 1.5
Sum CLA *c*,*c* isomers	≤ 1.5
CLA *c11*,*t13* isomer	≤ 0.2
Oleic acid	10 -20
Palmitic acid	≤ 4
Stearic acid	≤ 4

Linoleic acid	≤ 3
Saponification value (mg KOH/g)	185 -195
Iodine value	≥ 115
Peroxide value (mEq/kg)	≤ 1
Colour (Gardner)	≤ 4
Acid value (mg KOH/g)	≤ 2
Unsaponifiable matter (weight %)	≤ 1
Heavy metals (mg/kg)	
Pb	≤ 0.1
As	≤ 1
Hg	≤ 0.1
Cd	≤ 0.1
Total aerobic microbial Count	≤ 3000/g
Yeasts and moulds	≤ 300/g
Coliforms	≤ 10/g
Staphylococcus aureus	negative/10g
Salmonella spp.	negative/25g

[a)] relative area % of total fatty acids, determined by GC using a validated in-house method

Compositional data provided by the applicant for these CLA isomers in five batches of Tonalin® TG 80 are listed in Table 2.

Table 2: Compositional data on the two principal CLA isomers in Tonalin® TG 80.

	CLA c9,t11		CLA t10,c12		CLA total	
Lot nr.	area %	weight %	area %	weight %	area %	weight %
73123161	39.7	37.9	39.2	37.7	81.0	77.3
73113161	39.0	37.5	38.6	37.1	80.5	77.3
73003161	39.9	38.5	39.3	37.9	81.2	78.3
72843161	39.6	38.0	39.1	37.6	80.6	77.5
72223331	39.2	38.3	38.4	37.5	79.7	77.7

Analytical data on the contents of sterols in the novel food ingredient have not been provided. The applicant confirmed that only safflower oil is used as starting material in the production process. Owing to the multiple distillation steps, the sterol contents in the final product are expected to be very low and the sterol pattern may be different from that in the starting material. According to the applicant, a minor carry-over of traces of sterols may occur due to the use of the distillation unit for the production of plant sterols for food use.

Ethanol which is liberated by hydrolysis of the fatty acid ethyl esters formed by trans-esterification is removed in the course of the distillation process. According to the applicant, analyses of Tonalin® TG 80 demonstrated that the residual ethanol levels were below 50 ppm.

Stability

In order to ensure the stability of Tonalin® TG 80, the production process is performed at temperatures below 120°C and storage is in closed drums under nitrogen. The final product is stabilised by addition of 0.1 % natural mixed tocopherols (E306).

The stability of Tonalin® TG 80 has been tested upon storage up to 24 months in different types of containers (plastic pail, steel drum, glass bottle) under standardised conditions (20 - 40°C). The

parameters measured comprised fatty acid composition, isomer distribution, acid value, saponification value, peroxide value, unsaponifiable matter, colour and microbiological status. The stability properties were similar to those known from other oils containing high amounts of triglycerides of polyunsaturated fatty acids.

The applicant provided data on the stability of various products (orange juice, milk, yoghurt and nutrition bars) enriched with Tonalin® TG 80. Analysis of the CLA isomers did not reveal negative effects over the proposed shelf-life. In addition, it was shown that the application of pasteurisation (90°C, 2s) and Ultra-High Temperature (UHT) treatment (140°C, 4s) to milk products did not affect the distribution of CLA isomers.

2. Effect of the production process applied to the Novel Food

The starting material used for the manufacturing of Tonalin® TG 80 is food grade safflower oil, high in linoleic acid. The oil is subjected to chemical trans-esterification with ethanol to form the respective fatty acid ethyl esters. After distillation the isomerisation (conjugation) is achieved in the presence of an alkaline catalyst. The fatty acid ethyl esters are hydrolysed and the CLA are isolated as free fatty acids via distillation under reduced pressure. The free fatty acids are re-esterified with glycerol using a food-grade immobilised lipase as catalyst. After removal of the enzyme by filtration, the reaction mixture is distilled under reduced pressure to yield the crude CLA-rich oil.

The single steps of the process are procedures commonly applied in the isolation, refinement and modification of vegetable fats and oils. Information on key process parameters, e.g. type of enzymes employed or temperature conditions were provided in the dossier. According to the applicant, the production process is controlled by an HACCP system.

3. History of the organism used as the source of the Novel Food

The starting material for the manufacture of Tonalin® TG 80 is safflower oil meeting the general specifications laid down in the Codex Standard for Named Vegetable Oils Codex-*Stan 210*. Safflower oil species have been obtained by conventional breeding more than 20 years ago. The oil is widely used as replacement of or in combination with sunflower oil in various products.

4. Anticipated intake/extent of the use of the Novel Food

According to the applicant, Tonalin® TG 80 is intended for use as an ingredient in milk-, yoghurt- or fruit-type products. It is emphasised that this list of potential applications is not exhaustive. However, the use of Tonalin® TG 80 is not compatible with food manufacturing processes that require extensive heating (frying, baking, and grilling).

Referring to data from clinical studies on the efficacy of CLA in the reduction of body fat (Whigham et al., 2007; Gaullier et al., 2007; Lopez-Roman et al., 2007; Laso et al., 2007), the applicant suggests a daily intake of 4.5 g Tonalin® TG 80, corresponding to 3.5 g CLA. To control the consumption, the applicant proposes adequate labelling instructions and the restriction of the use of Tonalin® TG 80 to foods that can be easily divided into portions containing either a maximum of 3.5 g CLA, or a correspondingly smaller amount in case consumption of two or three portions per day is foreseen.

5. Information from previous exposure to the Novel Food or its source

Annual sales of CLA for the years 2006 – 2008 have been estimated to be approximately 170 – 180 tons in Europe (EFSA, 2010a).

Information provided by the applicant on the total CLA content in foods produced in Germany and in the US (Siems et al., 2001) is listed in Table 3.

Table 3: Total CLA content (mg/g fat) in foods produced in Germany and in the US.

Food category	CLA content (mg/kg fat)
Butter	4.7
Condensed milk	7.0
Milk, homogenised	5.5
Yoghurt, natural	5.1
Cottage cheese	4.5
Mozzarella cheese	4.7
Gouda cheese	6.3
Beef tallow	2.6
Roast beef	2.9
Ground meat	4.3
Veal, chop	5.6
Lamb, chop	2.7
Pork, chop	0.6
Sunflower oil	0.4
Canola oil	0.5
Corn oil	0.2
Olive oil	0.2

Natural contents of CLA isomers in conventional food sources are listed in Table 4.

Table 4: Natural contents of CLA isomers in conventional food sources.

Product	CLA isomers	CLA level (% of total fat)	Reference
CLA in meat			
Chicken, lamb, turkey, beef, pork	18:2 c9,t11	0.05 – 0.59	Chin et al., 1992
Beef	18:2 c9,t11	0.77	Shantha et al., 1995
Chicken, turkey, lamb, beef, pork, rabbit	18:2 c9,t11	0.11 – 1.20	Fritsche and Steinhart, 1998
Beef	18:2 c9,t11	0.61 -1.01	Fritsche and Steinhart, 1998
Beef	18:2 c9,t11	0.08 – 0.37	Ma et al., 1999
Bison, beef cattle, chicken	18:2 c9,t11 18:2 t10,c12	0.07 – 0.41 0.02 – 0.12	Rule et al., 2002
Beef	18:2 c9,t11	0.31	Griswold et al., 2003
Beef	18:2 c9,t11 18:2 t10,c12	0.522 0.005	Gillis et al., 2004
CLA in dairy products			
Milk	18:2 c9,t11	1.44 (summer) 0.64 (winter)	Collomb and Bühler, 2000
Milk	18:2 c9,t11	0.39	Abu-Ghazaleh et al., 2001
Milk	18:2 c9,t11	0.60	Chilliard et al., 2003
Milk (sheep)	18:2 c9,t11 18:2 t10,c12	1.73 0.04	Nudda et al., 2005
Milk	18:2 c9,t11 18:2 t10,c12	0.35 0.04	Chichlowski et al., 2005
Cheese	18:2 c9,t11	0.29 – 0.71	Chin et al., 1992
Cheese	18 :2 c9,t11	0.32 – 0.89	Shantha et al., 1995

Sheep cheese	18:2 c9,t11	1.69	Nudda et al., 2005
	18:2 t10,c12	0.04	
CLA in eggs			
eggs	18:2 c9,t11	0.24	Fogerty et al., 1988

The average intake of naturally occurring CLA from food is estimated to be about 0.3 g/day in Europe (EFSA, 2004). A supplementation of 3.5 g CLA from Tonalin® TG 80 would therefore lead to an 11.7-fold increase in CLA intake. The most abundant CLA isomer naturally occurring in foods is the *c*9,*t*11 isomer, accounting for more than 90 % of the dietary CLA intake (Bhattacharya et al., 2006). Thus an intake of 3.5 g CLA from Tonalin® TG 80 with a *c*9,*t*11: *t*10,*c*12 ratio of 1:1 would increase the intake of the *c*9,*t*11 and the *t*10,*c*12 isomers approximately 6- and 58-fold, respectively.

6. Nutritional information on the Novel Food

As demonstrated in animal studies, a large proportion of the ingested CLA is oxidised by the body, so that CLA is assumed to provide 9 kcal/g as other fatty acids. The energy content of 3.5 g CLA is not considered to have a big impact on long-term energy balance.

7. Microbiological information on the Novel Food

Considering the composition of Tonalin® TG 80, issues regarding microbiological contaminations are not to be expected. Microbiological data provided for three batches are shown in Table 2.

8. Toxicological information on the Novel Food

8.1. Non-human studies

8.1.1. Studies on absorption, distribution, metabolism, and excretion (ADME)

The metabolism of CLA has been widely studied and follows standard pathways known for fatty acids consumed as triglycerides.

8.1.2. Acute toxicity studies

According to the applicant an acute oral toxicity study in rats (strain unspecified) was performed using commercial beadlets of CLA methyl esters of unknown purity (Berven et al., 2002). The authors concluded that oral administration of CLA methyl esters was "non-toxic" based on an LD_{50} value of >2 g/kg body weight.

