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LIPID NUTRITION

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MAR 17 2004
OFFICE OF
FOOD ADDITIVE SAFETY

March 10, 2004

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Notice of a GRAS Exemption Claim
for Clarinol™ G-80 (vegetable oil conjugated linoleic acid ("CLA") preparation)

To Whom It May Concern:

The Lipid Nutrition division of Loders Croklaan B.V. hereby provides the following information pursuant to proposed 21 CFR 170.36(c)(1) (62 Fed. Reg. 18938, 18961; April 17, 1997):

GRAS Exemption Claim: Loders Croklaan B.V. ("Loders Croklaan") hereby claims that Clarinol™ G-80 (vegetable oil conjugated linoleic acid ("CLA") preparation), intended for use in yogurt products (and similar non-standardized products) at 1.3%, milk-based meal replacements at 0.7%, frozen or shelf-stable plate meals (with meat, poultry, or fish as a major ingredient) at 0.4%, bars (e.g., protein, breakfast, meal replacement, granola, power) at 4.0%, and low-caloric and reduced caloric salad dressings (and similar non-standardized products) at 2.7%, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Loders Croklaan has determined that this ingredient is generally recognized as safe ("GRAS") for such use, using scientific procedures

(i) ***Name and Address of Notifier***: Dr. Andreas Menzel
R&D Program Manager
Lipid Nutrition, Loders Croklaan B.V.
P.O. Box 4
1520 AA Wormerveer
The Netherlands
Tel 31 (0)75 629 2491
Fax 31 (0)75 629 2217

LIPID NUTRITION

(ii) ***Common or Usual Name of the Substance*:** Vegetable oil conjugated linoleic acid ("CLA") preparation.

(iii) ***Applicable Conditions of Use*:** For functional uses in certain foods, as described above.

(iv) ***Basis for the GRAS Determination*:** Scientific procedures.

(v) ***Statement of Availability*:** The data and information that are the basis for Loders Croklaan's GRAS determination are available for FDA review and copying at reasonable times at the offices of Kleinfeld, Kaplan and Becker, LLP, 1140 Nineteenth Street, NW, Washington, DC 20036 (Daniel R. Dwyer, Tel 202 223 5120), and such data and information will be sent to FDA upon request.

Additional required information, including a comprehensive summary of the data relied on to establish safety, is enclosed.

Sincerely,

Dr. Andreas Menzel

enclosure

RECEIVED
MAR 1 2004
OFFICE OF
FOOD ADDITIVE SAFETY

GRAS Notification

for

CLARINOL™ G-80 (VEGETABLE OIL CONJUGATED LINOLEIC ACID PREPARATION)

Loders Croklaan B.V.
Lipid Nutrition
Hogeweg 1
1521 AZ Wormerveer
The Netherlands

Loders Croklaan contact:
Dr. Andreas Menzel
R&D Program Manager
Loders Croklaan B.V.
Tel 31 (0)75 629 2491
Fax 31 (0)75 629 2217

Alternative contact:
Daniel R. Dwyer
Kleinfeld, Kaplan and Becker, LLP
1140 Nineteenth Street, NW
Washington, DC 20036
Tel 202 223 5120
Fax 202 223 5619

March 10, 2004

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List of abbreviations

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
ALAT	alanine aminotransferase
AlkP	alkaline phosphate
ALT	alanine aminotransferase
ApoB	apolipoprotein B
ASAT	aspartate aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
CLA	conjugated linoleic acid
CRP	C-reactive protein
EDI	estimated daily intake
EU	European Union
FFA	free fatty acids
GLP	good laboratory practice
HDL	high density lipoprotein
HOMA	Homeostasis Model Assessment (for insulin sensitivity)
HOSF	high oleic sunflower oil
IL-6	interleukin 6
ISI	insulin sensitivity index
LCD	low calorie diet
LDL	low-density lipoprotein
MUFA	mono-unsaturated fatty acids
NEFA	non-esterified fatty acids
NIDDM	non-insulin dependent diabetes mellitus
NOAEL	no observed adverse effect level
NS	not significant
OECD	Organization for Economic Co-operation and Development
OGTT	oral glucose tolerance test
PGE	prostaglandin E
PGF2α	prostaglandin F 2α
PPAR	peroxisome proliferator activated receptor
QUCKI	Quantitative Insulin Sensitivity Check Index
SD	standard deviation

SEM	standard error of the mean
SFA	saturated fatty acids
T4	thyroid hormone
TC	total cholesterol
TG	triglyceride
TNFα	tumor necrosis factor α
TSH	thyroid stimulating hormone
VLDL	very low density lipoprotein
WBC	white blood cells

Introduction

Pursuant to 62 Fed. Reg. 18938, 18960 (April 17, 1997) (proposed 21 CFR 170.36), Loders Croklaan B.V. ("Loders Croklaan") hereby claims that Clarinol™ G-80 (vegetable oil conjugated linoleic acid ("CLA") preparation), intended for use in yogurt, milk-based meal replacements, frozen or shelf-stable plate meals (with meat, poultry, or fish as a major ingredient), bars (e.g., protein, breakfast, meal replacement, granola, power), and low-calorie and reduced calorie salad dressings (and similar non-standardized versions of standardized foods), at levels ranging from 0.3 to 3.0 percent CLA (as described in section 4.4), is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Loders Croklaan has determined that this ingredient is generally recognized as safe ("GRAS") for such use, using scientific procedures.

This document accompanies the GRAS exemption claim required by proposed 21 CFR 170.36(c)(1) and provides the detailed information required by proposed 21 CFR 170.36(c)(2), (3), and (4). This GRAS determination is based on generally available and accepted scientific data, information, methods and principles, and is corroborated by information that is unpublished or otherwise not generally available. Loders Croklaan's conclusion is supported by the views of a panel of independent experts qualified by scientific training and experience to evaluate the safety of substances added to food ("Expert Panel"). Accordingly, this GRAS determination meets the requirements of section 201(s) of the Federal Food, Drug, and Cosmetic Act, 21 CFR sections 170.3 and 170.30, and the amendments to these rules proposed at 62 Fed. Reg. 18960.

Clarinol™ G-80 is an oil derived from safflower or other vegetable oil with a high proportion of two conjugated linoleic acid (CLA) isomers, specifically 9c,11t-CLA and 10t,12c-CLA, attached to glycerol. Loders Croklaan Lipid Nutrition currently sells Clarinol™ G-80 as an ingredient to the dietary supplement industry worldwide.

Conjugated linoleic acid (CLA) triglyceride is a natural food constituent that has emerged with the progress in analytical technology. CLA has always been present in human food, but was not detectible with former available analytical methods.

CLA was first discovered about 20 years ago, when Pariza and coworkers found that ground beef contains an anti-mutagenic factor that consisted of a series of conjugated dienoic isomers of linoleic acid (Ha et al., 1987; Pariza et al., 2001).

Later it was found that CLA is present in a number of foods, of which ruminant derived food such as beef, milk and cheese appear to have the highest content. Comparable amounts of CLA have been recorded for turkey meat, while vegetable oils, pork, and chicken meat contain much lesser amounts of CLA (Chin et al., 1992). The estimated human intake of CLA from these sources is approximately 200 mg/day (Ritzenthaler et al., 2001).

CLA would be added to food for its functional effect(s), intended for uses consistent with applicable U.S. law. The following describes general scientific information about potential functional effects.

Studies of CLA suggest that it may have a range of valuable properties. These include delaying the onset of type II diabetes, inhibiting and regressing atherosclerosis, improving the mineralization of bone, and modulating the immune system (reviewed by Belury, 2002). The anti-carcinogenic effects of CLA have been well-established, leading to the recognition of CLA by the National Academy of Sciences as the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals (Bauman, 2000).

One of the most widely reported effects of CLA is its influence on body composition. Body fat mass is decreased concomitantly with an increase in lean body mass (water, bones, musculature). Many studies have demonstrated that mice are extremely sensitive to these body fat mass reducing effects of CLA (Tsuboyama-Kasaoka et al., 2000; West et al., 2000). Low levels of CLA in the diet almost completely ablated fat tissue in mice. In humans, the effects of CLA on reducing body fat mass have also been demonstrated, although body fat loss was more gradual and moderate (Mougios et al., 2001; Smedman and Vessby, 2001; Thom et al., 2001). A dose-response study by Blankson et al. (2000) suggested that CLA intake required to obtain measurable changes in body composition in humans is in the range of about 3.0 g/day.

This level is higher than the current daily CLA intake of approximately 200mg from natural dietary sources. As stated above, natural dietary sources of CLA consist primarily of ruminant-derived products. The levels of CLA in these products are subject to change. CLA production in ruminants depends on the type of animal feed and shows seasonal variation. The concentration of CLA in milk and meat of cows grazing outside in summer may be about 2-5 times the concentration in milk produced by cows fed dry food (Dhiman et al., 1999; Lock and Garnsworthy, 2003). In the world of today, in-house breeding of cows and feeding them dry feed is

progressively common. With the expansion of this practice, CLA intake via the human diet may have been reduced over time. In addition, with the increased use of low-fat dairy products, CLA consumption may have decreased.

Experiments to increase the production of CLA in ruminants and their products by giving them specifically adapted feed are taking place in more than one location in the world (e.g., Bauman et al., 2000). The objective of these experiments is to find ways to increase the level of CLA in the human diet without intentional addition of CLA to food. However, the economic prospect of such practice is still doubtful. In addition, the level of CLA resulting from such practice would still be relatively low, and variable.

The intake of CLA expected to have a potentially beneficial functional effect on body composition is 10-15 times the average dietary intake today, and 2 times the highest daily intake recorded (McGuire and McGuire, 2002). Apart from low and variable CLA levels in natural dietary food sources, the isomeric composition of naturally occurring CLA is different from the isomeric composition most relevant to body composition. The fat mass-reducing effects of CLA can be attributed to the 10t,12c CLA isomer (reviewed by Banni, 2002), whereas the isomeric composition of CLA from ruminant-derived products consists of mainly the 9c,11t CLA isomer (Chin et al., 1992).

A potentially more consistent dietary intake of CLA can be obtained by adding Clarinol™ G-80 to specifically labeled and generally available food items intended for use as body weight management products. Clarinol™ G-80 contains an isomeric composition of CLA which has been demonstrated to decrease body fat mass. In addition, in a 52-week human trial, subjects taking Clarinol™ G-80 supplements while on a diet showed significantly reduced adverse events, indicating that Clarinol™ G-80 improves the ability to tolerate dieting (Whigham et al., 2003).

The isomeric composition of CLA is also of particular importance with regard to the safety for human consumption. As reviewed by Gaullier et al. (2002), CLA preparations consisting of equal proportions of the 9c,11t isomer and the 10t,12c isomer are most favorable, and high levels of *trans,trans* and other CLA isomers should be avoided. In addition, some production processes of CLA may introduce possible toxic by-products or contaminants in the CLA preparation. For example, in production processes using alkylates as a conjugation catalyst, the end product may contain elevated levels of dialkyl ketones

Clarinol™ G-80 contains the most favorable known isomeric composition, with regard to both safety and functional effect, and is the result of a careful and consistent production process. Its safety for human consumption has been well-established, as will be outlined in this document.

1 Identity and composition of Clarinol™ G-80

1.1 Description of the product

Clarinol™ G-80 is a colorless to slightly yellow oil obtained by conjugation of refined safflower oil (i.e. the oil from the seeds of *Carthamus tinctorius* L.) (or other appropriate vegetable oil). It is refined, bleached and deodorized to remove metal ions, unsaponifiables, and other undesirable components. It is a clear liquid at ambient temperatures (solidification point minus 6°C). The fatty acid composition of Clarinol™ G-80 consists of at least 78% conjugated linoleic acid (CLA) (i.e. 9-*cis*,11-*trans* (9*c*,11*t*), and 10-*trans*, 12-*cis* (10*t*,12*c*) octadecadienoic acid in approximately equal amounts).

1.2 Chemical identity of Clarinol™ G-80.

Chemical Name	:	Conjugated linoleoyl triglyceride
CAS Registry Number (CAS RN)	:	303101-61-3
CA Index Name	:	Octadecadienoic acid, 1,2,3-propanetriyl ester (9CI)[1]
Molecular Formula	:	$C_{57}H_{98}O_6$
Molecular weight	:	approximately 878
Trade name	:	Clarinol™ G-80
Synonyms	:	Octadecadienoic acid, 1,2,3-propanetriyl ester Conjugated linoleic acid triglyceride Triglyceride of conjugated linoleic acid Conjugated linoleic acid oil CLA triglyceride CLA glyceride CLA oil

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[1] Although there is a CAS RN clearly assigned to linoleic acid triglyceride (537-40-6), and another to conjugated linoleoyl triglyceride (303101-61-3), the distinction in naming is not always obvious. In both cases the name "octadecadienoic acid, 1,2,3-propanetriyl ester" may be referred to.

1.3 CAS Registry Numbers

Clarinol™ G-80 is a mixture of glycerides of fatty acids containing at least 78% glycerides of CLA. The CAS RN 303101-61-3 is applicable.

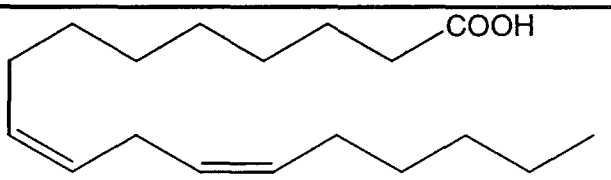
N.B. Oils like Clarinol™ G-80 are mixtures of a range of components characterized with the CAS RN of the main component. In such case the CAS RN is not only applicable for the main component, but for the oil as a whole (i.e. purity 100% not 78%). This is in contrast with preparations that consist of intentional mixtures of different chemical components, which have to be characterized with the CAS registry numbers of all components.

1.4 Product Outline and Structural Formula

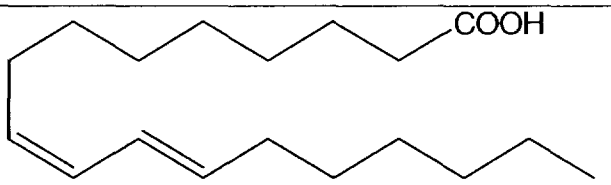
The oil used in the production of Clarinol™ G-80 contains triglycerides of at least 78% linoleic acid. Other acids present are mainly oleic (10-15%), palmitic, and stearic (max. 7%). Since the conjugation process only affects polyunsaturated components, the final product will be similar to safflower oil except that the linoleic acid is conjugated. The rest of the fatty acids will remain unchanged.

Clarinol™ G-80 is a mixture of glycerides of predominantly 9*c*,11*t*-CLA and 10*t*,12*c*-CLA in approximately a 1:1 ratio, together with other safflower fatty acids. In the figure (right), the free fatty acids structures are shown together with their CAS RN's. The difference between CLA and linoleic acid (9*c*,12*c* octadecadienoic acid; CAS RN 60-33-3) is that the double bonds are separated by a methylene group.

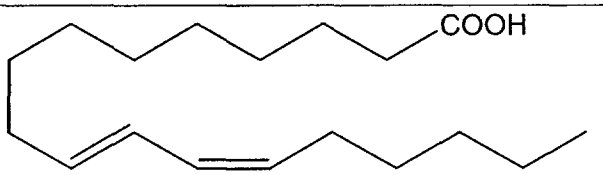
Chemical structure of the fatty acid isomers involved



(9-*cis*, 12-*cis*)-octadecadienoic acid (linoleic acid) (CAS RN 60-33-3)



(9-*cis*, 11-*trans*)-Conjugated linoleic acid (CLA) (CAS RN: 872-23-1)



(10-*trans*, 12-*cis*)-Conjugated linoleic acid (CLA) (CAS RN: 2420-44-2)

1.5 Trade name and Common or Usual Name

The trade name of the product described is "Clarinol™ G-80". The product has been on the market as a dietary supplement for several years in the US, Europe, and Asia. The common or usual name is considered to be "vegetable oil conjugated linoleic acid preparation."

2 Manufacture of Clarinol™ G-80

2.1 General

The process used for the production of Clarinol™ G-80 is an alkali-catalyzed conjugation process using safflower oil or fatty acids containing at least 76% linoleic acid as raw material. During the process the oil is saponified, and one of the double bonds in the linoleic acid is "flipped" in the direction of the second double bond. The thus conjugated linoleic acid (CLA) soap exists of merely 9*c*,11*t* and 10*t*,12*c* CLA isomers in a 1:1 ratio. Then the soap is neutralized, after which the fatty acids are re-esterified with glycerol, bleached and deodorized to form Clarinol™ G-80. An anti-oxidant is added at the refining stage (see also Figure 1).

2.2 Process outline

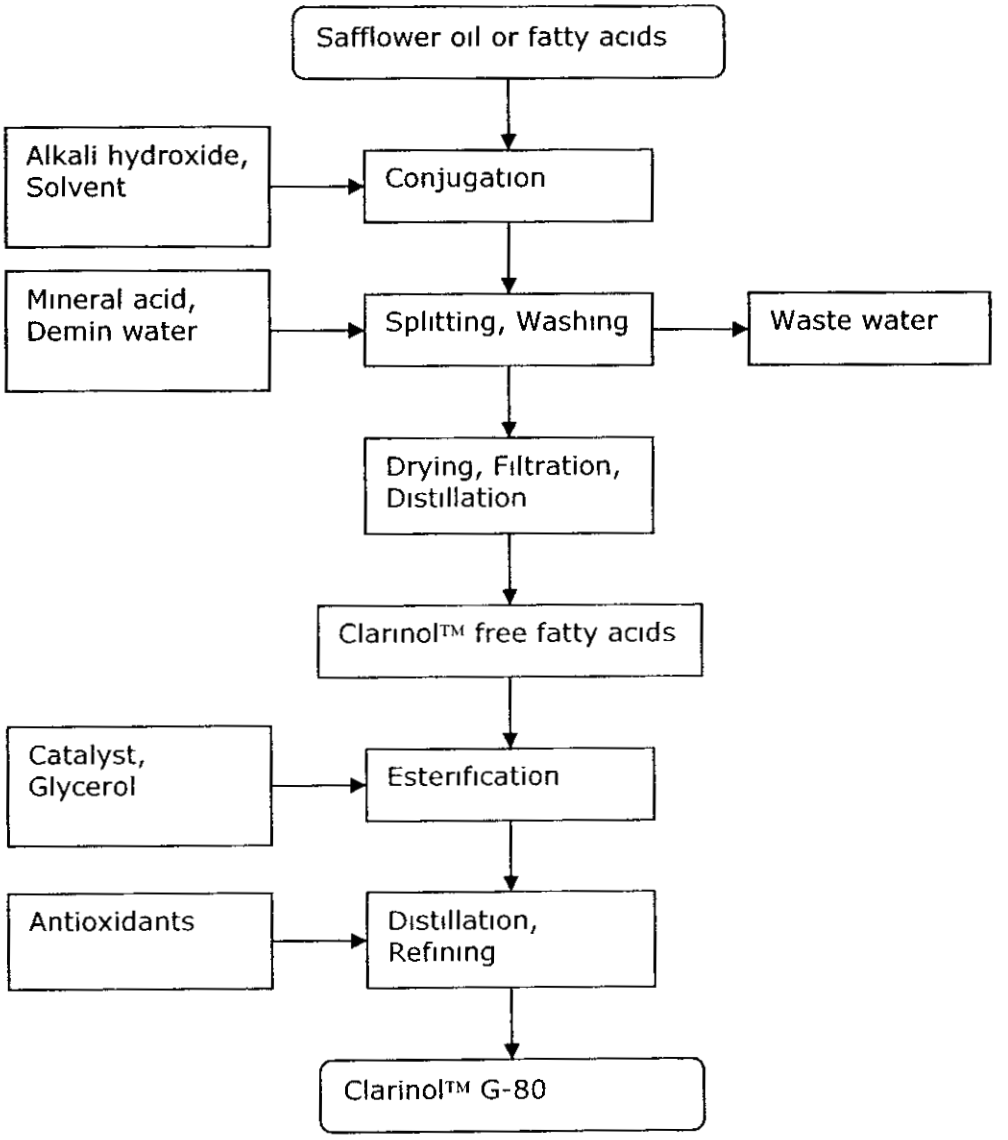
Safflower oil or free fatty acids from safflower oil serves as starting material for the production of Clarinol™ G-80. (Other appropriate vegetable oils may also be used.)

The conjugation is performed in a food-grade solvent and catalyzed by an alkali, under well controlled conditions. After conjugation, the substance is neutralised with acid. After washing and drying, the crude conjugated acid product is purified by short-path molecular distillation. The distillate is the free fatty acid CLA product Clarinol™.

For the preparation of the glyceride product the fatty acids from Clarinol™ are esterified with glycerol by a normal food-grade method. The crude product is further physically refined according to standard refining procedures (bleaching and deodorization) to give Clarinol™ G-80 (glycerides).

This process is consistent with other food industry practices.

Figure 1 Outline of production process for Clarinol™ G-80.



2.3 Specifications of the final product

Loders Croklaan uses the following manufacturing specifications for Clarinol™ G-80:

Specifications of Clarinol™ G-80	
CLA total (all isomers)	≥ 78 %
CLA (*c*9,*t*11 + *t*10,*c*12 isomer)	≥ 74 %
CLA *c*9,*t*11 and *t*10,*c*12 isomer	≥ 36 % each
CLA *trans,trans*	≤ 3 %
Saturated fatty acids	≤ 8 %
Free fatty acids	≤ 1 %
Diglycerides	≤ 25%
Monoglycerides	≤ 1 %
Water	≤ 0.1 %
Dialkyl ketones	≤ 100 ppm
Peroxide Value	≤ 1 meq/kg
Iron (Fe)	≤ 0.5 ppm
Nickel (Ni)	≤ 0.1 ppm
Copper (Cu)	≤ 0.05 ppm
Color yellow (Lovibond, cell 5¼")	≤ 10
Color red (Lovibond, cell 5¼")	≤ 2

2.4 Control of manufacture

Good Manufacturing Practice (GMP) is applied throughout the manufacturing process. As a consequence, control takes place by continuous monitoring. Sampling schemes and analysis schedules are applied to raw materials and end-products as well as products in all stages of processing.

Highly modern production facilities and electronic monitoring systems allow precise process control at all stages. Contact with air is excluded as the whole production of Clarinol™G-80 is carried out under nitrogen.

For the production of Clarinol™ G-80 a computer-assisted sampling routine is used. All samples are logged in the system under a specific sample code for each process step. The necessary analyses, including the specifications the sample has to comply with, are defined within this code. It becomes immediately obvious whether the results are within the specifications or not, making timely intervention possible.

Loders Croklaan B.V. is ISO 9001 and ISO 14001, as well as HACCP certified.

3 Regulatory status of Clarinol™ G-80

3.1 General

Conjugated linoleic acid (CLA) has been on the market as a dietary supplement in many countries all over the world since 1995. Both CLA fatty acids and CLA glyceride are sold for that purpose, although the labeling may not always clearly distinguish between them (both may be called CLA).

Clarinol™ G-80 (CLA glyceride) has not been used as a food ingredient so far. This document is intended to affirm that the use of Clarinol™ G-80 in food is safe when used in specific quantities.

The regulatory status of CLA, meeting the specifications outlined elsewhere in this document, is as follows:

3.2 The United States

CLA has been marketed as a dietary supplement for approximately five years, in constantly increasing amounts. In 2001 the use was estimated to be approximately 200 tons. CLA products are sold as soft gels or capsules, while an increasing number of supplement manufacturers use CLA as an ingredient in supplement bars and similar products.

Clarinol™ G-80 has also been used in dietary supplements mentioned, although in a lesser quantity than the CLA fatty acids.

Although not obliged to do so, Loders Croklaan has voluntarily submitted a New Dietary Ingredient (NDI) notification for Clarinol™ on April 14, 2003, grounding the use as a dietary supplement of Clarinol™ G-80 (glycerides), and Clarinol™ A-80 (fatty acids). FDA filed this notification on April 23, 2003 and stated its acceptance of the notification in a letter of July 8, 2003 to A. Menzel.

FDA has concluded that synthetically produced CLA is a component of certain foods, such as the CLA in processed vegetable oils (FDA letter to I.S. Bass, Mar. 12, 2003).

3.3 Europe

Conjugated linoleic acid has been on the market as a 'food supplement' (which is similar to dietary supplement in the US) in some EU countries since 1995. Since then the consumption of CLA has been growing significantly. Presently it is on the market as a food supplement in most European countries.

3.4 Other countries

CLA is also on the market as a 'food supplement' in Japan since 1998, in Thailand since 2000, and in Indonesia and S. Korea only recently.

In China, and Taiwan, there is CLA on the market, although of a clearly different quality than Clarinol™ G-80.

4 Conjugated Linoleic Acid in the Human diet

4.1 Introduction

Conjugated linoleic acid was first discovered about 20 years ago. Pariza and coworkers found that ground beef contained an anti-mutagenic factor that consisted of a series of conjugated dienoic isomers of linoleic acid (Ha et al., 1987; Pariza et al., 2001). Later it was found that CLA is present in a number of foods, in relatively low concentrations. The most important natural sources are (red) meat and milk, but also vegetable oils contain CLA, although in minor quantities (Chin et al., 1992).

CLA production in ruminants depends on the type of animal feed and also shows seasonal variation. The concentration of CLA in milk (and meat) of cows grazing outside in summer may be approximately 2-5 times the concentration in milk produced by cows fed dry food (Lock and Garnsworthy, 2003). Since in-house breeding of cows with the application of dry feed is progressively common in the present world it can be expected that the CLA intake has been reduced with the growth of bio-industry and the increased use of low fat (dairy) products.

4.2 Occurrence of CLA in foods.

Many different CLA isomers are present in fats and oils which are consumed in the normal diet. Sehat et al (1998) analyzed cheese, cow milk and beef for the presence of CLA isomers. They detected a range of isomers including 7t,9c-, 8t,10c-, 9t,11c-, 9c, 11t-, 10t,12c, 11c,13t-, 11t, 13c-CLA[2] in these three products. The predominant isomer was 9c,11t-CLA. Vegetable oils were found to contain equal amounts of 10t ,12c - and 9c,11t-CLA (Chin et al., 1992). Furthermore, used frying fats contain a mixture of CLA isomers classified as *trans,trans-* and *cis,trans* + *trans,cis*-isomers (Sebedio et al., 1988). Finally, partially hydrogenated vegetable oils contain CLA isomers as well (Banni et al., 1994; Dutton, 1979). A detailed investigation on CLA isomer formation during partial hydrogenation of soybean oil was done by Jung et al. (2002). They demonstrated the formation of many CLA isomers with 9c,11t- and 10t,12c-CLA among the main isomers formed during hardening.

[2] t=trans, c=cis

Chin et al. (1992) presented an overview of CLA concentrations measured in a large number of food products. They found the highest concentrations of naturally occurring CLA in meats from ruminants and dairy products, and also in ground fresh turkey. The concentrations found were between 2.5 and 6.7 mg/g fat, except for pork and chicken which only contained 0.6 and 0.9 mg/g fat respectively. It appears that red meat from ruminants contains more CLA than white meat from non-ruminants.

Table 1 Overview of occurrence of CLA in food items from human diet. (Chin et al., 1992).

Food	Total CLA (mg/g fat)	9*c*,11*t* CLA	10*t*,12*c* CLA	Food	Total CLA (mg/g fat)	9*c*,11*t* CLA
Meat				***Dairy products***		
Fresh ground	4.3	85		Homogenized milk	5.5	92
Beef round	2.9	79		Butter	4.7	88
Beef frank	3.3	83		Sour cream	4.6	90
Beef smoked	3.8	84		Plain yogurt	4.8	84
Veal	2.7	84		Ice cream	3.6	86
Lamb	5.6	92		Sharp cheddar	3.6	93
Pork	0.6	82		Mozzarella	4.9	95
Poultry				Colby cheese	6.1	92
Chicken	0.9	84		Cottage cheese	4.5	83
Fresh ground	2.5	76		Ricotta	5.6	84
Seafood				Brick	7.1	91
Salmon	0.3	n.a.[3]		Blue cheese	5.7	90
Lake trout	0.5	n.a.		Reduced fat Swiss	6.7	90
Shrimp	0.6	n.a.		Am. Processed	5.0	93
Vegetable				Cheez whiz™	5.0	92
Safflower	0.7	44	41	***Other products***		
Sunflower	0.4	38	37	Egg yolk	0.6	82
Canola	0.5	44	42	Lamb (strained)	8.8	81
Corn	0.2	39	37	Beef (strained)	6.8	74
Olive	0.2	47	40	Veal (strained)	6.8	78

The concentration of CLA found in vegetable oils was much lower (0.1-0.7 mg/g fat), with the highest values found for safflower oil. Comparable low concentrations were detected in a variety of both canned and uncooked sea foods (0.1-0.8 mg/g fat).

[3] not available

In meat and dairy products, 9*c*,11*t*-CLA was found to be the dominant isomer (76-95% of the total CLA detected). In a variety of vegetable oils analyzed, however, this particular isomer only contributed to approximately 40% of the CLA detected, while approximately an equal amount of 10*t*,12*c*-CLA was detected. Characterization of the CLA isomers in sea foods failed in this study due to technical problems.

It can be concluded that several different CLA isomers, including 9c,11t- and 10t,12c-CLA, are present in the daily diet through various food sources. Milk and dairy products are important dietary sources of nutrients, providing energy, high quality protein, and a variety of vitamins and minerals. However, fat reduction in milk products has reduced the intake of CLA.

4.3 Dietary intake of CLA.

For the general US population, Ritzenthaler et al. (2001) estimated the mean total dietary CLA intake to be 212 and 151 mg/day for men and women, respectively. This study, with 51 men and 51 women between ages of 18 and 60, compared 3-day food duplicates with dietary records and food-frequency questionnaires.

Earlier surveys, performed in US sub-populations, estimated the intake to be 137 and 52 mg/d in college-aged males and females, respectively (Ritzenthaler et al., 1998), or 291 and 15 mg/day in lactating women with high- and low-dairy diets (Park et al., 1999a). Herbel et al. (1998) measured 127mg/day as average daily CLA intake in a study with healthy young men and women.

The highest level indicated (i.e., 1000 mg/day) is that found in a Hare Krishna community in Australia. The relatively high level of ghee and butter consumption appeared to be the reason of this high intake. Also, measurements of the CLA content in the breast milk of women in this population resulted in relatively high values, and was clearly related to the high intake of CLA-containing products (McGuire and McGuire, 2002).

