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July 31, 2007

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JUL 31 2007

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

BY:.....

RECEIVED  
JUL 31 2007

Re: Notice of a GRAS Exemption Claim  
for Conjugated Linoleic Acid (CLA)-Rich Oil

To Whom It May Concern:

Enclosed please find three copies of a GRAS notification as described above. One copy of Attachments 1 and 2 are provided separately, in envelopes, because they contain confidential manufacturing information. If you need additional copies of Attachments 1 and 2, please let me know.

Sincerely,

Daniel R. Dwyer

Counsel to Lipid Nutrition B.V.

enclosure

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Re: Notice of a GRAS Exemption Claim  
for Conjugated Linoleic Acid (CLA)-Rich Oil

To Whom It May Concern:

On behalf of Lipid Nutrition B.V. ("Lipid Nutrition") and Cognis GmbH ("Cognis"), we hereby provide the following information pursuant to proposed 21 CFR 170.36(c)(1) (62 Fed. Reg. 18938, 18961; April 17, 1997):

**GRAS Exemption Claim:** Lipid Nutrition and Cognis hereby claim that Conjugated Linoleic Acid (CLA)-Rich Oil, intended for use in certain specified foods within the general categories of soy milk, meal replacement beverages and bars, milk products and fruit juices, at a level of 1.5 g CLA per serving, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because the companies have determined that this ingredient is Generally Recognized as Safe (GRAS) for such use, using scientific procedures.

**(i) Name and Address of Notifiers:**

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**(ii) Common or Usual Name of the Substance:** Conjugated Linoleic Acid (CLA)-Rich Oil

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**KLEINFELD, KAPLAN AND BECKER, LLP**

Office of Food Additive Safety  
July 31, 2007  
Page 2

**(iii) *Applicable Conditions of Use:*** As an ingredient in certain specified foods within the general categories of soy milk, meal replacement beverages and bars, milk products and fruit juices, at a level of 1.5 g CLA per serving.

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

**(v) *Statement of Availability:*** The data and information that are the basis for the GRAS determination are available for FDA review and copying at reasonable times at the offices of the undersigned, 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. Confidential exhibits containing manufacturing information from Lipid Nutrition and Cognis are provided separately.

This is a joint submission by two independent companies and it is requested that any formal communications be addressed separately to the two companies at the offices of their counsel.

Counsel for Cognis: Diane C. McEnroe  
Sidley Austin, LLP  
787 Seventh Avenue  
New York, New York 10019  
Tel. 212-839-5621  
Fax. 212-839-5599

Counsel for Lipid Nutrition: Daniel R. Dwyer (address above).

Informal communications (e.g., telephone calls with questions) may be directed either to Ms McEnroe or Mr. Dwyer.

Thank you for your attention to this matter.

Sincerely,

Daniel R. Dwyer

Counsel to Lipid Nutrition B V.

enclosure

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JUL 31 2007

BY:.....

**GRAS NOTIFICATION**

for

**CONJUGATED LINOLEIC ACID (CLA)-RICH OIL  
FOR USE IN CERTAIN FOODS**

***Submitted by:*** Lipid Nutrition  
P.O. Box 4  
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July 19, 2007

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# GRAS NOTIFICATION FOR CONJUGATED LINOLEIC ACID (CLA)-RICH OIL FOR USE IN CERTAIN FOODS

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## ABBREVIATIONS

3MH	3-methylhistidine
ACC	acetyl CoA carboxylase
ACO	acyl coenzyme A oxidase
ACS	acyl-CoA synthetase
ADME	absorption, distribution, metabolism, and excretion
AFABP	adipocyte fatty acid binding protein
AGPAT	acylglycerol phosphate acyltransferase
ALAT	alanine aminotransferase
ALBP	adipocyte lipid-binding protein
ALD	adrenoleukodystrophy
ALP	alkaline phosphatase
ALT	alanine aminotransferase
aP2	adipocyte fatty acid binding protein
ApoB	Apolipoprotein B
ApoB100	Apolipoprotein B100
ASAT	aspartate aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
BMI	body mass index
C	cholesterol
CCl <sub>4</sub>	carbon tetrachloride
CD	cluster of differentiation
CE	cholesteryl ester
CFU	colony forming units
cGMP	current good manufacturing practice
CHD	coronary heart disease
CLA	conjugated linoleic acid
CLA-EE	CLA-ethyl ester
CLA-ME	CLA methyl ester
CO <sub>2</sub>	carbon dioxide
CoA	Coenzyme A
CPT	carnitine palmitoyl transferase
CPT-I	carnitine palmitoyl transferase I
CRP	C-reactive protein
CYP4A1	microsomal cytochrome P450 4A1
DIN	2,3 diinor
ds	double strand
FA	fatty acid
FABP	fatty acid binding protein
FAS	fatty acid synthase
FAT	fatty acid transporter

FDA	Food and Drug Administration
FFA	free fatty acid
GC-MS	gas chromatography-mass spectrometry
GD	gestation day
GFAT	glutamine-fructose aminotransferase
GLP	good laboratory practices
GMP	good manufacturing practices
GPAT	glycerol phosphate acyltransferase
GRAS	generally recognized as safe
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
HF	high-fat
HOMA	Homeostasis Model Assessment
HOMA-R	homeostatis model for insulin resistance
IAUC	incremental area under the postprandial curve
IFN	Interferon
IFN- $\gamma$	interferon-gamma
IL	Interleukin
ILs	Interleukins
IOM	Institute of Medicine
ISI	insulin sensitivity index
ITT	insulin tolerance test
LA	linoleic acid
LCAD	mitochondrial long-chain acyl-CoA dehydrogenase
LD	lactation day
LDL	low density lipoprotein
LDL-C	low density lipoprotein cholesterol
LF	low-fat
L-FABP	liver fatty acid binding protein
Lp(a)	lipoprotein a
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LTB4	leukotriene B4
MCAD	mitochondrial medium-chain acyl-CoA dehydrogenase
MFD	milk fat deposition
mRNA	messenger RNA
MUFA	monounsaturated fatty acids
NADP	nicotinamide adenine dinucleotide phosphate
NAP	National Academy Press
NAS	National Academy of Sciences
NEFA	non-esterified fatty acids
NOAEL	no-observed-adverse-effect level
NR	not reported

NS	not significant
NTx	cross-linked N-telopeptides
ODC	ornithine decarboxylase
OECD	Organization for Economic Cooperation and Development
OGTT	oral glucose tolerance test
PAF-1	plasminogen activating factor-1
PBMC	peripheral blood mononuclear cell
PC	phosphatidylcholine
PCV2	porcine circovirus
PEPCK	phosphoenolpyruvate carboxykinase
PG	Prostaglandin
PGE2	plasma prostaglandin E2
PPAR	peroxisome proliferator-activated receptors
PPAR- $\alpha$	peroxisome proliferator-activated receptor <i>alpha</i>
PPAR- $\gamma$	Peroxisome proliferator activated receptor <i>gamma</i>
PUFA	polyunsaturated fatty acid
PUFAs	polyunsaturated fatty acids
QUICKI	Quantitative Insulin Sensitivity Check Index
RACC	reference amounts customarily consumed
REE	resting energy expenditure
RQ	respiratory quotient
S14	spot 14
SCAL	Mitochondrial short-chain acyl-CoA dehydrogenase
SCD	stearoyl CoA desaturase
SD	sorbitol dehydrogenase
SFA	saturated fatty acids
sICAM-1	serum-soluble intercellular adhesion molecule-1
SREBP	sterol responsive element-binding protein
ss	single strand
sVCAM-1	soluble vascular cell adhesion molecule-1
TCR	T cell antigen receptor
TG	Triglyceride
TNF	tumor necrosis factor
TNF $\alpha$	tumor necrosis factor- <i>alpha</i>
TSH	thyroid-stimulating hormone
UCP	Mitochondrial uncoupling proteins
UHT	ultra-high temperature
VAS	visual analog scale
VCAM-1	vascular cell adhesion molecule-1
VLCAD	Mitochondrial very long-chain acyl-CoA dehydrogenase
VLCD	very low calorie diet
VLDL	very low density lipoprotein
VLDL-C	very low density lipoprotein cholesterol

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WAT	white adipose tissue
WBC	white blood cell
WPR	within population range

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# GRAS NOTIFICATION FOR CONJUGATED LINOLEIC ACID (CLA)-RICH OIL FOR USE IN CERTAIN FOODS

## 1.0 INTRODUCTION

Pursuant to 62 Fed. Reg. 18938, 18960 (April 17, 1997) (proposed 21 CFR 170.36), Cognis GmbH and Lipid Nutrition B V. (the Companies) hereby claim that Conjugated Linoleic Acid (CLA)-Rich Oil, intended for use in certain specified foods within the general categories of soy milk, meal replacement beverages and bars, milk products and fruit juices, at a level of 1.5 g CLA per serving, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because the Companies have determined that this ingredient is Generally Recognized as Safe (GRAS) for such use, using scientific procedures (U.S. FDA, 1997).

This document accompanies the GRAS exemption claim required by proposed 21 CFR 170.36(c)(1) and provides the detailed information on general recognition of safety 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. The Companies' 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 (U.S. FDA, 1997).

CLA-Rich Oil is a food grade preparation derived from processed safflower oil. It consists of 78% total CLA isomers and at least 74% of an approximately 50:50 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers ("50:50 mixture").

In this document, quantities of CLA-Rich Oil (and other CLA-containing formulations) are expressed in terms of the quantity of CLA provided by those formulations (not including non-CLA ingredients in the formulations), unless stated otherwise.

Conjugated linoleic acid is the term given to a group of positional and geometric isomers of octadecadienoic acid. The conjugated double bonds (i.e., the 2 double bonds are separated by 1 single bond) can be present in either the *cis* or *trans* configuration and are present predominantly in positions 8 and 10, 9 and 11, 10 and 12, or 11 and 13. CLA occurs naturally in ruminant products such as beef, lamb, and dairy products due to the bacterial biohydrogenation of linoleic acid in the rumen, with the *cis*-9, *trans*-11 (C18 2 c9,t11) isomer accounting for more than 90% of CLA intake in the diet.

This dossier outlines the composition and manufacture of CLA, estimates consumer exposure under defined conditions of use, and documents the literature regarding the safety of CLA. It is based on a comprehensive and critical review of all relevant data and information. To ensure that all relevant data were reviewed, searches of the published scientific literature were performed by Cantox Health Sciences International through February 2007. Medline®, Agricola, JICSP-EPlus, Biosis Previews®, NTIS, and EMBASE served as the primary sources of published literature pertinent to the safety of CLA.