8.1.3. Subchronic and chronic toxicity studies

A subchronic (13-week) feeding study using male and female Wistar rats CrlGlxBrlHan:WI (10 animals per sex and group) and CLA methyl- and ethylesters (composition not further specified) as test materials was provided (BASF, 2002). According to the applicant the CLA esters were produced using the same CLA sources and manufacturing procedures as for Tonalin® TG 80. CLA methylester beadlets were administered in the diet at concentrations of 0.5, 1.5 and 5 % (w/w). This concentration corresponded to an intake from approximately 0.4 g/kg body weight in the low concentration group to 4 g/kg body weight in the highest concentration group. The control group received a standard rodent diet. An additional group received placebo beadlets (vehicle group). CLA ethylester beadlets were

only administered at a concentration of 5 % in the diet. No information was provided on the actual doses of CLA methyl- and ethylesters administered to the animals.

There was no mortality and body weight changes, food consumption and feed efficiency were comparable in all groups. Clinical (including ophthalmological) examinations revealed no CLA-related adverse effects. A functional observational battery (FOB) and motor activity tests did not reveal significant differences between groups. In haematological analysis the only statistically significant difference between the CLA and control groups was an increase in platelet counts in female rats of all groups receiving CLA methylesters. This effect was not dose-dependent. Urinalysis parameters were not changed whereas clinical chemistry analysis showed increased blood triglyceride levels in females of the high-dose group receiving CLA methylesters as well as CLA ethylesters. Determination of the weights of selected organs and tissues showed higher mean absolute kidney and higher relative liver weights in males of the high-dose group receiving CLA methylesters. Females of the high-dose CLA methylester group and of the CLA ethylester group had higher absolute and relative (in relation to body weight) liver weights as well as higher absolute kidney weights. In females of the high-dose CLA methylester group the absolute spleen weights were also increased compared with the control group. Histopathological examinations did not show relevant differences in relation to the control group.

The Panel notes that the effects observed in this study were also found in other subchronic rat studies after administration of different types of fats. Since no fat control group was included in this study, it is not possible to determine whether the administration of CLA methylesters or ethylesters induced substance-specific changes. The Panel considers that no conclusions can be drawn from this study in relation to the toxicity of CLA in rats.

The applicant presented another subchronic (13-week) feeding study in male and female Wistar outbred (Crl: (WI)WU BR) rats (20 rats per sex and group) using a commercial preparation of a CLA-rich oil (Clarinol® G80) claimed by the applicant to be essentially identical to Tonalin® TG 80 (O'Hagan and Menzel, 2003). The test material contained approximately 79 % CLA isomers, including equal amounts of the 9*cis*,11*trans* and 10*trans*,12*cis* CLA isomers, in the form of glycerides.

Control groups were administered either a high-fat (HF; 15 % w/w safflower oil) or a low-fat (LF; 7 % w/w safflower oil) basal diet. Test groups received the HF basal diet supplemented with 1, 5 or 15 % of the CLA-rich oil for 13 weeks, resulting in CLA intakes of approximately 0.48, 2.4 and 7.2 g/kg body weight per day for males and of 0.54, 2.7 and 8.2 g/kg body weight per day for females. Total added safflower oil and/or CLA-rich oil in all test diets was 15 %. The HF-diet was also supplemented with 10 % higher levels of protein, L-cysteine, cellulose, choline-bitartrate, minerals and vitamins to compensate for reduced food intake in rats fed a high calorie diet and to maintain a normal level of nutrient intake. At the end of the 13-week study period, recovery groups of 10 rats of each sex from each control group and from the high-level (15 %) CLA group were observed for further 4 weeks. Rats in the control groups were maintained on their respective diets, whereas rats in the 15 % CLA group were switched to the HF control diet.

The Panel notes that adverse effects on haematological parameters, liver enzyme activities, blood lipid concentrations and other clinical-chemistry parameters, organ weights and organ morphology have been already described in relation to the sub-chronic administration of high-fat diets to rats, and therefore considers that only changes in the CLA intervention groups in relation to the HF control diet are likely to be meaningful for the evaluation of CLA-related toxicity in this study.

According to the authors, there were no clinical signs or effects on survival attributed to CLA administration and ophthalmologic examinations did not reveal any treatment-related ocular changes. Food consumption was reported to be significantly decreased during days 7 to 14 of the treatment

period in the 15 % CLA group, which was attributed by the authors to reduced palatability of the diet. As a result, statistically significant decreases in body weight were observed in rats of both sexes in the 15 % CLA group at day seven and in females at day 14. Throughout the study, animals treated with high-dose CLA had consistently lower body weights than control animals and this effect was more pronounced in females. Feed conversion efficiency was unaffected. Water consumption was significantly lower in males and females consuming the high-dose CLA at week 12 relative to both control groups. According to the authors there was no indication of any effect on renal function in this group. Urinalyses revealed no treatment-related changes in urinary volume or density (data not provided).

According to the publication, there were no changes in haematological parameters considered by the authors to be an adverse effect related to CLA treatment. Mean erythrocyte cell volume was lower in females consuming the high-dose CLA compared with both control groups. As this was an isolated finding among the haematological parameters assessing red blood cell status, it was not considered toxicologically relevant. In males of the high-dose CLA group, white blood cell counts were statistically significantly lower compared with both control groups. The Panel notes that in the absence of further information the toxicological relevance of this finding remains unsolved.

With respect to liver function tests, increased activities of alkaline phosphatase (ALP) and alanine aminotransferase (ALAT) throughout the treatment period in males treated with 15 % CLA compared to both control groups were reported. The activity of aspartate aminotransferase (ASAT) was not significantly different from the HF control throughout the study. For female animals the situation was comparable though not fully consistent. Activity of sorbitol dehydrogenase (SD) was also increased in females receiving high-dose CLA at week 13 relative to both control groups. After a 4-week recovery period the changes in ALP, ALAT and ASAT were reversed in males with respect to both control groups, and those in ALAT and ASAT activities were reversed relative to the HF in the high-dose CLA females.

Compared with both control groups, plasma total cholesterol levels were significantly decreased in high-dose CLA males throughout the treatment period, while plasma triglyceride levels were significantly increased in females of the high-dose CLA group. In addition, both males and females in the high-dose CLA group were reported to have increased plasma albumin levels and an increased albumin:globulin ratio. The changes in plasma cholesterol, triglycerides and albumin were reported to be reversed at the end of the recovery period.

Males in the high-dose CLA group had decreased blood glucose concentrations at week 13 compared to both control groups, and increased insulin concentrations at week four (compared to both control groups) and week eight (compared to HF control), but not at week 13. Plasma glucose concentrations did not differ significant in females between CLA and control groups; however, insulin was increased at weeks eight and 13 relative to both control groups. At the end of the recovery period blood glucose concentrations were significantly lower in high-dose CLA males compared with high-fat controls with no differences in insulin concentrations between groups, whereas no significant differences between groups were reported in females for either blood glucose or insulin concentrations.

A number of changes in organ weights were observed. Compared to both control groups, male animals in the high-dose CLA group had a statistically significant increase in absolute and relative liver weights. In the mid-dose CLA group relative weights were increased in relation to both control groups, and absolute weights only in relation to the HF control group. Increased relative liver weights were observed in females of the high-dose CLA group in relation to both control groups and in the mid-dose CLA group in relation to the HF controls. After the recovery period these effects were reversed in males but females in the high-dose CLA group still had significantly increased relative liver weights compared with both control groups. In the high-dose CLA group absolute and relative spleen weights were increased in males and relative spleen weights in females compared to both

control groups. The increase in absolute spleen weight in males in relation to the HF controls was no longer significant after the recovery period. In addition, increased relative adrenal weights were observed in the high-dose CLA males compared to both control groups. No differences in adrenal weights in females or in pancreas weight in both sexes were reported in the high-dose CLA group relative to the HF group. Histopathological examination of the liver revealed hepatocellular vacuolation in males of all groups, which was most frequent in the HF control, the low- and mid-dose CLA groups. A relatively high incidence of hepatocellular hypertrophy was observed in high-dose CLA female rats (12/20 rats) and was almost completely reversed (2/20 rats) after the recovery period. According to the authors there were no histopathological changes in any other organ.

The authors suggested that the observed liver enlargement and hepatocellular hypertrophy were adaptive effects in response to the consumption of the CLA-rich oil. Based on these effects and on the effects on blood insulin concentrations, the authors considered the NOAEL to be 5 % CLA-rich oil in the diet, equivalent to 2433 and 2728 mg/kg body weight per day for males and females, respectively.

The Panel notes that the increase in blood concentrations of triglycerides, glucose, insulin and liver enzyme activities (particularly ALAT), and in the weight and morphology of some organs (particularly the liver) observed in the highest dose of CLA-rich oil (Clarinol™) compared to the HF control groups despite the reduction in total body weight (and probably body fat) observed in the high-CLA groups relative to the HF controls suggests that administration of the high dose of the CLA-rich oil induced a number of adverse (and metabolically related) effects which cannot be attributed to the administration of a high-fat diet *per se* but to the specific characteristics of this CLA-rich oil.

In a 36-week study (Scimeca, 1998) male weanling Fischer 344 rats (20 per group) were fed diets containing 0 % (control) or 1.5 % of a synthetic CLA preparation containing 85.5 % of a 50:50 % mixture of *c*9,*t*11 or *t*9,*c*11 and *t*10,*c*12 isomers, 4.3 % other CLA isomers, 7.1 % linoleic acid and 3.1 % other constituents (not specified). The intake of CLA was reported to range from 1970 to 467 mg/kg body weight per day from week 1 to week 36 (mean of 1218.5 mg/kg body weight per day). Animals fed CLA were reported not to show any clinical signs of toxicity, nor were there any differences in body weight gain or food consumption relative to the control group. Organ weight determinations showed statistically significantly reduced absolute and relative (i.e. in relation to body weight as well as brain weight) thymus weights and increased adrenal weights in the treatment group. According to the author, there were no significant treatment-related histopathological changes in the organs examined (including thymus and adrenal glands) or changes in haematological parameters. Therefore the NOAEL for CLA was determined to be 1218.5 mg/kg body weight per day, the only dose tested. The Panel considers that the relevance of this examination is limited because only one CLA dose was tested (in male rats only) and clinical-chemistry parameters were not examined.