Table 2 Estimated intake of CLA in humans (after McGuire and McGuire (2002)

Nation	**Subject**	**Method**	**CLA intake (mg/day)**	**Reference**
Australia	Adults	NR	500-1000	(Parodi, 1994)
U.S	Adults	NR	1000	(Ip et al., 1994)
U.S	Adults	Diet records	127	(Herbel et al., 1998)
Germany	Adult males	FFQ	430	(Fritsche and Steinhart, 1998)
Finland	Adults, High Dairy	Diet records	310	(Salminen et al., 1998)
	Adults, Low Dairy	Diet records	90	(Salminen et al., 1998)
U.S.	College-Aged Males	Diet records	137	(Ritzenthaler et al., 1998)
	College-Aged Females	Diet records	52	(Ritzenthaler et al., 1998)
U.S	Lactating Women, High Dairy	Diet records	291	(Park et al., 1999a)
	Lactating Women, Low Dairy	Diet records	15	(Park et al., 1999a)
Finland	Adult Women	FFQ	132	(Aro et al., 2000)

In conclusion the mean daily dietary CLA intake can be estimated at 210 and 150 mg/day for men and women in the general population with subgroups up to 300 mg/day. This is in line with the findings of other authors around the world (see Table 2).

4.4 Proposed addition of Clarinol™ G-80 to foods.

Please find below the proposed applications of Clarinol™ G-80. Information on estimated daily intake from these proposed uses is provided in section 5.9.2.

Food codes CSFII	Food Group	Intended % CLA	Intended % CLARINOL G-80
114	Yogurt	1	1.3
116	Milk-based meal replacements, fluid	0.5	0.7
281	Frozen or shelf-stable plate meals with meat, poultry, fish as major ingredient	0.3	0.4
4143	High protein bar	3	4
5354	Bars: Breakfast, Meal replacement, Granola, Power	3	4
832	Low-calorie and reduced calorie salad dressings	2	2.7

5 Safety review of Clarinol™ G-80

There is extensive supportive evidence of the safety of Clarinol™ G-80 for its proposed uses based on *in vitro,* animal and human studies and on a history of exposure through the human diet. This section covers the information reviewed by Loders Croklaan and an independent expert panel to evaluate the safety of Clarinol™ G-80.

5.1 Clinical trials on Clarinol™ G-80 and comparable CLA preparations

Twenty randomized, placebo-controlled, double-blinded trials in human subjects have been performed with Clarinol™ G-80 or CLA preparations comparable to Clarinol™ G-80 in their isomeric composition. The studies have been conducted on preparations with CLA in triglyceride form or in free fatty acid form. The results of these studies do not indicate a difference between the two forms on any safety parameters. As reviewed by Gaullier et al (2002), the safety of a CLA preparation is more related to its isomeric composition. Most human studies have been published in peer-reviewed scientific journals; some have been presented at professional meetings. An overview of all studies is given in Table 1.

The intake of CLA ranged from 0.7 to 6.8 g CLA/day, containing the c-9,t-11 and the t-10,t-12 isomers in a 50:50 ratio. The treatment periods ranged from 4 to 52 weeks. The trials were carried out in a range of population groups: body builders, healthy individuals, overweight and obese individuals, individuals with metabolic syndrome and individuals with non-insulin dependent diabetes mellitus. A short summary of the pivotal studies is given below.

A CLA preparation equivalent to Clarinol™ G-80 was studied in a randomized, double-blind study by Blankson et al. (2000) and Berven et al. (2000). The trial included 60 overweight and obese men and women (BMI 27.5 - 39.0 kg/m^2). The subjects were divided into five groups receiving placebo (9 g olive oil), 1.7, 3.4, 5.1 or 6.8 g CLA/day (free fatty acid form) for 12 weeks, respectively. Eight subjects withdrew from the study due to adverse events; however, no differences among treatment groups were found regarding adverse events.

Table 3 Overview of results of studies G-80 on Clarinol™ G-80 and CLA preparations similar to Clarinol™ in humans. These preparations are composed of equal amounts of the c-9,t-11 CLA isomer and the t-10,c-12 CLA isomer.

Reference	Subjects	Intake (g/day)	Length of study	Metabolic hormones	Liver parameters	Serum lipid profile	Other effects
Albers (2003)	healthy ♂, vaccinated for hepatitis B	1.7 (FFA)	12 weeks	glucose: ↔	↔	Total cholesterol: ↔ LDL cholesterol: ↔ HDL cholesterol: ↔ Triglycerides: ↔	treatment related adverse events: ↔
Atkinson (1999a)	obese, ♂ and ♀	2.7 (FFA)	26 weeks	n.a.	n.a.	n.a.	treatment related side events or adverse events: ↔
Basu (2000)	healthy, ♂ and ♀	4.2 (FFA)	12 weeks	n.a.	n.a.	n.a.	lipid peroxidation: ↑
Belury (2003)	subjects with type 2 diabetes mellitus	6 (FFA)	8 weeks	glucose: ↓ leptin: ↓	n.a.	n.a.	n.a.
Berven (2000) / Blankson (2000)	healthy overweight and obese, ♂ and ♀	1.7, 3.4, 5.1, 6.8 (FFA)	12 weeks	glucose: ↔ insulin: ↔	AST, ALT, bilirubin, g-glutamyltransferase: ↔	↔	blood safety parameters: ↔ vital signs: ↔ treatment related adverse events: ↔
Gaullier (2003)[2]	healthy overweight, ♂ and ♀	3.4 (FFA and TG)	52 weeks	glucose: ↔	n.a.	n.a.	treatment related adverse events: ↔
Hayden (2004)[1]	healthy, ♂ and ♀	2 (FFA)	5 weeks	glucose: ↔ insulin: ↔	↔	n.a.	kidney function: ↔

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Kamphuis (2003)	healthy overweight, ♂ and ♀	1.8, 3.6 (TG)	13 weeks	glucose: ↔ insulin: ↔	n.a.	↔	n.a.
Lambert (2003)[1]	healthy exercising, ♂ and ♀	3.9 (FFA)	12 weeks	glucose: ↔ insulin: ↓ (♀)	n.a.	cholesterol: ↔	treatment related adverse effects: ↔
Masters (2002)	Healthy lactating ♀	1.2 (?)	2 x 5 days	n.a.	n.a.	n.a.	Infant milk consumption: ↔ Total milk fat: ↓
Mougios (2001)	healthy, ♂ and ♀	0.7-1.4 (TG)	8 weeks	n.a.	n.a.	HDL cholesterol: ↓↔ Triglycerides: ↓	creatine kinase: ↔ treatment related adverse events: ↔
Noone (2002)	healthy, ♂ and ♀	3 (FFA)	8 weeks	glucose: ↔ insulin: ↔	↔	Triglycerides: ↓	n.a.
Petridou (2003)	healthy ♀	2.1 (TG)	45 days	leptin: ↔	ALT: ↔	total cholesterol: ↔ HDL cholesterol: ↔ triglycerides: ↔	n.a.
Risérus (2001)	obese middle-aged, ♂	4.2 (FFA)	4 weeks	glucose: ↔ insulin: ↔	n.a.	↔	treatment related adverse events: ↔
Risérus (2002a; 2002b)	obese middle-aged, ♂	3.4 (FFA)	12 weeks	glucose: ↔ insulin: ↔ leptin: ↔	n.a.	HDL cholesterol: ↓ other cholesterol: ↔ triglycerides: ↔ free fatty acids: ↔	insulin sensitivity: ↔ lipid peroxidation: ↑ α and γ tocopherol: ↔ IL6: ↔ CRP: ↔ TNFα: ↔
Rockway (2000)	moderately overweight, ♂	3.0 (FFA)	6 weeks	glucose: ↔	n.a	cholesterol: ↓ triglycerides: ↓	n.a.

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Smedman and Vessby (2001)	healthy, ♂ and ♀	4.2 (FFA)	12 weeks	glucose: ↑ (NS) insulin: ↔	AST and ALT: ↔	ApoB: ↑	n.a.
Song (2003)[2]	healthy	3 (TG)	12 weeks	glucose: ↔ insulin: ↔	↔	n.a.	CRP: ↔ TNFα: ↔
Thom (2001)	healthy exercising, ♂ and ♀	1.8 (FFA)	12 weeks	n.a.	n.a.	n.a.	treatment related adverse events: ↔
Traenor (2004)[1]	healthy elderly, ♂ and ♀	2 (FFA)	7 weeks	glucose: ↔ insulin: ↔	↔	n.a.	n.a.
Whigham (2003)[2]	healthy overweight, ♂ and ♀	6 (TG)	52 weeks	glucose: ↔ insulin: ↔	AST, ALT, AlkP: ↔	cholesterol: ↔ triglycerides: ↔	adverse events: ↓ T4, TSH: ↔ blood parameters: ↔

[1]These studies have not yet been published or presented
[2]These studies have not yet been published, but have been presented at professional meetings
All other studies have been published in peer-reviewed journals

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A number of parameters were analyzed, including blood lipids, haematology and chemistry at baseline, week 6 and week 12. Vital signs (blood pressure and pulse) and liver and kidney safety parameters (AST, ALT, bilirubin, g-glutamyltransferase) were measured at baseline and at week 12. A significant reduction of body fat was found in the 3.4 and 6.8 g/day CLA groups only (5.7% and 3.7% respectively). No significant changes were found in blood glucose or insulin levels. Some changes were observed within each group in haematological parameters, but none of the changes were considered clinically important. No clinically relevant changes in blood parameters, liver and kidney safety parameters or vital signs occurred.

A longer trial was conducted by Atkinson et al. (1999a; 1999b; 1998). Eighty obese subjects received 2.7 g/day placebo or CLA for 6 months. Significant changes in body fat were observed, with the placebo gaining fat mass and the CLA losing fat mass and increasing lean body mass. There were no treatment related major side effects or adverse events noted throughout the study.

Similar safety results were observed in more than 15 other published studies lasting from one to six months at various intake levels (reviewed by Gaullier et al., 2002).

These published data are further supported by generally available data from a human trial on Clarinol™ G-80 that has been discussed in scientific meetings. A 52-week clinical trial on Clarinol™ G-80 has been conducted at the University of Wisconsin Clinical Nutrition Clinic (Whigham et al., 2003). The study was conducted in overweight and obese individuals (BMI in range 27-35 kg/m^2) and was divided into three phases:

a. a weight loss phase on very low calorie diet (LCD) (±12 weeks)
b. a maintenance (of weight loss) phase (±16 weeks)
c. open label phase (± 24 weeks)

In the first two phases participants received either Clarinol™ G-80 or placebo (high oleic sunflower oil) capsules in addition to a weight loss/management and exercise program. The daily intake of Clarinol™ G-80 was 7.5 g, which is equivalent to 6.0 g/day of the CLA isomers. In phase 1, subjects were supplied with a primarily liquid LCD (Slim-Fast) providing 13 kcal/kg of desirable body weight (subject's weight at a BMI of 22 kg/m^2) over 12 weeks. Phase 1 ended when subjects had lost between

10% and 20% of initial body weight, or at 12 weeks, whichever came first. Phase 2 was a weight-maintenance/weight-regain phase lasting for an additional 16 weeks. During this time subjects initially were gradually reintroduced to solid food over a two-week transition period. Subjects were prescribed a calorie intake of about 25-30 kcal per kg of body weight at a BMI of 22 kg/m^2 to maintain weight loss.

In the third phase of the study all subjects were moved to the Clarinol™ G-80 group and continued with this treatment until the end of the study.

Of the 63 subjects who were initially randomized into the study, 17 (10 CLA, 7 placebo) dropped out before the end of the study at 52 weeks (Table 4). Nine subjects dropped out at or before the first follow up visit.

Table 4 Description of subjects who dropped out of the study.

Week of Drop	Reason for Drop	Treatment
baseline	Lost to follow-up	CLA
baseline	Too busy	CLA
baseline	Thyroid levels	CLA
baseline	Too busy	CLA
week 2	AEs related to LCD	CLA
week 2	AE-Rash	CLA
week 4	Couldn't take LCD	CLA
week 4	Lack of commitment	CLA
week 4	Too busy	CLA
week 8	Too busy	CLA
baseline	Too busy	placebo
baseline	Too busy	placebo
baseline	Couldn't take LCD	placebo
week 16	Pregnant	placebo
week 18	AE-hair loss	placebo
week 28	AE-lump in neck	placebo
week 38	Too busy	placebo

Subjects were scheduled for clinic visits every two weeks, in which vital signs, body weight, and body composition by Bioelectrical Impedance Analysis were recorded, the adverse event questionnaire was completed and food intake, exercise and adherence to the protocol was discussed. Pill counts were performed at each visit to monitor compliance in taking the supplements.

Safety parameters including clinical chemistry, liver and kidney function, fasting serum insulin and glucose levels were measured throughout the study. Levels of C-reactive protein and lipid peroxidation were not measured in this trial. Clarinol™ G-80 had no effect on liver function as there was no evidence of a treatment-related

increase of liver enzymes (Figure 2, Figure 3, Figure 4). In fact, levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) in the Clarinol™ G-80 group were significantly lower compared to control at week 6 and 12 of the study. No treatment-related effects were observed on fasting plasma insulin and glucose levels (Figure 5, Figure 6) and haematology parameters (

Table 6). Adverse events recorded by subjects through questionnaires that were completed at each biweekly visit overall were lower in the CLA group compared to placebo (Table 5). Significantly lower frequencies of skin rash, depression, irritability/anger, hair loss, and infection were noted in the CLA group versus the placebo group. This study has been presented in a scientific forum is submitted for publication (Whigham et al., 2003).

Table 5 List of adverse events included in questionnaire that was completed each biweekly visit throughout the 52-week trial on Clarinol™ G-80

1.Abnormal menses	8.Abdominal pain	15.Altered sense of time	22.Memory loss	29.Drowsiness
2.Bruising	9.Constipation	16.Anxiety	23.Binge eating	30.Insomnia
3.Shortness of breath	10.Diarrhea	17.Depression	24.Changes in vision	31.Fatigue
4.Chest pain	11.Dry mouth	18.Elevated mood	25.Headache	32.Vivid/weird dreams
5.Abnormal heart beats	12.Nausea/Vomiting	19.Irritability/Anger	26.Dizziness	33.Higher sex drive
6.Leg cramps	13.Heartburn	20.Nervousness	27.Sweating	34.Lower sex drive
7.Joint pains	14.Skin rash	21.Loss of concentration	28.Tremor	35.Hair loss

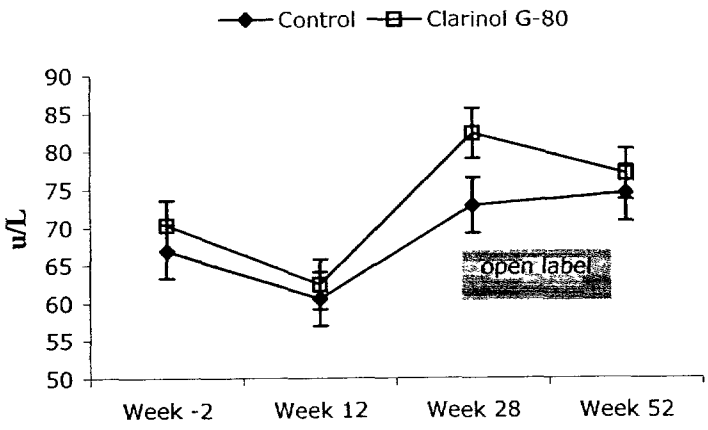

Figure 2 Plasma levels of alkaline phosphatase throughout a 52-week clinical trial on Clarinol™ G-80

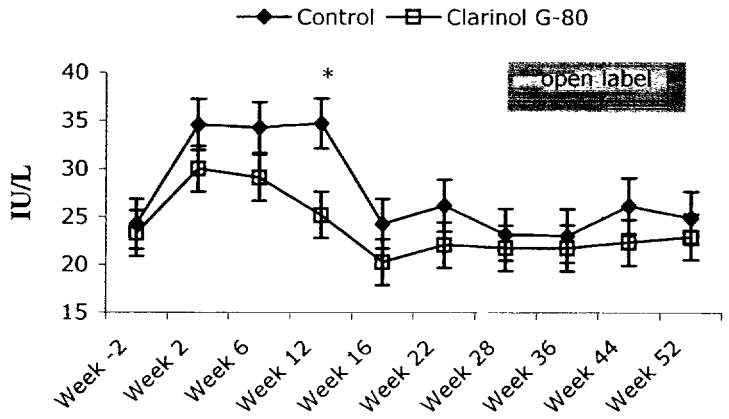

Figure 3 Plasma levels of aspartate aminotransferase throughout a 52-week clinical trial on Clarinol™ G-80

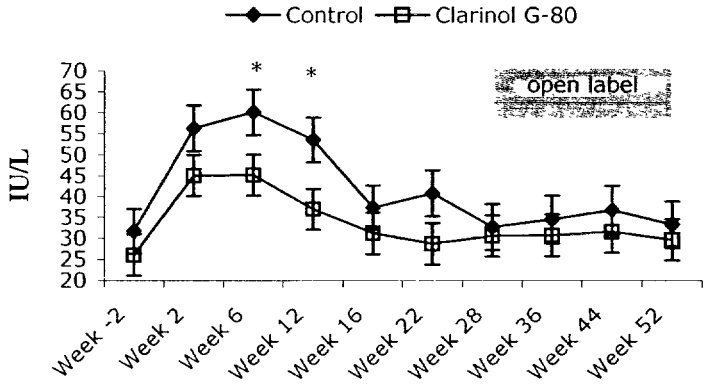

Figure 4 Plasma levels of alanine aminotransferase throughout a 52-week clinical trial on Clarinol™ G-80

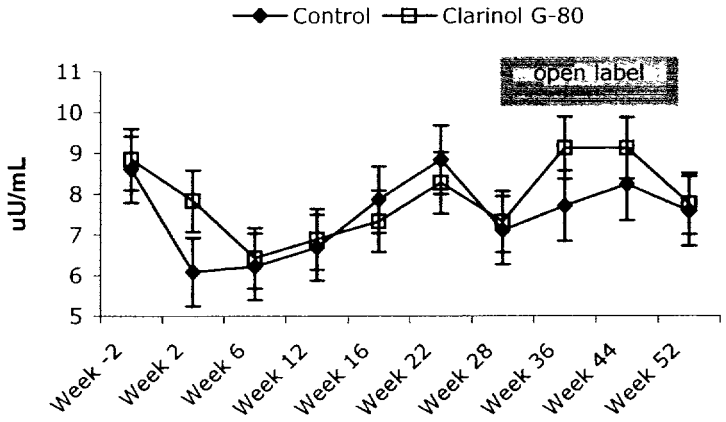

Figure 5 Fasting plasma insulin levels throughout a 52-week clinical trial on Clarinol™ G-80

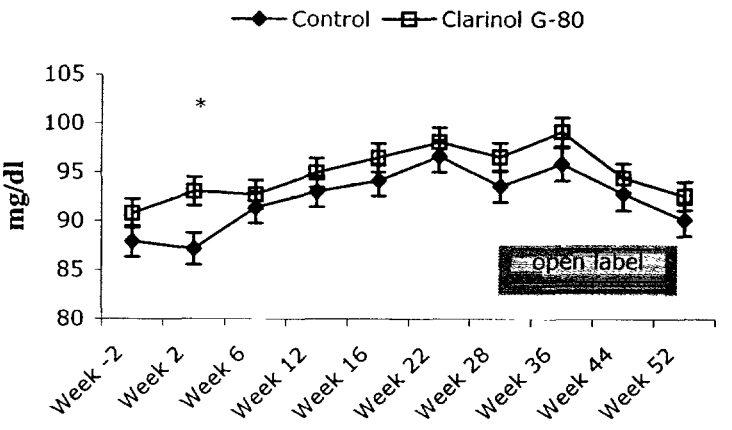

Figure 6 Fasting plasma glucose levels throughout a 52-week clinical trial on Clarinol™ G-80

Another long term study on a CLA preparation similar to Clarinol™ G-80 will be published shortly (Gaullier et al., 2004, in press; 2003, personal communication). Data from this study are also generally available, having been discussed in scientific meetings. One hundred eighty overweight and obese subjects were included in this randomized, double-blinded placebo controlled trial. Subjects were supplemented with 3.4 g/day CLA in triglyceride or free fatty acid form, or placebo (olive oil) for two years. No adverse events were related to the treatment with CLA. There were no significant differences between placebo and CLA supplemented groups in cholesterol and lipid profiles. Some changes were found in cholesterol, lipid profile, apolipoprotein A, aspartate aminotransferase, fasting glucose and glucohemoglobin and leuko- and thrombocyte levels in subjects supplemented with CLA compared to baseline levels or to placebo. The changes stayed within normal ranges and were not considered clinically significant.

Table 6 Hematology parameters throughout a 52-week clinical trial on Clarinol™ G-80

	Baseline			Week 12			Week 28			Week 52		
	Placebo	Clarinol™ G-80	p-value	Placebo	Clarinol™ G-80	p-value	Placebo	Clarinol™ G-80	p-value	Placebo	Clarinol™ G-80	p-value
Hematocrit	44.34 ± 0.75	45.45 ± 0.71	0.27	43.37 ± 0.75	44.67 ± 0.71	0.20	43.51 ± 0.77	43.96 ± 0.71	0.66	41.11 ± 0.79	42.79 ± 0.72	0.11
WBC	5.66 ± 0.30	6.16 ± 0.29	0.23	4.63 ± 0.30	5.19 ± 0.29	0.17	5.53 ± 0.32	6.55 ± 0.29	0.02	5.01 ± 0.32	5.48 ± 0.29	0.27
Lymphocytes	36.78 ± 2.30	41.17 ± 2.16	0.16	38.58 ± 2.30	41.49 ± 2.16	0.35	44.51 ± 2.44	41.19 ± 2.16	0.30	44.99 ± 2.45	49.35 ± 2.21	0.18
Neutrophils	50.44 ± 2.43	47.74 ± 2.28	0.41	49.29 ± 2.43	45.42 ± 2.28	0.24	44.13 ± 2.59	47.88 ± 2.28	0.27	41.06 ± 2.60	38.52 ± 2.33	0.46
Monocytes	7.34 ± 1.31	6.44 ± 1.23	0.61	7.08 ± 1.31	6.29 ± 1.23	0.66	5.21 ± 1.36	7.94 ± 1.23	0.14	7.17 ± 1.40	5.80 ± 1.25	0.46

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A clinical trial has been completed on Clarinol™ A-60 (Lambert and Goedecke, 2003). This preparation contains a CLA isomer content of 65.9% in the form of free fatty acids. The isomeric composition of Clarinol™ A-60 is similar to that of Clarinol™ G-80. The study examined the effect of Clarinol™ A-60 administration on substrate utilization, glucose and lipid metabolism, metabolic rate and body composition in regularly exercising individuals. The double blind, placebo controlled trial was conducted at the University of Cape Town. Sixty–four regularly exercising men and women aged 21-45 years were randomized into two groups receiving either 3.9 g/day Clarinol™ A-60 or 3.9g of olive oil for a period of 12 weeks (±49 and 65mg/kg body weight/day for males and females respectively). Two participants subsequently withdrew from the trial due to reasons unrelated to the treatment.

Treatment with Clarinol™ A-60 was associated with a small but statistically significant decrease in body fat levels in woman. Plasma glucose did not differ between the two groups. Plasma insulin concentrations were lower in females taking Clarinol™ A-60 compared to the women of the control group and this effect was statistically significant. Insulin sensitivity, as measured by the increment in glucose concentrations versus the increment in insulin concentrations and by the ISI and QUCKI models were not affected by treatment. Insulin resistance as measured using the HOMA model and fasting glucose/insulin ratio did not differ significantly between treatment and placebo group either. However, these indices may not be as sensitive to change in the extreme ranges, and in non-diabetic, non-obese, fit individuals. No treatment related effects were found on total cholesterol, LDL-cholesterol, and HDL-cholesterol.

In summary, none of the more than 20 human trials on Clarinol™ G-80 and comparable CLA preparations reported any significant treatment-related adverse events. In addition, no clinically significant effects of CLA were found on plasma glucose and insulin levels, liver and kidney function parameters and blood parameters. Two studies reported an increase in lipid peroxidation markers (Basu et al., 2000; Riserus et al., 2002a; 2002b) and one study reported a decrease of maternal milk fat (Masters et al., 2002) in subjects taking CLA supplements. The significance of these effects will be discussed in section 5.8.

5.2 Clinical trials on CLA preparations containing purified CLA isomers

Two human trials have studied CLA preparations containing the purified t-10,c-12 or the c-9,t-11 CLA isomer (free fatty acid form). The trial by Riserus et al (2002a; 2002b; 2003) studied the separate isomers in overweight men (BMI 27-39kg/m^2) with signs of the metabolic syndrome. Four groups received either 3.4 g control oil (olive oil), 3.4 g CLA mix, 3.4 g purified t-10.c-12 CLA isomers or 3.4 g purified c-9,t-11 CLA isomers for a period of 12 weeks. The CLA mix preparation was comparable to Clarinol™ G-80. Body fat and sagittal abdominal diameter decreased in the groups receiving the CLA mix and the purified t-10,c-12 CLA preparation, but not significantly compared to placebo. No effects on insulin and glucose levels were found in subjects receiving the CLA mix. However, a decrease in insulin sensitivity, a non-significant increase in fasting insulin levels and a non-significant increase in fasting glucose levels were observed in subjects receiving either of the purified CLA isomer preparations. Lipid peroxidation and levels of C-reactive protein (CRP) were also increased in this group, but no effects were found on TNFα, α-tocopherol, γ-tocopherol and IL-6. High density lipoprotein (HDL) cholesterol levels were lowered both by treatment with the t-10,c-12 CLA isomer and with the CLA mix. The increased insulin resistance was correlated to lipid peroxidation markers, although this correlation was very weak. No correlation was determined between insulin resistance and free fatty acid levels. The significance of the effects on insulin resistance, HDL cholesterol levels, CRP levels and lipid peroxidation markers will be discussed in section 5.8.

The findings of Riserus et al (2002a; 2002b; 2003) are in contrast to the results of a trial using comparable material, which was presented at the American Oil Chemist Society meeting of 2002 (Verboeket-van de Venne et al., 2002). The same purified isomer preparations used in the studies by Riserus et al (2002a; 2002b; 2003) was tested for 18 weeks in overweight middle-aged Europeans (BMI ~25kg/m^2). Subjects received 1.5 or 3 g/day of preparations containing either purified c-9,t-11 or t-10,c-12 CLA isomers, or high oleic sunflower oil. Changes in fat mass and fat-free mass were not significantly different between groups. Monthly monitoring of biological side effects, by assessment of haematological and clinical-chemical parameters, including plasma insulin and glucose concentrations, revealed no treatment related adverse effects.

5.3 Absorption, distribution, metabolism and excretion (ADME) properties of Clarinol™ G-80

No specific ADME studies have been conducted on Clarinol ™ G-80, but sufficient data are available from studies investigating the absorption, distribution and metabolism of the c-9,t-11 and t-10,c-12 CLA isomers (reviewed by Banni, 2002; Belury, 2002).

Clarinol™ G-80 is a triglyceride of predominantly c-9,t-11 and t-10,c-12 CLA, and a remainder of safflower oil fatty acids (oleic, palmitic and stearic acid). Therefore, the general absorption of Clarinol™ G-80 is comparable to that of most triglycerides. The triglyceride of CLA is split in the stomach by pancreatic lipase, forming a 2-mono-glyceride and two CLA free fatty acids. The 2-mono-glyceride and the two free fatty acids are then absorbed from the lumen of the proximal small intestine, facilitated by bile acids. The distribution and metabolism of the CLA isomers is comparable to that of oleic and linoleic acid. Similar to oleic acid, the incorporation of CLA occurs in neutral lipids. In animal studies on CLA, 85-100% of the ingested CLA isomers were absorbed from the gut and distributed mainly to brain, heart, liver, serum, spleen, kidney, lung, adipose tissue and adrenals (Kramer et al., 1998; Martin et al., 2000; Sergiel et al., 2001). One study found that lymphatic recovery of CLA in rats was 55% versus 80% for linoleic acid, although the distribution in lymph lipoproteins was similar (Sugano et al., 1997). With regard to its metabolism, CLA uses the same desaturation and elongation pathways as linoleic acid, specifically Δ5- and Δ6-desaturase and elongase, so that some competition between these fatty acids may take place. For example, a CLA intake-dependent reduction was found in arachidonic acid, a metabolite of linoleic acid. No adverse consequences of this decrease in arachidonic acid are known. In fact, the decrease in arachidonic acid may be related to the anti-carcinogenic properties of CLA, by inhibiting eicosanoid formation. Most of the CLA metabolites (conjugated diene (CD)18:3, CD20:3) are incorporated in neutral lipids. The CD20:4 metabolite of CLA is mainly incorporated in phospholipids, similar to linoleic acid. The metabolites of CLA can subsequently be partly or fully oxidized in peroxisomal ß-oxidation pathways (Banni, 2002). The metabolites of CLA likely play a role in the diverse biological activities of CLA, but since isolation of these metabolites is difficult, not much is known about their individual properties. Compared to linoleic acid, CLA is incorporated into tissues less and oxidized more. In rats, 24 hours after gastric

ingestion of either c-9, t-11 CLA, t-10,c-12 CLA or linoleic acid, ~70% of both CLA isomers were oxidized, compared to ~60% for linoleic acid (Sergiel et al., 2001).
The information on the ADME properties of CLA isomers present in Clarinol™ G-80 do not indicate any adverse effects of Clarinol™ G-80.

5.4 Sub-chronic toxicity studies on Clarinol™ G-80

A sub-chronic 13-week oral toxicity study was performed on Clarinol™ G-80 in male and female Wistar outbred (Crl: (WI)WU BR) rats (O'Hagan and Menzel, 2003). The study was conducted in accordance with the OECD principles of Good Laboratory Practice (GLP) and in accordance with OECD guideline 408 (adopted 1998).
Each experimental group consisted of 20 male and 20 female rats (
Table 7). One group was fed control AIN-93G diet with a standard fat content of 7% w/w in the form of the safflower oil while a second control group was fed a modified AIN-93G diet containing 15 % w/w safflower oil. The three test groups received modified AIN-93G diets containing 15% w/w fat made up from different levels of safflower oil (14%,10%, 0%) and Clarinol™ G-80 (1%, 5% or 15%). The study also included three recovery groups of 10 extra rats per sex in the two control groups and in the 15% Clarinol™ G-80 group. Rats in these groups were maintained on study for an additional 4 weeks after the 13-week treatment period. During this period rats in the control groups continued to receive their respective control diets while the rats in the 15% Clarinol™G-80 group were switched to the high-fat control diet.

Table 7 Level of safflower oil and Clarinol™ G-80 in the diet and number of rats in the 13-week oral toxicity study

Group name	Dietary level of safflower oil (%, w/w)	Dietary level of Clarinol™ G-80 (%, w/w)	Number of rats (male/female)	
			Main groups	Recovery groups
Low-Fat control	7	0	20/20	10/10
High-Fat control	15	0	20/20	10/10
1% Clarinol™ G-80	14	1	20/20	-
5% Clarinol™ G-80	10	5	20/20	-
15% Clarinol™ G-80	0	15	20/20	10/10

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There were no clinical signs or effects on survival that were related to treatment. Food consumption was significantly lower in males and females fed 15% Clarinol™ G-80 during the first two weeks on study due to reduced palatability of the 15%

Clarinol™ G-80 diet. The reduced food consumption resulted in a significant reduction in bodyweight in the 15% Clarinol™ G-80 fed animals during weeks 1 and 2 on study. No treatment related clinically significant effects were found on food conversion efficiency, water consumption, urinalysis parameters, and hematological parameters.