The Expert Panel consisted of the following individuals qualified by scientific training and experience to evaluate the safety of substances added to food:

- Joseph F. Borzelleca, PhD  
Medical College of Virginia
- David M. Klurfeld, PhD  
United States Department of Agriculture  
Agricultural Research Service
- Robert J. Nicolosi, PhD, CNS  
University of Massachusetts Lowell
- Walter H. Glinsmann, MD  
Glinsmann, Inc.
- John A. Thomas, PhD  
Indiana University School of Medicine

In addition, several experts in specialized fields provided advice to the expert panel relevant to assessing the safety of CLA. The information provided by these experts is provided in Appendix B.

A detailed summary of the data and information on which this GRAS determination is based is provided in the Summary and Conclusions of the Expert Panel, in Section 2.0.

The data and information summarized in this dossier demonstrate that CLA-Rich Oil, meeting appropriate food grade specifications and manufactured in accordance with current good manufacturing practice (cGMP), is GRAS, based on scientific procedures, under the conditions of intended use in foods, as described herein.

## **2.0 SUMMARY AND CONCLUSIONS OF THE EXPERT PANEL**

This section summarizes a determination by a panel of experts qualified by scientific training and experience to evaluate the safety of substances added to food ("Expert Panel") that CLA-Rich Oil is generally recognized as safe (GRAS) by scientific procedures for its intended use as described herein

CLA-Rich Oil is a food grade preparation derived from processed safflower oil. It consists of approximately 78% total conjugated linoleic acid (CLA) isomers and 74% of an approximately 50:50 mixture of cis-9,trans-11 and trans-10,cis-12 CLA isomers ("50:50 mixture"). CLA-Rich Oil is intended to be added to certain specified foods within the general categories of soy milk, meal replacement beverages and bars, milk products and fruit juices. CLA-Rich Oil would be added to these foods at a level of 1.5 g per serving.

### **MANUFACTURING AND SPECIFICATIONS**

CLA-Rich Oil is produced in accordance with current good manufacturing practice (cGMP) and is free from foreign materials and contamination. The materials and processes used to produce the substance are generally considered to be safe and suitable for the production of food ingredients. The manufacturing processes are adequately controlled to assure that the resulting products consistently meet appropriate food-grade specifications. Batch analyses confirm production of a consistent product.

### **SCIENTIFIC EVIDENCE OF SAFETY**

CLA occurs naturally in ruminant products such as beef, lamb, and dairy products. The cis-9,trans-11 CLA isomer accounts for more than 90% of CLA intake in the diet. Human intake of CLA in the diet is variable and is estimated at approximately 200 mg/day for adult Americans.

CLA preparations (e.g., in a 50:50 mixture as well as other isomeric forms) are the subject of many in-vitro, animal and human studies. In weighing the evidence of safety, human studies were accorded highest priority. The order of priority was:

1. Well-designed and properly executed human studies using a 50:50 mixture, of appropriate duration and dose;
2. Well-designed and properly executed preclinical studies using a 50:50 mixture in appropriate animal models and in-vitro, that considered metabolism, dose dependency and mechanism of observed effects, and
3. Additional clinical and preclinical data on other isomers, to demonstrate that the totality of the evidence has been reviewed.

The safety of CLA-Rich Oil is supported by extensive published data:

1. CLA is the subject of 32 clinical studies in which the 50:50 mixture of isomers was evaluated, of which the following studies are considered pivotal to this GRAS determination: Gaullier et al. (2004, 2005, 2007), Larsen et al. (2006), and Whigham et al. (2004). All of these studies have been published in peer-reviewed scientific journals. A comprehensive review of the clinical data demonstrates that consumption of CLA at levels of up to 6 g/day for up to 1 year (Larsen and Whigham) and 3.4 g/day for up to 2 years (Gaullier) is safe and is not associated with any significant adverse effects.
2. Animal and human studies demonstrate that CLA is likely to be absorbed across the human gastrointestinal tract similarly to cis-9,cis-12-linoleic acid, the most common linoleic isomer in the diet. The human metabolism of CLA is also similar to that of the more common isomer of linoleic acid.
3. Subchronic and chronic studies in rats demonstrate that CLA at 2,433 and 2,728 mg/kg body weight/day for males and females, respectively, for periods of 13 weeks to 18 months, produce no significant adverse effects.
4. Reproductive and developmental toxicity studies in rats and pigs demonstrate a lack of adverse effects on maternal food consumption and body weight, litter size, or offspring growth and development following exposure to CLA (0.25 to 2% in the diet, equivalent to 250 to 500 mg/kg body weight/day) throughout gestation, lactation, and/or during the post-weaning period.
5. *In vitro* assays demonstrate an absence of mutagenicity or genotoxicity.

The review of CLA-Rich Oil addressed several issues relevant to a safety assessment for CLA, as follows:

- Cardiovascular Disease Risk
  - Lipid Metabolism: A number of studies in humans and animals of CLA (50 50 mixture) report no evidence of adverse effects on blood lipid parameters. Certain studies report statistically significant changes from baseline or control values, but these effects are not considered to be clinically significant. We conclude, based on the weight of the evidence, that CLA (50 50 mixture) intake does not adversely affect lipid parameters associated with CVD risk
  - Markers of Inflammation: Human studies using the 50:50 mixture show no effect on markers of inflammation related to cardiovascular disease risk. Several studies report that the 50:50 mixture does not affect C-reactive protein (CRP) levels. One study suggests that a single CLA isomer (t10,c12) may increase

CRP levels, but not with the 50:50 mixture. Neither animal studies nor in-vitro studies support suppression of relevant inflammatory markers. Results relating to a single isomer are not relevant in assessing the safety of CLA-Rich Oil. We conclude, based on the weight of the evidence, that CLA (50:50 mixture) intake does not adversely affect markers of inflammation.

- **Markers of Oxidative Stress:** Some increase in isoprostanes following CLA intake has been observed in humans. The studies demonstrate that this effect was not due to oxidative stress because no markers of such stress other than isoprostanes increase with CLA consumption. The observed effect on isoprostanes appears to be metabolic: the evidence indicates that CLA competes with F2-isoprostanes for the same metabolic pathway, with the increase in F2-isoprostane levels being the result of a decrease in F2-isoprostane catabolism. We conclude, based on the weight of the evidence, that the observed effects on isoprostane levels do not represent a harmful effect of CLA (50:50 mixture).
- **Endothelial Function:** Based on the weight of the evidence representing studies in various human population groups, we conclude that CLA (50:50 mixture) demonstrates no significant adverse effects on endothelial function.
- **Insulin Sensitivity and Glucose Metabolism:** A number of human studies demonstrate no adverse effects of CLA (50:50 mixture) on glucose and insulin after periods of up to 6 months of CLA consumption at levels of 4 g/day. CLA does not affect insulin sensitivity in healthy, in overweight, or in obese or sedentary individuals. In one human study, an increase in insulin resistance was observed, but this was associated with the administration of only the t10,c12 isomer. We conclude, based on the weight of the evidence, that the 50:50 mixture of CLA isomers does not increase insulin resistance.
- **Maternal Milk Fat:** The effect of CLA on milk fat production has been studied in cows, rodents and pigs, but these data cannot be relied on as evidence of the effect of CLA on lipogenesis in humans due to a different mechanism of mobilization of fat stores during lactation in humans. One study in humans showed reduction of milk fat associated with CLA, whereas a more recent study from the same authors using the same protocol except for the use of higher doses and a larger cohort showed no effect. Based on the limited available data, there is no evidence to suggest that the consumption of CLA-containing foods by lactating women affects milk fat levels beyond the range of normal biological variation. We conclude, based on the weight of the evidence, that CLA (50:50 mixture) would not be harmful with respect to effects on milk fat levels.

## ESTIMATED EXPOSURE

The consumption of CLA from all intended food-uses of CLA-Rich Oil was estimated based on FDA's Guidance on Estimating Dietary Intake of Substances in Food (August 2006). This is the traditional approach to estimating intake of substances added to food and is based on historical consumption of the foods to which CLA-Rich Oil would be added.

CLA-Rich Oil will be added to foods for specific functional uses. Foods to which this ingredient is added will be labeled to disclose to consumers the presence of the ingredient and to provide directions and/or other relevant information about the functional uses. The functional uses will be a primary reason why consumers will purchase and consume the foods. In addition, such foods will be priced at a premium as compared to commodity foods that do not contain CLA because of the high cost of CLA-Rich Oil. Thus, foods to which CLA-Rich Oil is added will be specially labeled, marketed, and priced to reflect a functional use, and such foods will not be labeled, marketed, or priced like commodity foods.

Because the addition of CLA-Rich Oil will cause a food to be materially different from other commodity foods of its type, consumption of CLA-Rich Oil cannot be accurately estimated using solely a traditional consumption analysis that is based on historical consumption of the commodity foods. Instead, the estimate provided by the traditional analysis must be further evaluated by considering how CLA-Rich Oil-containing foods will be consumed for functional uses.

Using a traditional consumption analysis, the estimated mean eaters-only intake of CLA is 1.22 g/person/day (24.41 mg/kg body weight/day). The 90th percentile eaters-only intake of CLA is 2.33 g/person/day (49.65 mg/kg body weight/day). On an individual population basis, the estimated highest eaters-only intake of CLA by adults is in males, with a mean value of 1.46 g/person/day and a 90th percentile value of 3.00 g/person/day.

These consumption estimates are highly conservative for a number of reasons. In particular, they assume that all target foods that could contain CLA-Rich Oil will in fact be formulated with CLA-Rich Oil at the maximum level of use. Because CLA-Rich Oil is intended for functional uses that will alter the intended use (and price) of the foods to which it is added, it is likely that only a small number of the target foods will be formulated with CLA-Rich Oil.

Consumers who intentionally seek CLA and/or the labeled functional use will be able to select these foods based on their labeling. At this time, there is not a daily recommended intake level for CLA, but the proposed usage level is 1.5 g CLA per serving so that, if consumers choose to do so, they can conveniently obtain 3.0 g CLA by consuming two servings of the target foods. It is estimated that two servings of the target foods will be the most likely intake level for consumers who seek CLA, and that consumers would be unlikely to exceed this level over a long term, for the following reasons

- As the traditional consumption analysis indicates, consumption of sufficient target foods to obtain 3.0 g CLA represents a much higher than average level of intake of these foods (a level at or above the 90th percentile level of intake) Also, a recent analysis indicates that adult males consume about 1 serving of milk per day and adult females consume about 0.7 servings. Thus, although consumers may seek to supplement their diet with CLA and may experience a short period of time of increased intake, it is unlikely that they will significantly change their habitual intake levels of the target foods over a long period of time.
- The GRAS determination covers a narrow range of foods, i.e., a limited number of beverages, yogurt, and meal replacement bars. Because there is not a wide variety of target foods that could contain CLA-Rich Oil, and because few of the target foods would in fact be formulated with CLA-Rich Oil, it would be difficult for many consumers to eat more than two servings a day of such foods

In summary, for consumers who intentionally seek CLA-containing foods, consumption is estimated at 3.0 g CLA per day. It is possible that consumers who intentionally seek CLA-containing foods would eat more than two servings per day for a short time – for example, three servings per day would provide 4.5 g CLA. However, long-term consumption at this level is unlikely for the reasons discussed above. For consumers who consume the target foods based on historical intake patterns and who do not intentionally seek CLA, consumption is estimated based on 90th percentile intake at 2.33 g/day CLA, ranging up to 3.0 g/day for adult males. Estimated intake by children aged 3-11, eaters only, at the 90th percentile, is 1.95 g CLA per day.