The effects of long-term feeding of CLA were examined in Fischer 344 rats (Park et al., 2005). Weanling male rats were administered either a control diet (n=10) or a diet containing 1 % CLA (41.9 % *c*9,*t*11 and 43.5 % *t*10,*c*12; n=11; level of intake equivalent to a dose of approximately 1000 mg CLA/kg body weight per day), which was added to the diet at the expense of corn oil for a period of 18 months. Three animals of the CLA group and four animals of the control group died or were sacrificed in extremis before completion of the study. Despite a lower food consumption in the CLA-fed group, weight gain was not statistically significantly different in the CLA-fed group compared to controls. After 12 weeks three rats from each group were randomly selected to measure body fat distribution. There were no significant differences between groups in percentage body fat, empty carcass weight or percentage body water. Blood analyses at week 69/70 showed that blood glucose concentrations were significantly lower, and mean corpuscular volume was significantly higher, in the CLA-fed group compared to controls. Blood urea nitrogen and cholesterol concentrations were elevated beyond the normal range in both groups, but were not significantly different between groups. At necropsy there were no significant differences between the groups in organ weights. Microscopic analysis showed that all animals from both groups had chronic renal diseases (chronic interstitial

nephritis, nephrosis, and/or glomerulosclerosis). The chronic renal disease was explained by the authors as a side effect of the high protein content of the diets and was not considered to be CLA-related. One of the CLA-fed animals had an enlarged spleen, which was diagnosed as granular cell lymphoma. The incidences of pituitary or testicular tumors, prostatitis, lymphoma and other disorders were not significantly different between the groups. The Panel considers that the use of male animals only, the testing of a single dose of CLA, the limited number of animals available for the specific toxicological examinations, and the occurrence of severe renal disease in all animals greatly limits the relevance of this study to the evaluation.

The applicant summarised the results of a study with Beagle dogs fed CLA for 11 months. The test material incorporated into the diet at a level of 0.5 % was described as a product consisting of about 60 % of a 1:1 mixture of *c*9,*t*11 and *t*10,*c*12 CLA, minor amounts of other CLA isomers and about 40 % regular fatty acids. Groups of three male and five female dogs each received the diet containing CLA corresponding to a dose of approximately 65 – 85 mg/kg body weight per day or a control diet. According to the author administration of CLA had no effect on feed intake, growth and zoometric parameters of the dogs. Plasma concentrations of total cholesterol, HDL and LDL cholesterol, triglycerides and glucose did not differ between the groups on most occasions and a treatment trend was not apparent (Montaño Rivera, 2006).

8.1.4. Genotoxicity

CLA-rich oil (Tonalin FFA 80) was tested in five strains of *Salmonella enterica* Typhimurium (TA98, TA100, TA1535, TA1537 and TA102) up to concentrations of 5000 µg/plate in the presence and absence of metabolic activation (S9 mix) using the plate incorporation as well as the pre-incubation method (Cognis, 2003a). Using the pre-incubation method considerable toxicity was observed in tests without S9 mix using strains TA1537 and TA102. However, there was no increase in the number of revertant colonies in any of the five tester strains and the test material was considered to be not mutagenic in this assay.

The applicant also provided tests on gene mutations in mammalian cells (Cognis 2003b). In the mouse lymphoma *tk* assay using L5178Y cells the CLA-rich oil did not induce relevant increases in the numbers of mutant colonies up to a concentration of 20 µg/mL in the absence of S9 mix. In the presence of S9 mix the number of mutant colonies was twice as high as in the negative control at a concentration of 20 µg/mL after an incubation period of four hours in one culture. However, there was no such increase in the second culture and no relevant increase after incubation for 24 hours up to concentrations of 80 µg/mL. No shift of the ratio of small versus large colonies was observed. The Panel notes that due to the cytotoxicity of the test material the tested concentrations were relatively low but considers that the CLA-rich oil was not mutagenic in this assay.

In a mouse micronucleus assay linoleic acid methylester (designated by the applicant as Tonalin CLA methyl ester but not further specified) was administered twice, with a 24-hour interval between administrations, to male NMRI mice by i.p. injections at doses of 500, 1000 and 2000 mg/kg body weight (BASF, 2001). Bone marrow of the femora was prepared 24 hours after the second administration and evaluated after staining. There was no increase in the number of polychromatic erythrocytes containing micronuclei. The rate of micronuclei was in the same range as that of the negative control (olive oil) in all dose groups and also fell within the range of the historical control data. The ratio of polychromatic to normochromatic erythrocytes was in the same range as that of the control in all dose groups. No evidence of clastogenicity was found in this study.

In addition, the applicant refers to the results of genotoxicity tests using a commercial preparation of a CLA-rich oil (Clarinol™ G-80), which were summarised in a publication by O'Hagan and Menzel (2003). This CLA-rich oil was tested in five strains of *Salmonella enterica* Typhimurium (TA98, TA100, TA1535, TA1537 and TA102) in the presence and absence of metabolic activation (S9).

According to the authors the test material was not mutagenic up to the highest concentration of 5000 µg/plate. In a second study the CLA-rich oil was tested in human peripheral blood lymphocyte cultures in the presence and absence of metabolic activation. According to the authors the test material did not induce chromosome aberrations at concentrations up to 300 µg/mL.

The Panel concludes that the studies provide no indications that the CLA-rich oil is genotoxic.

8.1.5. Reproductive and developmental toxicity studies

A study on prenatal developmental toxicity using CLA methyl- and ethylester beadlets containing 39.2 % CLA methylesters and 37.7 % CLA ethylesters, respectively, as test materials (composition not further specified) was provided. According to the applicant the CLA esters were produced using the same sources and manufacturing procedures as for Tonalin TG 80. The test materials were administered to pregnant female rats (25 animals per group) from days six to 19 of gestation. CLA methylester beadlets were administered at doses of 100, 300 or 1000 mg/kg body weight per day. One control group received the vehicle alone (0.5 % carboxymethylcellulose in water) and one group received CLA placebo beadlets at a dose of 1000 mg/kg body weight per day. CLA ethylester beadlets were only administered at a dose of 1000 mg/kg body weight per day. On day 20 of gestation the dams were killed and their uteri removed for examination. There were no signs of toxicity in the pregnant rats receiving the test materials and no effects on gestational parameters. Examination of the foetuses did not reveal relevant differences between groups regarding the number of live foetuses per female, the ratio of male/female foetuses and mean foetal weight. External, soft tissue and skeletal examinations showed a low incidence of malformations and variations in all groups which were not dose-related and/or statistical significant and therefore not considered treatment-related. Thus the highest dose of CLA ester beadlets administered was considered as the no-observed adverse effect level (NOAEL) for maternal and prenatal developmental toxicity. (BASF, 2002)

Several reproductive and developmental toxicity studies in rats and pigs demonstrated a lack of adverse effects on maternal food consumption and body weight, litter size, or offspring growth and development following exposure to CLA (0.25 % to 2 % in the diet) throughout gestation, lactation, and/or during a post weaning-period (Bee, 2000; Chin et al., 1994; Poulos et al., 2001). Pup growth, as evidenced by increased body weights, was reported to be enhanced in rats fed 0.25 % and 0.5 % CLA (Chin et al., 1994). Chin et al. (1994) reported significant uptake of CLA in maternal mammary gland tissue and milk; however, this was not accompanied by any adverse effects.

8.1.6. Allergenicity

The applicant did not address potential allergenicity. However, based on the compositional data, its source, the production process, and the data from post marketing and clinical studies, the Panel considers it unlikely that Tonalin® CLA-rich oil elicits allergic reactions.

8.1.7. Risks for generation of cancer

Numerous studies have investigated potential carcinogenicity of CLA mixtures or pure isomers in animal models or cell cultures (Wahle et al., 2004; Kelley et al., 2007). A complex picture arises, depending on the cancer site and the model: both isomers reduced breast and forestomach tumorigenesis; the c9-t11 isomer did not affect the development of spontaneous intestinal or breast tumours, inhibited the cyclo-oxigenase pathway and had no effect on apoptosis, whereas the t10,c12 isomer increased the development of genetically induced mammary and intestinal tumors (though inhibiting *in vitro* the growth of most cancer cell types), inhibited the lipoxigenase pathway and induced the expression of apoptotic genes. In an *in vitro* Caco-2 model (human intestinal cancer cell line), the t10,c12 isomer had an impact on 918 genes involved in cell cycle, cell proliferation and

DNA metabolism, whereas the c9-t11 isomer had no effect on gene expression (Murphy et al., 2007). The relevance of these experimental findings for the safety assessment is unclear. In a cohort of Swedish women, CLA intake from natural sources was not associated with breast cancer after 17 years follow-up. The levels of intake of the naturally occurring c9-t11 isomer in this study are not comparable to those proposed in this application (Larsson et al., 2009).

Taken together with the results of toxicological studies, the Panel concludes that, based on the available evidence, CLA consumption does not raise a concern for cancer risk.

8.1.8. Additional *in vitro* and animal studies

The applicant presented an overview on additional *in vitro* and animal studies which identified areas of discussion in relation to effects observed for CLA.

8.1.8.1 Lipid metabolism parameters

There are several studies on the effects of a 50:50 CLA mixture and the individual c9,t11 and t10,c12 CLA isomers on lipid biology in mice. Wargent et al. (2005) observed a transient rise in triglycerides in mice fed CLA, which normalised after 5 weeks of treatment. Several studies revealed that the c9,t11 isomer either has no effect or significantly decreases serum triglycerides and that the t10,c12 CLA isomer appears to have variable, strain-dependent effects on serum triglycerides in mice (Degrace et al., 2003; Roche et al., 2002; de Roos et al., 2005).

In rabbits a reduction of total and LDL-cholesterol, triglycerides and atherosclerosis was reported after administration of a 50:50 CLA mixture (0.5 g CLA per rabbit per day) for 22 weeks (Lee et al., 1994). Other animal studies suggest that CLA administration inhibits cholesterol-induced atherosclerosis in rabbits (Kritchevsky et al., 2000; Kritchevsky, 2003).

In hamsters 50:50 CLA mixtures did not exhibit consistent effects on lipid metabolism.

8.1.8.2. Hepatic lipid accumulation and liver function

In a 90-day toxicological study in rats using 15 % ClarinolTM in the diet, an increase in serum hepatic enzymes (alkaline phosphatase and alanine aminotransferase) was reported throughout the treatment period, that was reversible during the recovery period; this effect was not seen with the 1 % or the 5 % dose (O'Hagan & Menzel, 2003).

Several studies report that feeding high concentrations of CLA to mice results in reduced adipose tissue accompanied by increased hepatic lipid accumulation (Tsuboyama-Kasaoka et al., 2000; Clement et al., 2002). The proposed mechanisms include (i) activation of peroxisome-proliferator-activated receptor (PPAR) regulated genes, (ii) increased plasma insulin and/or reduced leptin concentrations, and (iii) uptake of CLA into fat stores of the liver.