Clinical chemistry values were measured throughout the study. A number of parameters such as general enzymatic markers of liver function showed changes that were considered treatment-related at one or more time-points during the study in 15% Clarinol™ G-80 fed male and/or female rats. These effects were mostly reversed at the end of the recovery period. Absolute and relative liver weights were increased in male and female rats fed diets containing 15% Clarinol™ G-80. This was completely reversed in male rats at the end of the recovery period. Microscopic observations of the liver revealed hepatocellular vacuolation in both males and females from Clarinol™ G-80 treated and control groups. Therefore, this was likely due to consumption of high levels of oil. Hepatocellular hypertrophy was found in female rats fed 15% Clarinol™ G-80. The reversal of increased liver weights and hypertrophy was not fully complete in females at the end of the recovery period. Slight hypertrophy was still observed in 2 of the 10 females in this group. The effects of 15% Clarinol™ G-80 on liver weights, liver enzymes and hypertrophy were reversed during the recovery period, although the reversal was not complete in female rats. This indicated that the findings were an adaptive effect in response to feeding high levels (15%) of Clarinol™ G-80 in the diet. Such adaptive responses in the liver are not considered adverse (ECETOC, 2002) and are not thought to be relevant to human safety assessment. Small increases in liver weight were observed in the 5% Clarinol™ G-80 group but they were not accompanied by lesions or any changes in clinical chemistry and were therefore not considered a treatment-related effect.

Plasma insulin levels were measured at various time points during the study Table 8). Increased plasma insulin levels were observed in both males and females fed 15% Clarinol™ G-80. The onset of the increase occurred later in females than in males. In male rats the increase appeared to be transient and by the end of the 13-week experimental period there were no significant differences between high intake and control values.

Table 8 Plasma insulin levels in rats (µg/l) throughout the 13-week sub-chronic toxicity study on Clarinol™ G-80

	Week 4		Week 8		Week 13		Recovery	
	♂	♀	♂	♀	♂	♀	♂	♀
Low-Fat control	0.60	0.81	1.52	0.63	1.67	0.81	1.66	1.09
High-Fat control	0.46	0.78	0.93	0.71	1.44	0.93	1.88	1.00
1% Clarinol™ G-80	0.78	0.67	1.47	0.93	1.73	0.88	n.a.	n.a.
5% Clarinol™ G-80	0.63	0.58	1.20	0.93	1.80	0.88	n.a.	n.a.
15% Clarinol™ G-80	1.25**@@	1.03	2.17**@@	1.34**@@	2.21	2.17**@@	1.47	0.74

* p<0.05 **p<0.01 ***p<0.001 versus LF-control, @ P<0.05 @@ P<0.01 versus HF-control, n.a. not available

In male rats fed 15% Clarinol™ G-80, the data suggested a trend toward decreased glucose levels from week 8 (Table 9). At the end of the recovery phase, glucose levels in 15% Clarinol™ G-80 males remained significantly lower compared with low fat control rats. No treatment-related effect on plasma glucose levels was observed in female rats throughout the study. Therefore, glucose levels were not measured in females after the recovery period. The increased plasma insulin levels in males and females and the decreased glucose levels in the males may be related to a physiological adaptation to high levels (15%) of Clarinol™ G-80. The effects did not result in any adverse functional change e.g. on blood glucose or in the pancreas in both sexes.

Table 9 Plasma glucose levels in rats (µg/l) throughout the 13-week sub-chronic toxicity study on Clarinol™ G-80

	Week 4		Week 8		Week 13		Recovery	
	♂	♀	♂	♀	♂	♀	♂	♀
Low-Fat control	5.48	6.20	6.10	6.05	9.15	7.83	10.54	n.a.
High-Fat control	4.97	6.08	5.52	6.44	8.87	7.81	9.36	n.a.
1% Clarinol™ G-80	5.49	5.65	5.93	6.33	8.81	7.41	n.a.	n.a.
5% Clarinol™ G-80	5.62	5.29*	6.37@@	5.97	8.98	7.17	n.a.	n.a.
15% Clarinol™ G-80	5.10	5.37	5.46*	5.96	7.26**@@	8.02	8.05**	n.a.

* p<0.05 **p<0.01 ***p<0.001 versus LF-control, @ P<0.05 @@ P<0.01 versus HF-control, n.a. not available

In conclusion, it is uncertain whether the effects seen on the liver and clinical chemistry at the intake level of 15% Clarinol™ G-80 should be considered adverse. At this high level of dietary oil, consisting exclusively of Clarinol™ G-80, it is likely that the effects were caused by a nutritional imbalance. This is supported by the fact that most effects disappeared at the end of the recovery period. No treatment

related adverse effects were observed at an intake level of 5% Clarinol™ G-80. This was equivalent to a dietary intake of 2433 and 2728mg/kg bw/day for males and females respectively. The results of this study comply with a safety study on CLA methyl esters in pigs, in which a dietary level of 5% was demonstrated to be safe (Pfeiffer et al., 2003).

5.5 Genotoxicity studies on Clarinol™ G-80

Two *in vitro* mutagenicity studies were conducted on Clarinol™ G-80; a reverse mutation assay in five histidine-requiring strains of *Salmonella typhimurium* (an Ames test) and an *in vitro* cytogenetics assay in human lymphocytes (chromosome aberration test) (O'Hagan and Menzel, 2003). Both studies were conducted in accordance with GLP and in accordance with OECD guideline 471 and 473 respectively.

5.5.1 Ames test

In the Ames test, Clarinol™ G-80 was tested in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) in the presence and absence of metabolic activation (S9). Each concentration was tested in triplicate with and without S9. Positive and negative controls were included. In the first experiment, Clarinol™ G-80 was tested in strain TA100 at concentrations of 1.26, 6.29, 31.44, 157.2, 786 and 3930 µg/plate. Precipitation of the test article was observed on plates treated with 786 µg/plate and above, but there was no evidence of toxicity at any of the dose levels. In the remaining *Salmonella* strains, Clarinol™ G-80 was tested at concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate. Evidence of toxicity was observed in strain TA102 at the 1000 and 5000 µg/plate +/- S9. The test article was found to precipitate at concentrations of 1000 µg/plate and above.

The second experiment included a pre-incubation step (1 hour at 37 ± 1°C) before plating. In this experiment the following concentrations were tested: 51, 128, 320, 800, 2000 and 5000 µg/plate. Signs of toxicity were observed in TA102 at 5000 µg/plate in the presence of S9. Precipitation of the test article was observed on all plates treated with 2000 µg/plate and above.

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Clarinol™ G-80 did not produce an increase in revertant numbers that was indicative of mutagenicity. A small but statistically significant increase in revertant numbers was observed in some strains. However, these findings were not dose

related, occurring solely at either the lowest or an intermediate test dose and were not reproducible in comparable experiments.

In conclusion, Clarinol™ G-80 did not induce mutation in five histidine-requiring strains of *Salmonella typhimurium* in the presence and absence of S9 at concentrations up to and beyond its solubility limits.

5.5.2 In vitro chromosome aberration test

Clarinol™ G-80 was tested in an *in vitro* cytogenetics assay using human blood lymphocyte cultures. The material was tested in the presence and absence of S9. In the first experiment treatment was 3 hours followed by a 17-hour recovery period prior to harvest. The following dose levels were tested 128, 160 and 200 µg/ml. The highest dose level chosen was reported to induce a 3% and 4% reduction in mitotic index in presence and absence of S9 respectively. In experiment 2, treatment in the absence of S9 was 20 hours. In the presence of S9 it was 3 hours followed by a 17-hour recovery period prior to harvest. In this experiment dose levels of 192 (-S9) and 153.6 (+S9), 240 and 300 µg/ml were tested. The top dose level, 300 µg/ml, induced a 0% and 4% reduction in mitotic index in the absence and presence of S9 respectively. Positive and negative controls were included in both experiments. Each dose of test material was tested in quadruplicate cultures; controls were tested in duplicate.

In both experiments Clarinol™ G-80 -/+S9 produced frequencies of cells with structural aberrations which were similar to the concurrent negative controls. The numbers of cells with aberrations (excluding gaps) in all treated cultures fell within the historical negative control ranges.

In conclusion, Clarinol™ G-80 did not induce chromosome aberrations when tested in the presence and absence of S9 at concentrations up to 300 µg/ml.

5.6 Carcinogenicity of Clarinol™ G-80

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No specific carcinogenic studies have been performed on Clarinol™ G-80. The material did not produce any mutagenic effect in the *in vitro* genotoxicity studies. In addition, significant data are available on the anti-carcinogenic properties of the CLA isomers. In 1987, Pariza and co-workers found that ground beef contained an anti-mutagenic factor that consisted of a series of conjugated linoleic acid isomers (Ha et al., 1987). Since then, the inhibitory role of CLA isomers on at least the initiation and promotion stages of carcinogenesis has been well reported. Clarinol™ A-60, a free

fatty acid CLA preparation containing ~65% total CLA isomers with the same isomeric composition as Clarinol™ G-80, has been used in a study on mammary tumor metastasis in mice (Hubbard et al., 2000). Dietary Clarinol™ A-60 at levels as low as 0.1% and 0.5% significantly increased the latency and decreased the metastasis of mammary tumors in mice injected with tumor cells. In addition, the number of lung nodules was significantly decreased by Clarinol™ A-60. Although the mechanisms underlying the anti-carcinogenic effects of CLA isomers have not been fully elucidated, the anti-carcinogenic properties of CLA isomers have been well-established in several different tissues of both animals and humans (reviewed by Belury, 2002).

5.7 Reproductive toxicology of Clarinol™ G-80

Specific reproductive toxicology studies according to OECD guidelines have not been conducted on Clarinol™ G-80. A prenatal developmental toxicity study in rats, according to OECD guideline 414, was conducted on CLA methyl and ethyl esters in concentrations of up to 1 g/kg body weight/day, and did not find adverse effects (Richard, 2002). The material did not produce any adverse effects on reproductive organs in the 13-week sub-chronic toxicity study on Clarinol™ G-80 in rats. In addition, published studies assessing the effects of CLA on pregnancy and birth weight have reported no adverse reproductive effects of CLA isomers (Chin et al., 1994; Pietras et al., 2002; Poulos et al., 2000). Reproductive toxic effects of fatty acids in general are unlikely. The effects of CLA on gestation and lactation will be discussed further in section 5.8.

5.8 Literature review on Clarinol™ G-80

To date, more than 1500 studies on CLA isomers present in Clarinol™ G-80 have been published. It should be noted however, that the relevance of these studies on CLA to the safety of Clarinol™ G-80 strongly depends on the composition and purity of the CLA preparations used, as different CLA isomers have diverse biological activities.

As discussed in section 1, in human studies, a mixture of both c-9,t-11 and t-10,c-12 in a 50:50 ratio have been given in doses ranging from 0.7-6.8 g/day for periods of 8 weeks to 12 months. In these double blind, placebo-controlled human trials no adverse effects have been demonstrated as a result of supplementation with the 50:50 mixture of isomers. Nevertheless, a few published studies have raised

issues regarding potential safe use of CLA. In addition, four adverse events have been reported in Norway that as being potentially associated with consumption of CLA supplements. In 2004, a committee convened by the Norwegian Food Control Authority expressed the possibility that CLA could increase the risk of adverse effects relating to insulin resistance, cardiovascular disease, birth weight, and fat content of maternal milk.

Loders Croklaan and the members of an independent Expert Panel have considered the available studies and the views of the Norwegian committee carefully and, based on the totality of the scientific evidence available at this time and the appropriate weighing of the evidence most relevant to an evaluation of the safety of Clarinol™ G-80, Loders Croklaan concludes that Clarinol™ G-80 is safe, and generally recognized as safe, for its intended use. Each of the issues raised in the literature (including the issues considered by the Norwegian committee) is discussed below.

5.8.1 Effects of CLA on insulin and glucose metabolism

Human studies on Clarinol™ G-80 and comparable CLA preparations

A number of human trials have looked at the effects of the 50:50 mixture of CLA isomers on glucose and insulin levels. The 20 trials in healthy overweight or obese subjects, presented in table 1, did not find any differences in insulin or glucose metabolism as a result of supplementation with the mixture of CLA isomers. A trial investigating the long term effects of Clarinol™ in healthy overweight and obese subjects demonstrated that 7.5 g/day of the supplements containing 6.0 g/day of the 50:50 CLA mixture of isomers did not affect fasting glucose or insulin levels (Whigham et al., 2003). These results are supported by another one-year trial on a CLA preparation equivalent to Clarinol™ (Gaullier et al., 2003).

In a pilot study by Belury et al. (2002; 2003), type-2 diabetes patients received a 50:50 mixture of CLA isomers (6g/day). Insulin sensitivity was improved in patients receiving CLA and the effect was associated with the t-10,c-12 isomer (Belury, 2002; Belury et al., 2003). The insulin sensitizing effects of CLA have been well established in diabetic rats (Houseknecht et al., 1998).

Human studies with purified CLA isomers

Riserus et al. (2002a; 2002b) investigated the effects of CLA isomers in a mixture as in the previous studies and also the purified isomers individually in overweight men with symptoms of the metabolic syndrome. The subjects in the four arm study received 3.0 g/day of one of the following supplements:

1. 1 :1 mixture of both isomers
2. t-10,c-12 CLA
3. c-9,t-11 CLA
4. olive oil (placebo).

As demonstrated in all other published human trials no effects were seen on insulin sensitivity in those supplemented with the 50:50 mixture of isomers. However, a significant decrease in insulin sensitivity was observed in group 2 receiving purified t-10,c-12 CLA and group 3 receiving purified c-9,t-11 CLA isomer (Riserus et al., 2003). It is possible that the two isomers counteract each other's effect when given in a 50:50 ratio, by, for example, activating different PPAR-regulated enzymes (Brown et al., 2003). It is also possible that the background diet or non-CLA components in the purified isomer supplements are responsible for the effects found on insulin resistance.

The findings of Riserus et al. have been cited in a number of reviews (Gaullier et al., 2002; Larsen et al., 2003), but have only been found once by one group. In fact, the results are in contrast with a number of other human trials testing CLA preparations with one of the two active isomers. In an EU trial, presented at the American Oil Chemists Society Meeting 2002, purified isomer preparations similar to those used in the study by Riserus et al. were tested in overweight middle-aged Europeans for 18 weeks at 1.5 or 3.0 g/day (Verboeket-van de Venne et al., 2002). In this trial, no effects were found on insulin or on any other clinical-chemical parameters.

Furthermore, in two studies with healthy men and women, supplements containing c-9,t-11 CLA and t-10,c-12 CLA in a 80:20 ratio had no effect on glucose and insulin levels after 8 and 12 weeks (Albers et al., 2003; Noone et al., 2002). Although the effects demonstrated in Riserus' studies warrant further investigation, it is important to note that a CLA substance containing individual purified isomers is not the subject of this GRAS notification, and such a substance has not been made generally available for human consumption in dietary supplement form.

In summary, supplements purified in one of the active isomers increased insulin resistance in overweight and obese men with metabolic syndrome, but not in healthy normal or overweight subjects. The pure individual isomers are not available commercially and are therefore not available on the market for human consumption. In addition, supplements containing both active CLA isomers in a 50:50 ratio such as Clarinol™ did not show any adverse effects in any human trials, performed in a large

population range, including men with metabolic syndrome. In fact, supplements containing both CLA isomers in a 50:50 ratio improved insulin sensitivity in diabetic people. Based on these human trials, testing concentrations up to 6.0 g/day and study periods lasting up to one year, it is reasonably certain that the consumption of CLA supplements containing both CLA isomers in approximately equal proportions does not adversely affect insulin and glucose metabolism.

5.8.2 Effects of CLA on parameters of cardiovascular health

Serum lipid profiles have been measured in a number of human studies on CLA, but the results are inconsistent. This is largely due to the fact that most studies were designed to observe changes in body composition, not on cardiovascular health. The differences can be attributed to different doses, in different populations over different time spans.

One study by Noone et al (2002) was designed specifically to address the effect of CLA on parameters of cardiovascular health. No effects of CLA on the beneficial high density lipoprotein (HDL) cholesterol levels were found. However, CLA supplementation significantly improved plasma triacylglyceride and very low density lipoprotein (VLDL) metabolism in human subjects, indicating a cardio-protective effect of CLA that has also been shown in animal models. From the studies focusing on body composition, one reported a decrease of 2% in HDL cholesterol levels in subjects with metabolic syndrome receiving CLA compared with control (Riserus et al., 2002a; 2002b). Another study also reported a decrease in HDL cholesterol levels in CLA supplemented subjects; these changes were not significantly different from control groups, but reflected a change in time within the group and were not considered clinically important (Blankson et al., 2000; Mougios et al., 2001). In contrast, the study by Smedman and Vessby showed an increase in HDL cholesterol in both the control and CLA supplemented groups compared to baseline levels. Another study showed no changes on HDL cholesterol (Petridou et al., 2003). Levels of HDL cholesterol should always be considered in combination with the total serum lipid profile, including total and LDL cholesterol levels. For example, the study by Blankson et al (2000) reported a decrease in HDL cholesterol in combination with a decrease in total cholesterol, which is considered beneficial rather than adverse. Other serum lipid profile parameters were as inconsistent as HDL cholesterol levels throughout the studies, making firm conclusions unfeasible at this point.

The inconsistency between the studies on serum lipid profiles indicates that the changes may be related to other factors than CLA intake, such as diet and

exercise. Such factors are difficult to control in free-living subjects and are more easily studied in animal models. The effects of CLA on atherosclerosis, an important factor in development of cardiovascular disease, have been investigated extensively in rabbits by Kritchevsky et al (2000). His studies clearly showed that CLA could inhibit and even reverse established atherosclerosis.

Considering all the evidence, it is unlikely that CLA supplementation increases the risk to develop cardiovascular disease.

5.8.3 Effects of CLA on parameters of inflammation

In a study by Riserus et al, (2002b) and Basu et al (2000), CLA was reported to increase urinary prostaglandins, which are markers of lipid peroxidation. High levels of lipid peroxidation are associated with inflammation and oxidative stress, but also occur in fasting subjects, by the breakdown of fat (Richelle et al., 1999). A certain level of lipid peroxidation may actually be expected after supplementation with CLA, as most polyunsaturated fatty acids are more susceptible to this than for example monounsaturated fatty acids or saturated fatty acids. For example, supplementation with fish oil significantly increased plasma lipid peroxides in women (Meydani et al., 1991; Wander et al., 1996). The control oil used in the studies by Riserus et al (2002b) and Basu et al (2000) was olive oil, which contains high levels of the monounsaturated fatty acid oleic acid, which has been shown to work as an anti-oxidant (Bartsch et al., 2002). Reports on the anti-oxidative properties of CLA are inconsistent and tissue specific (Devery et al., 2001; Henriksen et al., 2003). For example, CLA has been reported to be oxidative unstable in air at high temperatures (50 and 90°C) (Yang et al., 2000). CLA has also been reported to induce lipid peroxidation in cancer cells, which was associated with the anti-carcinogenic effects of CLA. Work by Banni et al (1998) showed that CLA is not formed during free-radical attack of poly-unsaturated fatty acids and does not possess antioxidant activity. The inconsistency in results of oxidative stability studies on CLA may be attributed to the various conditions, such as pH of the medium, temperature, UV light, and interaction with other (food) components (Yang et al., 2000). The oxidation of Clarinol™ G-80 is prevented by production under nitrogen and addition of anti-oxidants.

The method used to measure lipid peroxidation in the studies by Basu and Riserus was a radio-immuno assay of urinary 15-keto-dihydro-prostaglandin $F_{2\alpha}$ and of 8-iso-prostaglandin $F_{2\alpha}$ formation. This method has not been widely used, complicating the interpretation of the relevance of these results. Levels of CRP also

increased in the study by Riserus et al (2002b), but not in the study by Song et al (2003). Levels of CRP can be influenced by BMI, gender, diet, drugs, exercise and alcohol intake (Clifton, 2003). Other markers of inflammation measured in the study by Riserus et al (2002b), such as IL-6, α- and γ-tocopherol and TNFα, are known to increase at early stages in inflammatory conditions. These markers were not affected in the study by Riserus et al (2002a; 2002b), indicating that CLA did not induce inflammation. In fact, many studies have now reported an anti-inflammatory effect of CLA (Albers et al., 2003; Bassaganya-Riera et al., 2003). Considering all the evidence, it is unlikely that CLA is a promoter of inflammation.

5.8.4 Effects of CLA on gestation and lactation

A study by Masters et al. (2002) found a decrease in milk fat in women supplemented with 1.2 g/day of CLA. There was no significant change in fatty acid composition of the milk fat, as measured by gas chromatography. It was suggested that the decrease in milk fat consumption may pose a risk for infant growth, although no significant effects on infant weights were found in this study. The milk fat content remained within normal ranges. The control used in this study was olive oil, which increased the milk fat content. In fact, both the increase of fat by olive oil and the decrease of fat by CLA were not significant compared to the washout period, in which no supplements were taken.

An *in vitro* study by Cheng et al (2003) in cells isolated from maternal intercotyledonary endometrium, fetal allantchorion and amnion from late pregnant ewes demonstrated that supplementation of linoleic acid and CLA affected the proportions and amounts of prostaglandins expressed by all three tissue types. It was suggested that these changes in expression may affect both the initiation and progression of parturition. Extrapolation from these *in vitro* studies to *in vivo* situations is difficult, especially between species. For example, it is unknown how much CLA is available in the cells studied. A study in male rats by Sergiel et al (2001) showed recovery of radioactivity after ingestion of 1-^{14}C-CLA isomers was highest in adipose, liver and adrenal tissue, and the lowest recovery was found in the testes, stomach and intestines, indicating a low availability of CLA isomers in these tissues. Furthermore, high levels of linoleic acid have been present in the human diet for years and have not been associated with adverse effects on parturition.

A study by Elias and Innis (2001) was conducted to determine associations of birth outcome with maternal and infant plasma concentrations of CLA and other fatty acids. Regression analysis indicated an inverse relation between infant plasma CLA

and birth length, birth weight and length of gestation. However, the pregnant women investigated in this study did not receive CLA supplements. The CLA measured in the plasma must therefore have originated from dietary intake from for example dairy and beef and consisted most likely of c-9,t-11 CLA isomer only. The investigators concluded that other dietary or lifestyle factors associated with high intakes of dairy fats rather than CLA intake itself may explain the relations.

The effect of CLA consumption on milk fat and birth outcome has been investigated more closely in rats (Chin et al., 1994), cows (Baumgard et al., 2002; Perfield et al., 2002) and pigs (Pietras et al., 2002; Poulos et al., 2000). Although the milk fat content was found to decrease, supplementation with CLA did not affect litter size nor induce apparent abnormalities (Baumgard et al., 2002; Chin et al., 1994; Perfield et al., 2002; Poulos et al., 2000). On the contrary, feeding CLA to the rat dams during gestation and lactation improved the postnatal body weight gain of pups. Pups that continued to receive the CLA-supplemented diet after weaning had significantly greater body weight gain and improved feed efficiency relative to control animals (Chin et al., 1994). It is possible that the decrease in milk fat was compensated by greater milk consumption in these animals. A prenatal developmental toxicity study according to OECD guideline 414 was performed on methyl and ethyl esters of CLA in rats and did not find any adverse effects either (Richard, 2002).

In conclusion, studies in different humans and different animal models showed that no adverse effects in offspring have been directly associated with CLA 50:50 supplementation or any resulting decreased milk fat content. Therefore, supplementation with CLA 50:50 is unlikely to be harmful for both lactation and gestation.

5.8.5 Effect of CLA on liver in mice

Many studies on CLA with mice models of different strains and some studies with hamsters demonstrated extreme sensitivity to the adipose tissue reducing effects of CLA. For example, dietary intake of relatively low levels of CLA caused complete ablation of the adipose tissue (Takahashi et al., 2003; Tsuboyama-Kasaoka et al., 2000; Xu et al., 2003). The rapid loss of adipose tissue in mice is repeatedly reported in combination with an increase in liver weight and lipid content, and an increase in insulin levels (Clement et al., 2002; Delany et al., 1999; Tsuboyama-Kasaoka et al., 2000; West et al., 2000). The effects on liver and insulin levels were not a direct effect of CLA, but a result of the development of lipodystrophy: the loss

of functional adipose tissue. As dietary fat cannot be taken up by the adipose tissue, the burden shifts to other tissues such as liver and muscle. The development of lipodystrophy also occurs in patients with chronic diseases, often in combination with muscle wasting (Schambelan et al., 2002). Lipodystrophy in mice supplemented with CLA is not found in combination with muscle wasting, on the contrary, CLA has been shown to increase lean body mass (Park et al., 1999b). Lipodystrophy is not at all seen in humans, because the metabolic rate of humans is much lower compared to mice, making humans less sensitive to the fat mass reducing effects of CLA (Terpstra, 2001). Human trials report fat mass reductions in the range of 1.0-7.0%, compared to fat mass reductions of 40-90% in mice. Accordingly, in all human trials on Clarinol™ G-80 and comparable CLA preparations, liver function parameters, plasma glucose and insulin levels were not affected (reviewed by Gaullier et al., 2002). Direct measurement of liver fat content in humans is a costly and invasive process, but levels of liver enzymes are validated parameters to measure liver function. It is reasonable to conclude that the adipose tissue in humans consuming Clarinol™ G-80 at our proposed levels retains its normal function. Lipodystrophy and resulting effects on liver function, plasma glucose and insulin levels are not expected.

5.8.6 Continuous literature research

In order to be aware of new published data on CLA, the PubMed database of the National Library of Medicine is searched every week for new publications. All published studies on CLA are listed on the website of the Food Research Institute of the University of Wisconsin (http://www.wisc.edu/fri/clarefs.htm) and this website is checked regularly.

5.9 Acceptable and estimated daily intake for Clarinol™ G-80

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5.9.1 Acceptable daily intake of Clarinol™ G-80

The acceptable daily intake of Clarinol™ G-80 is based on published human trials in which subjects have ingested Clarinol™ G-80 or comparable substances (i.e. with similar amounts and composition of CLA isomers). For example, in a study by Blankson et al. (2000) and Berven et al. (2000), four dose levels of CLA were added for three months to the diet of sixty healthy overweight or obese subjects: 1.7, 3.4, 5.1 and 6.8 g/day of CLA. No treatment-related effects were found on glucose and insulin levels, liver and kidney function parameters (AST, ALT, bilirubin, g-

glutamyltransferase), blood safety parameters and vital signs. No treatment-related adverse events were found. Similar safety results were observed in a number of other published studies lasting from one to three months at various intake levels. Similar safety results were also observed in a six-month study (at a lower dose, 2.7 g/day) that has been published in abstract form and presented in a scientific forum (Atkinson, 1999a, b; 1998).

The highest daily intake evaluated in these published studies was 6.8 g/day CLA. Since Clarinol™ G-80 contains 80% CLA isomers, this would correlate to a level of Clarinol™ G-80 of 8.5 g/day.

These published data are further supported by data from a generally available human trial on Clarinol™ G-80 that has been discussed in scientific meetings. This study evaluated the use of Clarinol™ G-80 for one year in 60 overweight and obese individuals. The daily intake of Clarinol™ G-80 was 7.5 g/day, equivalent to 6 g/day CLA. Clarinol™ G-80 had no effect on liver function as there was no evidence of a treatment-related effect on liver enzymes. Similarly, no treatment-related effects were observed on fasting plasma insulin and glucose levels and hematology parameters. This study will shortly be submitted for publication (Whigham et al., 2003). Additional supporting data are also available (e.g., Gaullier et al., (publication in press), involving an intake of 3.4 g/day CLA by 180 subjects for 52 weeks).

Based on the information from the one year study, as discussed above, an appropriate ADI is 6.0 g/day CLA, or 7.5 g/day Clarinol™ G-80.

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5.9.2 Estimated daily intake

The estimated daily intake of Clarinol™ G-80 was calculated by Exponent, Inc. using generally accepted techniques for estimating food consumption based on 2-day average daily intake of the foods to which Clarinol™ G-80 is proposed to be added (i.e., the "target foods"). Estimates were based on consumption data from USDA's 1994-1996, 1998 Continuing Survey of Food Intakes by Individuals/What We Eat in America (CSFII) and Exponent/Novigen's Foods Analysis and Residue Evaluation Program (FARE™) software. A 2-day average typically overestimates long-term (chronic) daily intake. The highest calculated intake, the 90th percentile for "user" males ages 20+, is 1.993 g/day CLA. This amount is less than one-third of the ADI.

It is possible that consumers seeking CLA would purposely eat higher levels of foods to which CLA has been added. Exponent's data indicate that consumers are highly unlikely to eat full servings of all of the target foods over any given two-day period. Further, such consumers would likely follow labeling instructions providing

guidance on recommended levels of use. Thus, even if a consumer were to purposely increase intake of the target foods, it is highly unlikely that they would do so in a manner that would exceed the ADI. To illustrate this, Table 10 shows estimated CLA levels in one serving of each of the target foods.

If, for example, a consumer were to ingest full servings of CLA-fortified yogurt, a frozen or shelf-stable plate meal, and a high-protein bar every day, he or she would still not exceed the ADI for CLA. This level of consumption is considered implausible in light of Exponent's data indicating that such consumption would greatly exceed even conservative, 90th percentile estimates of consumption.

Based on the information discussed above, an appropriate EDI is approximately 2 g/day CLA, or 2.5 g/day Clarinol™ G-80.

Table 10 Estimated CLA levels in target foods

Target Foods	Serving size (g)*	Intended % CLA	Quantity of CLA (g)
Yogurt	227	1	2.27
Milk-based meal replacements, fluid	341	0.5	1.71
Frozen or shelf-stable plate meals with meat, poultry, fish as major ingredient	500	0.3	1.5
High protein bar	70	3	2.1
Bars: Breakfast, Meal replacement, Granola, Power	50	3	1.5
Low-calorie and reduced calorie salad dressings	34	2	0.68

*Serving sizes are based on USDA survey data and reflect the largest servings actually consumed. These are larger than FDA serving sizes for these foods.

The discussion above does not address background consumption of dietary supplements of CLA. Some dietary supplements are labeled with a recommended dose of 3 g/day. This amount, together with the EDI discussed above, is below the ADI. If consumers seeking CLA purposely eat higher levels of foods to which CLA has been added, it is likely that they would use these foods as substitutes for (rather than in addition to) CLA from dietary supplements.