## SUMMARY AND CONCLUSION

A large number of published studies – including traditional toxicology studies and extensive human trials – have assessed the safety of CLA (50:50 mixture). The weight of the evidence strongly supports that this ingredient is safe at the levels used in the pivotal human studies: from 3.4 to 6 g CLA per day. These levels of consumption represent the maximum consumed in these studies and are not an upper safety limit. These levels of safe consumption indicate that the range of high consumption estimates discussed above are safe.

Based on a critical evaluation of the information summarized in this report, the Expert Panel concludes that CLA-Rich Oil, meeting appropriate food grade specifications described herein and produced by consistent and current good manufacturing practice, is safe for use as an ingredient in food as specified herein at levels of up to 1.5 g CLA per serving, with expected use of approximately two servings per day. Further, it is the Panel's opinion that qualified experts in the field would generally recognize that CLA-Rich Oil is safe for its intended use as specified herein. The Panel further concludes that CLA-Rich Oil is generally recognized as safe (GRAS) for its intended use based on scientific procedures, corroborated by the history of dietary

consumption of CLA, within the meaning of the Federal Food, Drug, and Cosmetic Act and FDA regulations.

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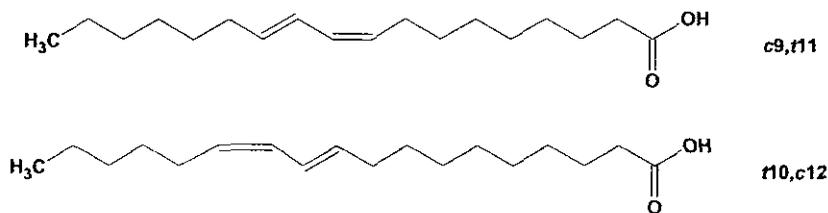
### 3.0 BACKGROUND

Conjugated linoleic acid (CLA) is a naturally occurring zoochemical found primarily in dairy products and ruminant tissue. CLA was first discovered about 20 years ago, when Pariza and coworkers found that ground beef contains an anti-mutagenic factor that consists of a series of conjugated dienoic isomers of linoleic acid (Ha *et al.*, 1987; Pariza *et al.*, 2001). The conjugated double bonds (*i.e.*, the 2 double bonds are separated by 1 single bond) can be present in either the *cis* or *trans* configuration and are present predominantly in positions 8 and 10, 9 and 11, 10 and 12, or 11 and 13.

CLA occurs naturally in ruminant products such as beef, lamb, and dairy products due to the bacterial biohydrogenation of linoleic acid in the rumen, with the *cis-9, trans-11* (C18:2 *c9,t11*) isomer accounting for more than 90% of CLA intake in the diet (Bhattacharya *et al.*, 2006). CLA production in ruminants depends on the type of animal feed and shows seasonal variation. The estimated human intake of CLA from the U.S. diet is approximately 200 mg/day (Ritzenthaler *et al.*, 2001).

Like many foods, CLA appears to have effects on the structure or function of the body based on its nutritive value, and a number of potentially functional effects have been studied for CLA consisting of an approximately 50:50 mixture of *cis-9,trans-11* and *trans-10,cis-12* CLA isomers (50:50 mixture). The CLA-Rich Oil produced by the Companies consists of approximately 78% total CLA isomers and 74% of the 50:50 mixture. The chemical structures of these isomers are shown in Figure 3-1, below

**Figure 3-1 Chemical Structure of the Two Primary CLA Isomers of CLA-Rich Oil**



From a nutritional and labeling perspective, CLA is not considered to be a *trans* fatty acid. The United States Food and Drug Administration (FDA) has stated, "Under FDA's definition conjugated linoleic acid would be excluded from the definition of *trans* fat." 68 Fed. Reg. 41433, 41462 (July 11, 2003) (U.S. FDA, 2003).

## **4.0 METHOD OF MANUFACTURE**

### **4.1 Manufacturing Process**

Please see Attachment 1 for a description of the confidential good manufacturing practices (GMP) manufacturing process for the CLA-Rich Oil product of Cognis GmbH, Tonalin® TG80. A complete description of the confidential GMP manufacturing process for the CLA-Rich Oil product of Lipid Nutrition, Clarinol™ G-80, is provided in Attachment 2. The processes for both materials can be summarized as follows: food grade linoleic acid-rich safflower oil triglycerides are saponified to free fatty acids and isomerised to form the CLA isomers under conditions of high pH and temperature; they are then concentrated and re-esterified with glycerol to form triglycerides, followed by extensive purification processes, which are proprietary. Due to the high temperature, highly lipophilic environment and the subsequent purification processes, no significant protein is present in the final product.

### **4.2 Product Specifications (Chemical and Microbiological)**

#### **4.2.1 Chemical Specifications**

The chemical specifications for CLA-Rich Oil are presented in Table 4.2.1-1 below. These specifications are for both the Cognis GmbH and Lipid Nutrition products.

<b>Specification Parameter</b>	<b>Specification</b>	<b>Method</b>
CLA total	≥ 78%	High resolution capillary gas chromatography
CLA (c9,t11 + t10,c12 isomers)	≥ 74%	High resolution capillary gas chromatography
CLA c9,t11 isomers	≥ 36%	High resolution capillary gas chromatography
CLA t10,c12 isomers	≥ 36%	High resolution capillary gas chromatography
CLA trans,trans	≤ 3%	High resolution capillary gas chromatography
Free Fatty Acid	≤ 1%	Titration method / ISO 660
Diglycendes	≤ 25%	by GC, rel. area % / High performance size exclusion chromatography
Monoglycendes	≤ 1%	by GC, rel. area % / High performance size exclusion chromatography
Water	≤ 0.1%	H-III 3a (92) / ISO 4317 / Karl-Fisher titration method
Peroxide Value	≤ 1 meq O <sub>2</sub> /kg	AOCS Cd 8b-90 (97)
Lead (Pb)	≤ 0.1 ppm	DIN EN 13805 / 14083 / Atomic absorption method
Arsenic (As)	≤ 0.1 ppm	DIN EN 13805 / 14083 / Atomic absorption method

#### 4.2.2 Microbiological Specifications

The microbiological specifications for CLA-Rich Oil are presented in Table 4.2.2-1 below.

<b>Specification Parameter</b>	<b>Specification</b>	<b>Method</b>
Total Viable Count	≤ 3000 cfu/g	ISO 4833
Yeast and mould	≤ 300 cfu/g	ISO 7954
<i>Escherichia coli</i>	Absent in 1 g	ISO 16649-2
<i>Salmonella</i>	Absent in 25 g	ISO 6579

### 4.3 Product Analysis

#### 4.3.1 Chemical Analysis of CLA-Rich Oil

Several lots of the products were analyzed to verify that the manufacturing process produced consistent products within product specifications. A summary of the physical and chemical product analyses for 3 non-consecutive lots of Tonalin® TG80 and Clarinol™ G-80 CLA-Rich Oils are presented in Tables 4.3.1-1 and 4.3.1-2, respectively.

<b>Table 4.3.1-1 Summary of the Chemical Product Analysis for 3 Lots of Tonalin® TG80 CLA-Rich Oil</b>				
Specification Parameter	Specification	Manufacturing Lot		
		Lot 63033121	Lot 62753121	Lot 62333121
CLA total	≥ 78%	79.3	80.0	80.3
CLA (c9,t11 + t10,c12 isomers)	≥ 74%	76.8	77.8	78.1
CLA c9,t11 isomers	≥ 36%	38.7	39.2	39.5
CLA t10,c12 isomers	≥ 36%	38.1	38.6	38.6
CLA trans, trans	≤ 3%	0.8	0.7	0.8
Free Fatty Acid	≤ 1%	0.80	0.75	0.90
Diglycerides	≤ 25%	11.2	13.8	14.2
Monoglycerides	≤ 1%	0.2	0.4	0.3
Water	≤ 0.1%	0.01	0.02	0.01
Peroxide Value	≤ 1 meq O <sub>2</sub> /kg	0.0	0.7	0.0
Lead (Pb)	≤ 0.1 ppm	0.07	< 0.05	< 0.05
Arsenic (As)	≤ 0.1 ppm	< 0.10	< 0.10	< 0.10

<b>Table 4.3.1-2 Summary of the Chemical Product Analysis for 3 Lots of Clarinol™ G-80 CLA-Rich Oil</b>				
Specification Parameter	Specification	Manufacturing Lot		
		6503	5166	5264
CLA total (all isomers)	≥ 78 %	78.3 %	80.4 %	79.5 %
CLA c9,t11+t10,c12 isomers	≥ 74 %	74.2 %	74.8 %	74.7 %
CLA c9,t11 isomers	≥ 36 %	36.6 %	36.7 %	36.6 %
CLA t10,c12 isomers	≥ 36 %	37.7 %	38.1 %	38.1 %
CLA trans, trans	≤ 3 %	1.2 %	2.4 %	2.0 %
Free Fatty Acids	≤ 1 %	0.71 %	0.64 %	0.73 %
Diglycerides	≤ 25 %	24.8 %	20.9 %	22.1 %
Monoglycerides	≤ 1 %	1.3 %	0.8 %	0.9 %
Water	≤ 0.1 %	0.02 %	0.01 %	0.05 %
Peroxide Value	≤ 1 meq/kg	0.3	0.0	0.0
Lead (Pb)	≤ 0.1 ppm	ND	ND	ND
Arsenic (As)	≤ 0.1 ppm	ND	ND	ND

ND = not detected

### 4.3.2 Microbiological Analysis of CLA-Rich Oil

Several lots of the products were analyzed to indicate that the manufacturing process produced consistent products within the limits of the required microbiological specifications. A summary of the microbiological product analyses for 3 non-consecutive lots of Tonalin® TG80 and Clarinol™ G80 CLA-Rich Oils are presented in Tables 4.3.2-1 and 4.3.2-2, respectively.