8.1.8.3 Markers of inflammation

Studies in animals demonstrate that CLA mixtures or individual isomers reduced inflammatory mediators, including TNF-α concentrations in rats (Sisk et al., 2001), decreased mRNA expression of the pro-inflammatory cytokines IL-6 and TNF-α in pigs (Changhua et al., 2005) and attenuated the development of inflammatory lesions in pigs (Hontecillas et al., 2002).

8.1.8.4 Insulin sensitivity and glucose metabolism

Various *in vitro* studies suggest that the t10,c12 CLA isomer is responsible for the loss of body fat and inhibits PPAR-γ, resulting in numerous downstream events leading to the down-regulation of genes related to insulin secretion and action (Granlund et al., 2003; Kang et al., 2003; Kennedy et al., 2005). Chung et al. (2005) observed in co-cultured human adipocytes and stromal vascular cells that the t10,c12 CLA-mediated suppression of insulin-stimulated glucose uptake at 24 h was associated with decreased total and plasma membrane glucose transporter 4 proteins. The authors showed that t10,c12 CLA promotion of NFkappa-B activation and subsequent induction of IL-6 were at least in part responsible for t10,c12 CLA -mediated suppression of PPAR-γ target gene expression and suppression of insulin sensitivity in mature human adipocytes.

Animal studies in rodents and pigs have documented varying effects of both, 50:50 CLA isomer mixtures and the single isomers on insulin sensitivity and glucose tolerance.

Poirier et al. (2005) administered a 1 % isomeric mixture of CLA by gavage to C57BL/6J female mice (approximately 1500 mg/kg body weight per day) for two to 28 days. Concentrations of leptin and adiponectin were reported to sharply decrease after two days of CLA feeding, whereas adipose tissue mass only decreased after six days. Hyperinsulinemia developed at day six and worsened up to day 28, in parallel with increases in hepatic lipid content.

Ohashi et al. (2004) examined the plasma concentrations and mRNA expression levels of several adipocytokines thought to be involved in the regulation of insulin sensitivity in normal C57BL, mildly obese/diabetic KK and morbidly obese/diabetic KKAy mice. CLA oil, 0.5 % (approximately 750 mg/kg body weight per day) and consisting of approximately 60 % of the 1:1 mixture (c9,t11:t10,c12) was administered by gavage for four weeks. An increase in liver weight with excess accumulation of triglycerides and insulin resistance associated with hyperglycaemia and hyperinsulinaemia in the CLA groups compared to placebo were reported. The authors concluded that feeding CLA promotes insulin resistance in obese/diabetic mice compared to normal control mice by inverse regulation of leptin, adiponectin and TNFα.

Bhattacharya et al. (2005) examined the effects of a low concentration of either safflower oil as control (0.5 %) or mixed isomers of CLA (0.4 % or approximately 600 mg/kg body weight per day) for 14 weeks along with treadmill exercise on body composition in male Balb/Cmice fed a high-fat diet (20 % corn oil). CLA consumption reduced fat mass and the fat mass decreased further with CLA and exercise. The effect was accompanied by decreased serum leptin concentrations and lower leptin mRNA expression in peritoneal fat. Serum insulin, glucose, TNFα and interleukin-6 were lower in CLA-fed mice than in controls. No increase in insulin resistance was observed in this study.

Tsuboyama-Kasaoka et al. (2000) investigated the effect of CLA on plasma leptin and insulin concentrations in female C57BL/6J mice. Mice were fed a low-fat control (n=14) diet (containing safflower oil) or a low-fat diet supplemented with CLA (1 % or 1500 mg/kg body weight per day; n=14) for up to eight months. Oral glucose tolerance testing conducted after 17 weeks of CLA supplementation revealed no difference in blood glucose concentrations compared to controls; however, after nine weeks marked insulin resistance was observed.

Clement et al. (2002) investigated the effects of CLA on plasma insulin and leptin concentrations in female C57BL/6J mice. Mice were fed a basal diet (control) or basal diet supplemented with c9,t11- or t10,c12-CLA isomers (0.4 %) for four weeks. Plasma leptin and insulin concentrations were unaffected by c9,t11-CLA treatment. However, plasma leptin concentration was reduced by approximately 47 % and plasma insulin concentration was increased by approximately 900 % in t10,c12-CLA-treated mice. Neither c9,t11 nor t10,c12-CLA-treatments altered the blood glucose concentrations.

Stangl (2000) looked at the effects of 1 %, 3 % and 5 % CLA compared to sunflower oil as control in male Wistar rats for five weeks and found that glucose concentrations were unaffected in the 1 % and 3 % CLA mixtures, but were elevated at the 5 % level compared to control. These data are in accordance with the results obtained by O'Hagan and Menzel (2003) who found that levels of the 50:50 CLA mixture at 15 % raised serum glucose concentrations in rats.

Bouthegourd et al. (2002) reported that the administration of a purified diet augmented with c9,t11 CLA isomer to 0.6 % or 50:50 CLA to 1.2 % to male Syrian hamsters for a period of six or eight weeks resulted in significantly higher plasma glucose concentrations in the group receiving the CLA mixed isomers compared with the other groups. Plasma insulin did not differ significantly between the groups.

Stangl et al. (1999) reported that the administration of basal diets containing 1.0 % of a CLA preparation containing 34.6 % c9,t11 CLA and 18.4 % t10,c12 CLA for a period of six weeks to adult female pigs resulted in non-significant increases in plasma insulin concentrations. Ramsay et al. (2001) found that the administration of 0.25 %, 0.5 %, 1.0 % and 2.0 % CLA preparations (25 % of the c9,t11 CLA and 35 % of the t10,c12 CLA isomer) to male and female crossbred grower pigs (Yorkshire x Landrace) had no effect on serum glucose and insulin concentrations.

Studies in mice using 0.5 % in the diet of either each purified isomer or a CLA mixture also show that t10,c12, but also the CLA mixture, induced insulin resistance, whereas the c9-t12 isomer prevented an increase of insulin resistance (Halade et al., 2009).

In conclusion, studies on the effects of CLA on insulin and glucose metabolism have been conducted both *in vitro* and in animal models. *In vitro* data suggest that the t10,c12 CLA isomer is involved in the regulation of fatty acid synthesis, the reduction of fat in adipocytes and the reduction of insulin sensitivity. Mice seem to be particularly sensitive to the effects of CLA on fat metabolism and are also a sensitive species with regard to insulin sensitivity. The extent of the effects of CLA on insulin resistance and also on markers of cardiovascular risk appears to be species-dependent, and at present results are difficult to extrapolate from animal studies to humans. Therefore the focus of the safety assessment needs to rely mainly on human studies and should include those parameters which were most affected in animal studies.

8.2. Human studies

Dyslipidemia (elevated LDL-cholesterol/elevated total:HDL-cholesterol ratio), hypertension, obesity, and diabetes are well-established and diet-related risk factors for CVD. Individuals with the "metabolic syndrome" or the "insulin resistance syndrome", characterised by hyperglycemia (or diagnosis of type 2 diabetes), abdominal obesity, dyslipidemia (elevated triglyceride and low HDL-cholesterol concentrations) and hypertension are at higher risk of CVD. The pathophysiologic mechanisms known to increase CVD risk in individuals with insulin resistance include formation of advanced glycation end products, hypertension, pro-inflammatory and prothrombotic states, dyslipidemia, and subclinical inflammation. Emerging risk factors for CVD are associated with many different biological systems such as those regulating platelets, coagulation, fibrinolysis, endothelial function, and the inflammatory response (Graham et al., 2007. In addition, enhanced oxidative stress and increased lipid peroxidation occurring either locally in the vessel wall or systemically as been implicated in the pathogenesis of atherosclerosis in humans. Determination of plasma concentrations and urinary excretion of some F(2)-isoprostanes (by immunometric assays or by mass-spectrometry), has been demonstrated to be a reliable approach to the assessment of lipid peroxidation, and therefore of oxidative stress *in vivo*. F2-isoprostanes are increased in association with a number of atherosclerotic risk factors, including cigarette smoking, hypercholesterolemia, diabetes mellitus, and obesity, among others. Recent evidence suggests their quantification may represent an independent

marker of atherosclerotic risk. A reduction in cardiovascular risk factors is associated with a decrease in F2-isoprostanes formation in humans (Minuz et al., 2006; Patrignani et al., 2005; Morrow, 2005).

Several intervention studies have been conducted in humans to investigate the effects of CLA intake on body weight and body fat, which have also assessed the impact of CLA administration on traditional and emerging risk factors for type 2 diabetes and cardiovascular disease such us insulin sensitivity, blood lipids, markers of inflammation, lipid peroxidation and vascular function, are described in the following sections to evaluate the safety of CLA consumption in humans.

8.2.1. Absorption, distribution, metabolism, and excretion (ADME)

Differences in the metabolism and effects of CLA between animal and human studies may be due to methodological differences, CLA dose and nature of the isomers, and species specificity (Plourde et al., 2008).

The few human studies that deal with CLA metabolism confirm a different metabolism of the two major CLA isomers. Only the *c*9,*t*11 isomer is found above the detection limit in plasma of healthy subjects consuming 1.4 g per day of a CLA 50:50 mixture for six months. The serum concentrations were slightly higher, with a large overlap, than the concentrations of this isomer in the serum of regular consumers of dairy products. In addition, CLA supplementation reduces the percentage of saturated fatty acids (34.1 % as compared to 38.6 %) in the plasma of control subjects not consuming dairy products or CLA supplements and the percentage of n-6 fatty acids (26.5 % and 32.1 % respectively), decreasing the n-6/n-3 ratio from 7.8 to 7.24 (Zlatanos et al., 2008).

8.2.2. Effects on insulin sensitivity and glucose metabolism

8.2.2.1. Insulin sensitivity

The hyperinsulinemic euglycemic glucose clamp test directly assesses whole body insulin-mediated glucose utilisation and is considered the gold standard method to assess changes in insulin sensitivity in intervention studies.

Three studies have assessed the effects of the CLA isomers *c*9-*t*11 and *t*10,*c*12, either alone or in combination, using the hyperinsulinemic euglycemic glucose clamp test (Risérus et al., 2004a; Risérus et al., 2002a; Syvertsen et al., 2007). All three studies have been conducted in obese individuals two of them in subjects with metabolic syndrome.