The discussion above also does not address background intake of CLA from the normal diet. Background intake has been estimated by Exponent (based on the same techniques discussed above) as being approximately 359 mg/day. The highest mean estimates reported in the literature are 212 mg/day for men and 151 mg/day for women (Ritzenthaler et al., 2001), and there is also a reported estimate of 291 mg/day for lactating women with high-dairy diets (Park et al., 1999). Background

intake is not included in our discussion of the ADI or EDI because both of these numbers are based on studies in populations consuming CLA *in addition to* normal diets, and therefore the ADI and EDI are established *in addition to* CLA present in the background diet.

6. Opinion of an Independent Expert Panel

Loders Croklaan convened an Expert Panel of independent biomedical scientists, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, to critically evaluate available information on Clarinol™ G-80 to determine if this ingredient is safe and generally recognized as safe (GRAS) by scientific procedures under its conditions of intended use. The members of the Expert Panel included Professor Joseph F. Borzelleca, Ph.D., Medical College of Virginia; Professor David M. Klurfeld, Ph.D., Wayne State University; Professor Robert J. Nicolosi, Ph.D., University of Massachusetts-Lowell; and Professor Ernst L. Schaefer, M.D., Tufts University School of Medicine.

Based on its independent and collective critical evaluation of the scientific data and information currently available, the Expert Panel concluded that Clarinol™ G-80, produced in accordance with current good manufacturing practice and meeting appropriate food-grade specifications, is safe for the intended uses described in this report and considered 6 g/day CLA (7.5 g/day Clarinol™ G-80) to be an appropriate and conservative ADI. The Panel further concluded that, based on such data and information, experts qualified by scientific training and experience would generally recognize that Clarinol™ G-80 is safe for such uses. Therefore, the Panel determined that Clarinol™ G-80 is GRAS based on scientific procedures for its intended uses. (A copy of the Expert Panel's report is available upon request.)

7. Conclusion

7.1 Scientific Summary

Clarinol™ G-80 contains the most favorable known isomeric composition of CLA, with regard to both safety and functional use, and is the result of a well-established production process. There is extensive supportive evidence of the safety of Clarinol™ G-80 for its proposed uses. Sufficient human trials on Clarinol™ G-80 and comparable CLA preparations are available to evaluate the safety of intake for CLA. None of the 20+ human trials on Clarinol™ G-80 and comparable CLA preparations reported any significant treatment-related adverse events. In addition, no clinically significant effects of CLA were found on plasma glucose and insulin levels, liver and kidney function parameters and blood parameters. Supplements purified in one of the active isomers increased insulin resistance in overweight and obese men with metabolic syndrome, but not in healthy normal or overweight subjects. Such pure individual isomers are not the subject of this GRAS notification and are not currently available commercially for dietary supplement use.

The safety of Clarinol™ G-80 is further supported by the numerous studies in *in vitro* and animal models. Studies investigating the absorption, distribution and metabolism of the c-9,t-11 and t-10,c-12 CLA isomers indicate no adverse effects of Clarinol™ G-80. A 13-week sub-chronic toxicity study in rats demonstrated no treatment related adverse effects at an intake level of 5% Clarinol™ G-80. This was equivalent to a dietary intake of 2433 and 2728mg/kg bw/day for males and females respectively. Treatment related effects at the intake level of 15% were likely related to nutritional effects rather than toxicity. Results from the Ames test and the chromosome aberration test demonstrate that Clarinol™ G-80 is not genotoxic. This is further supported by the many studies demonstrating anti-carcinogenic properties of CLA. There are no indications of reproductive toxicity of Clarinol™ G-80, as demonstrated in a number of animal models.

With respect to the appropriate interpretation of study results and reported adverse events, Loders Croklaan and an independent Expert Panel have considered carefully the extent to which the intended use of Clarinol™ G-80 could present any significant safety issue. Based on the totality of the scientific evidence available at this time and weighing the evidence most relevant to an evaluation of safety of Clarinol™ G-80, Loders Croklaan and the independent Expert Panel have concluded that this ingredient is safe for its intended use.

The acceptable daily intake of Clarinol™ G-80 is based on published human trials in which subjects have ingested Clarinol™ G-80 or comparable substances. One study showed safe administration of 6.8 g/day CLA for 12 weeks (equivalent to 8.5 g/day CLA preparation) and other studies of varying durations showed safe administration at lower levels. The safety of this intake level is further supported by the safe administration of 6 g/day CLA (7.5 g/day Clarinol™ G-80) for 12 months in the study by Whigham et al. (2003). Accordingly, an appropriate Acceptable Daily Intake (ADI) at this time is 6 g/day CLA (7.5 g/day Clarinol™ G-80). An appropriate Estimated Daily Intake was calculated to be approximately 2 g/day CLA, or 2.5 g/day Clarinol™ G-80, which is well below the ADI. Based on all of the considerations in this safety review, it is reasonably certain that the proposed use of Clarinol™ G-80 will not cause harm.

7.2 Summary of GRAS Status

GRAS status is based on common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances added to food. A GRAS evaluation through scientific procedures is based on "generally available and accepted scientific data, information, methods, or principles, which ordinarily are published and may be corroborated by unpublished scientific data, information, or methods." Proposed 21 CFR 170.30(b); 62 Fed. Reg. 18960 (April 17, 1997). *See also* 21 CFR 170.3(h); 170.30(a), (b). There must be a "consensus among qualified experts about the safety of the substance for its intended use." 62 Fed. Reg. 18940. This section summarizes why there is a basis for concluding that there is a consensus among qualified experts that there is reasonable certainty that Clarinol™ G-80 will not be harmful under the intended conditions of use.

7.2.1 The GRAS Determination is Based on Generally Available Information, and Corroborated by Unpublished Information

This GRAS notification sets forth the scientific data and information that is published or otherwise generally available on the safety of Clarinol™ G-80 and related compounds in humans and animals, and also refers to unpublished corroborative information.

7.2.2. The GRAS Determination is Based on a Consensus Among Qualified Experts

Based on a critical evaluation of the information summarized in this report, an independent panel of qualified experts convened by Loders Croklaan unanimously concluded that the use of Clarinol™ G-80, meeting appropriate specifications and produced by current good manufacturing practice, is safe for its intended use as an ingredient in certain foods. It is also the Expert Panel's opinion that qualified experts in the field would generally recognize that Clarinol™ G-80 is safe for such use.

As discussed above, the panel consists of individuals who are recognized as qualified experts in their fields (additional information on the qualifications of these individuals is available on request). As a result of the qualifications of these experts, their individual and collective opinions provide a strong basis for concluding that Clarinol™ G-80 is generally recognized as safe by experts qualified by scientific training and experience to evaluate its safety, as required by section 201(s) of the Federal Food, Drug, and Cosmetic Act.

Accordingly, Loders Croklaan concludes that its GRAS determination is based on a consensus among qualified experts that there is reasonable certainty that the substance will not be harmful under the intended conditions of use.

7.3 GRAS Determination

Based on the information summarized in this notification, Loders Croklaan determines that Clarinol™ G-80, intended for use in certain foods as described in this notification, is generally recognized as safe within the meaning of section 201(s) of the Federal Food, Drug, and Cosmetic Act; 21 C.F.R. 170.3 and 170.30; and the proposed rules described at 62 Fed. Reg. 18960.

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SUBMISSION END

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June 23, 2004

Paulette Gaynor
Felicia Williams
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification GRN 000148
Vegetable Oil Conjugated Linoleic Acid Preparation
Amendment: Responses to FDA questions

Dear Drs. Gaynor and Williams:

This letter amends the above-referenced GRAS notification for Clarinol G-80 Vegetable Oil Conjugated Linoleic Acid ("CLA") Preparation by providing a partial response to your questions conveyed during a conference call between you and our counsel Daniel Dwyer on June 7, 2004. The following paragraphs provide your questions (in italics) and our response.

QUESTION 1

Clarification is needed for the phrases "and similar non-standardized products" and "similar non-standardized versions of standardized foods" that are found on the letter dated March 10, 2004, and also on page 6 of the Notice. These phrases are applied differently on those pages. The March 10, 2004 letter applies the phrase "and similar non-standardized products" to two food categories within the listing of food categories. Page 6 of the Notice has the phrase "and similar non-standardized versions of standardized foods" at the end of the listing of the food categories. Is the intended use of CLA in both standardized foods and non-standardized foods, or in non-standardized foods that are similar to standardized foods – and to which food categories?

RESPONSE 1

Our list of foods in which CLA preparation is GRAS includes two terms that are standardized, "yogurt" and "low calorie/reduced calorie salad dressing." Our GRAS determination, however, is not intended to be limited to these standardized terms, but is intended to cover both all standardized versions of yogurt and low-calorie/reduced

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calorie salad dressing, as well as all non-standardized foods that substitute for and resemble the standardized foods.

QUESTION 2

The notice states that the anti-carcinogenic effects of CLA have been well-established, leading to the recognition of CLA by the National Academy of Sciences as the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals (reference is Bauman 2000, page 7 of the Notice). We did not see a reference for Bauman in the reference list for this particular article. We did, however, see two references for Bauman as the primary author in 2000. Please provide the reference and further elaborate on, or provide a basis for, the recognition of CLA by the National Academy of Sciences as the only fatty acid shown to unequivocally inhibit carcinogenesis in experimental animals.

RESPONSE 2

This statement in our Notice should have referenced the following book: *Carcinogens and Anticarcinogens in the Human Diet: A Comparison of Naturally Occurring and Synthetic Substances*, National Academies Press, Washington, D.C. (1996). This book is the work of the Committee on Comparative Toxicity of Naturally Occurring Carcinogens, Board on Environmental Studies and Toxicology, Commission on Life Sciences of the National Research Council. The text of the book is available online at: http://books.nap.edu/books/0309053919/html/R1.html. The following is the text discussing CLA, which can be found on pp. 85-86:

> "As linoleic acid is the only fatty acid shown unequivocally to enhance carcinogenesis, conjugated linoleic acid (CLA) is the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals (Ha et al. 1987, 1990; Ip et al. 1991, 1994). In contrast, the data indicating that omega-3 fatty acids may inhibit carcinogenesis are ambiguous (Pariza 1988), although these fatty acids appear to play a role in reducing the risk of heart disease in human populations (NRC 1989a). The major dietary sources of CLA are foods derived from ruminant animals, for example dairy products and beef (Chin et al. 1992).
>
> "CLA is effective as an anticarcinogen when present in the diet of experimental animals at levels as low as 0.05% to 0.1% (Ip et al. 1994). It appears to act via signal transduction pathways and effects on prostaglandin metabolism. In this regard, it has recently been shown that CLA is a growth factor for rats, possibly because it also modulates the catabolic effects resulting from immune stimulation (Chin et al. 1994). There are similarities in the effects of CLA and the effects of omega-3 fatty acids on mammals and birds; in general, CLA appears to be more potent (Miller et al. 1994)."

QUESTION 3

The specification for vegetable oil conjugated linoleic acid preparation is listed on page 16 of the Notice; however, a specification for lead was not provided. Please provide a lead specification.

RESPONSE 3

Lead is part of the standard contaminant specifications for Clarinol™ G-80. Indeed, it is Loders Croklaan's policy to assure that all of its products conform to detailed specifications to assure safety from potential contaminants, although these detailed standards were not included in the specifications listed on page 16 of the Notice. Our specification for lead is a maximum of 0.1 ppm, and the table on page 16 of the Notice is hereby amended to include this value.

QUESTION 4

On page 24 of the Notice, Loders Croklaan states that a CLA preparation equivalent to vegetable oil conjugated linoleic acid preparation was studied in a randomized double blind study by Blankson et al. in 2000 and Berman et al. in 2000. Additionally, eight subjects withdrew from the study due to adverse events. However, no differences among treatment groups were found regarding adverse events. What were the adverse events that the subjects experienced and which group or groups did the eight subjects participate in?

RESPONSE 4

More detailed information regarding these adverse events will be provided shortly.

QUESTION 5

Table 5 on page 30 of the Notice lists the adverse effects included in the questionnaire that were completed bi-weekly by the participants. However, it would be helpful if a break-out of these adverse effects was compiled.

RESPONSE 5

Sixty-four subjects were enrolled at baseline. Because the protocol involved a series of perturbations over a period of a year, the data analyses were meaningful only for subjects who remained in the protocol for at least the first phase of the study, which was a weight loss intervention. A total of 50 subjects met these criteria. Below is a table with a breakout of the reported adverse events.

Adverse event	CLA (*n* = 27)			Placebo (*n* = 23)			statistical significance[1]
	♂ (*n*=7)	♀ (*n*=20)	total	♂ (*n*=8)	♀ (*n*=15)	total	
Abnormal menses	n.a.	7	**7**	n.a.	5	**5**	NO
Bruising	0	0	**0**	1	0	**1**	NO
Shortness of breath	0	0	**0**	1	1	**2**	NO
Chest pain	0	0	**0**	1	1	**2**	NO
Abnormal heartbeats	1	1	**2**	0	0	**0**	NO
Leg cramps	3	2	**5**	2	5	**7**	ρ=0.0877 (♀, Chi) ρ=0.0540 (♀, pooled)
Joint pains	4	4	**8**	6	7	**13**	ρ=0.0548 (♂+♀, Chi)
Abdominal pains	1	6	**7**	2	6	**8**	NO
Constipation	3	12	**15**	3	5	**8**	NO
Diarrhea	2	3	**5**	3	5	**8**	NO
Dry mouth	3	5	**8**	0	3	**3**	ρ=0.0384[2] (♂, Chi)
Nausea and vomiting	0	7	**7**	1	7	**8**	NO
Heartburn	4	0	**4**	1	2	**3**	NO
Skin rash	0	1	**1**	1	5	**6**	ρ=0.0230 (♂+♀, Chi) ρ=0.0661 (♂+♀, pooled) ρ=0.0277 (♀, Chi) ρ=0.0588 (♀, pooled)
Altered sense of time	0	0	**0**	0	1	**1**	NO
Anxiety	2	3	**5**	2	2	**4**	NO
Depression	3	0	**3**	1	4	**5**	ρ=0.0141 (♀, Chi) ρ=0.0766 (♀, pooled)
Elevated mood	1	1	**2**	1	1	**2**	NO
Irritability or anger	2	2	**4**	2	5	**7**	ρ=0.0357 (♂+♀, pooled) ρ=0.0877 (♀, Chi) ρ=0.0433 (♀, pooled)
Nervousness	2	2	**4**	2	1	**3**	NO
Loss of concentration	1	1	**2**	1	3	**4**	NO
Memory loss	0	0	**0**	0	2	**2**	NO
Binge eating	3	0	**3**	1	2	**3**	NO

Change in vision	2	0	**2**	0	1	**1**	NO
Headache	1	11	**12**	4	9	**13**	NO
Dizziness	2	5	**7**	4	6	**10**	NO
Sweating	0	0	**0**	0	2	**2**	NO
Tremor	0	0	**0**	0	0	**0**	NO
Drowsiness	1	1	**2**	2	3	**5**	ρ=0.0662 (♂+♀, pooled)
Insomnia	0	5	**5**	1	5	**6**	NO
Fatigue	5	4	**9**	4	9	**13**	ρ=0.0154 (♀, Chi) ρ=0.0055 (♀, pooled)
Vivid dreams	1	1	**2**	0	1	**1**	NO
Higher sex drive	0	1	**1**	1	0	**1**	NO
Lower sex drive	0	0	**0**	1	1	**2**	NO
Hair loss	1	8	**9**	1	9	**10**	ρ=0.0238 (♂+♀, pooled) ρ=0.0104 (♀, pooled)
Other	3	7	**10**	4	11	**15**	ρ=0.0470 (♂+♀, Chi) ρ=0.0015 (♂+♀, pooled) ρ=0.0247 (♀, Chi) ρ=0.0030 (♀, pooled)
Infection	3	9	**12**	5	12	**17**	ρ=0.0354 (♂+♀, Chi) ρ=0.0036 (♂+♀, pooled) ρ=0.0365 (♀, Chi) ρ=0.0020 (♀, pooled)

[1]The data were analyzed for males and females separately and combined. Results are shown for the T-test Pooled Probability and the Chi Square test only.
[2]Significant Chi Square in males is probably due to 1 subject marking the AE on many visits, since the Pooled Probability is not significant.

QUESTION 6

On page 53 of the Notice, it states that a copy of the expert panel report is available upon request. We would like a copy of the report.

RESPONSE 6

A copy of this report is enclosed.

QUESTION 7

The notice does not discuss the mechanism or mechanisms of action of conjugated linoleic acid and why the effects observed are not adverse, and under what

circumstances they could become adverse. Please provide a discussion of the mechanism or the mechanisms of action of conjugated linoleic acid, why observed effects are not adverse, and under what circumstances they could become adverse.

RESPONSE 7

The mechanism of action underlying the effects of CLA on body composition have been studied mostly in mice. Studies with mice models of different strains (as well as some studies with hamsters) demonstrated extreme adipose tissue loss after feeding CLA in the diet. Dietary intake of relatively low levels of CLA caused complete ablation of the adipose tissue in mice (Takahashi et al., 2003; Tsuboyama-Kasaoka et al., 2000; Xu et al., 2003). Different modes of action of CLA on the decrease of adipose tissue have been suggested in these studies. Increased energy expenditure, a decrease in fat deposition in adipose tissue, inhibition of adipocyte differentiation, an increase in lipolysis, and apoptosis are all pathways that have been suggested as the underlying mechanism. These mechanisms are not mutually exclusive and may occur simultaneously in different tissues (Belury, 2002; Brown et al., 2003).

Human trials report fat mass reductions in the range of 1.0-7.0%, compared to fat mass reductions of 40-90% in mice. According to Terpstra (2001), the differences between mice and humans can be attributed mainly to the difference in metabolic rate. A key factor in the difference in metabolic rate between humans and mice may be the difference in abundance of and responsiveness to PPARα in the liver. Human PPARα shares many functional characteristics with the rodent receptors, in that they can be transcriptionally activated by peroxisome proliferators and regulate specific gene expression. In general, PPARs regulate a number of genes involved in lipid metabolism, adipocyte differentiation and apoptosis (Boitier et al., 2003; Holden and Tugwood, 1999) and CLA has been shown to be an activator of several PPARs (Brown et al., 2003; Moya-Camarena et al., 1999). However, on average, it has been estimated that human hepatocytes express PPARα at 5-10% of the levels found in rodent hepatocytes. In addition, there may be a qualitative difference between rodent and human PPARs (Holden and Tugwood, 1999).

The observed effects of CLA on body composition of humans are not adverse because the effects have been limited to, at most, modest but statistically significant decreases (1.0-7.0%) in body fat. These effects have been considered desirable by themselves for cosmetic reasons and because they may reduce the risk of overweight or obesity, and they have not been accompanied by any significant treatment-related adverse effects or clinically significant effects on plasma glucose and insulin levels, liver and kidney function, or blood parameters in a wide range of population groups. These effects in humans can be distinguished from those in mice because, based on repeated observations in a number of studies, their magnitude and extent is consistently much less than that in mice. Although the mechanism of action of CLA in humans has not been fully elucidated, that mechanism clearly functions differently

in humans than in mice, and the published literature provides a plausible discussion of differences in metabolic rate and differences in PPARs to explain why this is so.

In theory, the effects of CLA on body composition in humans would become adverse if there were to occur extreme and rapid reductions in fat mass resulting in a loss of functional adipose tissue. However, no such effects are known to have been observed with ingestion of any quantity of CLA, and a balanced analysis of all of the available animal and human data indicates that such effects are not expected.

QUESTION 8

We are requesting additional discussions on two issues that Loders Croklaan discussed in the Notice: (a) increase in lipid peroxidation markers; and (b) decrease in maternal milk fat. Detailed questions will be sent by fax.

RESPONSE 8

A detailed discussion on these issues will be provided shortly.

Respectfully submitted,

Louis H.T. Dederen
Safety and Regulatory Affairs Manager

cc: Daniel R. Dwyer
Kleinfeld, Kaplan and Becker

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Opinion of an Expert Panel on the GRAS Status of a Preparation of Vegetable Oil Conjugated Linoleic Acid Glycerides as a Food Ingredient

1. Introduction

Loders Croklaan BV convened an Expert Panel (Panel) of independent biomedical scientists, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, to critically evaluate available information on a preparation of Conjugated Linoleic Acid (CLA) glycerides derived from vegetable oil to determine if this ingredient is safe and Generally Recognized As Safe (GRAS) by scientific procedures under its conditions of intended use. This ingredient, known by the trade name "Clarinol™ G-80" and which the Panel refers to as "CLA preparation," consists primarily of an approximately equal proportion of two CLA isomers, 9*cis*,11*trans* (c9,t11) and 10*trans*,12*cis* (t10,c12).[1] The members of the Panel included Professor Joseph F. Borzelleca, Ph.D., Medical College of Virginia; Professor David M. Klurfeld, Ph.D., Wayne State University; Professor Robert J. Nicolosi, Ph.D., University of Massachusetts-Lowell; and Professor Ernst L. Schaefer, M.D., Tufts University School of Medicine. Professor Michael W. Pariza, Ph.D., University of Wisconsin, served as technical advisor to the Panel.

A thorough search of the scientific literature relevant to the safety of CLA preparation was conducted through 27 February 2004 by Loders Croklaan and the results were made available to the Panel; this included information that is published or otherwise generally available to the scientific community knowledgeable about the safety of substances added to food. Loders Croklaan also provided information on the manufacture and specifications of CLA preparation, exposure estimates, and unpublished safety and efficacy information. The Panel evaluated other information, published and unpublished, deemed appropriate or pertinent to the safety of CLA preparation.

Following independent, critical evaluation of all information deemed necessary, members of the Panel consulted by telephone. The Panel met with its technical advisor, scientists from Loders Croklaan, and counsel to Loders Croklaan in Washington, DC, on January 6, 2004. Presentations were made by scientists from Loders Croklaan and by counsel. The Panel went into executive session following collective critical evaluation of all information considered. The Panel continued to communicate by telephone and met again by conference call on 27 February 2004. The Panel unanimously concluded that CLA preparation is GRAS by scientific procedures.

2. Identity, Characterization, Composition

CLA preparation is a mixture for which there is no defined Chemical Abstracts Service (CAS) registry number or empirical structural formula. However, the CAS No. for

[1] CLA preparation is currently marketed by Loders Croklaan in the United States and other countries as a dietary supplement ingredient.

Octadecadienoic acid 1,2,3-propanetriyl ester is 303101-61-3, and the CAS Nos. for the two principal CLA isomers, c9,t11 and t10,c12, are 872-23-1 and 2420-44-2, respectively. The formula for c9,t11 and t10,c12 CLA is $C_{18}H_{32}O_2$ and the molecular weight is 280.452.

CLA preparation is an oil containing a high concentration of triglycerides of two specific Conjugated Linoleic Acid (CLA) isomers (c9,t11 CLA and t10,c12 CLA) which occur naturally in foods.

3. Manufacturing, Specifications, Batch Analyses

In summary, the production of CLA preparation involves (1) partial hydrolysis of safflower oil (or other source vegetable oil) (to the extent necessary depending on linoleic acid content); (2) alkali-catalyzed isomerization of linoleic acid; (3) distillation to isolate free fatty acids enriched in CLA; (4) esterification; (5) bleaching and deodorizing; and (6) the addition of a safe and suitable antioxidant as a preservative. All processing aids and additives are food-grade, safe and suitable for their intended use, and the manufacturing process is standard for such products, carried out consistently with requirements for current Good Manufacturing Practice (GMP).

CLA preparation is composed primarily of triglycerides, consisting of:

- not less than 78% CLA;
- not less than 74% of the total of c9,t11 and t10,c12 CLA isomers; and
- not less than 36% of each of the individual c9,t11 and t10,c12 CLA isomers.

Other specifications for CLA preparation have been reviewed by the Panel and determined to be appropriate. Batch analyses confirm that these specifications are consistently met.

4. Exposure: Background Consumption, Intended Uses, and Anticipated Consumption (Estimated Daily Intake (EDI))

CLA, including the c9,t11 and t10,c12 isomers, is present in relatively low concentration in many foods including meat (particularly meat from ruminants), milk, other dairy products, and vegetable oils. These components are both naturally occurring and produced by manufacturing processes (e.g., partial hydrogenation of soybean oil). Ritzenthaler, et al. (2001) estimated that the mean total dietary intake of CLA in the United States is 212 mg/day for males and 151 mg/day for females.

CLA preparation is proposed to be used as an ingredient in yogurt, milk-based meal replacements, bars (e.g., high protein, breakfast, meal replacement, granola, and power bars), low-calorie and reduced-calorie dressings for salad, and frozen or shelf-stable plate meals with meat, fish or poultry (i.e., the "target foods"). The estimated daily intake

(EDI) of CLA preparation was calculated by Exponent, Inc. using generally accepted techniques for estimating food consumption based on 2-day average intake of the target foods. Estimates were based on consumption data from USDA's 1994-1996, 1998 Continuing Survey of Food Intakes by Individuals/What We Eat in America (CSFII) and Exponent/Novigen's Foods Analysis and Residue Evaluation Program (FARE™) software. A 2-day average typically overestimates long-term (chronic) daily intake. The highest calculated intake from the proposed use, the 90th percentile for "user" males ages 20+, is 1.993 g CLA/day. This could result in a total daily intake of 2.5 grams of CLA preparation.

CLA preparation is proposed to be added to the target foods for potentially beneficial functional uses, which may include maintenance of appropriate body lean-to-fat ratios and other uses, for which there exists an extensive literature. The Panel reviewed this information but did not consider any potentially beneficial effects in evaluating the safety of CLA preparation.

5. Biological Data Pertaining to Safety

The data relevant to evaluating the safety and GRAS status of CLA preparation are derived primarily from studies in humans and supported by studies in animals

5.1. Absorption, Distribution, Metabolism and Excretion

In view of the composition of CLA preparation and based on the available data, the Panel considered the general ADME properties of this ingredient to be comparable to those of other triglycerides, with the exception of metabolic differences that are likely relevant to its studied functional effects. Based on published data, the triglyceride of CLA is split in the stomach by pancreatic lipase, forming a 2-mono-glyceride and two CLA free fatty acids. The 2-mono-glyceride and the two free fatty acids are then absorbed from the lumen of the proximal small intestine. In this regard, absorption of CLA isomers appears to be comparable to that of oleic and linoleic acid. Published studies further indicate distribution of the fatty acid moieties primarily to the brain, heart, liver, serum, spleen, kidney, lung and adipose tissue. With regard to metabolism, CLA uses the same desaturation and elongation pathways as linoleic acid, specifically Δ5- and Δ6-desaturase and elongase, so that some competition between these fatty acids may occur. For example, a CLA intake-dependent reduction was found in arachidonic acid, a metabolite of linoleic acid; however, no adverse consequences of this decrease in arachidonic acid are known. (In fact, the decrease in arachidonic acid may be related to anticarcinogenic properties of CLA that have been observed.) Most CLA metabolites (conjugated diene (CD)18:3, CD20:3) are incorporated in neutral lipids; the CD20:4 metabolite of CLA is incorporated mainly in phospholipids, similar to linoleic acid. The metabolites of CLA can subsequently be partly or fully oxidized in peroxisomal ß-oxidation pathways.

5.2. Acute and Subchronic Studies

The principal published animal study is a sub-chronic toxicity study in male and female Wistar outbred (Crl: (WI)WU BR) rats, 20 sex/group, which received 7% safflower oil (low-fat control), 15% safflower oil (high-fat control) or CLA preparation as a dietary admixture at concentrations of 1, 5 or 15% for 13 consecutive weeks followed by a 4-week recovery period (for 3 recovery groups of 10 rats/sex/group in the two control groups and at 15%). The NOAEL was reported to be 5%, equivalent to 2433 and 2728 mg/kg bw/day in male and female rats respectively. This was based on increased absolute and relative liver weights, increased liver enzyme activity and hypertrophy (completely reversed in males only after the recovery period), increased plasma insulin (reversible after recovery), and decreased glucose levels (males only and not completely reversible) at the 15% level. There were no histopathological findings in any of the animals receiving the CLA preparation, including those that received the 15% diet. This suggests that the NOAEL of 5% is a conservative one.

5.3. Long Term and Carcinogenicity Studies

There were no reported studies on the long term effects or potential carcinogenicity of CLA preparation in experimental animals. However, there were a number of published studies that have established anticarcinogenic effects in animals of CLA fatty acids and compounds similar to CLA preparation (for example, Ip, et al., 1991, 1994, 1999, 2002; Ha, et al., 1987; Belury, et al., 1996).

5.4. Genotoxicity

CLA preparation was evaluated for mutagenicity in the Ames Test (strains TA 98, T100, TA 1535, TA 1537 and TA 102) and it did not induce mutations in the presence or absence of S9 fractions (metabolic activation). CLA preparation was tested in an *in vitro* cytogenetics assay using human blood lymphocyte cultures and it did not induce chromosome aberrations in the presence or absence of S9 fraction.

5.5. Human/Clinical Studies

In evaluating the safety of CLA preparation, the Panel considered all relevant animal and human data, applying a weight-of-the-evidence approach. The Panel reviewed approximately 20 clinical studies that are published or generally available that involved the administration to human subjects of CLA preparation and related materials consisting of approximately equal ratios of the c9,t11 and t10,c12 isomers. Although many of these studies were primarily designed to evaluate potentially beneficial functional effects, the studies also included assessment of safety parameters and involved a number of different subject populations (including healthy, healthy overweight and obese, healthy lactating, type 2 diabetics), dosages (1.7- 6.8 grams/day), and durations of use (10 days to 13 weeks). In general, these studies were adequate and well-controlled for their purposes, and the Panel gave them appropriate weight based on the extent to which they evaluated

safety parameters most directly relevant to the administration of approximately equal ratios of the c9,t11 and t10,c12 isomers.

In particular, the Panel considered the published study by Blankson et al. (2000) to be especially relevant, as it evaluated numerous safety parameters in overweight subjects for 12 weeks; the highest intake was 6.8 g/day of CLA consisting primarily of equal proportions of the c9,t11 and t10,c12 isomers (equivalent to 8.5 g/day CLA preparation). This study raised no significant safety issues. The Panel also considered the generally available studies by Atkinson (1998), Whigham (2003), and Gaullier (2003) to be relevant, as they evaluated longer durations of use at lower intake levels (26 weeks at 2.7 g/day CLA, 52 weeks at 6 g/day CLA, and 52 weeks at 3.4 g/day CLA, respectively) and similarly raised no significant safety issues.[2] The Panel further considered a number of other published and generally available human studies to be relevant and consistent with these conclusions.

5.6. Other Issues Addressed by the Panel

The Panel considered the following issues.