<b>Table 4.3.2-1 Summary of the Microbiological Product Analysis for 3 Lots of Tonalin® TG80 CLA-Rich Oil</b>				
Specification Parameter	Specification	Manufacturing Lot		
		Lot 63033121	Lot 62753121	Lot 62333121
Total Viable Count	≤ 3000 cfu/g	< 100	< 100	< 100
Yeast and mould	≤300 cfu/g	< 100	< 100	< 100
<i>Escherichia coli</i>	Absent in 1 g	Absent	Absent	Absent
<i>Salmonella</i>	Absent in 25 g	Absent	Absent	Absent

<b>Table 4.3.2-2 Summary of the Microbiological Product Analysis for 3 Lots of CLARINOL™ G-80 CLA-Rich Oil</b>				
Specification Parameter	Specification	Manufacturing Lot		
		1213	1354	1423
Total Viable Count	≤ 3000 cfu/g	< 100	< 100	< 10
Yeast and mould	≤300 cfu/g	< 10	< 10	< 10
<i>Escherichia coli</i>	Absent in 1g	Absent	Absent	Absent
<i>Salmonella</i>	Absent in 25 g	Absent	Absent	Absent

CFU = colony forming units

### 4.4 Stability Testing

Please see Attachment 1 for a description of the stability testing results for Tonalin® TG80. A complete description of the stability testing results for Clarinol™ G-80 is provided in Attachment 2. The results indicate that Clarinol™ G-80 is stable for at least 36 months, if stored dry, in the unopened original packaging, preferably at a temperature of 10 to 20°C / 50 to 68°F, away from strong odors and not in direct sunlight. Testing on Tonalin® TG80 has shown that it is stable over a storage period of 24 months at up to 25°C in steel drums or plastic pails. Additional studies have demonstrated the stability of CLA-Rich Oil in various food matrices (orange juice, milk, yogurt and nutrition bars) and following pasteurization and ultra-high temperature (UHT).

## 5.0 INTENDED USE OF CLA-RICH OIL IN FOOD

CLA-Rich Oil is intended for use in specific foods within the following general food categories: beverages and beverage bases, grain and pasta products, milk and milk products, and processed fruits and fruit juices. The specific foods in which CLA-Rich Oil is intended for use, and the levels of use, are described in Appendix A and summarized in Table 5-1. CLA-Rich Oil would be added to these foods at a level of 1.5 g CLA per labeled serving.

Estimated consumption of CLA-Rich Oil from the intended food uses is described in Section 6.0. In estimating consumption, food codes representative of each intended food use were chosen from the USDA CSFII 1994-1996 and the 1998 consumption survey (USDA, 2006) and were grouped in food use categories according to 21 CFR §170.3. Serving sizes were assigned according to 21 CFR §101.12, Reference Amounts Customarily Consumed per Eating Occasion (RACC). Product-specific adjustment factors were developed based on data provided in the standard recipe file for the USDA CSFII 1994-1996 and the 1998 survey (USDA, 2006). All food codes included in the current intake assessment are listed in Appendix A.

Food Category	Intended Food-Use	RACC* (g or mL)	Maximum CLA Level (g/ serving)	Maximum Use-Level (%)
Beverages and Beverage Bases	Specific Soy Milk Beverages	240	1.5	0.625
	Specific Meal Replacement Beverages	240	1.5	0.625
Grain Products and Pasta	Meal Replacement Bars	40	1.5	3.75
Milk and Milk Products	Specific Flavored Milk Products	240	1.5	0.625
	Milk (Filled)	240	1.5	0.625
	Specific Yogurt Products	225	1.5	0.667
Processed Fruits and Fruit Juices	Specific Fruit Juice Products	240	1.5	0.625

\* RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12).

## 6.0 ESTIMATED DIETARY CONSUMPTION OF CLA FROM INTENDED FOOD USES OF CLA-RICH OIL

### 6.1 Background Dietary Intake of CLA

For the general U.S. population, Ritzenthaler *et al.* (2001) estimated the mean background dietary CLA intake (from all isomers) 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. This study is the most relevant for purposes of this GRAS determination because it is well designed, completely reported, and uses a sample that is representative of the general population.

Earlier surveys, performed in U.S. sub-populations, estimated intake to be 137 and 52 mg/day 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.*, 1999). Herbel *et al.* (1998) reported 127 mg/day as the average daily CLA intake in a study with healthy young men and women.

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

NR = Not reported

Thus, the mean daily dietary CLA intake from the background diet can be estimated at 212 and 151 mg/day for men and women, respectively, in the general U.S. population, with subgroups consuming up to 300 mg/day. This is in line with the findings of other authors around the world (see Table 6.1-1).

The highest level reported (*i.e.*, 1,000 mg/day) is that found in a Hare Krishna community in Australia. High levels of ghee and butter consumption appeared to be the reason for this high intake. Also, measurements of the CLA content in the breast milk of women in this population resulted in relatively high values, and were related to the high intake of CLA-containing products (McGuire and McGuire, 2002).

As previously noted, the most abundant isomer in dietary CLA is the *c9,t11* isomer, which accounts for more than 90% of CLA intake in the diet (Bhattacharya *et al.*, 2006). This is different from CLA-Rich Oil, which consists of approximately equal proportions of the *c9,t11* isomer and the *t10,c12* isomer. Because background intake levels are low, they do not significantly affect the intakes from the intended food uses of CLA-Rich Oil.

## **6.2 Estimated Consumption of CLA from Intended Food Uses of CLA-Rich Oil**

The consumption of CLA from all intended food-uses of CLA-Rich Oil was estimated based on FDA's Guidance on Estimating Dietary Intake of Substances in Food (August 2006) (U.S. FDA, 2006). This is the standard approach to estimating intake of substances added to food and results in an eaters-only 90<sup>th</sup> percentile estimated intake of 2.33 g CLA/person/day. This estimate is based on historical consumption of the foods to which CLA-Rich Oil would be added.

CLA-Rich Oil will be added to foods for specific functional uses. Foods to which this ingredient is added will be labeled to disclose to consumers the presence of the ingredient and to provide directions and/or other relevant information about the functional uses. The functional uses will be a primary reason why consumers will purchase and consume the foods. In addition, such foods will be priced at a premium as compared to commodity foods that do not contain CLA because of the high cost of CLA-Rich Oil. Thus, foods to which CLA-Rich Oil is added will be specially labeled, marketed, and priced to reflect a functional use, and such foods will not be labeled, marketed, or priced like commodity foods. In this regard, CLA-Rich Oil is similar to plant sterols in the sense that the ingredient is added to food for a functional use that defines the intended use of the food to which it is added.

Because the addition of CLA-Rich Oil will cause a food to be materially different from other commodity foods of its type, consumption of CLA-Rich Oil cannot be accurately estimated using solely a traditional consumption analysis that is based on historical consumption of the commodity foods. Instead, the estimate provided by the traditional analysis must be further evaluated by considering how CLA-Rich Oil-containing foods will be consumed for functional uses. This analysis is provided below.

### **6.2.1 Consumption Estimate of CLA Based on Historical Food Use**

Consumption based on historical intake of the foods to which CLA-Rich Oil would be added was estimated using the USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (USDA

CSFII 1994-1996) and the 1998 Supplemental Children's Survey (USDA CSFII 1998) (USDA, 2006). When combined, these surveys provide the best available data for evaluating food use and food consumption patterns in the U.S., containing 4 years of data on individuals selected *via* stratified, multistage area probability sampling of American households within all 50 states. USDA CSFII (1994-1996, 1998) survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (day 1 and day 2) throughout all 4 seasons of the year. Data was collected in person, a minimum of 3 days apart, on different days of the week, to achieve the desired degree of statistical independence. USDA CSFII (1994-1996) contains 2-day dietary food consumption data for more than 15,000 individuals of all ages, and 1-day data for 16,103 individuals. USDA CSFII (1998) contributes consumption data from an additional 5,559 children from birth through 9 years of age to data reported for 4,253 children of the same ages within USDA CSFII (1994-1996). The overall USDA CSFII (1994-1996, 1998) response rate for individuals selected for participation in surveys was 81.5 and 77.5% for day 1 and day 2, respectively.

In addition to information on the types and quantities of foods being consumed, USDA CSFII (1994-1996, 1998) contains physiological and demographic information from individual participants in the survey, such as sex, age, self-reported height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. USDA sample weights were developed and incorporated with USDA CSFII (1994-1996, 1998) data to correct for potential under-representation of intake which results from variability in samples due to survey design, non-response, or other factors.

Estimates for the daily intake of CLA represent projected averages over 2 days (day 1 and day 2) of USDA CSFII (1994-1996, 1998) data. Individual consumption data were collated by computer and the resulting distributions were analyzed statistically. Eaters-only intake refers to the intake of CLA by individuals consuming food products to which this ingredient has been added, hence the 'eaters-only' designation. Individuals were considered users if they consumed 1 or more food products, in a category for which CLA is intended to be added, on either day 1 or day 2 of the survey. The analysis assumes that all foods in the target food groups are formulated with the highest possible level of CLA.

Calculations for the mean and 90<sup>th</sup> percentile all-person and eaters-only intakes, and percent consuming were performed for each of the individual intended food-uses of CLA. Similar calculations were used to determine the estimated total intake of CLA from all intended food-uses combined. In both cases, the per person and per kilogram body weight intakes were reported for the following population groups:

- children, ages 3 to 11;
- female teenagers, ages 12 to 19,
- male teenagers, ages 12 to 19,

female adults, ages 20 and up;  
 male adults, ages 20 and up; and  
 total population (all population and gender groups combined).

The estimated consumption of CLA in the U.S. by population group (g/person) from all intended food-uses is summarized in Table 6.2-1. Table 6.2-2 presents this data on a per kilogram body weight basis. A complete description of the consumption estimates and methodology is provided in Appendix A.

<b>Table 6.2-1 Summary of the Estimated Daily Intake of CLA from All Intended Food Categories in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)</b>					
Population Group	Age Group (Years)	% Consumers	Actual # of Total Users	Eaters-Only Consumption	
				Mean (g)	90 <sup>th</sup> Percentile (g)
Child	3-11	31.4	1,982	1.03	1.95
Female Teenager	12-19	23.5	165	1.25	2.22
Male Teenager	12-19	25.0	174	1.28	2.67
Female Adult	20 and Up	23.9	1,092	1.16	2.13
Male Adult	20 and Up	19.7	935	1.46	3.00
Total Population	All Ages	24.3	5,002	1.22	2.33

<b>Table 6.2-2 Summary of the Estimated Daily per Kilogram Body Weight Intake of CLA from All Intended Food Categories in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)</b>					
Population Group	Age Group (Years)	% Consumers	Actual # of Total Users	Eaters-Only Consumption	
				Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
Child	3-11	31.4	1,982	40.96	84.22
Female Teenager	12-19	23.5	165	22.53	40.08
Male Teenager	12-19	25.0	174	20.76	39.32
Female Adult	20 and Up	23.9	1,092	17.87	33.73
Male Adult	20 and Up	19.7	935	17.76	36.53
Total Population	All Ages	24.3	5,002	24.41	49.65

The estimated mean eaters-only intake of CLA is 1.22 g/person/day (24.41 mg/kg body weight/day) (Tables 6.2-1 and 6.2-2). The 90<sup>th</sup> percentile eaters-only intake of CLA is 2.33 g/person/day (49.65 mg/kg body weight/day).