A randomised, double-blind, placebo-controlled trial (Risérus et al., 2004a) for 12 weeks with insulin sensitivity as primary end point was performed in 25 abdominally obese men with signs of the metabolic syndrome, including insulin resistance, receiving daily supplementation with either placebo (3.4 g olive oil, n=12) or CLA (3.4g *c*9,*t*12, isomer, n=13). Insulin sensitivity was assessed using the euglycaemic hyperinsulinemic clamp technique. It was calculated that 13 subjects per group would be needed to detect a mean difference between groups in insulin sensitivity index (*M*/*I*) of 1 unit with a power of 0.80 at a significance level of 0.05 with the use of the unpaired *t* test. In the *c*9,*t*12 CLA isomer group, compared to placebo, baseline-adjusted insulin sensitivity was significantly decreased (-14 %), whereas fasting plasma glucose and insulin did not change significantly.

A randomised, double-blind, placebo-controlled trial (Risérus et al., 2002a) for 12 weeks with change in serum cholesterol as primary end point was performed in 57 abdominally obese men with signs of the metabolic syndrome, including insulin resistance, receiving daily supplementation with either placebo (3.4 g olive oil, n=19), CLA (3.4 g *t*10,*c*12 isomer, n=19) or CLA (3.4 g of a 50:50 mixture of *c*9-*t*11 and *t*10,*c*12 as 4.5 g CLA oil, n=19). Insulin sensitivity was assessed using the euglycaemic

hyperinsulinemic clamp technique. The Panel notes that, based on the calculations above (Risérus et al., 2004a), sample size is adequate to detect a mean difference between groups in insulin sensitivity index (*M*/*I*) of 1 unit with a power of 0.80 at a significance level of 0.05. There was no significant effect on baseline-adjusted stimulated insulin sensitivity or fasting plasma glucose, insulin or HbA_{1c} in the CLA-mix group compared to placebo. In the *t*10,*c*12 CLA isomer group, compared to placebo, baseline-adjusted insulin sensitivity was decreased and fasting plasma glucose was increased. Plasma insulin and HbA_{1c} were unaffected. An additional publication on this study (Risérus et al., 2004b) shows that the intervention with the *t*10,*c*12 CLA isomer increased proinsulin, proinsulin/insulin ratio and C-peptide concentrations compared to controls and that changes in pro-insulin correlated with impaired insulin sensitivity (r = -0.58) independently of insulin changes, C-peptide, glucose, adiponectin and BMI. Hyperproinsulinemia also correlated with adiponectin concentrations. The Panel notes that hyper-proinsulinemia has been proposed as an independent predictor of diabetes and CVD.

As part of a randomised, double-blind, placebo-controlled trial (Syvertsen et al., 2007) with changes in body composition as primary end point in 118 overweight or obese adults receiving daily supplementation with either placebo (4.5 g olive oil) or CLA (3.4 g of a 50:50 mixture of *c*9-*t*11 and *t*10,*c*12) for 6 months, a sub-population of 49 subjects participated in an euglycemic hyperinsulinemic clamp study at baseline and after six months of intervention (26 CLA, 23 placebo). No significant differences in baseline-adjusted insulin sensitivity were observed between the CLA and placebo groups. No effect on fasting serum insulin or glucose concentrations, on HOMA values or on HbA_{1c} and insulin C-peptide were observed. The authors concluded that 3.4 g CLA (mixture of 2 isomers) did not affect glucose metabolism or insulin sensitivity in overweight or obese subjects.

The Panel notes that 3.4 g/CLA given as isomolar combination of the *c*9-*t*11 and *t*10,*c*12 does not appear to significantly affect insulin sensitivity for up to three months in insulin-resistant men (Riserus et al., 2002a) and for up to six months in overweight/obese men and women (Syvertsen et al., 2007). The Panel also notes that both the *c*9-*t*11 (Risérus et al., 2004a) and the *t*10,*c*12 isomers (Risérus et al., 2002a; Risérus et al., 2004b) given alone at doses of 3.4 g per day, could have an adverse effect on insulin sensitivity in obese males with insulin resistance. These changes in insulin sensitivity do not appear to induce changes in fasting plasma glucose or blood glucose control (HbA1c) in the short-term in these non-diabetic subjects. No data are available on the effects of the single isomers on insulin sensitivity in obese women and in normal weight subjects of both sexes, neither on the long-term effects of the CLA mixture.

8.2.2.2. Surrogate markers of insulin sensitivity

Most of the available RCTs on the effects of CLA on insulin-mediated glucose disposal have used surrogate indexes for insulin sensitivity/resistance that are derived from blood insulin and/or glucose concentrations either under fasting conditions (steady state), such as the quantitative insulin sensitivity check index (QUICKI) or the homeostasis model assessment (HOMA), or during an OGTT or a standardized meal test (e.g., insulin sensitivity index (ISI). Whereas steady state indices are quick, inexpensive and reliable for use in large population studies, they all suffer from important limitations, including poor precision, which limits their use in intervention studies. Dynamic tests offer at the same time information about insulin secretion and insulin action.

Surrogate markers of insulin sensitivity using dynamic tests

Besides the study by Syvertsen et al., 2007 discussed above, two studies investigated the effects of CLA on dynamic surrogate markers of insulin-mediated glucose disposal (Eyjolfson et al., 2004; Moloney et al., 2004). The randomised, double-blind, placebo-controlled trial by Eyjolfson et al. (2004) investigating the effects of CLA (3.0 g of a 50:50 mixture of *c*9-*t*11 and *t*10,*c*12, n=10) and placebo (3 g safflower oil, n=6) for eight weeks in 16 non-diabetic sedentary subjects could not be

evaluated by the Panel because no direct comparison between intervention (CLA) and control groups was reported with respect to outcome variables.

A randomised, double-blind, placebo-controlled trial (Moloney et al., 2004) for eight weeks was performed in 32 overweight, diet-controlled diabetic type 2 subjects receiving daily supplementation with either placebo (3.0 g palm oil/soybean oil blend, n=16) or CLA (3.0 g of a 50:50 mixture of *c*9-*t*11 and *t*10,*c*12, n=16). Sample size was estimated by the ability to detect a 20-30 % difference in triacylglycerol concentrations assuming a type I error of 0.05 and a power of 0.9. Insulin sensitivity was assessed by using an oral glucose tolerance test and different insulin sensitivity indexes were calculated from fasting glucose and insulin concentrations (QUICKI, HOMA-IR) and from glucose and insulin concentrations at different time points during the OGTT (ISI composite, oral glucose insulin sensitivity index (OGIS). Baseline-adjusted fasting blood glucose and basal insulin resistance (HOMA-IR) were increased at the end of the CLA treatment, whereas baseline-adjusted stimulated oral glucose insulin sensitivity (OGIS) and ISI were reduced compared to placebo. Fasting serum insulin, basal insulin sensitivity measured as QUICKI and HbA_{1c} remained unchanged.

The Panel notes that insulin sensitivity assessed by dynamic tests (ISI composite and OGIS), which offer at the same time information about insulin secretion and insulin action, may be decreased primarily due to an increase in fasting plasma glucose without compensatory increases in insulin concentrations in type 2 diabetic subjects on treatment with CLA. The Panel also notes that the length of the intervention (eight weeks) is too short to assess changes in blood glucose control using the HbA1c, and that no studies using surrogate markers of insulin sensitivity assessed by dynamic tests are available in normal weight, non-diabetic individuals.

Other surrogate markers of insulin sensitivity

Several studies in normal weight, overweight and obese subjects have assessed the effects of the CLA mixture or the two CLA isomers alone on fasting blood glucose and insulin concentrations, and on the HOMA-IR index derived from them. The Panel notes that, whereas the HOMA-IR index is quick, inexpensive and reliable for use in large population studies, it suffers from important limitations, including poor precision, which greatly limits its use in intervention studies.

A randomised, double-blind, placebo-controlled trial over 16 weeks (Raff et al., 2009) was performed in 81 healthy postmenopausal women (primarily normal weight, but also including some overweight and obese) receiving daily supplementation with either 4.5 g CLA (1:1 mixture of both isomers, n=25), 4.7 g of the *c*9-*t*11 isomer (n=24) or placebo (olive oil, n= 26). No significant treatment-related differences were observed between groups in fasting serum glucose or insulin, or calculated HOMA-IR. *Post hoc*-analyses showed that fasting serum insulin concentrations were greater in the CLA-mix group (34 %) than in the control group (P = 0.02) in subjects with the highest waist circumference tertile only. The Panel notes the small number of subjects per sub-group in the *post hoc*-analyses.

In another study by Taylor et al (2006), a total of 40 overweight subjects (BMI >27 kg/m2) were randomised to receive 4.5 g per day of an isomeric mixture of CLA or 4.5 g per day olive oil for 12 weeks following a double-blind design. No significant treatment-related differences were observed between groups in fasting serum glucose or insulin, or calculated HOMA-IR.

In a further double-blind, placebo-controlled 13-week study, 92 overweight subjects with hypercholesterolemia were randomly allocated to one of three treatment groups which received a placebo dairy product or the same product enriched with 3 g c9, t11 CLA-isomer or 3 g t10, c12 CLA-isomer. No differences between the control and treated groups were noted with regard to changes in fasting blood glucose, insulin or the HOMA-IR values calculated from them (Naumann et al., 2006).

Further studies with CLA administration for 6 – 24 months do not show changes in fasting plasma glucose and insulin concentrations, the calculated HOMA-IR, or in HbA1c concentrations between intervention and control values. The doses used in these studies vary from about 3.4 to 6 g per day CLA and the number of subjects from about 40 to 180 subjects of both sexes, with up to 60 subjects per study group (Gaullier et al., 2004, 2005, 2007) and generally less than 20 subjects per group (Watras et al., 2006; Whigham et al., 2004; Larsen et al., 2006). In two studies, insulin and C-peptide were measured in addition to insulin and glucose. Also these parameters remained unaffected by the CLA treatment (Gaullier et al., 2005, 2007).

A meta-analysis provided by the applicant (Herrmann, 2009) assessed the effect of the 1:1 CLA mixture on fasting blood glucose (16 studies) and insulin (15 studies) concentrations, and on the HOMA-IR index (seven studies), in RCT published between 2000 and 2008 conducted in normal-weight, overweight and obese subjects. Most of the studies described above were included in the meta-analysis. No significant differences between the CLA and control groups on fasting blood glucose or insulin concentrations were observed, neither on the HOMA-IR.

The Panel notes that the data in normal weight and obese, non-diabetic subjects do not support an effect of CLA on fasting blood glucose or insulin concentrations, neither on basal insulin resistance.