5.6.1. Insulin Resistance

One study investigated the effects of c9,t11 and t10,c12 CLA isomers in a 50:50 mixture, and also investigated each isomer individually, at 3.0 g/day. A significant decrease in insulin sensitivity was observed in the groups receiving individual purified t10,c12 CLA and c9,t11 CLA. (Riserus et al., 2003). However, this effect was not observed in the group receiving the 50:50 mixture, and differences in insulin or glucose metabolism were not observed in any of the other published and generally available studies in which a 50:50 mixture of these isomers was administered (including a trial in men with metabolic syndrome). It is not possible to definitively reconcile these results; possibly the two-isomer combination eliminates effects of the individual isomers, for example, by activating different PPAR-regulated enzymes (Brown et al., 2003). It is also possible that the background diet or non-CLA components in the purified isomer supplements were responsible for the observed effects.

Moreover, in a trial presented at the American Oil Chemists Society Meeting in 2002, purified isomer preparations similar to those used in the study by Riserus et al. (2003) were tested in overweight middle-aged Europeans for 18 weeks at 1.5 or 3.0 g/day (Verboeket-van de Venne et al., 2002). In this trial, no observed adverse effects were reported on insulin sensitivity or on any other clinical-chemical parameter evaluated. Also, in two studies with healthy men and women who received supplements containing c9,t11 CLA and t10,c12 CLA in an 80:20 ratio at a daily dose of 3 grams for 8 weeks or 1.6 grams for 12 weeks, no adverse effects on glucose and insulin levels were reported (Noone et al., 2002; Albers et al., 2003.)

[2] *See also* the discussion of these studies in section 6, below.

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The CLA preparation under consideration consists of approximately equal proportions of the c9,t11 and t10,c12 isomers and the Panel concludes that the weight of the evidence strongly indicates that it does not adversely effect insulin or glucose metabolism.

5.6.2. Risk of Cardiovascular Disease

The Panel considered the potential relationship of CLA preparation to cardiovascular disease because CLA is considered by some to be a *trans* fatty acid (although CLA is not defined in the United States as a *trans* fatty acid), and because CLA has been reported to decrease (2%) high-density lipoprotein (HDL) cholesterol levels in subjects with metabolic syndrome (Riserus et al., 2002a; 2002b).

In a published study by Noone et al. (2002; using 50:50 and 80:20 isomeric blends of c9, t11 and t10, c12; 3 gram/day for 8 weeks), which was designed specifically to address the effect of CLA on biomarkers of cardiovascular disease, no adverse effects of CLA on HDL cholesterol levels were observed. However, CLA supplementation significantly improved plasma triacylglyceride levels and very low density lipoprotein (VLDL) metabolism in human subjects, which are positive effects from the perspective of cardiovascular health (similar to those shown in animal models). A study by Smedman (2001; using 50:50 isomeric blend of c9,t11 and t10, c12; 4.2 grams/day for 12 weeks) showed an increase in HDL cholesterol in both the control and CLA-supplemented groups compared to baseline levels. A study by Petridou et al. (2003) showed no changes in HDL cholesterol levels. In this study, seventeen sedentary women with BMI<30kg/m2 and aged 19-24 received 2.1 g CLA for 45 days and placebo (soybean oil) for 45 days with no washout period in between, in a randomized double-blind cross-over design. The CLA preparation contained a 50:50 blend of c9,t11 and t10, c12 CLA isomers. The study by Blankson et al. (2000; using a 50:50 isomeric blend of c9,t11 and t10, c12; 1.7 to 6.8 grams/day for 12 weeks) reported a non-significant decrease in HDL cholesterol in combination with a decrease in total cholesterol, which is considered beneficial rather than adverse.

The inconsistencies among the studies that evaluated serum lipid profiles suggest that the changes reported may be related to factors other than CLA intake, such as diet and exercise. Based on the weight of the evidence presented, the Panel considers it unlikely that intake of CLA preparation will have any significant effect on the risk of cardiovascular disease.

5.6.3. Effects on Parameters of Inflammation

In studies by Riserus et al. (2002b) and Basu et al. (2000), CLA was reported to increase urinary prostaglandins, which are markers of lipid peroxidation. High levels of lipid peroxidation are associated with inflammation and oxidative stress, but also occur in fasting subjects by the breakdown of fat (Richelle et al., 1999). Indeed, a certain level of lipid peroxidation may be expected after supplementation with CLA, as most polyunsaturated fatty acids are more susceptible to this effect than, for example, monounsaturated fatty acids or saturated fatty acids. (Similarly, supplementation with

fish oil significantly increased plasma lipid peroxides in women (Meydani et al., 1991; Wander et al., 1996).) Further, the control oil used in the studies by Riserus et al. (2002b) and Basu et al. (2000) was olive oil, which contains high levels of the monounsaturated fatty acid oleic acid that may have functioned, by comparison, as an antioxidant in these studies. An anti-inflammatory effect of CLA has been reported (Albers et al., 2003; Bassaganya-Riera et al., 2003).

Based on the weight of the evidence presented, the Panel considers it is unlikely that CLA preparation will have any significant effect on the promotion of inflammation.

5.6.4. Effects on Gestation and Lactation

Masters et al. (2002) reported a decrease in milk fat in women supplemented with 1.2 g of CLA/day but this decrease was non-significant when compared with the 5-day washout period during which no supplements were given. There was an increase in milk fat associated with the control, olive oil. There were no significant changes in fatty acid composition of the milk, other than the CLA isomers, of the mothers that received CLA preparation or olive oil. Some reports have correlated CLA concentration in the triglycerides of umbilical cord blood with reduced birth weight and height. CLA supplementation was not included in these studies, and the reports concluded that other dietary or lifestyle factors associated with high intakes of dairy fats may explain the relationship.

There have been no reported adverse effects of CLA on litter size or other reproductive/development parameters evaluated in animal studies. There is no known association between CLA intake and observed adverse effects on gestation or lactation.

The Panel considered the likelihood that any minor decrease in maternal milk fat would be adequately compensated for by natural adjustments in consumption patterns by infants.

Based on the weight of the evidence presented, the Panel considers it unlikely that intake of CLA preparation will adversely effect the outcome of reproduction.

5.6.5. Effects on Liver of Mice

A number of studies indicate that mice are extremely sensitive to the observed adipose tissue-reducing effects of CLA. Dietary intake of relatively low levels of CLA was associated with complete ablation of the adipose tissue in some studies, in combination with an increase in liver weight and lipid content and an increase in insulin levels. These effects on liver and insulin levels appear to be a result of the development of lipodystrophy, i.e., the loss of functional adipose tissue. These effects have not been reported in humans. Possible explanations include the difference in metabolic rate (the metabolic rate of humans is much lower than that of mice; human trials report fat mass reductions in the range of 1.0-7.0%, as compared to 40-90% in mice); effects on peroxisome proliferator-activated receptor-gamma (PPAR-gamma), and changes in

SCD-1 (a delta-9 desaturase). Consistently, humans using CLA preparation and comparable ingredients show no detectable effects on liver function parameters, plasma glucose levels, and insulin levels.

Based on the weight of the evidence presented, the Panel considers it highly unlikely that intake of CLA preparation will have an adverse effect on normal human adipose tissue function and related liver function.

5.6.6. Reported Adverse Events

The Panel is aware that four adverse events have been reported in Norway that may be associated with consumption of CLA supplements, including one event of pancreatitis, one event of liver function test disturbances and liver steatites, and two events of pulmonary hypertension. Although these are serious events, a causal relationship between these events and CLA has not been established. In addition, intake levels and compositions of CLA were not reported. This limited information was evaluated in light of (1) the absence of reports of adverse events from the human trials conducted with CLA preparation and similar substances, and (2) the lack of any adverse events in reports from the United States and other countries where CLA has been marketed in significant quantities for several years as a dietary supplement ingredient.

The Panel recommends continued monitoring for adverse events from the use of CLA preparation and for any new food ingredient.

Based on the weight of the evidence presented, the Panel concludes that CLA preparation is safe for its proposed use.

6. Discussion and Summary

The above is based on scientific data and information that are published or otherwise generally available to the relevant scientific community and critically evaluated by the Panel.

The absorption, distribution, metabolism and excretion (ADME) of CLA preparation is similar to linoleic acid. CLA preparation is relatively non-toxic orally when administered acutely or subchronically (dietary administration for 13 weeks). The NOAEL in the subchronic study was 5% in the diet, equivalent to 2433 and 2728 mg/kg bw/day for male and female rats respectively. CLA preparation was non-genotoxic in two *in vitro* assays.

Data from placebo-controlled and randomized human trials including a trial in which 6.8 grams of CLA isomers/day were consumed for 12 weeks and a 52-week trial in which 6 grams of CLA isomers/day were consumed failed to identify any adverse effects on any of the parameters evaluated.

Consideration of three studies requires explanation. The study by Atkinson et al. (1998) has been described in a book chapter, presented at a scientific meeting and accepted for publication in a peer-reviewed journal; the studies by Whigham et al. (2003) and Gaullier et al. (2003) have been presented at scientific meetings and have been submitted for publication. Although these three studies have not yet been published in scientific journals, their relevant information has been made generally available to the scientific community knowledgeable about the safety of substances added to food. Therefore, the Panel considers these three studies appropriate for consideration in conducting a GRAS determination. The results corroborate the findings of Blankson, et al. (2000).

For example, Whigham et al. (2003) evaluated the efficacy and safety of CLA preparation in an obese population that received 6.0 g/day CLA isomers (7.5 g/day CLA preparation) for 12 months. A number of safety parameters were evaluated every two weeks, including fasting insulin and glucose levels, liver and kidney function, and hematological and clinical chemistry. There were no adverse effects reported due to the CLA preparation. These data are consistent with other data, including shorter-term data on higher intake levels (e.g., Blankson et al. 2000, 6.8 g/day CLA for 3 months, equivalent to 8.5 g/day CLA preparation).

The Panel also considered the views of a scientific committee convened by the Norwegian Food Control Authority (2004). This committee expressed the possibility that CLA could increase the risk of adverse effects relating to insulin resistance, cardiovascular disease, birth weight, and fat content of maternal milk. The Panel considered these views carefully and, based on the totality of the scientific evidence available at this time and the appropriate weighing of the evidence most relevant to an evaluation of the safety of CLA preparation, the Panel respectfully differs with the committee on its assessment of the significance of any risk posed by the proposed use of CLA preparation. Each of the safety issues considered by the committee is addressed in the discussion above.

7. Acceptable Daily Intake

An Acceptable Daily Intake (ADI) is determined from a critical evaluation of available human and animal safety data. Human data are preferred since extrapolation is not necessary. The available information on CLA isomers supports the safety of these isomers as sources of CLA. No significant adverse health effects were reported in a clinical study at doses up to 6.8 g CLA isomers/day for 12 weeks (equivalent to 8.5 g/day CLA preparation). This is corroborated by the safe administration of 6 g CLA isomers/day (7.5 g/day CLA preparation) for 12 months in the study by Whigham et al. (2003). The Panel considers 6 g/day CLA (7.5 g/day CLA preparation) an appropriate and conservative ADI .

8. Conclusion

Based on its independent and collective critical evaluation of the scientific data and information currently available, the Panel concludes that CLA preparation, produced in accordance with cGMP and meeting appropriate food-grade specifications, is safe for the intended uses described herein.

The Panel further concludes that, based on such data and information, experts qualified by scientific training and experience would generally recognize that CLA preparation is safe for such uses.

Therefore, the Panel has determined that CLA preparation is GRAS based on scientific procedures for the intended uses described herein.

Joseph F. Borzelleca, Ph.D. 19 March 2004
Medical College of Virginia

Robert J. Nicolosi, Ph.D.
University of Massachusetts-Lowell
March 25, 2004

David M. Klurfeld, Ph.D. March 30 2004
Wayne State University

Ernst L. Schaefer, M.D.
Tufts University School of Medicine
23 March 2004

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CURRICULUM VITAE

NAME

Sebastiano Banni

BIRTH DATE

ADDRESS

Università degli Studi di Cagliari
Dipartimento di Biologia Sperimentale
Cittadella Universitaria
09042 Monserrato (Cagliari), Italy

Phone +39/070-675-4128 (office)
+39/070-675-4033 (lab)
FAX +39/070-675-4032

E-MAIL BANNI@VAXCA1.UNICA.IT

ACADEMIC INFORMATION

1999-2004

Teaching activities on Cellular Pathology, Cellular Physiology, Physiology of Nutrition for undergraduate and postgraduate students of the Faculty of Sciences at University of Cagliari, Cagliari and University of Sassari , Italy.

1997-2004

Permanent position as Assistant Professor in the Department of Experimental Biology, University of Cagliari.

1993-1999

Series of seminars of General Pathology for undergraduate and postgraduate students of the Faculty of Sciences at University of Cagliari, Cagliari Italy.

1991

Received the Doctorate of Research in Experimental and Molecular Pathology discussing a thesis on "characterization of some unusual fatty acids in animal tissues".

1990

Permanent position as a research technician in the Department of Experimental Biology, Section of Experimental Pathology, University of Cagliari, Chaired by Prof. F.P. Corongiu

1987-89

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Spent the Doctorate of Research as a Research Fellow in the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh PA, USA, under the supervision of Prof. Benito Lombardi, working on a project on conjugated dienes in choline deficient rats

1987

Examination to attend the quadrennial Italian Doctorate of Research in Experimental and Molecular Pathology directed by Prof. Mario Umberto Dianzani.

1985

Annual practicum in the Institute of Pharmacology (Cagliari) and General Pathology (Cagliari) in fulfillment of the State requirement for preparation for the ability to practice as a biologist.

State examination to qualify to practice as a biologist.

1984

Graduated, with honors, in Biological Sciences from Cagliari University,
November 1981-March 1984 : experimental project on the determination and separation of some aflatoxins using an improved HPLC procedure in fulfillment for the honors degree.

1979

5 years High school (Science) in Cagliari

SCIENTIFIC INFORMATION

SIGNIFICANT SCIENTIFIC CONTRIBUTION ON CLA RESEARCH

1) Absorption and deposition in rat tissues of low level of dietary CLA from partially hydrogenated oils
2) Metabolization of CLA to CD 18:3, CD 20:3 and CD 20:4
3) CLA and oxidative stress
4) Increase of retinol levels in liver, plasma and mammary tissue by dietary CLA
5) Decrease of linoleic acid metabolites in mammary tissue by dietary CLA
6) Correlation between changes in lipid metabolism and CLA anti-carcinogenic activity in mammary tissue
7) CLA enriched butter and vaccenic acid exerts the same preventive activity versus mammary cancer in rodents, as does synthetic CLA.
8) Identification of CLA peroxisomal beta oxidation products in in vivo and in vitro models and humans

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16) S. Banni, L. Lucchi, A. Baraldi, B. Botti, G. Cappelli, F.P. Corongiu, M.A. Dessi, A. Tomasi and E. Lusvarghi
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Nephron 72, 177-183, 1996

17) S. Banni, M.S. Contini, E. Angioni, M. Deiana, M.A. Dessi, M.P. Melis, G. Carta and F.P. Corongiu
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18) V.B. Ritov, S. Banni, J.C. Yalowich, B.W. Day, H.G. Claycamp, F.P. Corongiu and V.E. Kagan
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Biochim Biophys Acta 1283, 127-140, 1996

19) V. A. Tyurin, G. Carta, Y. Y. Tyurina, S. Banni, B.W. Day, F. P. Corongiu and V.E. Kagan
Peroxidase-catalyzed oxidation of beta carotene in HL-60 Cells and in model Systems: Involvement of phenoxyl radicals.
Lipids 32, 131-142, 1997

20) Aruoma, O.I., Deiana, M., Jenner, A., Halliwell,B., Banni,S., Corongiu, F.P., Dessì,M.A., Aeschbach,R.
Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low density lipoprotein
J. Agricol Food. Chem. 46, 5181-5187 1998

21) S. Banni, E. Angioni, G. Carta, M. Deiana, M.A. Dessi, M.P. Melis, S. Scrugli and F.P. Corongiu
Assessment of lipid peroxidation in biological systems. In "Advances in Free Radicals in Disease IV"; R. Pizzala, A. Tomasi and V. Vannini Eds., La Golardica Pavese, Pavia. pp. 55-69, 1999

22) M. Deiana, O.I.Aruoma, M.D.P.Bianchi, J.P.E.Spencer, H. Kaur, B.Halliwell, R.Aeschbach, S. Banni, M.A.Dessi, F.P. Corongiu
Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol.
Free Rad. Biol. Med.. 26, 762-769, 1999

23) B. Botti, S. Banni, A.Bini, M.A. Dessì, F.P. Corongiu, A. Tomasi and V. Tannini
Glutathione,and ascorbate redox state and vitamin E in rat liver in vivo following CCl4, 1,2 dibromoethane, phorone and diethylmaleate treatment
Intl J Med Biol Envviron 27, 151-159, 1999

24) E. Angioni, G. Lercker, N. G. Frega, G. Carta, M. P. Melis, E. Murru, S. Spada and S. Banni
UV spectral properties of lipids as a tool for their identification.
Eur. J. Lipid Sci. Technol. 104, 65-68 2002

25) M.A. Dessì, M. Deiana, B.W. Day, A. Rosa, S. Banni and F.P. Corongiu.
Oxidative stability of polyunsaturated fatty acids: Effect of squalene

Eur. J. Lipid Sci. Technol. 104, 506-512 2002

INVITED SPEAKER, CHAIRMAN AND SEMINARS

1994

1) Seminar on " Characterization of conjugated diene-fatty acids from different sources in animal and human tissues " at Free Radical Research Group, Division of Biochemistry, Medical & Dental School Guy's & St Thomas's Hospital chaired by Prof. C. Rice-Evans.1994

2) Lecture on "Conjugated diene fatty acids in human and animal tissues" at "Unesco/Costam SFRR-ASIA workshop on nutrition, lipids, health and disease" held in Penang, Malesia from 1 to 3 September 1994

1996

3) Co-chaired with Dr. Huth the technical session on "Conjugated linoleic acid (CLA). Chemistry and Biology" at 87th Annual Meeting of the AOCS held in Indianapolis 1996. In the same session I have been invited to present an oral communication by the title "Organ deposition and metabolism of CLA in animal tissues"

4) Member of the scientific commettee and co-chairman of the session Health aspects of oxidation and antioxidants of the 1st meeting of the AOCS European section to be held in Dijon (France) 19-20 september 1996

1997

5) Chaired technical session on "General edible applications technology II" at 88th Annual Meeting of the AOCS. 1997

6) Lecture on "Conjugated linoleic acid. In vivo metabolism " at "CLA Forum" held in Madison, Wisconsin, USA, 13-14 August 1997

1998

7) Lecture on "the dietary source and the biological effects of CLA" at the first annual research/industry meeting organized by the Centre Europeén des Sciences du gout (CESG) to be held in Dijon November 25 & 26, 1998.

1999

8) Co-chaired with Dr. Scimeca the technical session on "Biological activity of conjugated linoleic acid" at 90th Annual Meeting of the AOCS held in Orlando 1999. In the same session I have been invited to present an oral communication by the title "Changes in lipid metabolism in association with CLA anti-carcinogenic activity."

9) Lecture on " Biological activities CLA " at the meeting of the Italian Society of Pharmacology. Firenze (Italy) 1999.

10) Co-chaired with Dr. Whale the session on "Conjugated linoleic acid (CLA)" at 40th ICBL Meeting held in Dijon (France)- Sept. 14-17 1999
Co-author of 47 Abstracts (30 as a first author) presented in National (13) and International (34) Meetings

2000

Seminar on "Conjugated Linoleic Acids and Chemoprevention of Breast Cancer" at the Department of Occupational and Environmental Health, University of Pittsburgh, Pittsburgh PA, USA.

Award for the form, content and presentation for an oral communication presented at 92nd Meeting of the American Oil Chemists' Society held in San Diego USA May 2000.

Invitato Speaker on "Metabolism of Conjugated Linoleic Acid in Relation to its Anticarcinogenic Activity" at 220th Meeting of the American Chemical Society. Washington, USA, August 2000.

2001
Invited speaker on "Lipid metabolism modulation by dietary CLA" at the 1st International Conference on Conjugated linoleic acid (CLA) held in Alesund, Norway, 10-13 June 2001.

Invited speaker on "Conjugated linoleic acid" at the Meeting organized by EU on "Functional foods: health benefits and safety issues" held in Brussels, 20-21 June 2001.

Invited speaker on "Modulation of lipid metabolism and vitamin A nutrition by conjugated linoleic acid" at the "Fatty acid and cell signalling" Meeting held in Gargnano, 2-3 September 2001.

2002
Invited speaker on "CLA metabolism" at the Meeting Perspectives on conjugated linoleic acid research, current status and future directions, organized by the Division of Nutrition Research Coordination (DNRC) of the National Institute of Health held in Bethesda, Maryland, USA il 15-16 Maggio 2002. (Web site con abstracts in PDF: http://ods.od.nih.gov/news/conferences/cla.pdf)

Invited speaker on "Modulation of lipid metabolism by naturally occurring conjugated linoleic acid in relation to its beneficial activities" 3rd Meeting Euro-Mediterraneo Cheese Art, held in Ragusa (Italy), 4-9 June 2002.
(Web: http://www.cheeseart.corfilac.it/conferenze_c.htm)

Invited speaker on "Lipid metabolism changes by dietary CLA in relation to its beneficial activities" at the Fifth International Congress on Essential fatty acids and eicosanoids, held in Taipei, Taiwan, 29 August-2 September 2002.
(Web: www.5thicefae2002.org)

Invited speaker on "Conjugated linoleic acid isomers (CLA): good for everything?" session Nutrition and Health of Congrilait 2002, 26th IDF World Dairy Congress held in Paris, France, 24 – 27 September, 2002. (Web: http://www.congrilait2002.com)

BEST ORIGINAL COPY

2003

Invited speaker on "Retinol tissue levels are positively correlated to dietary conjugated linoleic acid" at the12th European Meeting on Fat-Soluble Vitamins held in Rieti il 13-15 March 2003

Seminar on "Recent advances on Conjugated Linoleic Acid (CLA) metabolism in influencing vitamin A levels and fatty acid pattern in different tissues" at the Department of Human Nutrition, Ohio State University, Columbus, OH, USA.

Invited speaker on " CLA Accumulation and Metabolism in Some Human Pathological States" at the 94th Annual Meeting dell'American Oil Chemists' Society held in Kansas City, MO, USA, 5 May 2003.

Invitated speaker on " Different metabolism of conjugated linoleic acid (CLA) isomers. Is this the discriminant for their peculiar biological effects?" at the "Fatty acid and cell signalling" meeting held in Washington, DC, USA, 15-17 July 2003.

Invitato speaker on " Mechanism of action and immune-modulatory actions of conjugated linoleic acid" at the European Association of Animal Production (EAAP) meeting, held in Roma, Italy, 31 August 2003.

Invitato as a Keynote Speaker on "CLA and human health-An overview" at the "CLA and human health" meeting held in Toronto (Canada) il 21-22 November 2003.

2004

Invitato speaker on "CLAs as precursors of a new family of PUFAs"
At the 6th International Society for the Study of Fatty Acids & Lipids (ISSFAL) meeting held in Brighton (UK), 27 June to 1 July 2004. (web http://wheldonevents.co.uk/issfal/)

Invitato speaker at the Conference on "CLA in Human Health: Mechanisms and Biomarkers" October 21st - 23rd, 2004, Winnipeg, Manitoba, Canada.

Invitated speaker on "CLA metabolism in humans" at the 10th World Congress on Clinical Nutrition that will be held in Pearl Village, Phuket, Thailand, 30 November al 3 December 2004 .
(web www.psu.ac.th/news/wccn.htm)

SCIENTIFIC SOCIETY ACTIVITIES

Member of " Società Italiana di Patologia", "International Society for Free Radical Research" , "American Oil Chemists' Society" and "European Section of the American Oil Chemists' Society"

Member at large of the European section of the AOCS from 1996-1997.

President of the European Section of the AOCS from 1997-1998 .

LAB VISITING

1) 1985. Semestral grant from Fidia Research Laboratories for a project on the determination of homovanillic and dihydroxy phenyl acetic acid in rat and human plasma to evaluate the dopaminergic activity of the central nervous system.

2) 1985. Co-working in a research project on free radical activation of various carcinogenic compounds in vivo and in rat isolated hepatocytes using electron spin resonance and the spin trapping technique (Institute of General Pathology, University of Modena).

3) 1986. Co-working in a research project on free radical activation of Carbon Tetrachloride in vivo and lipid peroxidation, using second derivative spectra and Nuclear Magnetic Resonance techniques (Institute of General Pathology and Department of Chemical Sciences, University of Cagliari, Italy).

4) 1987. Annual fellowship from "Associazione Italiana per la Ricerca sul Cancro" to spend in the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh PA for a common project with Prof. Lombardi on lipid peroxidation and choline deficiency in rats.

5) 1987-90. Spent the Doctorate of Research as a Research Fellow in the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh PA, USA, under the supervision of Prof. Benito Lombardi, working on a project on conjugated dienes in choline deficient rats

6) 1993.Two months as a research fellow in the laboratories of Prof. Lombardi at the Department of Pathology and of Prof. Day at the Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh PA working on a project on the characterization of conjugated linoleic acid and its metabolites by HPLC equipped with on line diode array and mass spectrometer detectors in rat tissues.

7)1995. Three months as a research fellow in the laboratories of Prof. Lombardi at the Department of Pathology and of Prof. Day and Prof. Kagan at the Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh PA working on a project on the metabolism of conjugated linoleic acid in choline-deficient rats .

8) 1996. One month as a research fellow in the laboratories of Prof. Day and Prof. Kagan at the Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh PA working on a project on the fatty acid peroxidation in human leukemia cells

ORGANIZED MEETINGS

Co-organized with Prof. F.P. Corongiu and Dr. M.A. Dessi an International Meeting on "Free Radicals in Nutrition" held in Cagliari (Italy) 1992, Satellite of the Biannual Meeting of the International Society of Free Radical Research to be held in Torino (Italy).

Co-organized with Prof. F.P. Corongiu and Prof. M.A. Dessi the 2nd meeting of the AOCS European section to be held in Cagliari (Italy) 1-4 october 1998

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[redacted]

CURRENT COLLABORATIONS

Dr. Giovanni Piredda, Istituto Zootecnico Caseario, Sassari, Italy
research on: development of new dairy products enriched with conjugated linoleic acid (CLA)

Dr. Leonardo Lucchi, Policlinico Università di Modena, Italy
research on: metabolism of conjugated linoelic acid (CLA) in hemodylasis and chronic renal failure patients

Prof. Anna Iannone and Prof. Aldo Tomasi, Università di Modena, Italy
Research on: Development of new analytical methods to study PUFA oxidation by free radicals

Dr. Clement Ip, Roswell Park Cancer Institute, Buffalo, NY, USA
Research on: Absorption and metabolism of conjugated linoleic acid (CLA) in animal models of mammary carcinogenesis

Prof. Martha Belury, Department of Human Nutrition Ohio State University, Columbus, OH, USA
Research on: PPAR activation by dietary CLA and related changes on lipid metabolism.

Prof. Anna Petroni, Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano.
Research on: Degradative metabolism via peroxisomal beta oxidation in adrenoleukodistrophy patients.

Dr. Barbara Batetta, Università di Cagliari, Italy.
Research on: Modualtion of lipid metabolism and eicosanoid formation during atherosclerosis

MAIN RESEARCH GRANTS IN THE LAST 10 YEARS

1997-1998
1) Biannual grant from Dairy Management Inc. USA, for a project in collaboration with Dr. C. Ip from Roswell Park Cancer Institute, Buffalo, NY and Dr. D. Baumann from Cornell University, NY, on Mammary cancer prevention by milk fat-conjugated linoleic acid

1997-2000
2) Triannual grant from the European Community "Concerted action" on Conjugated linoleic acid

1999-2000
Biannual grant from Cattlemen and Beef Association, USA, in collaboration with Martha Belury, Department of Human Nutrition Ohio State University, Columbus, OH, USA.

2000-2003
3) Triannual grant from the local regional gouvernement of Sardinia on the development of new dairy products enriched with conjugated linoleic acid (CLA)

2003-2006
Triannual grant from the European Community within the 5th Framework Programme, Key Action Food, Nutrition and Health, n.# QLK1-2002-02362.

BOOK EDITED

Co-edited with Prof. F.P. Corongiu, Prof. M.A. Dessi and Dr. C. Rice-Evans the volume VII in the Richelieu Free Radical Series, "Free Radicals and Antioxidants in Nutrition"
The Richelieu Press, London. 1993.

REFEREE IN JOURNALS

J. Nutr.; Lipids; Brit. J. Nutr.; Eur.J.Clin. Nutr.; Biochim.Biophys. Acta; J. Lipid Res. , J. Am. Oil Chem. Soc.; Eur. J. Lipid Sci. Technol.; J. Chrom.; Pharmacol. Res.
Associate editor of the Newsletter of the International Society for the Study of Fatty Acids and Lipids (ISSFAL).

LIPID NUTRITION

RECEIVED
AUG - 2
OFFICE OF
FOOD ADDITIVE SAFETY

July 14, 2004

Paulette Gaynor
Felicia Williams
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification GRN 000148
Vegetable Oil Conjugated Linoleic Acid Preparation
Amendment: Responses to FDA questions

Dear Drs. Gaynor and Williams:

This letter amends the above-referenced GRAS notification for Clarinol G-80 Vegetable Oil Conjugated Linoleic Acid ("CLA") Preparation by providing a partial response to your questions conveyed during a conference call between you and our counsel Daniel Dwyer on June 7, 2004. The following paragraph provides your question 4 (in italics) and our response.

QUESTION 4

On page 24 of the Notice, Loders Croklaan states that a CLA preparation equivalent to vegetable oil conjugated linoleic acid preparation was studied in a randomized double blind study by Blankson et al. in 2000 and Berman et al. in 2000. Additionally, eight subjects withdrew from the study due to adverse events. However, no differences among treatment groups were found regarding adverse events. What were the adverse events that the subjects experienced and which group or groups did the eight subjects participate in?

RESPONSE 4

The human trials on CLA by Berven et al. (2000) and Blankson et al. (2000) were conducted with CLA material manufactured by Natural, Inc.[1] The material should be very similar to Clarinol™ G-80, as we understand that Natural was using the same production process for their CLA products as Loders Croklaan B.V. during the time period when the studies were conducted. Although there may be differences in

[1] Our GRAS notice (p. 24) suggests that this was a single trial. However, further review of the reports indicates that these were two separate trials.

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starting materials and storage conditions between the two products, these should be of no major importance.