On an individual population basis, the estimated highest eaters-only intake of CLA is in male adults, with a mean value of 1.46 g/person/day (17.76 mg/kg body weight/day), and a 90<sup>th</sup> percentile value of 3.00 g/person/day (Tables 6 2-1 and 6 2-2).

These consumption estimates are highly conservative for a number of reasons. In particular, they assume that all target foods that could contain CLA-Rich Oil will in fact be formulated with CLA-Rich Oil at the maximum level of use. Because CLA-Rich Oil is intended for functional uses that will alter the intended use (and price) of the food to which it is added, it is virtually certain that only a small number of the target foods will be formulated with CLA-Rich Oil. (Experience with plant sterols is similar in that, although plant sterols can be added to almost all food categories, they have in fact been added to very few foods )

### **6.2.2 Consumption Estimate of CLA Based on Intended Use**

CLA-Rich Oil will be added to foods for specific functional uses and the target foods will be labeled to disclose to consumers the presence of the ingredient and to provide directions and/or other relevant information about the functional uses. Therefore, the labeling of these foods will distinguish them from commodity foods that do not contain CLA. Consumers who intentionally seek CLA and/or the labeled functional use will be able to select these foods based on their labeling.

At this time, there is not a daily recommended intake level for CLA, but the Companies have proposed a level of 1.5 g CLA per serving so that, if consumers choose to do so, they can conveniently obtain 3.0 g CLA by consuming 2 servings of the target foods. The Companies estimate that two servings of the target foods will be the most likely intake level for consumers who seek CLA, and that consumers would be unlikely to exceed two servings per day over a long term, for the following reasons:

- As the analysis in Section 6.2.1 indicates, consumption of sufficient target foods to obtain 3.0 g CLA represents a much higher than average level of intake of these foods (a level at or above the 90<sup>th</sup> percentile level of intake). Thus, although consumers may seek to supplement their diet with CLA and may experience a short period of time of increased intake, it is unlikely that they will significantly change their habitual intake levels of the target foods over a long period of time.
- The GRAS determination covers a narrow range of foods, *i.e.*, a limited number of beverages, yogurt, and meal replacement bars. Because there is not a wide variety of target foods that could contain CLA-Rich Oil, and because very few of the target foods would in fact be formulated with CLA-Rich Oil, it would be difficult for many consumers to eat more than two servings a day of such foods. [This is similar to FDA's analysis of likely consumption of plant sterol-containing foods; see 65 Fed. Reg. 54685, 54707]

(September 8, 2000) (U.S. FDA, 2000). In addition, experience with plant sterols suggests that few of the target foods will in fact be formulated with CLA.]

It is possible that consumers who intentionally seek CLA-containing foods would eat more than 2 servings per day for a short time – for example, 3 servings per day would provide 4.5 g CLA. However, long-term consumption at this level is unlikely for the reasons discussed above.

Foods containing CLA-Rich Oil are intended for use by adults but consumption by children is possible. As discussed above, the estimated intake by children aged 3 to 11, eaters only, at the 90<sup>th</sup> percentile, is 1.95 g CLA per day.

### **6.3 Post-Market Surveillance**

Data from a post-market surveillance study conducted in 2006 in Spain following the launch of a range of food products supplemented with Tonalin TG80 CLA-Rich Oil (milk, yogurt, and juice) provide some information that is relevant to the consumption analysis discussed above, although these data might not be predictive of U.S. consumption because of differences in labeling and eating habits. This study interviewed 1,235 consumers of CLA-Rich Oil supplemented products. Eighty-three percent of consumers were women and the average age of respondents was 41 years. For the yogurt product containing 1.5 g CLA-Rich Oil per serving, 93% of consumers consumed up to 2 servings per day. For juice and milk products where the level of CLA-Rich Oil was the same, the percentage of consumers eating up to 2 servings per day were 88 and 87% respectively. For both product categories combined, about 6% consumed three servings per day; about 3% consumed four servings per day, and less than 1% consumed more than 4 daily servings.

In addition, the post-market surveillance study included data on adverse effects: 98% of consumers reported no adverse effects; the most commonly reported effect was diarrhea, followed by nausea and dyspepsia. Because this was not a controlled study, it is not known whether any of the reported adverse effects were actually attributable to CLA-Rich Oil.

## **7.0 INFORMATION TO ESTABLISH THE SAFETY OF CLA-RICH OIL**

### **7.1 Introduction**

When reviewing the safety of fats such as CLA-Rich Oil, it is important to highlight some fundamental principles.

- First, the selection of test species is important when studying levels of fat in the diet. Laboratory animals, rodents in particular, do not have the same level of adipose tissue, and therefore the ability to store fat, as humans – a phenomenon that is of particular relevance to CLA studies conducted in mice. Consequently, effects that are often

observed in animal models, such as the accumulation of fat in a variety of organs, are not necessarily relevant to predicting toxicity in humans. Humans are the most suitable model upon which to base accurate conclusions on the safety of CLA.

- Second, the safety evaluation should focus on the relevant isomeric form of CLA, i.e., the 50:50 mixture. Other isomeric forms do not always produce comparable effects and data derived from such studies should be treated with caution.
- Third, both animal and human studies must be well designed, conducted and reported. There should be multiple doses, adequate duration of exposure, and a sufficient number of subjects to provide statistical power to evaluate comparative safety based on valid endpoints and markers.
- Fourth, there should be a clear understanding the absorption and metabolic fate of CLA-Rich Oil.

Based on these principles, the order of priority in weighing the evidence of safety of CLA is as follows:

1. Well-designed and properly executed human studies using a 50:50 mixture, of appropriate duration and dose;
2. Well-designed and properly executed preclinical studies using a 50:50 mixture in appropriate animal models and in-vitro, that considered metabolism, dose dependency and mechanism of observed effects, and
3. Additional clinical and preclinical data on other isomers, to demonstrate that the totality of the evidence has been reviewed.

To summarize the relevant data:

- The metabolism of CLA has been widely studied and reported, and follows the standard pathway of dietary triglycerides. Numerous clinical trials have evaluated the effects of the 50:50 mixture and a number of other isomers on similar parameters. A comprehensive review of the clinical data has demonstrated that consumption of 50:50 CLA isomers (CLA-Rich Oil) at doses of up to 6 g/day for up to 1 year (Whigham *et al.*, 2004, Larsen *et al.*, 2006) and 3-4 g/day for up to 2 years (Gaulhier *et al.*, 2004, 2005, 2007) is safe and has no significant effects on cardiovascular parameters (lipid metabolism, markers of inflammation, and markers of oxidative stress), insulin sensitivity and glucose, or maternal milk fat. For these “pivotal” studies, the levels of consumption represent the maximum dose consumed, rather than absolute safety endpoints. A single oral dose of up to approximately 15 g of CLA-Rich Oil (containing up to approximately 9 g of CLA isomers) in bioavailability studies has revealed no adverse events.

- Preclinical data have demonstrated an absence of significant toxicological, mutagenic, or reproductive and developmental effects. The no-observed-adverse-effect level (NOAEL) for the 50:50 mixture in the rat, based on a 13-week feeding study, was reported to be 5% in the diet, the highest level fed, which is equivalent to 2,433 and 2,728 mg/kg body weight/day for males and females, respectively. The same authors also reported the absence of the mutagenic potential of CLA, in two *in vitro* assays (reverse mutation and chromosomal aberration in human lymphocytes) (O'Hagan and Menzel, 2003). Such observations on the absence of mutagenicity/genotoxicity are further supported by chronic studies conducted by Park *et al* (2005) who examined the effects of long-term feeding of male Fischer 344 rats with a diet containing 1% CLA (41.9% c9,t11 and 43.5% t10,c12) (1,000 mg/kg body weight/day) for a period of 18 months. On a molecular structure and metabolic level, it is clear that CLA would not represent a carcinogenic risk above that of normal dietary triglycerides.
- Reproductive and developmental toxicity studies in rats and pigs also have demonstrated a lack of adverse effects on maternal food consumption and body weight, litter size, and offspring growth and development following exposure to CLA (0.25 to 2% in the diet) throughout gestation, lactation, and/or during a post-weaning period (Chin *et al.*, 1994; Bee, 2000; Poulos *et al.*, 2001).

The weight of the evidence strongly supports that CLA-Rich Oil is safe at the levels used in the pivotal studies. The following sections provide a detailed discussion of the totality of the generally available clinical and preclinical study data

## **7.2 Absorption, Distribution, Metabolism, and Excretion (ADME)**

### **7.2.1 Absorption and Distribution**

#### *7.2.1.1 Overview*

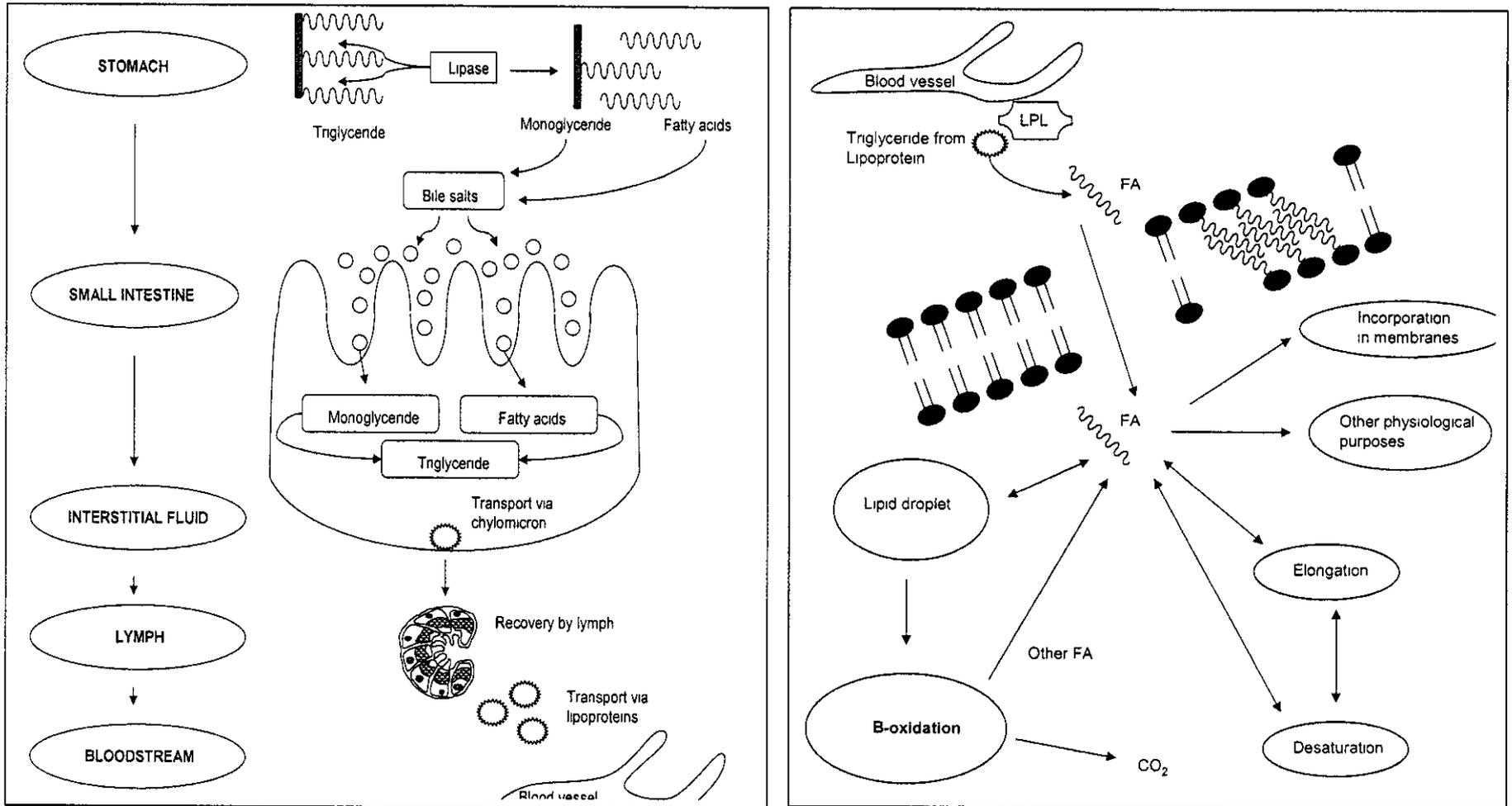
The CLA 50:50 mixtures are triglycerides of predominantly c9,t11 and t10,c12 CLA, and a small proportion of other fatty acids. Therefore, the general metabolic fate of CLA is comparable to that of any triglyceride, as summarized below and in Figure 7.2.1.1-1, and is considered to be similar to that of linoleic acid.