Besides the study by Moloney et al. (2004), only one RCT (cross-over design) has investigated the effects of CLA (6.4 g per day, n=16) on blood glucose and insulin concentrations, and on the HOMA-IR index derived from them, in 32 type-2 diabetic subjects. The intervention lasted 16 weeks and safflower oil was used as control (n=16). Fasting glucose concentrations as well as the HOMA-IR significantly increased in the CLA group compared to controls, whereas fasting insulin concentrations did not differ between groups (Norris et al., 2009). The Panel notes that the dose of CLA used in this study is double than proposed by the applicant.

8.2.2.3. Blood glucose control

Some of the studies described in the previous sections report the effects of CLA (1:1 mixture) on blood concentrations of HbA1c, which is a reliable marker of long-term blood glucose control, and show no effect of CLA on HbA1c. However, most studies have been conducted in non-diabetic subjects and for periods of less than three months. The Panel considers that these studies are insufficient to exclude an adverse effect of CLA on long-term blood glucose control, particularly in type-2 diabetic subjects.

8.2.2.4. Conclusions on insulin sensitivity and glucose metabolism

The administration of a 1:1 isomer mixture of CLA to normal weight, overweight and obese non-diabetic subjects does not appear to have medium-term adverse effects on insulin sensitivity and blood glucose control at the proposed conditions of use. For the observed adverse effects on insulin sensitivity in relation to either isomer when administered alone in males with abdominal obesity, the available evidence does not provide an explanation for this observation. With respect to type-2 diabetic subjects, the Panel considers that the evidence provided does not establish the safety of 3-6 g per day of CLA in type 2 diabetic subjects under the proposed conditions of use, since the CLA mixture appears to show an adverse effect on both static (HOMA-IR) and dynamic (ISI, OGIS) surrogate markers of insulin sensitivity as well as on fasting blood glucose compared to the control fat and no studies on long-term blood glucose control (e.g., HbA1c) are available.

8.2.3. Effects on blood lipids and lipoproteins

8.2.3.1. Blood lipids

Many studies using the proposed CLA mixture investigated the effect of CLA on blood lipids in different target populations (normal weight, overweight, obese, metabolic syndrome) with CLA intakes ranging from 0.7 to 6.8 g per day and duration of the intervention ranging from four weeks to two years. The applicant provided a meta-analysis of randomised controlled trials (RCT) addressing the effects of CLA on LDL- and HDL- cholesterol concentrations (26, studies, 1228 subjects, 671 treated and 541 controls), and on blood concentrations of triglycerides (27 studies, 1282 subjects), and on the LDL:HDL-cholesterol ratio (only 14 studies provided suitable data for this analysis). The RCT included were retrieved from a literature search on several databases starting from around 1980 up to 1 March 2009 (Clifton, 2009).

According to this meta-analysis, blood HDL-cholesterol concentrations were reduced significantly in the CLA intervention group (by 5-6 %) compared to placebo (usually olive oil or safflower oil rich in oleic acid). This effect was statistically significant in four of the studies. Removal of one study at a time showed no undue influence of any one study on the overall estimate. Cumulative analysis showed that persistent significance was reached after 13 studies were included in the analysis but the effect size increased progressively with every additional study added. There was no apparent dose/response relationship or influence of the treatment duration. No significant differences were observed for changes in LDL-cholesterol, triglyceride concentrations, or the LDL:HDL-cholesterol ratio.

A more recent meta-analysis of RCT on the effects of the 1:1 CLA mixture on blood lipids (search from 1965 to April 2006) included 25 studies (only 17 RCT already included in the meta-analysis by Clifton, 2009) published between 2000 and 2008 with a total of 646 subjects in the CLA group and 517 subjects in the control group (sample sizes between 16 and 180 subjects, age between 19 and 65 years) with a duration of the intervention between 4 and 52 weeks and CLA doses between 1.1g per day and 4.5g per day (Herrmann, 2009). Only three studies included subjects with BMI <25kg/m^2, whereas 12 studies included only subjects with BMI >27 kg/m^2. Most of the studies used olive oil (n=9), safflower oil (n=3), or sunflower oil (n=4) as control fat. The overall effect of CLA on HDL-concentrations (19 studies) was neither significant in the total population nor in the 12 studies in subjects with BMI >27 kg/m^2. When only the eight studies using olive oil as placebo were considered, HDL-concentrations were significantly lower in the CLA group. Blood concentrations of triglycerides were also higher in the CLA group compared to placebo in the overall population (n =18), in subjects with BMI >27 kg/m^2, and in the studies using olive oil as placebo. No significant differences were observed for changes in LDL-cholesterol concentrations. Dose-response relationships were not assessed in this meta-analysis.

The Panel notes that the results of the two meta-analyses above show no effect of the 1:1 CLA mixture on LDL-cholesterol concentrations or the LDL:HDL-cholesterol ratio at the proposed conditions of use.

The Panel also notes that a significant (although modest) HDL-cholesterol lowering effect and a significant (although modest) triglyceride raising effect of the 1:1 CLA mixture cannot be excluded.

The applicant argues that oleic acid has been shown to increase HDL-cholesterol concentrations and to decrease blood concentrations of triglycerides in the meta-analysis by Mensink et al. (2003) and therefore the use of oleic acid as placebo could have been responsible for the observed effects of CLA on blood lipids. However, the Panel notes that the comparison nutrient in that meta-analysis was carbohydrates, which although they appear to have no effect on LDL-cholesterol concentrations, tend to decrease HDL-cholesterol and to increase triglycerides to a certain extent, whereas most fatty acids

increase HDL-cholesterol and decrease triglyceride concentrations compared to carbohydrates (Mensink et al., 2003). Changes in HDL- and triglyceride concentrations (in the CLA group compared to control fats) of the magnitude reported in the two meta-analyses (Clifton, 2009; Herrmann, 2009) are unlikely to have an impact on coronary heart disease risk (Briel et al., 2009), but these changes in the lipid profile are typically associated with the insulin-resistance syndrome, supporting the notion that long-term changes in insulin sensitivity associated with the chronic administration of CLA cannot be excluded.

The Panel considers that consumption of the 1:1 CLA mixture under the proposed conditions of use has no significant effect on LDL-cholesterol concentrations, and that the magnitude of the changes observed in HDL- and triglyceride concentrations is unlikely to have an impact on coronary heart disease risk.

8.2.3.2. Lipoproteins

In a long-term study, Gaullier et al. (2004) reported a slight but significant increase in lipoprotein(a) concentrations, from 0.24 g/L to 0.28 g/L after 12 months 3 g per day CLA, and 0.30 g/L after 24 months (Gaullier et al. 2005). The Panel notes that the values remain below the generally accepted cut-off value of 0.30 g/L. Gaullier et al. (2007) also noted an increase in lipoprotein(a) from 0.364 g/L to 0.386 g/L in 55 subjects taking 3.4 g per day CLA for six months; a rise of similar magnitude was observed in the control group (+ 0.023 g/L as compared to + 0.028 g/L in the treated group).

8.2.4. Markers of lipid peroxidation

8.2.4.1. $F_{2\alpha}$ isoprostanes

Enhanced oxidative stress and increased lipid peroxidation occurring either locally in the vessel wall or systemically is implicated in the pathogenesis of atherosclerosis in humans. Determination of plasma concentrations and urinary excretion of some F(2)-isoprostanes (by immunometric assays or by mass-spectrometry), has been demonstrated to be a reliable approach to the assessment of lipid peroxidation, and therefore of oxidative stress *in vivo*. F2-isoprostanes are increased in association with a number of atherosclerotic risk factors, including cigarette smoking, hypercholesterolaemia, diabetes mellitus, and obesity, among others. In addition, recent evidence suggests their quantification may represent an independent marker of atherosclerotic risk. A reduction in cardiovascular risk factors is associated with a decrease in F2-isoprostanes formation in humans. The potential contribution of these compounds to the pathophysiology of the vascular damage and atherosclerosis has not yet been defined (Minuz et al., 2006; Patrignani et al., 2005; Morrow, 2005).

Nine RCTs have assessed the effects of CLA (given as the only intervention and compared to a placebo fat) on lipid peroxidation using plasma (Taylor et al., 2006; Basu et al., 2000b) or urinary F2-isoprostanes (Basu et al., 2000a; Basu et al., 2000b; Etzdorf, 2008; Risérus et al., 2002b; Risérus et al., 2004c; Raff et al., 2008; Tholstrup et al., 2008). Most of the studies have used radioimmunoassay for analysis of F2- isoprostanes with a reported very low cross-reactivity (usually <1 %) with other metabolites of arachidonic acid.

Plasma $F_{2\alpha}$ isoprostanes

Basu et al. (2000b) performed a RCT to investigate the effects of consuming the 1:1 CLA isomer mix (4.2g per day) on plasma 8-*iso*-prostaglandin $F_{2\alpha}$ in 28 healthy men using olive oil as placebo (n =25) for three months. Data were available only in 34 out the 53 subjects completing the study. Plasma 8-*iso*-prostaglandin $F_{2\alpha}$ significantly increased in the CLA groups compared to controls. In another

study, a total of 40 overweight subjects (BMI >27 kg/m2) were randomised to receive 4.5g per day of an isomer mixture CLA or 4.5g per day olive oil for 12 weeks following a double-blind design. Plasma F2-isoprostanes significantly increased after CLA supplementation compared to olive oil (Taylor et al., 2006).