Information on the adverse events in the trial by Berven et al. (2000) is available publicly in the following paper: "Safety of conjugated linoleic acid (CLA) in overweight or obese human volunteers." European Journal of Lipid Science and Technology 102: 455-462. The following is an outline of the relevant information in this paper:

The trial included 60 healthy overweight or obese subjects randomized into a placebo group and a 3.4g/day CLA group. Registration of adverse events was taken together with a clinical examination before and after the treatment (at baseline and week 12). Fifty-five of the 60 randomized subjects completed the study. In the CLA group, two withdrawals were registered: one due to lack of interest and one due to bad oral smell and bad smell of perspiration. In the placebo group 3 subjects withdrew: one due to lack of interest, one due to cessation of treatment during holiday, and one terminated the treatment due to gastrointestinal bleeding. Thus, 28 subjects in the CLA group and 27 subjects in the placebo group completed the study.
Three subjects in each group experienced one or more adverse events. Of these, 2 subjects (one in each group) withdrew from the study due to the adverse events. (As noted above, one subject (in the placebo group) was withdrawn by the investigator due to gastrointestinal bleeding, while the other subject (in the CLA group) withdrew on his own due to bad oral smell and smell of perspiration.) For the other subjects, no actions were taken due to adverse events. All adverse events were of mild to moderate character. The most frequent adverse events were gastrointestinal origin. All adverse events were regarded as possibly related to the administration of CLA or placebo.

Information on the adverse events in the trial by Blankson et al. is available publicly in the following paper: "Conjugated linoleic acid reduces body fat mass in overweight and obese humans." Journal of Nutrition 130: 2943-2948. The following is an outline of the relevant information in this paper:

The trial included 60 healthy overweight or obese subjects, randomized into one placebo group (olive oil) and 4 CLA groups (1.7, 3.4, 5.1 or 6.8 g/day CLA). Adverse events were monitored throughout the study. For every adverse event, a rating of severity, frequency, treatment relation, action taken and subject outcome was recorded. Forty-seven subjects completed the study after 8 subjects withdrew during the first 6 weeks of the study and another 5 withdrew from weeks 6 to 12. Eight subjects withdrew due to adverse events; others did not report for follow-up visits. The frequency of adverse events was 60% and no significant differences among the different treatment groups were reported. One of these adverse events was serious because the subject was hospitalized due to a relapse of asthma, but this adverse event was not judged to be treatment-related (the subject was in the CLA 3.4g/day group). Of all other adverse events reported, one was considered to be severe (fatigue); the remainder were of a mild-to-moderate character. The most

frequent adverse events were gastrointestinal symptoms, and may have been treatment-related. The numbers of possibly treatment-related adverse events were 3, 5, 9, 8 and 11 in the placebo, 1.7, 3.4, 5.1 and 6.8 g/day CLA groups, respectively. Of these 36 adverse events, 20 were gastrointestinal symptoms. Altogether, 55% of the adverse events were considered to have a possible connection to the study treatment. Although the number of possibly treatment-related adverse events in the placebo group was numerically lower than that in each of the CLA groups, the investigator reported no statistically significant difference in such events among the groups. As pointed out above, adverse events resulted in eight subject withdrawals; seven of these were in active treatment groups and one in the placebo group. The other subjects had transient adverse events that disappeared during continuous treatment without any dose adjustments. Both withdrawal rates and the occurrence of adverse events were quite high in the study. The authors state that this may be caused by the number of capsules that were to be taken each day (12 capsules). The dominant nuisances reported were of gastrointestinal origin. These events could have been caused by the capsules or the oil per se rather than the high content of CLA.

Thank you for your consideration of this information.

Respectfully submitted,

Andreas Menzel
Head Research and Development

cc: Daniel R. Dwyer
Kleinfeld, Kaplan and Becker

REFERENCES

Berven, G. et al. 2000. Safety of conjugated linoleic acid (CLA) in overweight or obese human volunteers. European Journal of Lipid Science and Technology 102: 455-462.

Blankson, H. et al. 2000. Conjugated linoleic acid reduces body fat mass in overweight and obese humans. Journal of Nutrition 130: 2943-2948.

AM

LIPID NUTRITION

July 23, 2004

Paulette Gaynor
Felicia Williams
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification GRN 000148
Vegetable Oil Conjugated Linoleic Acid Preparation
Amendment: Responses to FDA questions

Dear Drs. Gaynor and Williams:

This letter amends the above-referenced GRAS notification for Clarinol G-80 Vegetable Oil Conjugated Linoleic Acid ("CLA") Preparation by providing a partial response to your questions conveyed during a conference call between you and our counsel Daniel Dwyer on June 7, 2004. The following paragraph provides part (a) of your question 8 (in italics) and our response. (Copies of referenced scientific publications are provided in an accompanying binder.)

QUESTION 8
We are requesting additional discussions on two issues that Loders Croklaan discussed in the Notice: (a) increase in lipid peroxidation markers; and (b) decrease in maternal milk fat. (Additional discussion was provided in a facsimile dated June 7, 2004.)

RESPONSE 8 (a)

At your request, we have prepared an extensive review on the issue of observed increased F_2-isoprostane levels after CLA intake. This review was prepared with the help of an expert in the field of CLA and oxidative stress research, Dr. Sebastiano Banni. Dr. Banni is Assistant Professor at the Dipartimento di Biologia Sperimentale, Università degli Studi di Cagliari, Italy. (A copy of Dr. Banni's curriculum vitae is enclosed.)

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SUMMARY

Our review makes the following key points:

- Although dietary intake of CLA has been associated with relatively small increases in observed F_2-isoprostane levels, it is unlikely that CLA causes significant oxidative stress based on the following:
  - Other PUFAs such as fish oil do not increase F_2-isoprostane levels, and it is unlikely that the single extra double bond in CLA, as compared with oleic acid, would cause a significant difference in effects on F_2-isoprostanes.
  - The oxidative stability of CLA has been demonstrated in a number of tissues.
  - There is no indication that CLA induces an inflammatory state.
  - Significant evidence exists that CLA decreases oxidative stress and inflammation; for example:
    - The precursors of F_2-isoprostanes (arachidonic acid and prostaglandins) have been demonstrated in many studies to be decreased by CLA.
    - Studies indicate that CLA has no adverse effects on the development of diabetes.
    - CLA has been reported to have anti-atherogenic properties in the rabbit and hamster.

- It is much more likely that CLA does not cause a significant increase in the formation of F_2-isoprostanes.
  - By competing with isoprostanes for metabolic pathways, CLA likely causes a decline in metabolism of already-formed F_2-isoprostanes.
  - As a result, levels of F_2-isoprostanes *appear* higher in the presence of CLA (even though it is unlikely that they are actually higher) because fewer F_2-isoprostanes are metabolized into a different form.

- Isoprostane levels are not a definitive marker for oxidative stress.
  - Although the presence of disease is associated with elevated F_2-isoprostane levels in humans, an increase in F_2-isoprostanes does not necessarily imply the presence of increased oxidative stress or increased risk of any disease.
  - Small increases in F_2-isoprostane levels (at the levels observed in human trials of CLA) are not necessarily suggestive of oxidative stress because no threshold has been established at which the level of F_2-isoprostanes is indicative of this condition.
  - Other markers for oxidative stress must be considered and, as noted above, various data suggest that CLA does not cause oxidative stress.

Accordingly, based on the weight of the evidence, Loders Croklaan concludes that there is reasonable certainty that the observed effects on F_2-isoprostane levels do not represent

a harmful effect of CLA preparation under its intended conditions of use. This conclusion is based on scientific procedures, common knowledge about the substance throughout the scientific community, and the views of qualified experts.

DISCUSSION

Our review will begin with a discussion of the data referred to by FDA, followed by some background information on the formation of F_2-isoprostanes and a discussion of possible mechanisms by which CLA may increase the observed levels of F_2-isoprostanes. Lastly, an evaluation of the method used to measure lipid peroxidation is provided, followed by an overall conclusion on the relevance of the increased F_2-isoprostanes for the safety of CLA preparation.

Discussion of Data Referred to by FDA

The study by Smedman and Vessby et al. (2001) investigated the effects of 12 week CLA supplementation on body composition and fatty acid composition of serum lipids and thrombocytes in healthy humans. There was a 3.8% reduced body fat proportion in subjects supplemented with CLA. The change in fatty acid composition in the CLA supplemented subjects suggested an increase in Δ5-desaturase activity and a decrease in Δ6- and Δ9-desaturase activity. The enzyme Δ5-desaturase is involved in the formation of arachidonic acid from di-homo-γ-linolenic acid (figure 1). Levels of di-homo-γ-linolenic acid were indeed decreased, but arachidonic acid levels were not affected. To our knowledge, this study did not measure F_2-isoprostanes in the urine or plasma. Rather, this study referred to the study by Basu et al. (2000b) in the introduction and discussion.

It appears that Basu et al. (2000a) is the same clinical trial as published by Riserus et al. (2001). In this study, levels of both 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ were significantly increased in abdominally obese men supplemented with CLA for 1 month, but not in men supplemented with the control oil, olive oil. As will be explained in more detail later, 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ are products of lipid oxidation of arachidonic acid; 8-iso-$PGF_{2\alpha}$ is formed by non-enzymic free radical induced lipid peroxidation and 15-keto-dihydro-$PGF_{2\alpha}$ is formed by enzymic cyclo-oxygenase-catalyzed oxidation. The effects of CLA were completely reversed after 2 weeks. Plasma levels of arachidonic acid and isoprostanes were not reported. The non-enzymic conversion appeared to be affected more. Nevertheless, plasma levels of anti-oxidants (α- and γ-tocopherol), which work as hydrogen atom donors and thereby as a chain breaking of free radical induced lipid peroxidation, were not affected. Levels of products of the enzymic lipid peroxidation of arachidonic acid, thromboxanes, were not affected either. As will be discussed later, the use of olive oil may not be a good placebo since it has been shown to decrease F_2-isoprostane levels due to the presence of anti-oxidants (Visioli et al., 2000).

The most recent study by Smedman et al. (2004) investigated the regulatory mechanisms behind the increased lipid peroxidation observed after CLA supplementation in healthy

humans. The study investigated two different CLA preparations, one with an isomeric composition comparable to Clarinol™ G-80 (CLA mix) and one with mainly the t-10,c-12 CLA isomer (CLA10,12). There was no group taking placebo oil in this trial. The study investigated the effects of α-tocopherol and a cyclo-oxygenase-2 inhibitor on the effects of CLA. Both plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ were increased by both CLA supplements, but the increase was greater in the CLA10,12 group, suggesting an isomer-specific effect. The levels were not affected by the supplementation with α-tocopherol. On the contrary, several studies have shown that supplementation with Vitamin E is able to decrease isoprostane levels (Cyrus et al., 2003; Dietrich et al., 2002).

The levels of 15-keto-dihydro-$PGF_{2\alpha}$ were not affected by supplementation with the COX-2 inhibitor, apart from a smaller increase in subjects receiving CLA10,12 and the COX-2 inhibitor. This suggests that the induction of enzymatically catalysed lipid peroxidation by CLA10,12 may be partially mediated by COX-2.

In reviewing all the information, it is clear that CLA increases observed plasma and urinary levels of F_2-isoprostanes (table 1), and this effect seems to be stronger after supplementation with t-10,c-12 CLA. The increases are larger than can be expected from the day to day variation of ~40% (Helmersson and Basu, 2001). However, it is not clear what the relevance of these increased levels is. None of the studies have clarified or suggested a mechanism of action by which CLA may trigger increased oxidative stress.

Table 1 Results from different human trials on urinary levels of 8-iso-PGF2α and 15-keto-dihydro-PGF2α before and after treatment with CLA preparations similar to Clarinol™ G-80.

trial	**8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)**			**15-keto-dihydro-$PGF_{2\alpha}$ (nmol/mmol creatinine)**		
	before CLA	after CLA	% change	before CLA	after CLA	% change
Basu (2000a)	0.2	1.01	405	0.21	0.42	100
Basu (2000b)	0.47	1.85	407 (293)[a]	0.7	1.3	129 (85)[a]
Riserus (2002b)	0.16	0.41	156	0.39	0.53	35
Smedman (2004)	0.24	0.65	171	0.46	0.79	72

[a]Values in brackets are recalculated from the data reported in the publication.

Background information on lipid oxidation

Increased concentrations of lipid oxidation products are found in most, if not all, human diseases. Lipid peroxidation often occurs late in the injury process. Many assays are available to measure lipid peroxidation, but no single assay is an accurate measure of the whole process (Griffiths et al., 2002).

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Because of their divinyl-methane structure, polyunsaturated fatty acids (PUFAs) are susceptible to hydrogen abstraction by free radical attack, originating a pentadienyl radical (Halliwell and Gutteridge, 1989). This results in the rearrangement of the double bond to conjugated diene and, in the presence of O_2, in the formation of fatty acid hydroperoxides (Porter et al., 1984). It should be stressed that oxygen addition on the pentadyenyl moiety is diffusion controlled, hence the conjugated diene hydroperoxide is the only secure marker of lipid peroxidation in a biological sample (Porter et al., 1984). Once formed, PUFA hydroperoxides may further undergo both enzymatic and non-enzymatic reactions forming a host of compounds, a class of which is the isoprostanes. Increasing appreciation of the causative role of oxidative injury in many disease states places great importance on the reliable assessment of lipid peroxidation. The incorporation of molecular oxygen into PUFA to yield hydroperoxides as the primary initial product is the focus of intense current interest in biomedical and pharmacological research. The basis for the interest rests with the potential role of lipid peroxidation and peroxidation products in the degeneration associated with aging and in the pathogenesis of several clinically significant diseases (Halliwell and Gutteridge, 1989).

Increased understanding of the occurrence of lipid peroxidation, its injury potential, and its pathogenic role in several disease states demands quantitative methods which are diagnostic of the process and meet basic analytical criteria regarding accuracy, reliability, sensitivity, and specificity. The most direct approach for the assessment of lipid peroxidation, quantification of the primary products, is in practice complicated by the labile, fleeting nature of these species. Consequently, detection of lipid peroxidation has relied largely on indirect methods.

One of the most used indirect methods is the detection of 8-iso-$PGF_{2\alpha}$ isoprostanes. Measurement of F_2-isoprostanes has been employed to a great degree over the last decade to detect and quantify lipid peroxidation in a variety of biological matrices.
The F_2-isoprostanes are enzymatic or non-enzymatic lipid oxidation products of arachidonic acid. Their formation involves a number of steps as depicted in figure 1 and 2. Tissue arachidonic acid pools originate from the diet and from hepatic and extrahepatic pathways from linoleic acid in a number of steps involving desaturase and elongase enzymes (figure 1). Arachidonic acid is then incorporated into cell membranes, mostly phospholipids.

A condition of oxidative stress or other specific stimuli can result in two different scenarios (figure 2). Free radicals generated during oxidative stress may attack the esterified arachidonic acid (and other fatty acids), eventually resulting in the formation of a range of esterified F_2-isoprostanes, such as 8-iso-$PGF_{2\alpha}$. The F_2-isoprostanes may then be released from the phospholipid through the action of phospholipases (Bessard et al., 2001; Li et al., 1999; Morrow et al., 1992). Alternatively, oxidative stress may trigger the activation of phospholipase 2. Subsequently, arachidonic acid may be released from carbon-2 of the phospholipid. This may be followed by conversion to a series of potent eicosanoids through the action of cyclooxygenase, forming prostaglandins and thromboxanes (Klein et al., 1997). Prostaglandins may subsequently be metabolized,

forming 15-keto-dihydro-$PGF_{2\alpha}$ and other metabolites (Granstrom and Samuelsson, 1971).

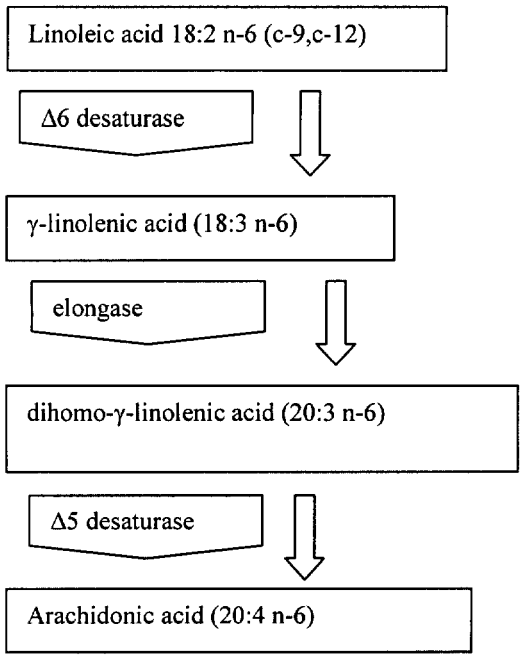

Figure 1. Conversion of linoleic acid into arachidonic acid

The two different F_2-isoprostanes 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ measured in CLA trials are regularly referred to as non-enzymatic (free radical induced) and enzymatic derived isoprostanes, respectively, although cyclooxygenase-2 dependent formation of small amounts of 8-iso-$PGF_{2\alpha}$ has been reported (Klein et al., 1997). Both 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ may be further metabolized by combinations of ß-oxidation, double bond reduction, alcohol group oxidation, ω-hydroxylation and ω-oxidation, leading to a myriad of metabolites, which are eventually excreted in the urine (Basu, 1998c; Chiabrando et al., 1999; Granstrom and Samuelsson, 1971).

Possible mechanisms for the CLA related increase of lipid peroxidation products

There are several theoretical mechanisms of action that may account for the observed effects of CLA on F_2-isoprostane levels, including the following:

1) CLA may increase oxidative stress by
    a. generating an increase in free radicals
    b. causing a pathological state with an inflammatory component
2) CLA may interfere with the synthesis or metabolism of F_2-isoprostanes by
    a. increasing lipolysis
    b. increasing arachidonic acid or prostaglandin availability or metabolism
    c. competing with the metabolism of F_2-isoprostanes.

As discussed below, it is unlikely that CLA causes significant oxidative stress. It is much more likely that, by competing with F_2-isoprostanes for metabolic pathways, CLA causes F_2-isoprostane levels to *appear* higher – even though it is unlikely that they are actually higher – because fewer F_2-isoprostanes are metabolized into a different form.

1) Discussion of the Possibility that CLA May Increases Oxidative Stress

Altered generation of F_2-isoprostanes has been reported in a variety of syndromes associated with oxidative stress. Examples include diseases with an inflammatory component, but also aging, alcohol and cigarette smoking have been shown to increase F_2-isoprostane generation (Lawson et al., 1999). On the one hand, the generation of free radicals may catalyze peroxidation of arachidonic acid, forming esterified F_2-isoprostanes that may be released from the membrane by lipases. On the other hand, a state of inflammation may induce cyclooxygenase 2, increasing the formation of prostaglandins from free arachidonic acid (Klein et al., 1997). In this case, oxidative stress may be a secondary effect to inflammation (Helmersson et al., 2004).

The possibility for CLA to be involved in either enzymatic or non-enzymatic lipid peroxidation are discussed below.

a. We have investigated the possibility for CLA to generate free radicals, and as such increase lipid peroxidation of arachidonic acid. In our notice, we suggested that increased lipid peroxidation may have been a harmless side effect from a higher intake of dietary poly-unsaturated fatty acids. The study by Richelle et al. (1999) demonstrated that levels of isoprostanes were not confounded by the dietary lipid content. However, the effect of type of dietary fatty acids (poly-unsaturated versus mono-unsaturated fatty acids) was not investigated in this study. We cited two papers that demonstrated an increase in lipid peroxides by measuring TBARS or the more specific TBA-MDA adducts after fish oil supplementation (Meydani et al., 1991; Wander et al., 1996). In the study by Meydani et al (1991), an increase in lipid peroxide levels was found in plasma from women taking fish oil supplements. An increase in both plasma and urinary lipid peroxides were found in a study by Wander et al. (1996). Urinary, but not plasma, lipid peroxides decreased with increased levels of α-tocopherol, but only as measured by the TBARS method. Levels of plasma arachidonic acid decreased after supplementation with fish oil. Using the same TBARS or TBA-MDA adduct method, other studies have found similar increases in plasma or urinary lipid peroxides after a high intake of PUFA, either from fish oil or other sources (Allard et al., 1997; Foulon et al., 1999; Jenkinson et al., 1999; Nelson et al., 1993), although other biomarkers of oxidative stress, such as breath alkanes and selenium-dependent glutathione peroxidase (Se-GSHPx) were not affected. However, as has been rightly noted by the FDA, the method of measuring malonaldehyde with the TBARS method is highly inaccurate and should be combined with other methods of measuring lipid peroxidation.

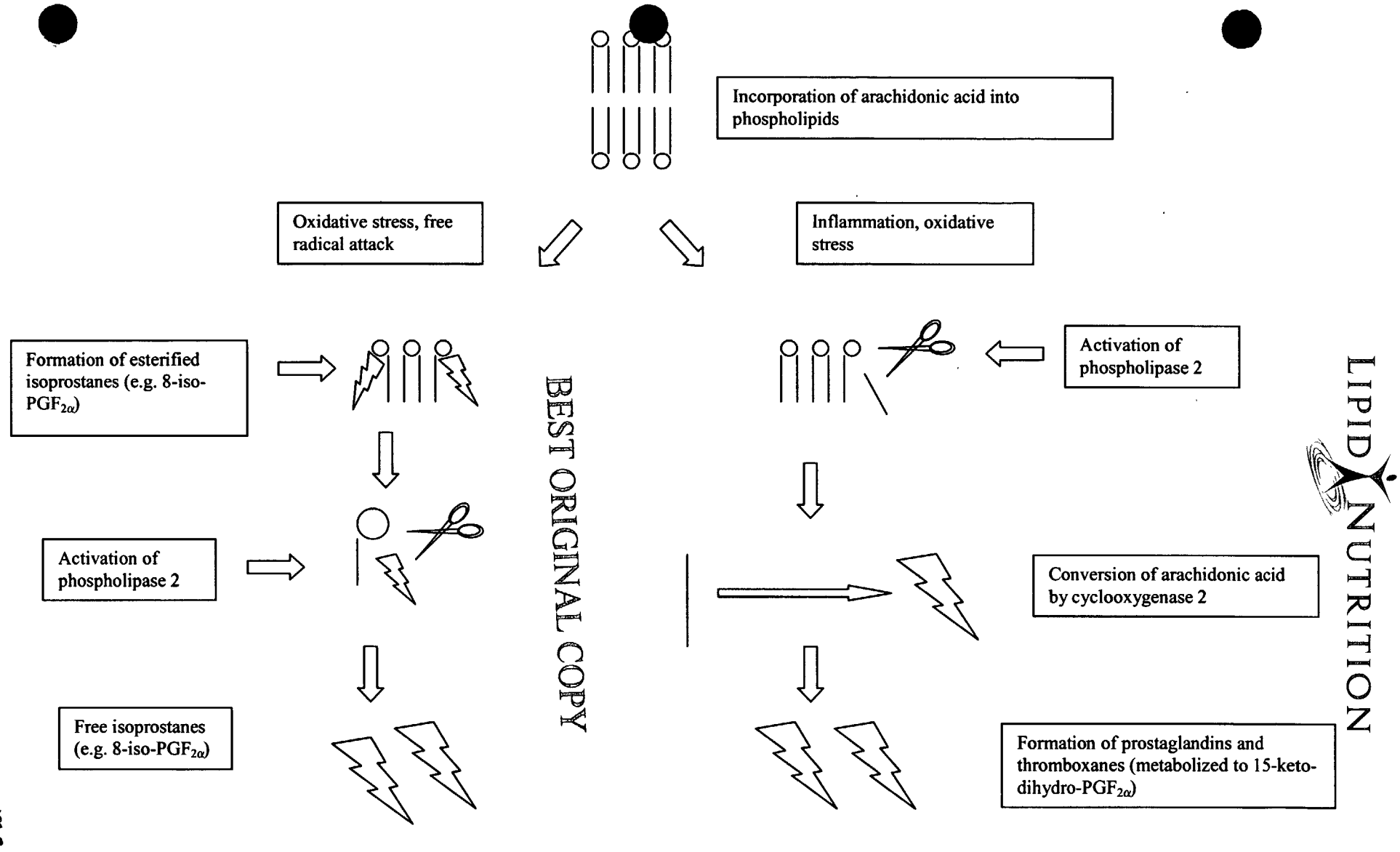

Figure 1. Pathway of non-enzymatic and enzymatic 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ formation.

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A number of studies have investigated the effects of different fatty acids on the excretion of isoprostanes. It has been noted by Basu et al. (2000b) that the choice of oleic acid instead of linoleic acid as a control oil in a study on CLA should be of no major importance for the results of increased lipid oxidation, as an earlier study showed that there was no significant difference in the urinary concentration of F_2-isoprostane between subjects consuming diets high in linoleic acid or oleic acid (Turpeinen et al., 1998). However, it has been shown that components of olive oil other than oleic acid have high anti-oxidant capacity (Bartsch et al., 2002). Indeed, a study by Visioli et al. (2000) has demonstrated that intake of olive oil decreased isoprostane levels, due to the presence of natural catecholic phenols.

The effects of three different types of oil supplements on lipid peroxidation have been compared by Higdon et al. (2000), using three different methodologies: the measurement of F_2-isoprostanes, malondialdehyde (MDA) and thiobarbituric reactive substances (TBARS) in plasma of post-menopausal women. Levels of plasma free F_2-isoprostanes appeared to be decreased after fish oil supplementation compared to sunflower oil. Levels were also decreased, but not significantly, after supplementation with safflower oil compared to sunflower oil. However, when levels were corrected for levels of arachidonic acid, there were no significant differences between any of the supplementations. Plasma MDA levels showed a similar trend as the free F_2-isoprostanes, but differences were eliminated when corrected for total plasma PUFA concentrations. In contrast, plasma TBARS levels showed a different trend, with a significant increase in TBARS after supplementation with fish oil, even after correction for total plasma PUFA concentrations. There were no differences in TBARS levels between supplementation with safflower and sunflower. The results of this study suggest that the increase in lipid peroxidation after fish oil supplementaion observed with the TBARS assay could be due to increased TBARS other than MDA or increased MDA formation from EPA and DHA. Oxidative stress as measured by plasma protein carbonyl concentrations were not increased after fish oil supplementation. Other studies have shown a decrease in urinary isoprostanes after fish oil supplementation (Mori et al., 1999; Mori et al., 2000).
The findings of the different studies measuring lipid peroxidation after fish oil supplementation demonstrate clearly the need to apply more than one relevant assay when assessing the effect of increased PUFA intake on *in vivo* lipid peroxidation in humans. To our knowledge, no other assays have been used to measure the effects of CLA on lipid peroxidation in humans. Animal studies have measured lipid peroxidation in different tissues by measuring TBARS, protein carbonyls or conjugated dienes and did not find an increase or even a decrease after supplementation with CLA (Corino et al., 2002; Kritchevsky et al., 2000; Sugano et al., 1998; Sugano et al., 1997; Teachey et al., 2003; Wilson et al., 2000). Although the TBARS measuring malondialdehyde is considered relatively inaccurate, this method usually leads to an over- rather than an underestimation of free radical damage (Griffiths et al., 2002). In a study in rats, lower levels of malondialdehyde were detected in mammary gland which have been associated with the concomitant anti-carcinogenic activitiy of CLA (Ip et al., 1991). However, in a following study it has been demonstrated that feeding CLA massively decreased linoleic acid metabolites, gamma linolenic, eicosatrienoic and arachidonic acid concentration and

therefore the decrease of malondialdehyde could be ascribed to a decrease of its substrates (Banni et al., 1999).

It should be stressed therefore, that when manipulating the diet by changing dietary polyunsaturated fatty acids, it may also change the absorption, deposition in different lipid classes and metabolism of all PUFAs which are the substrates of lipid peroxidation. This becomes even more important when a single byproduct from one single PUFA is taken into consideration. Although the measurement of urinary isoprostanes appears to be a more accepted method of measuring lipid peroxidation products, there are a number of issues that should be considered when using this method, as will be discussed in a later section.

It is unknown how CLA could generate an increase in free radicals other than through its double bonds. However, work by Banni et al. (1998) demonstrated that CLA was not involved in free radical reactions in liver tissues from lamb and rats. This is further supported by the study of Smedman et al. (2004). Although they found an increase in plasma and urinary isoprostane levels, these levels were not decreased after supplementation with vitamin E, as shown in other studies where Vitamin E supplementation was able to decrease isoprostane levels in smokers (Dietrich et al., 2002) and in experimental atherosclerosis in mice (Cyrus et al., 2003), which is expected after supplementation with a potent liposoluble antioxidant.

In summary, the increase in generation of free radicals after CLA due to a higher intake of polyunsaturated fatty acids is likely insignificant. Since dietary intake of other PUFAs such as fish oil did not increase isoprostanes, it is unlikely that one extra double bond of CLA compared to oleic acid is fully responsible for the observed increase in lipid peroxidation products. The oxidative stability of CLA has been demonstrated in a number of tissues and it is therefore unlikely that CLA increases oxidative stress lipid peroxidation products due to directly increasing the generation of free radicals.

b. The increase in lipid peroxidation products after CLA supplementation may have been the result of CLA directly causing a disease with an inflammatory component, such as atherosclerosis or diabetes. The inflammatory state may have induced cyclooxygenase 2, resulting in an increase in prostaglandins and derivates. As has been discussed in our notice, markers of inflammation, such as α-tocopherol, γ-tocopherol, interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) were not affected by CLA supplementation in human trials finding an increase in lipid peroxidation products (Riserus et al., 2002a; Riserus et al., 2002b). In the study by Riserus et al. (2002b), C-reactive protein (CRP) levels were significantly increased after CLA supplementation compared to baseline levels, but lower compared to placebo. The CRP level measurements in all groups were highly variable, making the value of these data questionable. In two unpublished studies in healthy subjects on Clarinol™ G-80 and Clarinol™ A-80 (fatty acid form), CRP levels were not affected after supplementation with 3 g/day for 12 weeks and 2g/day for 5 weeks, respectively (Peterson et al., 2004; Song et al., 2004). Two animal studies showed a decrease in TNFα levels in rats and mice after dietary CLA intake (Akahoshi et al., 2002; Yamasaki et al., 2003), while others showed no change (Sisk et al., 2001). In general, no

adverse effects of CLA on inflammatory markers have been reported. In fact, several *in vitro* studies have shown that CLA decreased cyclooxygenase activity in different cell cultures, although this may have been related to a decreased availability of arachidonic acid, as will be discussed later (Eder et al., 2003; Iwakiri et al., 2002; Yu et al., 2002). The effects of CLA on the development of diseases with inflammatory components further demonstrate no adverse effects. As discussed in our notice, studies with Clarinol™ G-80 and similar CLA preparations have demonstrated no adverse effects on the development of diabetes, including in men with metabolic syndrome (Riserus et al., 2002a). In fact, a recently published human trial indicates a beneficial effect of CLA on insulin sensitivity in sedentary subjects (Eyjolfson et al., 2004), supporting the results found earlier by Belury et al. (2002) in subjects with non-insulin dependent diabetes melitus (NIDDM). The findings of CLA improving insulin sensitivity have been shown in a number of studies with diabetic Zucker rats and have even been attributed to a decrease in oxidative stress by CLA (Henriksen et al., 2003; Houseknecht et al., 1998; Teachey et al., 2003). Furthermore, it appears that oxidative stress is a later process in the development of diabetes, possibly secondary to inflammation (Helmersson et al., 2004). As discussed previously, several inflammatory markers were not affected in studies with CLA.