Approximately 95 to 98% of dietary fat is ingested as triglycerides (Linder, 1991). In general, ingested triglycerides are enzymatically hydrolyzed by pancreatic lipase in the upper small intestine into free fatty acids and 2-monoglycerides (Linder, 1991; Linscheer and Vergroesen, 1994; Guyton and Hall, 1996; IOM, 2005). These end products are then incorporated into bile acid micelles for diffusion to the interior of the intestinal epithelial cells (enterocytes). Within the enterocytes, the monoglycerides are reacylated, leading to the formation of new triglycerides. Reconstituted triglycerides, together with phospholipids, cholesterol, and apoproteins, are then incorporated into chylomicrons (87% triglycerides, 9% phospholipids, 3% cholesterol, 1%

apoprotein), which are released into the lymphatic duct and into the blood. As they pass through the capillaries of adipose tissue and the liver, chylomicrons are removed from the circulating blood by lipoprotein lipase, which hydrolyzes the contained triglycerides and phospholipids, releasing free fatty acids to the tissues for metabolism. The chylomicron remnants are taken up primarily by the liver *via* specific receptors and endocytosis (Linder, 1991; Krummel, 1996). Following cellular uptake, fatty acids are re-esterified into triglycerides and phospholipids for storage as a source of energy for the body or as structural components of cell membranes (Linder, 1991; Linscheer and Vergroesen, 1994; Krummel, 1996; IOM, 2005). Incorporation of fatty acids into membrane phospholipids and the lipid configuration of the membrane affect the physiochemical characteristics of the membrane and in turn influence the function of various membrane proteins such as hormone receptors, ion channels, and enzymes (Horrobin, 1992; Graham *et al.*, 1994).

The degree of fatty acid absorption may vary depending on the ingested form (*i.e.*, triglyceride *versus* ethyl ester *versus* free fatty acid). Fatty acids in triglyceride form have been reported to be absorbed more completely than those in ethyl ester form (El Boustani *et al.*, 1987), but not as completely as free fatty acids (Lawson and Hughes, 1988a,b).

**Figure 7.2.1.1-1 Chemical Schematic Overview of the Absorption, Distribution, Metabolism, and Excretion of Triglycerides (e.g., 50:50 CLA mixtures)**



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### 7.2.1.2 Studies in Rats

Sergiel *et al.* (2001) compared the absorption and metabolic oxidation of the *c9,t11* and *t10,c12* CLA isomers by administering a single radiolabeled dose of the isomers to semi-fasting male rats (strain not reported). Rats were placed in metabolism cages and  $^{14}\text{CO}_2$  was collected for a period of 24 hours. At the end of the experiment, 85 to 95% of the ingested radioactivity was recovered.  $^{14}\text{CO}_2$  Production was similar for both isomers and 72 and 70% of the dose of radioactivity from *c9,t11* and *t10,c12* CLA isomers, respectively, was recovered in  $^{14}\text{CO}_2$ . These values were higher than that obtained for radiolabeled linoleic acid (60% recovery in  $^{14}\text{CO}_2$ ). Sergiel *et al.* (2001) reported that incorporation of the two CLA isomers was similar to linoleic acid in most tissues with the exception of the brain, heart, adrenals, testes and carcass, where higher levels of linoleic acid were incorporated. These results indicate similar metabolism of both isomers, although competition between the isomers could not be evaluated in this study. It is unknown to what extent the metabolites of the CLA isomers contribute to its physiological properties.

The lymphatic absorption and tissue distribution of a mixed isomer preparation of CLA was studied in male Sprague-Dawley rats (Sugano *et al.*, 1997). In lymph cannulated rats, lymphatic recovery of total CLA was approximately 55% of a test dose of 200 mg CLA (mixed isomers in free fatty acid form), compared to 80% of a test dose of 200 mg of linoleic acid during the 24 hours post dosing, suggesting a lower level of absorption of total CLA in comparison to linoleic acid. Eighty percent of the absorbed dose of total CLA was carried in chylomicrons, the remaining 20% in very low density lipoproteins. Approximately 95% of total CLA absorbed was incorporated in triglycerides and 5% in phospholipids. A similar pattern was observed with linoleic acid. No adverse effects of CLA were reported in this study.

The isomeric CLA compositions of maternal diet, milk, and liver of suckling Sprague-Dawley rats were compared in a study by Yang *et al.* (2002). The proportion of each isomer in the milk was not different from that in the maternal diet, suggesting similar absorption of all CLA isomers. This is in contrast with the results by Sugano *et al.* (1997), who reported preferential absorption of the *trans,trans* CLA isomers. However, different extraction methods were used and may account for the differing results. The CLA isomeric composition in the liver phospholipids and triglycerides of suckling rats was different from that in the maternal diet and milk. Total *cis,trans* isomers were lower in the liver compared to the milk and diet, whereas total *trans,trans* isomers were higher. As the absorption was reported to be similar for all CLA isomers, the higher accumulation of *trans,trans* isomers in the liver may be due to slower metabolism of these CLA isomers. Incorporation of total CLA isomers in liver phospholipids was relatively low, less than 1.0% of total fatty acids. Approximately 70% of total CLA consisted of *cis,trans* isomers, and approximately 20% consisted of *trans,trans* isomers. These levels of *trans,trans* isomers in the liver did not result in adverse effects in the suckling rats.

### 7.2.1.3 *Studies in Rabbits*

CLA is a chemical isomer of linoleic acid. Linoleic acid is passively diffused across rabbit small intestinal brush border membrane vesicles (Ling *et al.*, 1989).

### 7.2.1.4 *Studies in Pigs*

In a study by Kramer *et al.* (1998), pigs (Landrace boar) were fed a CLA preparation composed of several different isomers, primarily *c9,t11*; *t8,c10*, *t10,c12* and *trans,trans* CLA isomers. No difference was observed between the isomeric composition in the commercial CLA preparation and the isomeric composition in adipose tissue, heart, and liver, suggesting that the relative absorption and distribution of all the CLA isomers was very similar. The total CLA content in tissue lipids ranged from 1 to 6%, with a higher content in neutral lipids compared to phospholipids.

### 7.2.1.5 *Human Studies*

As previously mentioned, CLA is an isomer of linoleic acid (*cis*-9, *cis*-12 linoleic acid), and it is likely that CLA and common linoleic acid are absorbed across the human gastrointestinal tract by similar mechanisms (Nilsson and Melin, 1988; Ling *et al.*, 1989; Gore *et al.*, 1994; Minich *et al.*, 1999). Gore *et al.* (1994) reported that uptake of linoleic acid into isolated intestinal cells was carrier mediated.

The relative bioavailability of CLA from CLA-Rich Oil (Tonalin™ TG80) or in free fatty acid form was assessed in healthy male volunteers (n=12). Each subject received two treatments as single oral administrations. The first was CLA-Rich Oil (14.9 g containing 8.9 g CLA) administered on visit 2. After a washout period of 9 to 14 days, CLA-free fatty acid (14.7 g containing 11.4 g -with a balanced isomer ratio) was administered on visit 4. The bioavailability of CLA was measured in the serum as area under the curve (AUC)<sub>0-24h</sub>. The authors reported that CLA in the form of free fatty acid was 1.15-1.19 times greater than CLA in the triglyceride form, however they noted that this might be due to the high load of acid, in the case of the free fatty acid form, changing the rate of absorption and disposition. The bioavailability of the CLA *c9, t11* was reported to be 1.25 to 1.27 times better than the *t10,c12* isomer. The CLA reached a peak concentration at 2 to 8 hours (depending on the isomer and form) and after 24 hours there was only a small fraction left. Half-lives of the isomers in the two forms ranged between 16 and 29 hours. No adverse events were noted during the trial period of 10 to 15 days (Høye *et al.*, 2004).

A number of studies have found a relationship between diet and the occurrence of CLA isomers in human serum, milk, and adipose tissue. In a study by Park *et al.* (1999), breastfeeding women consumed high fat dairy foods for 1 week. Dietary rumenic acid (*c9,t11* CLA) intake and content in the milk was greater during the high- compared to the low-dairy period. A strong relationship between the intake of bovine milk fat and the occurrence of CLA in human adipose

tissue was reported by Jiang *et al.* (1999). Plasma lipid and phospholipid concentration of CLA was significantly higher (19 to 27%) in men following supplementation with cheddar cheese, compared to initial concentrations (Huang *et al.*, 1994). The increase in CLA concentrations in plasma, milk, and adipose tissue is linked to dietary intake of CLA, indicating that CLA isomers are well-absorbed in the human body.

## 7.2.2 Metabolism

### 7.2.2.1 Overview

Regulation of lipid metabolism is controlled by the liver and involves catabolism of triglycerides for use as energy, desaturation, and elongation of fatty acids, and synthesis of triglycerides and phospholipids (Krummel, 1996). The metabolism of absorbed fatty acids depends, in part, upon their chemical structure. Medium-chain length fatty acids are used mainly as energy sources ( $\beta$ -oxidation), longer-chain saturated and monounsaturated fatty acids are generally stored (*i.e.*, in adipose tissue as triglycerides), and polyunsaturated fatty acids (PUFAs) are typically taken up by cells for incorporation into membrane phospholipids (see Section 7.2.1) (Linder, 1991).