Urinary $F_{2\alpha}$ isoprostanes

In the study by Risérus et al., (2004a), 25 abdominally obese men with the metabolic syndrome were randomised to consume either 3 g per day of the *c*9,*t*12 CLA isomer or placebo (olive oil) for 12 weeks. Urinary 8-*iso*-prostaglandin $F_{2\alpha}$ was used as a marker of nonenzymatic lipid peroxidation, whereas urinary 15-*keto*-dihydroprostaglandin $F_{2\alpha,}$ a major metabolite of prostaglandin $F_{2\alpha}$, was used as a marker of enzymatic lipid peroxidation. 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ is also a powerful marker of systemic inflammation. Urinary 8-*iso*-prostaglandin $F_{2\alpha}$ and 15-*keto*-dihydroprostaglandin increased significantly in the CLA group compared to placebo. In another study from the same group (Risérus et al., 2002b), a total of 60 abdominally obese men with the metabolic syndrome were randomised to consume either 3.4 g/day CLA (isomer mixture), 3.4 g/day of the purified CLA isomer *t*10,*c*12, or placebo (olive oil) for 12 weeks. Urinary isoprostanes (8-iso $PGF_{2\alpha}$ and 15-*keto*-dihydroprostaglandin $F_{2\alpha}$) significantly increased in the CLA groups compared to placebo, and the increase was significantly higher in the CLA isomer *t*10,*c*12 group than in the group receiving the CLA isomer mixture. Changes in urinary 8-iso $PGF_{2\alpha}$ significantly correlated with changes in insulin resistance and with changes in C-reactive protein even after adjustment for all other variables including smoking. Similarly, in a study by Basu et al. (2000a), a significant increase of both 8-*iso*-prostaglandin $F_{2\alpha}$ and 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ in urine was observed after one month of daily CLA intake (4.2 g per day, n=14) as compared to the control group (olive oil, n=10) in middle age men. The same group (Basu et al., 2000b) performed another RCT to investigate the effects of consuming the 1:1 CLA isomer mix (4.2g per day) on urinary8-*iso*-prostaglandin $F_{2\alpha}$ and 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ in 28 healthy men using olive oil as placebo (n =25) for three months. A significant increase was observed in both urinary8-*iso*-prostaglandin $F_{2\alpha}$ and 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ in the CLA group compared to placebo. In the study by Raff et al., (2008), 38 healthy young men were randomised to consume either 115 g per day of CLA-butter (5.5 g per day of CLA oil, both isomers) or CLA-free butter for 5 weeks. Urinary8-*iso*-prostaglandin F2 was used as a marker of nonenzymatic lipid peroxidation and significantly increased in the CLA group compared to placebo. Tholstrup et al. (2008) assessed the effects of 5.5 g per day of either the CLA mixture (4.6 g per day CLA), the CLA *c*9,*t*11 isomer (5.1 g per day) or olive oil for 16 weeks in 75 post-menopausal women (about 25 per group). Urinary 8-*iso*-prostaglandin $F_{2\alpha}$ significantly increased in the CLA groups compared to placebo, and significantly more in the CLA mix group than in the *c*9,*t*11 CLA isomer group.

The mechanism by which CLA could increase plasma and urinary concentrations of $F_{2\alpha}$ isoprostanes has been investigated *in vitro* (Stachowska et al., 2008) and *in vivo* (Smedman et al., 2004). Smedman et al., (2004) randomised 60 men and women to take a cyclo-oxygenase (COX)-2 inhibitor (rofecoxib, 12 mg per day), alpha-tocopherol (200 mg per day) or no treatment (control) for two weeks. The three groups were subsequently randomised to consume either the 1:1 CLA mix (3.4 g per day) or the t10,c12 CLA isomer (4g per day) for four weeks in addition to the basal treatment. Plasma and urinary concentrations of 8-*iso*-prostaglandin $F_{2\alpha}$ and 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ significantly increased after CLA administration, with a significantly larger increase in the t10,c12 CLA isomer group than in the CLA mixture group. The increase of plasma 8-*iso*-prostaglandin $F_{2\alpha}$ was significantly higher with the t10,c12 CLA isomer than with the 1:1 CLA mix (3.4 g per day). Plasma concentrations of isoprostanes were not affected by the supplementation with alpha-tocopherol, but no increase was observed when both the CLA mix and the t10,c12 CLA isomer were consumed together with the COX-2 inhibitor. Treatment with the COX-2 inhibitor significantly suppressed the increase of 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ in the t10,c12 CLA isomer group. No other changes were

observed due to either COX-2 inhibitor or alpha-tocopherol treatment in any of the CLA groups with respect to urinary isoprostanes.

The Panel notes that plasma and urinary F2-isoprostanes consistently and markedly increase with the administration of the CLA mixture in humans. An increase in markers of nonenzymatic (8-*iso*-prostaglandin $F_{2\alpha}$) lipid peroxidation could partly result from a reduced catabolism of isoprostanes (i.e., 8-*iso*-prostaglandin $F_{2\alpha}$) in peroxisomes due to a competition with CLA (Iannone et al., 2009). However, the extent to which this mechanism may contribute to increased plasma and urinary concentrations of isoprostanes in subjects consuming CLA has not been quantified in humans. A contribution of CLA to increased lipid peroxidation and systemic inflammation (i.e, 15-*keto*-dihydroprostaglandin $F_{2\alpha}$) is suggested by the data presented.

Plasma concentrations of 8-*iso*-prostaglandin $F_{2\alpha}$ isoprostanes observed after CLA administration in the studies described are generally >1.0 nmol/mmol creatinine (Basu et al., 2000a and 2000b; Risérus et al., 2002b; Smedman et al., 2004) or >1.0 μg/L (Tholstrup et al., 2008; Raff et al., 2008), which is clearly above mean basal values recently reported in 588 healthy subjects from three EU countries including smokers (from 0.17 to 0.28 nmol/mmol creatinine) (Basu et al., 2009), where only eight subjects had values > 0.60 nmol/mmol creatinine and only two subjects has values > 1.0 nmol/mmol creatinine.

8.2.4.2. Other markers of lipid peroxidation

As stated by the applicant, CLA supplementation in animal models has not been shown to affect other measures traditionally used to assess lipid peroxidation, such as TBARS, MDA, oxidation lag time of LDL particles *ex vivo* or muscle antioxidant enzymes (e.g., catalase, glutathione peroxidase). However, the Panel considers that, when used alone, and not simultaneously with urinary F-2 isprostanes, TBARS, MDA, oxidation lag time of LDL particles *ex vivo* or muscle antioxidant enzymes are not reliable markers of lipid peroxidation (EFSA, 2010b).

In humans, Basu et al., (2000b) did not observe a statistically significant change in MDA assessed by HPLC and fluorescence detector after CLA administration, and Etzdorf (2008) did not observe an increase in serum concentrations of oxidised LDL measured by the ELISA method. The Panel notes that the data presented on markers of lipid peroxidation in humans other than isoprostanes are too limited to draw any conclusions.

8.2.4.3. Conclusion on markers of lipid peroxidation

The Panel considers that the data presented suggest an increase in lipid peroxidation as a result of cyclo-oxygenase-mediated inflammation and oxidative stress associated with the consumption of the 1:1 CLA mixture in humans at the conditions of use proposed by the applicant.

8.2.5. Markers of systemic (subclinical) inflammation and adipokines

A consistent increase in cyclooxygenase-mediated inflammation (i.e., assessed by concentrations of 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ in urine) has been observed in association to CLA consumption in humans (see section 8.2.4).

Increased concentrations of CRP were observed in a few studies (Risérus et al., 2002b; Smedman et al., 2005; Watras et al., 2006; Gaullier et al., 2007) but not in other studies with similar CLA exposure (Moloney et al., 2004; Tricon et al., 2004; Naumann et al., 2006; Ramakers et al., 2005; Song et al., 2005). In the study by Risérus et al. (2002b) CRP was increased significantly in subjects ingesting the t10, c12 CLA-isomer (3.4 g per day) but only insignificantly in subjects consuming the 1:1 mix of the

c9, t11 and t10, c12 CLA-isomers. No effects of the t10, c12 CLA-isomer (up to 2.5 g per day) on CRP were observed by Tricon et al. (2004).

In a recent meta-analysis presented by the applicant (Herrmann, 2009), the 1:1 CLA mixture showed a significant increase in CPR concentrations compared to the control group (six studies considered). Five of the studies were performed in subjects with BMI >27 kg/m^2. In that meta-analysis, the effects of CLA on other markers of systemic (namely TNF-alpha, IL-6) and endothelial (namely ICAM-1, VCAM-1) inflammation could not be assessed due to the small number of studies available, from which the results were inconsistent.

Adiponectin was unaffected by CLA treatment (Gaullier et al., 2007; Taylor et al., 2006). TNF-alpha and IL-6 were generally not affected by CLA treatment (Moloney et al., 2004; Risérus et al., 2002b; Taylor et al., 2006; Watras et al., 2006; Gaullier et al., 2007; Smedman et al., 2005).

The Panel notes that none of these studies has been designed to address the effects of CLA on subclinical inflammation and that the results are inconsistent. However, an increase in 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ and possibly in CRP concentrations has been observed following CLA intake.

8.2.6. Vascular function

The applicant acknowledges that the vascular endothelium is a key regulator of vascular homeostasis and it plays a central role throughout the atherosclerotic disease process. Few studies evaluated a limited number of markers of endothelial function (Taylor et al., 2006; Raff et al., 2006; Watras et al. 2007; Pfeuffer et al., 2007).

One study investigated the effects of CLA on endothelial function by flow-mediated dilation (FMD) measurements (Taylor et al., 2006). A total of 40 overweight subjects (BMI >27 kg/m^2) were randomised to receive 4.5 g per day of an isomeric mixture CLA or 4.5 g per day olive oil for 12 weeks in a double-blind design. A 2-tailed P value <0.025 was considered significant for FMD measurements after Bonferroni correction (secondary outcome). FMD significantly decreased in the CLA group compared to placebo.

In a recent study, Pfeuffer et al (2009, unpublished) report no effect of the CLA mixture (1:1, 4.5 g per day, n=21) on fasting and post-prandial endothelial function assessed by finger volume pulse waves (PAT index) as compared to a safflower oil control (4.5g per day, n=21) in overweight and obese males, despite the significant reduction in body weight observed in the CLA group during the four weeks of intervention (-1.43 kg vs 0.04 kg).

Another study presented by the applicant addressed the effects of CLA-rich butter (5.5 g per day CLA oil, both isomers, 4.6 g pre day CLA, n=15), vaccenic acid (VA)-rich butter (3.6 g per day, n=21)) and butter low in VA and CLA (n=19) for five weeks in healthy men (Raff et al., 2006). Changes in isobaric arterial elasticity measured by an oscillometric method were not different between groups. The Panel notes that CLA was compared in this study with vaccenic acid (a *trans* fatty acid) and with butter (rich in saturated fatty acids), whose effects on endothelial function and arterial compliance are not neutral (Mozaffarian et al., 2004 and 2009; Siddiqui et al., 2008; Harvey et al., 2008).

The Panel considers that the data presented suggest a possible adverse effect of the CLA 1:1 isomer mixture on vascular function.

8.2.7. Vascular damage

Possible adverse effects of CLA consumption on lipid peroxidation, subclinical inflammation and vascular function have been described in sections 8.2.4, 8.2.5., and 8.2.6, respectively.

Impaired endothelial function, subclinical inflammation, and increased lipid peroxidation have all been associated with an increased risk of CVD (Graham et al., 2007; Minuz et al., 2006; Patrignani et al., 2005; Morrow, 2005). Although the potential contribution of these factors to the pathophysiology of vascular damage and atherosclerosis has not been defined yet, any adverse effects on the arterial wall are to be expected in the long-term.