In addition, as discussed in our notice, CLA has been reported to have anti-atherogenic properties in the rabbit. The FDA expressed doubt whether the rabbit was an appropriate model to measure effects of CLA on atherosclerosis because

1) The lesions seen in rabbits differ in severity, histology and location from the lesions seen in humans.
2) The rabbit has to be fed a diet extremely high in fat and cholesterol to produce these lesions
3) The effects of CLA on blood lipids are inconsistent with an anti-atherosclerotic effect

However, the positive effects of CLA on the course of experimental atherosclerosis has also been established in hamsters fed hypercholesterolemic diets (McLeod et al., 2004; Nicolosi et al., 1997; Wilson et al., 2000). In addition, feeding rabbits a diet high in cholesterol is still an accepted and highly used model for studying the development of atherosclerosis. A critical assessment of the model concluded that diet-induced hypercholesterolemia is useful for detection of agents interfering with the adsorption, degradation and excretion of cholesterol, but not those interfering with the biosynthesis of cholesterol (Vogel and Vogel, 1997). Several mechanisms of action of CLA with regard to atherosclerosis have been proposed, none of which involve interference with the biosynthesis of cholesterol (reviewed by McLeod et al., 2004). In conclusion, despite inconsistent results on plasma lipid or lipoprotein profiles, CLA appears not to be a risk factor for the development of cardiovascular disease.

Overall, the data on the effects of CLA are relatively consistent, and the weight of evidence clearly exhibits no adversely affected markers for inflammation, diabetes and cardiovascular risk in humans taking CLA supplements similar to Clarinol™ G-80. This

indicates that an increase in inflammatory related diseases by CLA is not a plausible mechanism of action for the observed increase in isoprostanes.

2) Discussion of the Possibility that CLA May Interfere with the Synthesis or Metabolism of Isoprostanes

As an increase in oxidative stress or inflammation does not seem to occur after CLA intake, alternative explanations were investigated to explain the increase in levels of isoprostanes. The most logical explanation would comprise a direct effect of CLA on the synthesis or metabolism of isoprostanes. A few possiblities are discussed below.

a. As demonstrated in a study by Richelle et al. (1999), levels of urinary isoprostanes were increased after one day of fasting, and may have been caused by an increased lipolysis in the adipose tissue rather than oxidative stress. Lipolysis has been suggested as one of the mechanisms of action for the CLA related reduction of adipose tissue (reviewed by Evans et al., 2002), and this may be one cause for the observed increase in urinary isoprostanes. This mechanism of action would not necessarily involve an increase in oxidative stress, but may reflect a release of F_2-isoprostanes from the adipose tissue or other tissues.

Alternatively, CLA may modulate lipase enzymes, thereby increasing the production of prostaglandins from arachidonic acid or the release of isoprostanes from phospholipids. As discussed by Tsikas et al. (2003), a decrease or increase in urinary excretion of 8-iso-$PGF_{2\alpha}$ by pharmacological or nutritional events might feign reduction or elevation in oxidative stress due to unrelated changes in phospholipase activity and this possibility must be considered in clinical studies dealing with oxidative stress. Not much information is available on the effects of CLA on phospholipase activity, although one *in vitro* study in human aortic endothelial cells found a decreased phospholipase activity after exposure to CLA (Eder et al., 2003).

Other lipases such as heparin releasable lipoprotein lipase (HR-LPL) have been shown to be differentially regulated by c-9,t-11 and t-10,c-12 CLA isomers in 3T3-L1 adipocytes, depending on the concentration used (Lin et al., 2001; Pariza et al., 2001). On the other hand, lipoprotein lipases were not affected in two studies with mice (Degrace et al., 2003; Warren et al., 2003). A modulation of lipases would explain the increase in isoprostanes without increasing oxidative stress. However, it is currently unknown what role the modulation of lipases plays in the increase of isoprostanes by CLA.

b. It is conceivable that CLA or its metabolites interact with the synthesis, incorporation or metabolism of the precursor of F_2-isoprostanes, arachidonic acid. The possibility that CLA may have increased the availability of arachidonic acid and concomittantly its lipid peroxidation products was investigated. However, it appears CLA is more likely to decrease arachidonic acid levels. Both CLA isomers have been shown to use the same metabolic pathway as linoleic acid (figure 1) (Banni, 2002). CLA may compete with linoleic acid for $\Delta 5$- and $\Delta 6$-desaturase and elongase enzyme systems, resulting in a decreased synthesis of arachidonic acid. Indeed, this was clearly

demonstrated in a study in rats, where a 50% decrease in arachidonic acid was found in mammary tissue after up to 1% dietary CLA. After 1%, there was no further decrease in any linoleic acid metabolites. These effects of CLA were tissue specific, as they were not found in the liver and plasma (Banni et al., 1999).

The tissue specific effects of CLA appear to be due to the level of incorporation of CLA and its metabolites in different tissues. Similar to oleic acid, conjugated linoleic acid is incorporated mainly in the neutral lipid fraction, so higher levels of CLA are incorporated in tissues rich in neutral lipids (Banni et al., 2001). One conjugated diene (CD) 20:4 metabolite of c-9,t-11 CLA was also incorporated in phosphatidylserine and phospathidylinositol lipid fraction, thus competition of this metabolite with arachidonic acid may also take place at the level of tissue incorporation. Indeed, in rats, less arachidonic acid was found in the phosphatidylserine fraction of the liver after a high c-9,t-11 CLA intake than after a low intake, but there was no significant difference in other lipid fractions (Banni et al., 2001). Phosphatidylinositol appears to be the major source for eicosanoid biosynthesis. However, the concentration of arachidonic acid in phosphatidylinositol is many times higher than that of CD20:4, so that the uptake of arachidonic acid will unlikely be affected in this fraction (Banni et al., 2001). Nevertheless, other studies have shown a decrease of arachidonic acid and eicosanoids, including prostaglandins in different tissues after CLA supplementation, including in liver phospholipids (Kavanaugh et al., 1999; Kramer et al., 1998; Sebedio et al., 2001; Sugano et al., 1998). The differences in concentration of arachidonic acid and CLA metabolites are likely related to the dietary intake of linoleic acid, with lower levels of linoleic acid increasing the competitive position of CLA, resulting in a lower tissue concentration of arachidonic acid (Banni et al., 2001).

In summary, a high intake of CLA will lead to a higher concentration of CLA and its metabolites mostly in neutral lipids, which have a high concentration of oleic acid and a low concentration of linoleic acid. The arachidonic acid concentration is likely to decrease in these tissues. In tissues high in phospholipids, such as plasma and liver, the high concentrations of linoleic acid would prevent CLA from decreasing arachidonic acid concentrations, but would not necessarily increase arachidonic acid concentrations.

On the other hand, a high dietary intake of linoleic acid would compete with CLA for the enzymatic pathways, leading to an increased concentration of arachidonic acid and possibly its lipid peroxidation products. Unfortunately, the dietary intake of linoleic acid (and arachidonic acid) has not been reported in any of the studies measuring isoprostanes. To our knowledge, the increased levels of F_2-isoprostanes after supplementation with CLA have not been corrected for levels of the parent compound, arachidonic acid.

Alternatively, CLA may interact directly with the formation of prostaglandins from arachidonic acid by modulating cyclooxygenase activity. Other fatty acids have also been shown to modulate the expression and activity of this key enzyme (Watkins and Seifert, 2000). To our knowledge, no *in vivo* studies are available investigating the effects of CLA on cyclooxygenase activity. Several *in vitro* studies have shown that CLA decreases cyclooxygenase activity in different cell cultures, possibly related to the decreased

availability of arachidonic acid or competition between CLA metabolites and arachidonic acid (Eder et al., 2003; Iwakiri et al., 2002; Yu et al., 2002). As discussed before, many studies have also shown a decrease in prostaglandins after CLA intake (Kavanaugh et al., 1999; Kramer et al., 1998; Sugano et al., 1998).

Overall, many studies show a decrease in arachidonic acid and derived prostaglandins in a number of tissues after CLA intake. It therefore appears that the observed increases in levels of isoprostanes are caused by an increased availability of isoprostanes rather than an increased production of isoprostanes. The apparent reason why isoprostanes are more available is discussed further below.

c. As depicted in figure 2, 8-iso-$PGF_{2\alpha}$ is initially formed from free radical oxidation of arachidonic acid esterified in lipid membranes. A portion of 8-iso-$PGF_{2\alpha}$ remains esterified in the lipid membranes, whereas another portion is released by the action of phospholipases. Free 8-iso-$PGF2_{2\alpha}$ is excreted in the urine as such, or it is metabolized further by ß-oxidation in peroxisomes to 2,3-Dinor-5,6-dihydro-8-epi-$PGF_{2\alpha}$ or 2,3-dinor-8-epi-$PGF_{2\alpha}$ (Basu, 1998a; Chiabrando et al., 1999). In addition, 15-keto-dihydro-$PGF_{2\alpha}$ is also metabolized further by ß-oxidation in peroxisomes (Basu, 1998c). It has recently been hypothesized that both isomers of CLA may be metabolized by partial ß-oxidation in peroxisomes (Banni et al., 2004; Sebedio et al., 2001). It is therefore conceivable that CLA competes with F_2-isoprostanes for metabolism in the peroxisomes. A higher affinity for CLA would be reflected by an increase in free F_2-isoprostanes levels, both in plasma and in urine. Furthermore, it has been shown that t-10,c-12 CLA is by far more efficiently ß-oxidized in peroxisomes than c-9,t-11 CLA (Banni et al., submitted). This may imply that t-10,c-12 CLA would decrease F_2-isoprostane catabolism more strongly and thereby increase free F_2-isoprostanes. In this respect, one would have to assess the urinary excretion of the major metabolites of F_2-isoprostanes and/or plasma esterified levels of F_2-isoprostanes. The studies showing an increase in free F_2-isoprostane levels after CLA intake did not include these measurements. Although currently little direct evidence is available to support this mechanism of action behind the observed increase of F_2-isoprostanes, it is a very plausible explanation. This mechanism would not involve an increase of oxidative stress by a generation of free radicals or by the development of inflammatory diseases, which both have been shown unlikely to occur as a result of CLA intake. Moreover, this mechanism would in general not even involve an increase in the formation of isoprostanes, which is supported by the fact that precursors of F_2-isoprostanes have been shown to decrease as a result of CLA intake. Rather, it appears that CLA increases the availability of F_2-isoprostanes by competing with their metabolism in the peroxisomes.

Evaluation of the radioimmunoassay on prostaglandin $F_{2\alpha}$-derived isoprostanes.

As discussed earlier, the measurement of F_2-isoprostanes is increasingly used as a method to assess lipid peroxidation. While there are several papers on 8-iso-$PGF_{2\alpha}$ formation during the lipid peroxidation process, 15-keto-dihydro-$PGF_{2\alpha}$ is mainly referred to in the literature as a $PGF_{2\alpha}$ metabolite and not as an enzymatic derived F_2-isoprostane. More

studies are warranted to validate 15-keto-dihydro-$PGF_{2\alpha}$ as marker of enzymatic lipid peroxidation.

There are two underlying assumptions implicit in the use of the F_2-isoprostane measurement to assess lipid peroxidation:

1) an operative and quantitative relationship exists between lipid peroxidation and F_2-isoprostane formation;
2) product formation is diagnostic of the presence and amount of fatty peroxides.

However, these conclusions are limited in practice by several important considerations:
- F_2-isoprostane yield as a result of lipid peroxidation varies with the peroxidation stimulus;
- only certain lipid oxidation products i.e. Hydroperoxy-eicosatetraenoic acid (HPETE) yield F_2-isoprostanes;
- F_2-isoprostanes are only one of several end products of fatty peroxide formation and decomposition;
- the peroxidation environment influences both the formation of lipid derived F_2-isoprostane precursors and their formation to F_2-isoprostanes;
- F_2-isoprostanes themselves are biologically active substances which can be oxidatively and metabolically degraded.

The studies reporting an increase in F_2-isoprostanes after CLA intake have used a radioimmunoassay (Basu, 1998b, c). In this assay, rabbit polyclonal antiserum was developed and used. While the author reported little to no cross reaction to other isoprostanes other than the intended 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$, the number of F_2-isoprostanes tested was limited. It should be pointed out that almost all work suggesting that F_2-isoprostanes are a marker of oxidative stress, have used the electron capture negative ionization gas chromatography-mass spectrometry (GC-MS) method. Comparison of this method with the radioimmunoassay used by Basu et al (1998b) is problematic, as both methods do not correlate well and do not measure the same compounds (Bessard et al., 2001; Proudfoot et al., 1999). Up to 64 isomers in four structural classes can be generated by free radical attack of arachidonic acid (Lawson et al., 1999). It has therefore been suggested that immunoassays should be considered as semi-quantitative indices of F_2-isoprostanes (Cracowski et al., 2002). Other works have gone on to show that there are multiple classes of F_2-isoprostanes and depending on the class of urinary F_2-isoprostanes, different diseases may be indicated (Li et al., 1999).

The finding of detectable levels of F_2-isoprostanes in all normal animal and human biological fluids indicates that there is certain level of ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses, even in the normal state (Roberts and Morrow, 1997). In other words, no threshold has been established at which the level of F_2-isoprostanes indicates an increased oxidative stress.

Another major shortcoming of the method used in the CLA trials is that only free F_2-isoprostanes have been measured. It has been noted by several authors that the vast

majority of F_2-isoprostanes is esterified in phopholipids rather than in free form (Bessard et al., 2001; Li et al., 1999; Roberts and Morrow, 2000). Due to the possible interference of CLA with the release and metabolism of F_2-isoprostanes, it is necessary to measure both free and esterified levels (in plasma) of F_2-isoprostanes to adequately address whether CLA indeed increases the formation of F_2-isoprostanes.

In summary, although the measurement of isoprostanes as an indication of oxidative stress is a widely used method, caution should be taken in interpreting the results of these measurements. In the case of CLA, much evidence exists that the observed increase in isoprostanes is unlikely the result of oxidative stress.

Overall conclusion on the increased levels of prostaglandin $F_{2\alpha}$-derived isoprostanes.

The question of whether observed increased F_2-isoprostanes reflect a harmful effect of CLA depends on whether the increase results from increased oxidative stress, or even from the development of an inflammatory state. Although it is well recognized that a higher level of F_2-isoprostanes is found in most human diseases, an increase does not necessarily imply increased oxidative stress. In general, nothing is known about the relevance of a small increase as observed in human trials on CLA, as no threshold has been established at which the level of F_2-isoprostanes indicates increased oxidative stress. In the case of dietary intake of CLA, an increase in F_2-isoprostanes is likely unrelated to oxidative stress altogether. The generation of free radicals is unlikely directly due to CLA intake. In addition, there are no indications that CLA induces an inflammatory state. The findings of increased oxidative stress have been based on a single method measuring F_2-isoprostanes and cannot be related to other markers of oxidative stress or inflammation, making the evidence rather weak. In fact, more evidence exists that CLA decreases oxidative stress and inflammation. The precursors of isoprostanes, arachidonic acid and prostaglandins, have been demonstrated in many studies to be decreased by CLA. Therefore, a more logical explanation for an increase in isoprostane levels after CLA intake would be an increased availability rather than an increased formation of F_2-isoprostanes. The most logical explanation for the increased availability of F_2-isoprostanes appears to be the result of an inhibition of their metabolism by competition with CLA.

It is likely, then, that CLA does not cause a significant increase in the formation of F_2-isoprostanes. Rather, by competing with F_2-isoprostanes for metabolic pathways, CLA likely causes a decline in metabolism of already-formed F_2-isoprostanes. As a result, levels of isoprostanes *appear* higher in the presence of CLA (even though it is unlikely that they are actually higher) because fewer F_2-isoprostanes are metabolized into a different form.

Accordingly, based on the weight of the evidence, Loders Croklaan concludes that there is reasonable certainty that the observed effects on F_2-isoprostane levels do not represent a harmful effect of CLA preparation under its intended conditions of use. This conclusion is based on scientific procedures, common knowledge about the substance throughout the scientific community, and the views of qualified experts.

LIPID NUTRITION

Thank you for your consideration of this information.

Respectfully submitted,

Andreas Menzel
Head, Research and Development

Cc: Daniel R. Dwyer
Kleinfeld, Kaplan and Becker

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CURRICULUM VITAE

NAME

Sebastiano Banni

BIRTH DATE

ADDRESS

Università degli Studi di Cagliari
Dipartimento di Biologia Sperimentale
Cittadella Universitaria
09042 Monserrato (Cagliari), Italy

Phone +39/070-675-4128 (office)
+39/070-675-4033 (lab)
FAX +39/070-675-4032

E-MAIL BANNI@VAXCA1.UNICA.IT

ACADEMIC INFORMATION

1999-2004

Teaching activities on Cellular Pathology, Cellular Physiology, Physiology of Nutrition for undergraduate and postgraduate students of the Faculty of Sciences at University of Cagliari, Cagliari and University of Sassari , Italy.

1997-2004

Permanent position as Assistant Professor in the Department of Experimental Biology, University of Cagliari.

1993-1999

Series of seminars of General Pathology for undergraduate and postgraduate students of the Faculty of Sciences at University of Cagliari, Cagliari Italy.

1991

Received the Doctorate of Research in Experimental and Molecular Pathology discussing a thesis on "characterization of some unusual fatty acids in animal tissues".

1990

Permanent position as a research technician in the Department of Experimental Biology, Section of Experimental Pathology, University of Cagliari, Chaired by Prof. F.P. Corongiu

1987-89

Spent the Doctorate of Research as a Research Fellow in the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh PA, USA, under the supervision of Prof. Benito Lombardi, working on a project on conjugated dienes in choline deficient rats

1987

Examination to attend the quadrennial Italian Doctorate of Research in Experimental and Molecular Pathology directed by Prof. Mario Umberto Dianzani.

1985

Annual practicum in the Institute of Pharmacology (Cagliari) and General Pathology (Cagliari) in fulfillment of the State requirement for preparation for the ability to practice as a biologist.

State examination to qualify to practice as a biologist.

1984

Graduated, with honors, in Biological Sciences from Cagliari University,
November 1981-March 1984 : experimental project on the determination and separation of some aflatoxins using an improved HPLC procedure in fulfillment for the honors degree.

1979

5 years High school (Science) in Cagliari

SCIENTIFIC INFORMATION

SIGNIFICANT SCIENTIFIC CONTRIBUTION ON CLA RESEARCH

1) Absorption and deposition in rat tissues of low level of dietary CLA from partially hydrogenated oils
2) Metabolization of CLA to CD 18:3, CD 20:3 and CD 20:4
3) CLA and oxidative stress
4) Increase of retinol levels in liver, plasma and mammary tissue by dietary CLA
5) Decrease of linoleic acid metabolites in mammary tissue by dietary CLA
6) Correlation between changes in lipid metabolism and CLA anti-carcinogenic activity in mammary tissue
7) CLA enriched butter and vaccenic acid exerts the same preventive activity versus mammary cancer in rodents, as does synthetic CLA.
8) Identification of CLA peroxisomal beta oxidation products in in vivo and in vitro models and humans

MOST SIGNIFICANT RESEARCH PAPERS PUBLISHED ON CLA

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Detection of conjugated diene fatty acid isomers in liver lipids of rats fed a choline devoid diet indicates that the diet does not cause lipoperoxidation.
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9) S. Banni, and J.C. Martin
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A. Tomasi, F. Ursini and V. Vannini Eds., Cleup, Padova. pp.207-215, 1994.

6) S. Banni, G. Carta, M.S. Contini, E. Angioni, M. Deiana, M.A. Dessi, M.P. Melis and F.P. Corongiu
Conjugated diene fatty acids in human and animal tissues.
In: "Nutrition, Lipids, Health and Disease. A.S.H. Ong, E. Niki and L. Packer, Eds.
AOCS Press Champaign, IL. pp. 218-224, 1995

000092

7) E. Murru, E. Angioni, G. Carta, M. P. Melis and S. Banni
Conjugated linoleic acid isomers (CLA): good for everything?
Sci. Aliments 22, 371-380 2002

8) E. Murru, E. Angioni, G. Carta, M. P. Melis, S. Spada and S. Banni
Reversed-Phase HPLC Analysis of Conjugated Linoleic Acid and Its Metabolites. In: Advances in conjugated linoleic acid research, Volume 2. J.L. Sebedio, W.W. Christie, and R. Adlof Eds. AOCS Press Champaign, IL. 94-100 2003

9) M. P. Melis, E. Murru, E. Angioni, G. Carta and S. Banni
Does metabolism of conjugated linoleic acid play a role in its biological activities? In: Essential fatty acids and eicosanoids: Invited papers from the Fifth International Congress. H.V. Huang et al. Eds. AOCS Press Champaign, IL. 339-342 2003

PAPERS ON LIPID PEROXIDATION

1) T. Trenti, B. Botti, M.A. Dessì, S. Banni and A. Masini
Structural and functional properties of rat liver mitochondria in experimental iron overload: II. Membrane potential and Ca transport.
IRCS Med. Sci. 14, 839-840, 1986

2) F.P. Corongiu, S. Banni, P. Scano and M.A. Dessì.
Evidence for carbon tetrachloride-induced lipid peroxidation "in vivo" by 23Na relaxation times T1.
Bioelectrochem. Bioenergetics 18: 179-186, 1987

3) S. Banni, M. Casu, F.P. Corongiu, M.A. Dessì, A. Lai and V. Vannini.
Na-23 Spin-lattice relaxation times of livers of normal and CCl4 treated rats.
Bull. Magn. Res. 9: 92, 1987

4) S. Banni, M.Casu, F.P.Corongiu, M.A.Dessì, A.Lai and C.Meloni
NMR spin-lattice relaxation times of intracellular Na-23 on rat livers and related lipid peroxidation following CCl4 intoxication.
Chem.-Biol. Interact. 63: 207-214, 1987

5) F.P. Corongiu, M.A. Dessì, S. Banni, F. Bernardi, M.P.Piccardi, M. Del Zompo and G.U. Corsini.
MPTP fails to induce lipid peroxidation "in vivo".
Biochem. Pharmacol. 36: 2251-2253, 1987

6) A. Tomasi, E. Albano, S. Banni, B. Botti, F.P. Corongiu, M.A. Dessì, A. Iannone, V. Vannini and M.U. Dianzani.
Free-radical metabolism of carbon tetrachloride in rat liver mitochondria. A study of the mechanism of activation.
Biochem. J. 246: 313-317, 1987

7) F.P. Corongiu, S. Banni, S. Vargiolu and M.A. Dessì.
The role of vitamin E in controlling the production of cis,trans and trans,trans diene hydroperoxides during carbon tetrachloride induced lipid peroxidation in rat liver.
In: Advances in free radical in disease. Eds. F.P. Corongiu, A. Tomasi and V. Vannini, Cleup, Padova. pp. 33-38, 1987

8)F.P. Corongiu, S. Banni, A. Bini, M. Casu, M.A. Dessì, A. Iannone, A. Lai, C. Meloni, A. Tomasi and V. Vannini.
A novel approach to the study of the lipid peroxidation and late stages of the ensuing lesions by 23NaT1 and31P-NMR.
In: Free Radicals: Methodology and Concepts. Free radical series, C. Rice Evans and B. Halliwell eds. Richelieu Press, London. pp 287-305,1988

9) F.P. Corongiu, S. Banni and M.A. Dessì.
Conjugated dienes detected in tissue lipid extracts by second derivative spectrophotometry.
Free. Rad. Biol. Med. FORUM, 7, 183-186, 1989

10) A. Masini, T. Trenti, S. Banni, D.Gallesi and U. Muscatello
Reduced glutathione depletion and lipid hydroperoxide formation in rat liver mitochondria following acute ethanol intoxication
In: Free Radicals and antioxidants in nutrition, Richelieu free radical series,
F.P. Corongiu, S. Banni, M.A. Dessì and C. Rice-Evans eds.
Richelieu Press London. pp. 287-301, 1993

11) L. Lucchi, S. Banni, B. Botti, G. Cappelli, G. Medici, M.P. Melis, A. Tomasi, V. Vannini and E. Lusvarghi
Conjugated diene fatty acids in patients with chronic renal failure: evidence of increased lipid peroxidation?
Nephron 65; 401-409, 1993

12) F.P. Corongiu and S. Banni
Detection of conjugated dienes by second derivative ultraviolet spectrophotometry
Meth. Enzymol. 233, 303-310, 1994

13) F.P. Corongiu, S. Banni, and B. Lombardi
Quantitation of conjugated dienes by second-derivative UV spectroscopy
In: In Vitro toxicity indicators, Methods in Toxicology, Volume 1B, C.A. Tyson and J.M. Frazier eds., Academic Press, pp. 415-419, 1994

14) A. Tomasi, S. Banni, A. Bini, M.A. Dessi, A. Iannone
A critical overview on spin trapping, salicylate oxidation and conjugated diene determination as probes for the detection of free radical reactions "in vivo".
In: Free radicals in the environment, medicine and toxicology. Richelieu free radical series, H. Nohl, H. Hesterbauer and C. Rice-Evans eds. Richeliu Press, London, pp. 265-286, 1994

15) B. Botti, S. Banni, A. Bini, M.A. Dessì, F.P. Corongiu, A. Iannone, V. Vannini and A. Tomasi.
Liver glutathione, ascorbate and vitamin E redox equilibria following CCl4, 1,2 dibromoethane, and GSH depletion in vivo in the rat.
In "Advances in Free Radicals in Disease III";
A. Tomasi, F. Ursini and V. Vannini Eds., Cleup, Padova, pp.181-191, 1994.

16) S. Banni, L. Lucchi, A. Baraldi, B. Botti, G. Cappelli, F.P. Corongiu, M.A. Dessi, A. Tomasi and E. Lusvarghi
No direct evidence of increased lipid peroxidation in hemodialysed patients
Nephron 72, 177-183, 1996

17) S. Banni, M.S. Contini, E. Angioni, M. Deiana, M.A. Dessi, M.P. Melis, G. Carta and F.P. Corongiu
A novel approach to study linoleic acid autoxidation :importance of simultaneous detection of the substrate and its derivative oxidation products
Free Rad Res 25, 43-53, 1996

18) V.B. Ritov, S. Banni, J.C. Yalowich, B.W. Day, H.G. Claycamp, F.P. Corongiu and V.E. Kagan
Non-Random peroxidation of different classes of membrane phospholipids in live cells detected by metabolically integrated cis-parinaric acid
Biochim Biophys Acta 1283, 127-140, 1996

19) V. A. Tyurin, G. Carta, Y. Y. Tyurina, S. Banni, B.W. Day, F. P. Corongiu and V.E. Kagan
Peroxidase-catalyzed oxidation of beta carotene in HL-60 Cells and in model Systems: Involvement of phenoxyl radicals.
Lipids 32, 131-142, 1997

20) Aruoma, O.I., Deiana, M., Jenner, A., Halliwell,B., Banni,S., Corongiu, F.P., Dessì,M.A., Aeschbach,R.
Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low density lipoprotein
J. Agricol Food. Chem. 46, 5181-5187 1998

21) S. Banni, E. Angioni, G. Carta, M. Deiana, M.A. Dessi, M.P. Melis, S. Scrugli and F.P. Corongiu
Assessment of lipid peroxidation in biological systems. In "Advances in Free Radicals in Disease IV"; R. Pizzala, A. Tomasi and V. Vannini Eds., La Golardica Pavese, Pavia. pp. 55-69, 1999

22) M. Deiana, O.I.Aruoma, M.D.P.Bianchi, J.P.E.Spencer, H. Kaur, B.Halliwell, R.Aeschbach, S. Banni, M.A.Dessi, F.P. Corongiu
Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol.
Free Rad. Biol. Med.. 26, 762-769, 1999

23) B. Botti, S. Banni, A.Bini, M.A. Dessì, F.P. Corongiu, A. Tomasi and V. Tannini
Glutathione,and ascorbate redox state and vitamin E in rat liver in vivo following CCl4, 1,2 dibromoethane, phorone and diethylmaleate treatment
Intl J Med Biol Envviron 27, 151-159, 1999

24) E. Angioni, G. Lercker, N. G. Frega, G. Carta, M. P. Melis, E. Murru, S. Spada and S. Banni
UV spectral properties of lipids as a tool for their identification.
Eur. J. Lipid Sci. Technol. 104, 65-68 2002

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25) M.A. Dessì, M. Deiana, B.W. Day, A. Rosa, S. Banni and F.P. Corongiu.
Oxidative stability of polyunsaturated fatty acids: Effect of squalene

Eur. J. Lipid Sci. Technol. 104, 506-512 2002

INVITED SPEAKER, CHAIRMAN AND SEMINARS

1994

1) Seminar on " Characterization of conjugated diene-fatty acids from different sources in animal and human tissues " at Free Radical Research Group, Division of Biochemistry, Medical & Dental School Guy's & St Thomas's Hospital chaired by Prof. C. Rice-Evans.1994

2) Lecture on "Conjugated diene fatty acids in human and animal tissues" at "Unesco/Costam SFRR-ASIA workshop on nutrition, lipids, health and disease" held in Penang, Malesia from 1 to 3 September 1994

1996

3) Co-chaired with Dr. Huth the technical session on "Conjugated linoleic acid (CLA). Chemistry and Biology" at 87th Annual Meeting of the AOCS held in Indianapolis 1996. In the same session I have been invited to present an oral communication by the title "Organ deposition and metabolism of CLA in animal tissues"

4) Member of the scientific commettee and co-chairman of the session Health aspects of oxidation and antioxidants of the 1st meeting of the AOCS European section to be held in Dijon (France) 19-20 september 1996

1997

5) Chaired technical session on "General edible applications technology II" at 88th Annual Meeting of the AOCS. 1997

6) Lecture on "Conjugated linoleic acid. In vivo metabolism " at "CLA Forum" held in Madison, Wisconsin, USA, 13-14 August 1997

1998

7) Lecture on "the dietary source and the biological effects of CLA" at the first annual research/industry meeting organized by the Centre Europeén des Sciences du gout (CESG) to be held in Dijon November 25 & 26, 1998.

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1999

8) Co-chaired with Dr. Scimeca the technical session on "Biological activity of conjugated linoleic acid" at 90th Annual Meeting of the AOCS held in Orlando 1999. In the same session I have been invited to present an oral communication by the title "Changes in lipid metabolism in association with CLA anti-carcinogenic activity."

9) Lecture on " Biological activities CLA " at the meeting of the Italian Society of Pharmacology. Firenze (Italy) 1999.