Energy production from long-chain fatty acids generally occurs in the mitochondria via  $\beta$ -oxidation and is initiated by the lipase-dependent hydrolysis of triglycerides to free fatty acids. Fatty acids are transported across the mitochondrial membrane in the form of acyl-carnitine and subsequently undergo sequential removal of 2-carbon units until the final product, acetyl Coenzyme A (CoA), is formed and enters the citric acid cycle for energy production (Linscheer and Vergroesen, 1994)

The metabolism of CLA isomers is very similar to that of linoleic acid (Banni, 2002). CLA is metabolized by 2 distinct pathways, desaturation, and oxidation. Desaturation of CLA has been investigated more extensively than oxidation; however, both are well-recognized metabolic pathways for CLA (Sébédio *et al.*, 2003). In animal and human tissues, the *t*10,*c*12 and *c*9,*t*11 CLA isomers undergo  $\Delta$ 6 desaturation, elongation and further  $\Delta$ 5 desaturation forming 18:3, 20:3 and 20:4 fatty acids. Both isomers may also be oxidized in the  $\beta$ -oxidation pathway. Studies investigating the metabolism of different CLA preparations are discussed below.

### 7.2.2.2 Studies in Rats

CLA is desaturated by  $\Delta$ 6 desaturase and  $\Delta$ 5 desaturase (Banni *et al.*, 1996; Belury and Kempa-Steczko, 1997; Sébédio *et al.*, 1997, 2001, Liu and Belury, 1998). The metabolites produced are dependent upon the type of fatty acids present in the diet (Sébédio *et al.*, 1997). For instance, when CLA is fed to rats deficient in dietary linoleic and linolenic acids, the *c*9,*t*11 and *c*10,*t*12 CLA isomers are converted into conjugated isomers of arachidonic acids (*i.e.*, 8,11,13-20:3 and 8,12,14-20:3, respectively) (Banni, 2002). These conjugated arachidonic acid-type metabolites are further desaturated into *c*5,*c*8,*c*11,*t*13-20:4 and *c*5,*c*8,*t*12,*c*14-20:4 for *c*9,*t*11 and *c*10,*t*12 CLA isomers, respectively. When rats were fed a fatty acid rich diet, the

c9,t11 CLA isomer was converted into the c8,c11,t13-20:3 as the final end product (Banni *et al.*, 2001). The c10,t12- CLA isomer was converted into c6,t10,c12-18:3 and into 6,c10-16:2 (Banni *et al.*, 2001). CLA metabolites are incorporated into the lipid component(s) of adipose tissue, liver, heart, and kidney (Sébédio *et al.*, 2003).

CLA is also metabolized to carbon dioxide (CO<sub>2</sub>) by  $\beta$ -oxidation *in vivo* in rats (Sergiel *et al.*, 2001) Sergiel *et al.* (2001) demonstrated that 70% of the total oral dose of c9,t11-CLA or t10,c12-CLA was converted into CO<sub>2</sub> over a 24-hour period. A significant proportion of CLA administered orally was oxidized to CO<sub>2</sub> *in vivo*

In a study by Martin *et al.* (2000), the  $\beta$ -oxidation pathways of both individual CLA isomers and a mixture were investigated. Male Wistar rats were fed a 6% fat diet, containing 1% of c9,t11 CLA isomers, 1% of t10,c12 CLA or an equal mixture of both isomers for 6 weeks. The activity of 2 rate-limiting enzymes in  $\beta$ -oxidation of CLA was measured: mitochondrial carnitine palmitoyl transferase I (CPT-I) and peroxisomal acyl coenzyme A oxidase (ACO). In the liver, these activities were not modified after dietary intake of any of the CLA isomers. In the adipose tissue, CPT-I enzyme activity was induced by both isomers. Kinetic studies conducted on hepatic mitochondrial CPT-I and peroxisomal ACO using CoA derivatives of the pure isomers suggested a greater metabolism of the t10,c12 CLA in peroxisomal pathways.

The same researchers studied the metabolites of the CLA isomers further (Sébédio *et al.*, 2001). Similar to the previous study by Martin *et al.* (2000), each isomer (c9,t11- and t10,c12-CLA) was given separately in the diet of Wistar rats for 6 weeks. The t10,c12 isomer was primarily metabolized into conjugated 16:2 and 18:3 fatty acids, while the c9,t11 isomer was preferentially metabolized into a conjugated 20:3 isomer. Thus, the c9,t11- and the t10,c12-CLA isomers are metabolized differently. It was also reported that the isomers have distinct effects on the fatty acid profiles of the liver. Consumption of the t10,c12 CLA isomer resulted in a decrease of 38% in liver lipid content compared to the control group. The c9,t11 CLA isomer also resulted in a decrease in liver lipid content, although this was not statistically significant. The t10,c12 CLA isomer resulted in an increase of the saturated fatty acid stearic acid and a decrease in the mono-unsaturated fatty acid oleic acid, indicating an inhibition of  $\Delta 9$  desaturase; however, a similar response was not observed with the c9,t11 CLA isomer. Consumption of both CLA isomers produced a decrease in the arachidonic acid content in liver phospholipids, and the t10,c12 CLA isomer resulted in an increase in long-chain PUFA. The effects of the individual CLA isomers and their metabolites on liver fatty acid profiles may be involved in the biological properties of CLA, although the exact impact of these effects has not been fully elucidated

Further information on the metabolism of the individual isomers was reported by Gruffat *et al.* (2003). Rat liver slices were incubated with purified radioactive t10,c12 CLA, or c9,t11 CLA. Uptake was similar for both isomers and 50% of the isomers incorporated in the cells were oxidized. Only 3 0% of total oxidized CLA was fully oxidized to CO<sub>2</sub>. The isomers that were

partly or not oxidized were esterified and incorporated into neutral and polar lipids, secreted into very low density lipoprotein (VLDL) lipids, or converted into conjugated 18:3, a metabolite with as yet unknown biological properties. The respective metabolism for both isomers was not significantly different, except that the secretion as part of VLDL particles was higher for *t*10,*c*12 CLA. However, it should be noted that the effects of enzyme competition between the different isomers could not be analyzed in this study.

#### 7.2.2.3 Human Studies

In serum of human subjects taking supplements of CLA (6 g/day, containing ~37% *c*9,*t*11 CLA and ~39% *t*10,*c*12 CLA), accumulation of the  $\Delta$ 6 desaturase metabolite of the *t*10,*c*12 CLA isomer was reported to be higher compared to that of the *c*9,*t*11 CLA isomer, further indicating more rapid metabolism of the *t*10,*c*12 isomer (Belury, 2002). Similarly, a significant increase in *c*9,*t*11 CLA content of the plasma lipids was reported after intake of Clarinol™ A80 supplements, containing a similar isomeric composition. The *t*10,*c*12 isomer was not detectable, indicating that this isomer is more likely to be metabolized *via* desaturation and elongation pathways or oxidized in  $\beta$ -oxidation pathways (Noone *et al.*, 2002).

### 7.2.3 Excretion

#### 7.2.3.1 Studies in Rats

Sergiel *et al.* (2001) demonstrated that radiolabeled metabolites of CLA are excreted *via* expired air, urine, and feces in rats (strain not specified). In this study, the primary route of elimination was expired air (*i.e.*, 70% of the total dose was excreted in air) over a 24-hour period after oral administration. Urine and feces accounted for 1.3 to 2% and <0.5%, respectively, of the total dose. The extent to which CLA metabolites were excreted in expired air was time-dependent, reaching a plateau after 12 hours.

### 7.2.4 Summary and Conclusions (ADME)

Like most fatty acids, CLA is well absorbed across the gastrointestinal mucosa. It is also widely distributed throughout the body, metabolized *via* oxidation and desaturation, and the metabolites extensively excreted from the body in expired air, and lesser amounts in urine and feces.

## 7.3 Preclinical Toxicological Studies

### 7.3.1 Introduction

Numerous studies were conducted in animals to investigate the potential preclinical toxicity of CLA. The published studies presented in the sections below are related primarily to CLA preparations that consist of a 50:50 mixture of the *c*9,*t*11 and *t*10,*c*12 isomers (*i.e.*, relevant to

CLA-Rich Oil). Studies on the potential subchronic, chronic, and reproductive and developmental toxicity of CLA are summarized in Table 7.3-1.

### 7.3.2 Acute Toxicity Studies

An acute oral toxicity study in rats (strain unspecified) was performed using CLA (Tonalin<sup>®</sup>) beadlets consisting of CLA methyl ester (CLA-ME) of unknown purity (Berven *et al.*, 2002). Berven *et al.* (2002) concluded that oral administration of CLA-ME to rats was "non-toxic" based on an LD<sub>50</sub> value of >2 g/kg. This is the limit dose recommended by the Organization for Economic Cooperation and Development (OECD) guidelines [Guideline no. 425 (OECD, 2006)].

### 7.3.3 Subchronic Toxicity Studies

#### 7.3.3.1 Studies in Rats

Berven *et al.* (2002) reported that there were no observed adverse effects in rats (strain not specified) fed "beadlet formulations" containing 50,000 ppm or 5 g/kg day CLA-ME or CLA-ethyl ester (CLA-EE) for an unspecified period of time. No further details were available.

O'Hagan and Menzel (2003) evaluated the subchronic oral toxicity of CLA in a 13-week oral toxicity study in male and female Wistar outbred (CrI: (WI)WU BR) rats (20 rats/sex/group). The study was conducted in accordance with the OECD principles of Good Laboratory Practices (GLP) and in accordance with OECD guideline 408 (OECD, 1998a,b). Control rats were administered either a high-fat (HF) (15% w/w safflower oil) or low-fat (LF) (7% w/w safflower oil) basal diet. Test groups received the HF basal diet supplemented with 1, 5, or 15% Clarinol<sup>™</sup> G-80 (containing 79% CLA 50:50 mixture) for 13 weeks, resulting in CLA intakes of 0.48, 2.4, or 7.2 g/kg, respectively, for males, and 0.54, 2.7, and 8.2 g/kg for females. Total added safflower oil and/or Clarinol<sup>™</sup> G-80 content in all test diets was 15%. At the end of the 13-week study period, recovery groups of 10 rats/sex from each control group and from the high-level CLA group (*i.e.*, 15% Clarinol<sup>™</sup> G-80) were observed for a further 4 weeks. Rats in the control groups were maintained on their respective diets, while rats in the 15% CLA group were switched to the HF control diet for a period of 4 weeks to assess the reversibility of any observed effects. The HF-diet also was supplemented with 10% higher levels of protein, L-cysteine, cellulose, choline-bitartrate, minerals, and vitamins to compensate for reduced food intake in rats fed a high calorie diet and maintain a normal level of nutrient intake. Body weights and food and water consumption were monitored throughout the study. Ophthalmoscopic observations were performed prior to treatment and after 87 days of treatment in rats of the control and highest dose group. Blood was collected for hematology and clinical chemistry analysis on treatment day 22 (males), day 24 (females), day 51 (females), and day 52 (males) and at the end of the 90-day treatment period and the 4-week recovery period. In the last week of the treatment period, urine was collected from 10 rats/sex from all groups for urinalysis and to evaluate the concentrating ability of the kidneys. These measurements were repeated in all male rats in the recovery groups at the end of the recovery period. Clinical assessments were

performed weekly throughout the 90-day treatment period, and neurobehavioral tests were conducted in the last week of the treatment period. At the end of the treatment and recovery periods, animals were euthanized, gross pathological examinations were performed, organs were removed, weighed, and prepared for histopathology, and microscopic evaluations of the tissues were performed.