No data on the effects of CLA intake on vascular damage and atherosclerosis have been provided in humans.

8.2.8. Liver function and liver steatosis

As stated in section 8.1.6.2, an increase in serum liver enzymes and increased fat accumulation in the liver have been described in rats (O'Hagan & Menzel, 2003) and mice (Tsuboyama-Kasaoka et al., 2000; Clement et al., 2002) following the administration of feedings containing high doses of CLA. The proposed mechanisms for these effects include the activation of PPAR-regulated genes, an increase in plasma insulin and/or reduced leptin concentrations, and the uptake of CLA into the liver fat stores.

A case report of a severe toxic hepatitis, confirmed by elevated serum transaminases and liver biopsy, occurring in a healthy woman, without prior antecedent, 14 days after starting CLA supplementation (mixture of c9,-t11 and t10,c12, 3x3 g daily) has been published (Ramos et al., 2009); serum enzymes returned to normal within two months after CLA discontinuation. After elimination of viral and other possible toxic causes, the causality by CLA was scored as probable. The Panel notes that the subject consumed three times the proposed dose and that this is the only report that has been published more than ten years after the commercialisation of CLA dietary supplements with annual sales in Europe which have been reported to be 170 – 180 tons CLA for the years 2006 - 2008 (EFSA, 2010a).

A human RCT investigating the effects of the CLA isomers *c*9-*t*11 and *t*10,*c*12 consumed alone at doses of 1.5 g per day and 3 g per day each for four months compared to a high oleic sunflower oil (3 g per day, placebo) on body composition in overweight males and females (15 subjects in the placebo and the two CLA *t*10,*c*12 randomisation arms, 18 subjects in the two CLA *c*9-*t*11 randomisation arms) reports no changes in any of the study groups in liver size or liver ultrastructure during the intervention assessed by ultrasound (Malpuech-Brugere et al., 2004). In a more recent study by Iwata et al. (2007), 60 overweight and obese volunteers were randomised to consume 3.5 g per day of the CLA mixture, 6.8 g per day of the CLA mixture or placebo (safflower oil, 10.8 g per day) for 12 weeks. Liver steatosis was assessed by ultrasound and no significant changes were observed between groups during the study. The Panel notes that, whereas ultrasonography is an established screening technique for detecting moderate or severe fatty infiltration of the liver, it is not a reliable method for the quantification of fat stores.

Some human intervention studies report on the effects of CLA on hepatic enzymes. Out of seven studies including measures of liver function (Gaullier et al., 2004 and 2005; Berven et al., 2000, Whigham et al., 2004; Watras et al., 2006, Malpuech-Brugere et al., 2004), only Gaullier et al. (2004 and 2005) reported a significant increase in ASAT in the CLA group compared to the control group. In the study by Gaullier et al. (2005) with up to 24 months of CLA consumption (3.4 g per day), two out of the 134 subjects had above normal increased activities of both transaminases (ASAT and ALAT) at the end of the study, which returned to baseline levels four weeks after of ending the consumption of CLA. In the publication by Iwata et al. (2007) described above, ALAT concentrations

significantly increased in the 6.8 g per day CLA group compared to placebo, and GGT significantly increased in the 3.5 and 6.8 g per day CLA groups compared to placebo when only subjects with normal enzyme activities at baseline were taken into account. The Panel notes the post-hoc nature of the analysis and the small number of subjects included.

The Panel notes that most of the intervention studies with CLA conducted in humans do not report adverse effects on liver function enzymes at the proposed conditions of use.

The Panel considers that, whereas no acute or mid-term (up to six months) effects can be expected in humans in relation to the consumption of CLA on markers of liver function.

8.2.9. Impact on milk secretion and content

Some studies in pigs (Harrell et al., 2002, Poulos et al., 2004) and one human study in 9 lactating women (Masters et al., 2002) raised concern that CLA can decrease milk fat content. Due to the cross over design of the human study (CLA effect was not studied during the same period of lactation) and the low number of subjects, the relevance of such a finding is unclear. More recent studies performed either with CLA mixtures (Mosley et al., 2007; 50:50 CLA 2 or 4 g per day) in 36 lactating women or with pure isomers in 12 lactating women (Hasin et al., 2005; 750 mg per day of each isomer) showed no effect on milk composition. Using naturally occurring CLA in cheese Ritzenthaler et al. (2005) also found no effect on milk in a study enrolling 36 lactating women for three weeks, but the doses (160 or 346 mg per day of the c9-t11 isomer) were lower than those proposed by the applicant. Total CLA content of milk fat is increased with CLA consumption both in animals and in women (Moutsioulis et al., 2008). In animal studies, CLA in milk did not result in impairment of the growth of the progeny (Poulos et al., 2004).

8.2.10. Adverse events

Clinical studies, generally performed in a small number of subjects, only showed rare and moderate adverse events, with the same frequency in the control and treated groups. Some of these events, especially, in the gastrointestinal tract, have been attributed to the ingestion of the gelatine capsules rather than to CLA itself. In a post-market survey on CLA containing foods, consumers in Spain reported adverse effects in approximately 2 % (25 cases among 1235 consumers), mostly digestive symptoms such as diarrhoea, nausea and dyspepsia (Anadón et al., 2006). Owing that most consumers were under pharmacological treatment for different conditions at the time of CLA supplementation, causality remains unclear.

DISCUSSION

The applicant provided sufficient information regarding the production, the composition, the stability, and the estimated intake of Tonalin® TG 80 CLA-rich oil.

In vitro data suggest that the *t*10,*c*12 CLA isomer is involved in the regulation of fatty acid synthesis and mediating suppression of insulin sensitivity in mature human adipocytes. This isomer has also been reported to be responsible for undesirable effects on fat and glucose metabolism *in vivo*. Mice seem to be particularly sensitive to the effects of CLA on fat and glucose metabolism. However the extent of the effects of CLA on insulin sensitivity, but also on hepatic fat accumulation and markers of cardiovascular risk appears to be species-dependent. The focus of the safety assessment therefore relies mainly on human studies. The available data from non-human studies do not indicate a risk for genotoxicity, reproductive toxicity, carcinogenicity or allergenicity.

The administration of the 1:1 isomer mixture of CLA to normal weight, overweight and obese non-diabetic subjects does not appear to have adverse effects on insulin sensitivity, blood glucose control or liver function at the proposed conditions of use for up to six months. Effects of CLA consumption over periods longer than six months on insulin sensitivity and liver steatosis have not been adequately addressed in humans. With respect to type-2 diabetic subjects, the evidence provided does not establish the safety of CLA under the proposed conditions of use, since the CLA 1:1 isomer mixture appears to adversely affect both static (HOMA-IR) and dynamic (ISI, OGIS) surrogate markers of insulin sensitivity as well as fasting blood glucose and no studies on blood glucose control (e.g., HbA1c) are available for periods of consumption beyond eight weeks. Under the proposed conditions of use, CLA has no effect on LDL-cholesterol concentrations or the LDL:HDL-cholesterol ratio, and the magnitude of the changes observed in HDL- and triglyceride concentrations are unlikely to have an impact on CVD risk. However, the observed increase in plasma and urinary concentrations of isoprostanes, which may indicate an increase in lipid peroxidation, and the increase in some markers of subclinical inflammation (i.e., 15-*keto*-dihydroprostaglandin $F_{2\alpha}$ and possibly CRP) associated with CLA consumption, together with the limited data available on the effects of CLA on vascular function may indicate a potential for vascular damage (i.e., atherosclerosis) in the longer term. No data on effects of CLA intake on the arterial wall have been provided in humans.

The Panel considers that CLA consumption does not appear to have adverse effects on insulin sensitivity, blood glucose control or liver function for up to six months, and that observed effects on blood lipids are unlikely to have an impact on CVD risk. Long-term effects of CLA intake on insulin sensitivity and the arterial wall have not been adequately addressed in humans. The evidence provided does not establish the safety of CLA consumption by type-2 diabetic subjects under the proposed conditions of use.

CONCLUSIONS

The Panel concludes that the safety of Tonalin® TG 80, an oil with approximately 80 % CLA 1:1 mixture of *t*9,*c*11 and *t*10,*c*12 isomers, has been established for the proposed uses at intakes of 4.5 g per day (corresponding to 3.5 g CLA), for up to six months. The safety of CLA consumption for periods longer than six months has not been established under the proposed conditions of use. The safety of CLA consumption by type-2 diabetic subjects has not been established.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the European Commission to the European Food Safety Authority with the request for an opinion on the safety of the safety of 'CLA (Conjugated Linoleic Acid) - rich Oil' as food ingredient.
2. Application for the approval as a novel food of Tonalin® TG 80 CLA-rich Oil under Regulation (EC) No 258/97 for the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients.
3. Initial assessment report carried out by Spain: Initial Assessment of the Application for the Authorisation of Tonalin™ CLA-Rich Oil under *Article 4* of the Novel Food Regulation (EC) No. 258/97.
4. Member States' comments.
5. Response to by the applicant to the initial assessment report and the additional Member States comments.

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GLOSSARY AND ABBREVIATIONS

ALAT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
ASAT	Aspartate Aminotransferase
BMI	Body Mass Index
CLA	Conjugated Linoleic Acid
COX	Cyclo-Oxygenase
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DNA	Deoxyribonucleic Acid
FMD	Flow-Mediated Dilation
FOB	Functional Observational Battery
GGT	Gamma-Glutamyl Transferase
GM	Genetically modified
HACCP	Hazard Analysis Critical Control Points
HDL	High Density Lipoprotein
HF	High-fat
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
ICAM	Inter-Cellular Adhesion Molecule 1
IL	Interleukine
ISI	Insulin Sensitivity Index
LDL	Low Density Lipoprotein
MDA	Malondialdehyde
mRNA	messenger Ribonucleic Acid
NOAEL	No Observed Adverse Effect Level
OGIS	Oral Glucose Insulin Sensitivity Index
OGTT	Oral Glucose Tolerance Test
PPAR	Peroxisome-Proliferator-Activated Receptor

QUICKI	Quantitative Insulin Sensitivity Check Index
RCT	Randomised Controlled Trial
SCF	Scientific Committee on Food
SD	Sorbitol Dehydrogenase
TBARS	Thiobarbituric Acid Reactive Substances
TNF	Tumor Necrosis Factor
UHT	Ultra-High Temperature
VA	Vaccenic Acid
VCAM	Vascular Cell Adhesion Molecule