BEST ORIGINAL COPY

10) Co-chaired with Dr. Whale the session on "Conjugated linoleic acid (CLA)" at 40th ICBL Meeting held in Dijon (France)- Sept. 14-17 1999
Co-author of 47 Abstracts (30 as a first author) presented in National (13) and International (34) Meetings

2000

Seminar on "Conjugated Linoleic Acids and Chemoprevention of Breast Cancer" at the Department of Occupational and Environmental Health, University of Pittsburgh, Pittsburgh PA, USA.

Award for the form, content and presentation for an oral communication presented at 92nd Meeting of the American Oil Chemists' Society held in San Diego USA May 2000.

Invitato Speaker on "Metabolism of Conjugated Linoleic Acid in Relation to its Anticarcinogenic Activity" at 220th Meeting of the American Chemical Society. Washington, USA, August 2000.

2001
Invited speaker on "Lipid metabolism modulation by dietary CLA" at the 1st International Conference on Conjugated linoleic acid (CLA) held in Alesund, Norway, 10-13 June 2001.

Invited speaker on "Conjugated linoleic acid" at the Meeting organized by EU on "Functional foods: health benefits and safety issues" held in Brussels, 20-21 June 2001.

Invited speaker on "Modulation of lipid metabolism and vitamin A nutrition by conjugated linoleic acid" at the "Fatty acid and cell signalling" Meeting held in Gargnano, 2-3 September 2001.

2002
Invited speaker on "CLA metabolism" at the Meeting Perspectives on conjugated linoleic acid research, current status and future directions, organized by the Division of Nutrition Research Coordination (DNRC) of the National Institute of Health held in Bethesda, Maryland, USA il 15-16 Maggio 2002. (Web site con abstracts in PDF: http://ods.od.nih.gov/news/conferences/cla.pdf)

Invited speaker on "Modulation of lipid metabolism by naturally occurring conjugated linoleic acid in relation to its beneficial activities" 3rd Meeting Euro-Mediterraneo Cheese Art, held in Ragusa (Italy), 4-9 June 2002.
(Web: http://www.cheeseart.corfilac.it/conferenze_c.htm)

Invited speaker on "Lipid metabolism changes by dietary CLA in relation to its beneficial activities" at the Fifth International Congress on Essential fatty acids and eicosanoids, held in Taipei, Taiwan, 29 August-2 September 2002.
(Web: www.5thicefae2002.org)

Invited speaker on "Conjugated linoleic acid isomers (CLA): good for everything?" session Nutrition and Health of Congrilait 2002, 26th IDF World Dairy Congress held in Paris, France, 24 – 27 September, 2002. (Web: http://www.congrilait2002.com)

000097

2003

Invited speaker on "Retinol tissue levels are positively correlated to dietary conjugated linoleic acid" at the12th European Meeting on Fat-Soluble Vitamins held in Rieti il 13-15 March 2003

Seminar on "Recent advances on Conjugated Linoleic Acid (CLA) metabolism in influencing vitamin A levels and fatty acid pattern in different tissues" at the Department of Human Nutrition, Ohio State University, Columbus, OH, USA.

Invited speaker on " CLA Accumulation and Metabolism in Some Human Pathological States" at the 94th Annual Meeting dell'American Oil Chemists' Society held in Kansas City, MO, USA, 5 May 2003.

Invitated speaker on " Different metabolism of conjugated linoleic acid (CLA) isomers. Is this the discriminant for their peculiar biological effects?" at the "Fatty acid and cell signalling" meeting held in Washington, DC, USA, 15-17 July 2003.

Invitato speaker on " Mechanism of action and immune-modulatory actions of conjugated linoleic acid" at the European Association of Animal Production (EAAP) meeting, held in Roma, Italy, 31 August 2003.

Invitato as a Keynote Speaker on "CLA and human health-An overview" at the "CLA and human health" meeting held in Toronto (Canada) il 21-22 November 2003.

2004

Invitato speaker on "CLAs as precursors of a new family of PUFAs"
At the 6th International Society for the Study of Fatty Acids & Lipids (ISSFAL) meeting held in Brighton (UK), 27 June to 1 July 2004. (web http://wheldonevents.co.uk/issfal/)

Invitato speaker at the Conference on "CLA in Human Health: Mechanisms and Biomarkers" October 21st - 23rd, 2004, Winnipeg, Manitoba, Canada.

Invitated speaker on "CLA metabolism in humans" at the 10th World Congress on Clinical Nutrition that will be held in Pearl Village, Phuket, Thailand, 30 November al 3 December 2004 .
(web www.psu.ac.th/news/wccn.htm)

SCIENTIFIC SOCIETY ACTIVITIES

Member of " Società Italiana di Patologia", "International Society for Free Radical Research" , "American Oil Chemists' Society" and "European Section of the American Oil Chemists' Society"

Member at large of the European section of the AOCS from 1996-1997.

President of the European Section of the AOCS from 1997-1998 .

000098

LAB VISITING

1) 1985. Semestral grant from Fidia Research Laboratories for a project on the determination of homovanillic and dihydroxy phenyl acetic acid in rat and human plasma to evaluate the dopaminergic activity of the central nervous system.

2) 1985. Co-working in a research project on free radical activation of various carcinogenic compounds in vivo and in rat isolated hepatocytes using electron spin resonance and the spin trapping technique (Institute of General Pathology, University of Modena).

3) 1986. Co-working in a research project on free radical activation of Carbon Tetrachloride in vivo and lipid peroxidation, using second derivative spectra and Nuclear Magnetic Resonance techniques (Institute of General Pathology and Department of Chemical Sciences, University of Cagliari, Italy).

4) 1987. Annual fellowship from "Associazione Italiana per la Ricerca sul Cancro" to spend in the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh PA for a common project with Prof. Lombardi on lipid peroxidation and choline deficiency in rats.

5) 1987-90. Spent the Doctorate of Research as a Research Fellow in the Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh PA, USA, under the supervision of Prof. Benito Lombardi, working on a project on conjugated dienes in choline deficient rats

6) 1993.Two months as a research fellow in the laboratories of Prof. Lombardi at the Department of Pathology and of Prof. Day at the Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh PA working on a project on the characterization of conjugated linoleic acid and its metabolites by HPLC equipped with on line diode array and mass spectrometer detectors in rat tissues.

7)1995. Three months as a research fellow in the laboratories of Prof. Lombardi at the Department of Pathology and of Prof. Day and Prof. Kagan at the Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh PA working on a project on the metabolism of conjugated linoleic acid in choline-deficient rats .

8) 1996. One month as a research fellow in the laboratories of Prof. Day and Prof. Kagan at the Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh PA working on a project on the fatty acid peroxidation in human leukemia cells

ORGANIZED MEETINGS

Co-organized with Prof. F.P. Corongiu and Dr. M.A. Dessi an International Meeting on "Free Radicals in Nutrition" held in Cagliari (Italy) 1992, Satellite of the Biannual Meeting of the International Society of Free Radical Research to be held in Torino (Italy).

Co-organized with Prof. F.P. Corongiu and Prof. M.A. Dessi the 2nd meeting of the AOCS European section to be held in Cagliari (Italy) 1-4 october 1998

CURRENT COLLABORATIONS

Dr. Giovanni Piredda, Istituto Zootecnico Caseario, Sassari, Italy
research on: development of new dairy products enriched with conjugated linoleic acid (CLA)

Dr. Leonardo Lucchi, Policlinico Università di Modena, Italy
research on: metabolism of conjugated linoelic acid (CLA) in hemodylasis and chronic renal failure patients

Prof. Anna Iannone and Prof. Aldo Tomasi, Università di Modena, Italy
Research on: Development of new analytical methods to study PUFA oxidation by free radicals

Dr. Clement Ip, Roswell Park Cancer Institute, Buffalo, NY, USA
Research on: Absorption and metabolism of conjugated linoleic acid (CLA) in animal models of mammary carcinogenesis

Prof. Martha Belury, Department of Human Nutrition Ohio State University, Columbus, OH, USA
Research on: PPAR activation by dietary CLA and related changes on lipid metabolism.

Prof. Anna Petroni, Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano.
Research on: Degradative metabolism via peroxisomal beta oxidation in adrenoleukodistrophy patients.

Dr. Barbara Batetta, Università di Cagliari, Italy.
Research on: Modualtion of lipid metabolism and eicosanoid formation during atherosclerosis

MAIN RESEARCH GRANTS IN THE LAST 10 YEARS

1997-1998
1) Biannual grant from Dairy Management Inc. USA, for a project in collaboration with Dr. C. Ip from Roswell Park Cancer Institute, Buffalo, NY and Dr. D. Baumann from Cornell University, NY, on Mammary cancer prevention by milk fat-conjugated linoleic acid

1997-2000
2) Triannual grant from the European Community "Concerted action" on Conjugated linoleic acid

1999-2000
Biannual grant from Cattlemen and Beef Association, USA, in collaboration with Martha Belury, Department of Human Nutrition Ohio State University, Columbus, OH, USA.

2000-2003
3) Triannual grant from the local regional gouvernement of Sardinia on the development of new dairy products enriched with conjugated linoleic acid (CLA)

2003-2006
Triannual grant from the European Community within the 5th Framework Programme, Key Action Food, Nutrition and Health, n.# QLK1-2002-02362.

BOOK EDITED

Co-edited with Prof. F.P. Corongiu, Prof. M.A. Dessi and Dr. C. Rice-Evans the volume VII in the Richelieu Free Radical Series, "Free Radicals and Antioxidants in Nutrition"
The Richelieu Press, London. 1993.

REFEREE IN JOURNALS
J. Nutr.; Lipids; Brit. J. Nutr.; Eur.J.Clin. Nutr.; Biochim.Biophys. Acta; J. Lipid Res. , J. Am. Oil Chem. Soc.; Eur. J. Lipid Sci. Technol.; J. Chrom.; Pharmacol. Res.
Associate editor of the Newsletter of the International Society for the Study of Fatty Acids and Lipids (ISSFAL).

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LIPID NUTRITION

August 13, 2004

RECEIVED
AUG 18 2004
OFFICE OF
FOOD ADDITIVE SAFETY

Paulette Gaynor
Felicia Williams
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification GRN 000148
Vegetable Oil Conjugated Linoleic Acid Preparation
Amendment: Responses to FDA questions

Dear Drs. Gaynor and Williams:

This letter amends the above-referenced GRAS notification for Clarinol™ G-80 Vegetable Oil Conjugated Linoleic Acid ("CLA") Preparation by providing a partial response to your questions conveyed during a conference call between you and our counsel Daniel Dwyer on June 7, 2004. The following paragraph provides part (b) of your question 8 (in italics) and our response.

QUESTION 8
We are requesting additional discussions on two issues that Loders Croklaan discussed in the Notice: (a) increase in lipid peroxidation markers; and (b) decrease in maternal milk fat. (Additional discussion was provided in a facsimile dated June 7, 2004.)

RESPONSE 8 (b)

At your request, we have prepared a more thorough discussion on the effects of a 50:50 CLA isomer mix on lactating and pregnant women and their infants.

The studies that have investigated this issue most thoroughly have been done in animals. Of these studies, those in rats and piglets are most relevant because these animals are particularly effective human surrogates.

Two studies have investigated the effects of CLA during pregnancy in rats (Chin et al., 1994; Poulos et al., 2001). The study by Chin et al. (1994) was previously discussed in our notice (page 48), where we noted that feeding CLA to the rat dams during gestation and lactation improved the postnatal body weight gain of pups. In its facsimile of June 7, 2004, FDA observed that the isomeric composition of the CLA preparation used in this study was not specified. However, personal communication with Dr. M. Pariza, corresponding author of the paper, revealed that the composition was virtually identical

to the CLA that is the subject of our notice; it consisted of an almost equal mixture of c-9,t-11 and t-10,c-12 CLA with virtually no other CLA isomers.

Similar findings in rats have been reported by Poulos et al. (2001). A diet enriched with 0.5% CLA supplement (50:50 mixture) was fed to pregnant Sprague-Dawley rats from day 7 of pregnancy and continued through day 21 of lactation when the pups were weaned. A subgroup of pups were fed either CLA or control diets from weaning until 11 weeks of age. There were no differences in the number of pups born per litter in CLA-fed and control fed dams. Female pups exposed to CLA were significantly heavier than those of dams fed control diets at weaning. CLA had no effect on liver weight in either sex. Male pups exposed to CLA throughout gestation and lactation, as well as post-weaning, were the heaviest, fastest growing and most feed efficient of all treatment groups, whereas males never exposed to CLA in any of these phases of development were smallest.

The findings of improved growth after CLA intake in rats are supported by findings of studies in pigs. Similar to humans, milk is the main food of piglets in the first weeks of life. Therefore its composition is of major importance for proper growth and development. It has been demonstrated many times that feeding young animals diets deficient in polyunsaturated fatty acids leads to stunted growth and disease, presumably by a decrease in thyroid hormone secretion and subsequent increase in cholesterol levels. To this end, Pietras et al. (2002) conducted a study in pigs to determine to what extent the addition of CLA to the diets of sows in the late stage of pregnancy affects thyroid hormone and cholesterol levels in the blood of piglets. Sows received diets supplemented with 4% of a CLA preparation containing four CLA isomers (approximately 15% each of c-9,t-11; c-11,t-13; t-8,c-10; and t-10,c-12) from day 90 of pregnancy to day 21 of lactation. The CLA supplementation had no effect on the average milk consumption or body weight of piglets. Plasma thyroxine and triiodothyronine contents in experimental piglets were significantly higher than in the control animals, whereas plasma total cholesterol, HDL and LDL cholesterol were significantly lower. The authors focused on the relationship between these two observations, concluding that "This suggests that the blood cholesterol level of piglets fed conjugated linoleic acid in the colostrum and milk of their dams may depend on the level of thyroid gland activity."

In a study by Bee et al. (2000), piglets were born to and reared by sows fed a diet with linoleic acid or with 2% of a commercial CLA preparation containing a 50:50 ratio of both isomers. Compared to sows fed a control diet, sows fed a CLA-enriched diet tended to have lower fat content in the mature milk. Nevertheless, the piglets born to and reared by sows fed the CLA diet had greater feed intake, daily weight gain and final body and warm carcass weight than piglets reared by sows fed a linoleic acid diet. Neonatal growth was unaffected by the nutrition of the dams (Bee, 2000). When sows were fed

CLA only during lactation, there were no differences in the growth rate of piglets, despite a decrease in milk fat and changes in fatty acid composition (Harrell et al., 2000).

A number of studies have evaluated the effects of CLA on lactation and pregnancy in cows and the results are generally consistent with those in rats and piglets. With respect to lactation, studies have shown a decrease in milk fat content, without changes in milk yield, milk lactose and milk protein content (Bernal-Santos et al., 2001; Chouinard et al., 1999; Loor and Herbein, 2000; Medeiros et al., 2000; Perfield et al., 2002). Ratios of $C_{14:0}$ to $C_{14:1}$, $C_{18:0}$ to $C_{18:1}$, $C_{18:1}$ to $C_{18:2}$ and $C_{18:2}$ to $C_{20:4}$ fatty acids increased after CLA intake, indicating a reduced *de novo* fatty acid synthesis and desaturation of fatty acids (Baumgard et al., 2002; Chouinard et al., 1999; Loor and Herbein, 2000; Peterson et al., 2002). Subsequent studies in cows have demonstrated that the decrease in milk fat content is mainly due to the inhibition of milk fat synthesis by the t-10,c-12 CLA isomer (Baumgard et al., 2000; Baumgard et al., 2002; Baumgard et al., 2001; Peterson et al., 2002). This isomer appears to reduce the expression of genes that encode for enzymes involved in circulating fatty acid uptake and transport, *de novo* fatty acid synthesis, desaturation of fatty acids and triglyceride synthesis (Baumgard et al., 2002).

The effects of CLA intake during pregnancy in cows has been studied by Perfield et al. (2002). Calcium salts providing 30.4 g/day of CLA consisting of four isomers (9.2% t-8,c-10; 25.1% c-9,t-11; 28.9% t-10,c-12; 16.1% c-11,t-13) were supplemented to pregnant cows from day 79 of pregnancy and continued for 140 days until dry off. Cows receiving CLA had a lower milk fat content, but milk yield, protein and lactose content were unaffected. According to the authors, "Changes in body weight gain, body condition score, and net energy balance were not significant and imply no differences in cows fed the CLA supplement in replenishment of body reserves in late lactation. Likewise, maintenance of pregnancy, gestation length, and calf birth weight were unaffected by treatment." (Abstract.)

In addition to the studies discussed above, one study has also been conducted in mice. The effects of individual CLA isomers on lactation in CD-1 mice were studied by Loor et al. (2003). On day 4 postpartum, lactating mice with litters of 8 pups each were randomly assigned to control (rapeseed oil) or diets enriched with 1% stearic acid, vaccenic acid, c-9,t-11 CLA or t-10,c-12 CLA. These diets were fed until day 15 postpartum. Milk fat concentration was greater in the dams fed stearic acid compared with those fed vaccenic acid, c-9,t-11 CLA or t-10,c-12 CLA. Feeding t-10,c-12 CLA resulted in the lowest concentration of milk fat. The body and liver weight of the pups nursing from the dams fed t-10,c-12 CLA were significantly lower compared with the other pups. Although the authors suggest that the single-stomached animals such as mice may serve as models for humans, it is questionable whether this is true in case of studies of CLA. As discussed in our notice (page 48/49), mice are unlike humans in that they have a much higher metabolic rate and are much more sensitive to the adipose tissue

reducing effects of t-10,c-12 CLA (Terpstra, 2001). Indeed, the dams fed t-10,c-12 CLA had lower live body weights, which may partly explain the lower body weights of the pups from this group. Further, the mice were not fed a blend of isomers (e.g., a 50:50 blend) but were fed either the c-9,t-11 or t-10,c-12 isomers separately. Thus, they were not exposed in a manner identical to the piglets and rats. Accordingly, extrapolation of the findings from this study to humans consuming a blend of approximately 50:50 CLA isomers is not appropriate.

Two relevant studies have been conducted in humans: Masters et al. (2002) and Elias and Innis (2001).

The study by Masters et al. tested the hypothesis that CLA supplementation would increase milk CLA concentration and decrease milk fat content in humans. Breastfeeding women (n = 9) participated in this double-blind, placebo-controlled, crossover study divided into three periods: intervention I (5 d), washout (7 d), and intervention II (5 d). Women were randomized to treatment order. During each intervention period, women consumed 1.5 g of CLA supplement (containing approximately a 50:50 blend of c-9,t-11 and t-10,c-12 isomers) or placebo (olive oil) daily; during the washout period, no supplements were consumed. Milk was collected by complete breast expression on the final day of each period; milk output was estimated by 24-h weighing on the penultimate day of each intervention period. Milk c-9,t-11 and t-10,c-12 CLA concentrations were greater ($P < 0.05$) during the CLA treatment period as compared to the placebo period. Milk fat content was significantly lower during the CLA treatment, as compared to the placebo treatment ($P < 0.05$). As compared with the washout period, milk fat content with CLA was lower, but not significantly, and milk fat content with placebo was higher, but not significantly. Data indicate no effect of treatment on milk output. There was no significant change in fatty acid composition of the milk fat (apart from CLA), and the milk fat content remained within normal ranges. There was no effect of treatment on mean infant milk consumption.

This study is consistent with the data from animal studies in that it suggests that CLA may reduce maternal milk fat content. Because milk fat content remained within normal ranges, the study suggests that supplementation with CLA may have effects on milk fat that are not significantly different from normal dietary variations. In addition, because there was no significant change in fatty acid composition of the milk fat, no effect on mean infant milk consumption, and no variation of milk fat content outside normal ranges, the study does not suggest that there would be any significant effect of CLA on nursing infants. As discussed above, the relevant animal studies do not suggest any adverse effects of CLA on infants (and indeed they suggest that infants may thrive); however, this study did not assess infant weight or other effects on infants.

The authors of this study state that their findings "may be of public health importance" and recommend that lactating women not consume CLA supplements. In addition, in explaining why the study had a small sample size the authors state that they "decided that enrolling more women was not ethically justifiable." These comments suggest that the study identified risks to the health of nursing mothers or infants associated with CLA, but we respectfully disagree that this is the case. CLA is a normal constituent of the diet; the c-9,t-11 isomer is particularly abundant in milk and beef fat, and the t-10,c-12 isomer is found in small quantities particularly in vegetable oil. As such, it is virtually always consumed by mothers and infants. The levels of CLA evaluated in this study are higher than those consumed in the normal diet (dietary intake of CLA is described in our notice at pp. 62-63), at levels approaching those proposed in our notification. In this study, these levels resulted in milk fat content within normal ranges, no significant change in fatty acid composition of the milk fat (apart from CLA), and no effect on mean infant milk consumption. Although milk fat content in the study was reduced, a significant body of animal data suggests that such a reduction by CLA is not associated with adverse effects in infants. Because this study did not assess effects on infants, it does not provide a scientifically adequate basis for disregarding the information from animal studies that suggest no adverse effects on infants, and therefore does not justify the author's advice regarding consumption of CLA supplements. For these reasons, we conclude that this study does not indicate that CLA presents a risk to the health of the nursing mother or child.

Elias and Innis (2001) evaluated the associations of birth outcome with maternal and infant plasma concentrations of *trans* fatty acids (TFA), CLA, arachidonic acid (AA) and docosahexaenoic acid (DHA). The study did not involve the addition of CLA to the diet, but considered only existing dietary intake. Among other things, the study reported that infant plasma cholesteryl ester TFA and CLA and triacylglycerol CLA concentrations were significantly inversely related to the length of gestation, and that plasma concentration of infant cholesteryl ester CLA was inversely related to infant birth weight and birth length. No significant relations were found between maternal plasma TFA or CLA and the length of gestation or infant birth weight or length.

In interpreting these data, the authors stated that the observed effects are not consistent with what is known about CLA isomers: effects on lipids have been attributed to the t-10,c-12 isomer, whereas the c-9,t-11 isomer makes up almost all of the CLA in dairy fats. The authors concluded, "This suggests that other dietary or lifestyle factors associated with high intakes of dairy fats during pregnancy, rather than CLA intake itself, may explain the inverse relations found in our study between CLAs and infant birth length and weight. Further studies are needed to determine whether the different CLA isoforms have biological activity relevant to fetal growth and to explain the apparent preferential assimilation of CLAs into fetal compared with maternal plasma triacylglycerols."

In our view, this study is important but does not provide information that, at this time, can be used in an analysis of the safety of CLA. Although the study observed effects in infants that were statistically associated with CLA levels, the authors were careful to point out that the data suggest that other causative factors may be responsible for observed effects. Therefore, this study is not inconsistent with the observed lack of significant effect of CLA on offspring in the animal studies and with the observed lack of significant effect of CLA on fatty acid composition of milk fat, mean infant milk consumption, and variation of milk fat content outside normal ranges in the study by Masters et al.

It is important to note that this study also found inverse associations between dietary TFA and both DHA in triacylglycerols and AA in cholesteryl esters of newborn infant plasma. (AA and DHA are critically important in fetal and infant growth and central nervous system development.) Further, the study found a significant inverse relation between the newborn infant plasma concentration of TFA in cholesteryl esters and the length of gestation. However, it would be inappropriate to conclude that these data, by themselves, call into question the safety (or GRAS status) of TFA for nursing mothers and infants. Similarly, the study data do not by themselves call into question the safety of CLA.

Finally, we note that the authors conclude that more definitive studies of dietary intakes of TFA and CLA and their relation to subsequent infant growth and development are needed to define appropriate dietary fat intakes during pregnancy. We agree and, consistent with our responsibility to ensure that the food ingredients we market are safe, we will continue to evaluate new scientific information relevant to this issue.

In conclusion, in evaluating the potential effects of a 50:50 CLA isomer mix on lactating and pregnant women and their infants, the greatest weight must be given to the animal studies discussed above – particularly those in rats and piglets which are effective human surrogates. These studies were carefully done and the results are consistent in a variety of studies and animal species. These studies indicate strongly that CLA is safe for nursing mothers and infants. Relatively less weight should be given to the human data because the studies are fewer in number and the results less definitive. Even so, the results of the human studies are generally consistent with the results of the animal studies, particularly in that they do not suggest that CLA effects milk fat in a way that is different from normal dietary variation.

Accordingly, based on this weighing of the evidence, and taking into consideration all of the other data supporting the safety of CLA as discussed in our notice, we conclude that there is reasonable certainty that CLA preparation will not be harmful under its intended conditions of use. This conclusion is based on scientific procedures, common knowledge about the substance throughout the scientific community, and the views of qualified experts. In addition, we note that CLA preparation is intended for use in foods for special

dietary use in the management of body weight. These are normally not consumed by pregnant women or lactating mothers.

Thank you for your consideration of this information. If FDA has further questions or comments about the information presented in this letter, we request that we meet to discuss this information in more detail.

Respectfully submitted,

Andreas Menzel
Head, Research and Development

cc: Daniel R. Dwyer
Kleinfeld, Kaplan and Becker

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LIPID NUTRITION

August 31, 2004

RECEIVED
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OFFICE OF
FOOD ADDITIVE SAFETY

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRN No. 148
Request that FDA cease evaluating a Notice of GRAS Exemption Claim for Clarinol™ G-80 (vegetable oil conjugated linoleic acid preparation)

To Whom It May Concern:

By letter dated March 10, 2004, Loders Croklaan B.V. submitted to FDA a Notice of a GRAS Exemption Claim for Clarinol™ G-80 (vegetable oil conjugated linoleic acid ("CLA") preparation) (GRN No. 148).

We request that FDA cease evaluating the notice at this time while we clarify certain technical information, with the understanding that we intend to submit, in the future, an extended GRAS notice for Clarinol.

Thank you for your attention to this request.

Sincerely,

Andreas Menzel
R&D Program Manager

cc: Antonia Mattia, Ph.D. (HFS-255)
Rudolph Harris, Ph.D.

Lipid Nutrition is a division of Loders Croklaan

www.lipidnutrition.com

SU

THOMAS O. HENTELEFF
RICHARD S. MOREY
KINSEY S. REAGAN
PETER R. MATHERS
ANTHONY L. YOUNG
ANNE V. MAHER
BONNIE A. BEAVERS
DANIEL R. DWYER
GLENN E. DAVIS
STACY L. EHRLICH
JENNIFER A. DAVIDSON
STACEY L. VALERIO
JONATHAN M. WEINRIEB

OF COUNSEL:
HARVEY A. SUSSMAN
WILLIAM J. HARDY

LAW OFFICES
KLEINFELD, KAPLAN AND BECKER, LLP
1140 NINETEENTH STREET, N.W.
WASHINGTON, D.C. 20036-6606
TELEPHONE (202) 223-5120
FACSIMILE (202) 223-5619
www.kkblaw.com

WEST COAST OFFICE:
ONE MARKET STREET
STEUART TOWER, SUITE 1450
SAN FRANCISCO, CA 94105-1313
TELEPHONE (415) 538-0014
FACSIMILE (415) 538-0016

VINCENT A. KLEINFELD
1907-1993
ALAN H. KAPLAN
1930-2001

January 18, 2005 2005-555

Via Facsimile 301-436-2964

Antonia Mattia, Ph.D.
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Vegetable Oil Conjugated Linoleic Acid Preparation (CLA)

Dear Dr. Mattia:

On September 22, 2004, Loders Croklaan and FDA held a conference call to discuss technical requirements relating to a withdrawn GRAS notice, GRN 148, to facilitate the future submission of new GRAS notice for CLA. In preparation for the submission of such a GRAS notice, we would like to request a meeting with FDA. At this meeting, we would propose to discuss a detailed outline of the points to be addressed in the notice, and obtain FDA's comments on those points, to ensure that our outline fully addresses FDA's observations made during the September 22nd conference call. We believe that this exchange of information will assist both the company and FDA to focus on the highest priority issues that need to be addressed in the new notice.

We would propose a meeting to be held on February 16 or 17, if possible, and request that you schedule approximately two hours for the discussion. If these dates do not work, please propose alternatives during the same time period. I will follow up by telephone to confirm the date. Prior to the meeting, we will provide a more detailed agenda and list of attendees.

Thank you for your continued assistance with this matter.

With kind regards,

Daniel R. Dwyer /

Counsel to Loders Croklaan B.V.

SU

Binion Williams, Felicia B

From: Daniel Dwyer [DDwyer@kkblaw.com]
Sent: Friday, January 28, 2005 8:36 AM
To: felicia.binionwilliams@fda.hhs.gov
Cc: Mattia, Antonia; Harris, Rudolph; Menzel, Andreas; Dederen, Louis; O'Shea, Marianne
Subject: RE: Request for meeting re GRAS notification

Felicia B. Williams
FDA/CFSAN/OFAS

Dear Dr. Williams:

This confirms a meeting between FDA and Loders Croklaan B.V. on February 17, 2005, from 9:00 to 11:00 a.m. to discuss a planned GRAS notification for conjugated linoleic acid (CLA). The following is a brief agenda:

1. Background and research update on CLA (presented by Loders Croklaan).

2. Review of outline for GRAS notification by Loders Croklaan, and discussion with FDA as to whether the outline addresses the issues raised during the September 22, 2004 conference call between FDA and the company. Key topics in the outline will include:

   a. CLA and cardiovascular risk factors
   b. CLA and insulin resistance
   c. CLA and maternal milk fat
   d. General recognition of CLA as safe by qualified experts

3. Conclusion and discussion of tentative plans for GRAS notification.

The following individuals, as well as myself, will attend the meeting on behalf of Loders Croklaan.

-- Andreas Menzel, Ph.D., R&D Program Manager
-- Marianne O'Shea, Ph.D., Nutrition & Technical Services Manager
-- Sandra Einerhand, Ph.D., Senior Nutritionist
-- Jennifer Causey, Ph.D., Nutritionist

Thank you for your assistance in scheduling this meeting.

Kind regards,

-Dan

Daniel R. Dwyer
KLEINFELD, KAPLAN and BECKER, LLP
1140 Nineteenth Street, NW
Washington, DC 20036
202.223.5120

Counsel to Loders Croklaan B.V.

-----Original Message-----
From: Daniel Dwyer
Sent: Tuesday, January 18, 2005 1:20 PM
To: 'Mattia, Antonia'
Cc: 'Menzel, Andreas'; 'Harris, Rudolph'

000605

Subject: Request for meeting re GRAS notification

Antonia Mattia, Ph.D.
FDA/CFSAN/OFAS

Dear Dr. Mattia,

Attached please find a request for a meeting from Loders Croklaan relating to conjugated linoleic acid preparation. This has also been sent to you by fax.

Thank you for your assistance.

Kind regards,

-Dan

Daniel R. Dwyer
KLEINFELD, KAPLAN and BECKER, LLP
1140 Nineteenth Street, NW
Washington, DC 20036
202.223.5120

Counsel to Loders Croklaan B.V.

000606

Reference List for Industry Submission, GRN 000148

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NA- Not applicable

Pages	Author	Title	Publish Date	Publisher	BIB_Info
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