The authors reported no clinical signs or effects on survival attributed to CLA administration, nor were there any significant effects noted upon ophthalmoscopic examination (O'Hagan and Menzel, 2003). Food consumption was reported to be significantly decreased during days 7 to 14 of the treatment period in the 15% CLA group, which was attributed by the authors to poor palatability of the diet. As a result of the reduced food consumption, statistically significant decreases in body weight were observed in rats of both sexes in the 15% CLA group at day 7 and in high-level (15% CLA) females at day 14. Feed conversion efficiency was unaffected. Water consumption was significantly lower in high-level males and females at week 12 relative to both control groups. The authors reported that the reasons for reduced water intake were not clear and that there was no indication of any effect on renal function in this group. Urinary volume and urine density were not significantly different from control values, nor were there any significant effects on urinalysis parameters. Furthermore, there were no significant treatment-related effects on hematological parameters.

Clinical chemistry analysis revealed increased levels of alkaline phosphatase (ALP) and alanine aminotransferase (ALAT) throughout the treatment period in 15% CLA males and females compared to both control groups, and increased aspartate aminotransferase (ASAT) at week 13 and throughout the treatment period in high-level male and female rats, respectively, compared to the LF controls. Sorbitol dehydrogenase (SD) also was reported to be increased in high-level females at week 13 relative to both control groups. The changes in ALP, ALAT, and ASAT were reported to be reversible, with the exception of ALAT and ASAT in the high-level females, which were significantly higher only relative to the low fat controls at the end of the recovery period. Compared to both control groups, plasma cholesterol was significantly decreased in males in the high level group, while plasma triglycerides were significantly increased in females of the high-level group. In addition, both males and females in the high-level group were reported to have increased plasma albumin levels. The changes in plasma cholesterol, triglycerides, and albumin were reported to be reversible at the end of the recovery period. Males in the high-level group had decreased blood glucose at week 8 (compared to LF control) and week 13 (compared to both control groups) and increased insulin at week 4 (compared to both control groups) and week 8 (compared to high fat control). Insulin was not significantly different from controls at week 13. Females in the 15% CLA group were reported not to have any significant changes in plasma glucose; however, insulin was increased at weeks 8 and 13 relative to both control groups. However, insulin levels in both sexes of the 15% CLA group were not significantly different from controls at the end of the recovery period. A number of changes in organ weights were observed, and included increased relative liver weights in males of the 5

and 15% CLA groups and females of the 15% CLA group, increased absolute (15% CLA males) and relative liver weights (high-level males and females), increased absolute (15% CLA males) and relative spleen weights (15% CLA males and females), increased relative adrenal weights (15% CLA males and females), and increased relative pancreas weights (15% CLA females). Histopathological examination of the liver revealed hepatocellular vacuolation, which was significant in males of the HF control group and 1% CLA group and was reported to be reversible in the 1% CLA males. The incidence of hepatocellular hypertrophy was significantly higher in 15% CLA female rats (12/20 rats) and was almost completely reversible (2/10 rats). There were no histopathological changes in any other organ. The authors suggested that the observed liver enlargement and hepatocellular hypertrophy were adaptive effects in response to the consumption of high levels of Clarinol™ G-80. Although such adaptive effects in the liver may not be considered adverse, based on these effects and the effects on blood insulin levels, which were regarded as transient (O'Hagan and Menzel, 2003), using a self-described conservative approach, the authors reported the NOAEL to be 5% Clarinol™ G-80 in the diet, which is equivalent to 2,433 and 2,728 mg/kg body weight/day for males and females, respectively.

Scimeca (1998) conducted a 36-weeks study investigating the potential subchronic toxicity of CLA. Male weanling Fischer 344 rats (20/group) were fed diets containing 0 (control) or 1.5% of a synthetic CLA preparation containing 85.5% of a 50:50 mix of *c9,t11*- or *t9,c11* and *t10,c12* isomers, 4.3% other CLA isomers, 7.1% linoleic acid, and 3.1% other constituents (not specified). Food consumption and body weights were recorded and physical examinations were performed weekly throughout the study. At the end of the study period, rats were euthanized and necropsied, organ weights were measured and examined histologically, and hematological and clinical chemistry parameters were measured. The intake of CLA was reported to range from 1,970 to 467 mg/kg body weight/day from week 1 to week 36 (mean of 1,218.5 mg/kg body weight/day). Animals fed CLA were reported not to show any clinical signs of toxicity, nor were there any differences in body weight gain or food consumption relative to the control group. Likewise, there were no significant compound-related histopathological or microscopic changes in the organs, or changes in hematological or clinical chemistry parameters. Based on the results of this study, the NOAEL for CLA was determined to be 1,218.5 mg/kg body weight/day, the only dose tested.

#### 7.3.3.2 *Studies in Dogs*

Berven *et al.* (2002) reported that the administration of CLA-ME beadlets (50,000 ppm or 1.25 g/kg body weight/day) to dogs (strain and study duration not specified) produced no adverse effects. Consumption of beadlets containing 50,000 ppm CLA-EE was reported to result in mild liver impairment. However, histopathological examination of the liver did not reveal any untoward morphological effect. No further information on these studies was available.

### 7.3.3.3 Studies in Pigs

The administration of CLA (as an oil containing 60% of CLA isomers) at concentrations of 0, 0.48, or 0.95% in the diet (approximately 0, 100, or 200 mg/kg body weight/day) to pigs (strain not specified) revealed no histopathological effects (Cook *et al.*, 1998). Non-treatment related morphological changes, apparently due to the method of euthanasia or agona, were observed (Cook *et al.*, 1998). No further details were available.

### 7.3.4 Chronic Toxicity / Carcinogenicity Studies

Park *et al.* (2005) examined the effects of long-term feeding of CLA in Fischer 344 rats. Weanling male rats were administered either a control diet (n=10) or a diet containing 1% CLA (41.9% c9,t11 and 43.5% t10,c12) (n=11) (approximately 1,000 mg/kg body weight/day) for a period of 18 months. Body weight and food consumption were measured weekly and twice weekly, respectively. After 12 weeks, 3 animals from each group were randomly selected, euthanized, and subjected to body fat analysis and water content. At the end of the study period, all animals were euthanized and examined for gross pathological changes. Appropriate tissues were examined histopathologically. Organ weights, clinical chemistry, and hematological parameters were measured. Weight gain, survival rate, and water consumption did not differ between treatments; however, food consumption was significantly lower in the CLA-fed group compared to controls. Body fat analysis and water content at 12 weeks revealed no significant difference between groups in percentage body fat, empty carcass weights, or percentage body water. Blood glucose levels were significantly lower and mean corpuscular volume was significantly higher in the CLA-fed group compared to controls. Blood urea nitrogen and cholesterol levels were elevated beyond the population range in both groups, but were not significantly different between groups. Protein was detected in the urine of animals from both groups; however, the protein levels in the CLA-fed rats were significantly lower than that of the control group. There were no significant differences between groups in organ weights. All animals from both groups had chronic renal diseases (chronic interstitial nephritis, nephrosis, and/or glomerulosclerosis). The chronic renal failure was thought by the authors to be due to the high protein content of the diets and was not considered to be compound-related. The incidences of pituitary or testicular tumors, prostatitis, or lymphoma were not significantly different between groups. Based on the results of this study, the NOAEL was considered to be 1% CLA in the diet (1,000 mg/kg body weight/day), the only dose tested.

Based on the above study and knowledge of the structure and metabolic fate of CLA, one would not anticipate that CLA-Rich Oil would represent an increased carcinogenic risk, an observation supported by the results of mutagenicity studies discussed below.

### 7.3.5 Mutagenicity

The mutagenic potential of Clarinol™ G-80, a preparation containing 79% CLA isomers, was tested in two *in vitro* assays by O'Hagan and Menzel (2003). In the first assay, Clarinol™ G-80

was tested in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA102). Each concentration was tested in triplicate in the presence or absence of metabolic activation (S9). Positive and negative controls were included. Clarinol™ G-80, tested at concentrations up to 5,000 µg/plate, was not mutagenic.

In the second assay, the potential for Clarinol™ G-80 to induce chromosome aberrations was tested in human peripheral blood lymphocyte cultures in the presence and absence of metabolic activation (O'Hagan and Menzel, 2003). Clarinol™ G-80 did not induce chromosome aberrations when tested at concentrations up to 300 µg/mL (O'Hagan and Menzel, 2003).

### 7.3.6 Reproductive and Developmental Toxicity Studies

#### 7.3.6.1 Studies in Rats

Berven *et al.* (2002) reported no signs of reproductive or developmental toxicity in rats fed CLA at doses of up to 1 g/kg body weight/day. No further details were available.

Chin *et al.* (1994) investigated the effects of CLA on neonatal growth and development in Fischer 344 rats in 2 experiments. In the first experiment, female rats were mated and then fed either a basal diet (corn oil - control) or basal diet supplemented with CLA (0.5% or 500 mg/kg) (n=20 per group). On day 20 of gestation, 10 rats/group were euthanized and liver, mammary gland, skeletal muscle and abdominal adipose tissues were collected. Fetuses were removed, weighed, and examined grossly for abnormalities. The remaining rats continued on their respective diets and were allowed to deliver at term. In the second experiment, this same dosing regimen was followed throughout gestation and lactation; however, 2 additional groups were included. A third group was fed the basal diet supplemented with 0.25% CLA (*i.e.*, 250 mg/kg) during gestation and lactation, while a fourth group was fed the basal diet throughout gestation, followed by the basal diet supplemented with 0.5% CLA during lactation only. Immediately after weaning, pups were fed the same diets as their mothers for a period of 8 to 10 weeks. Body weights and food intakes were monitored weekly.

In the first experiment, dietary supplementation with 0.5% CLA had no effect on maternal food intake, body weights, mammary gland weights, or liver weights. There were no significant differences between groups in litter size, fetal body weights, fetal liver and brain weights, or gross evidence of fetal abnormality. Mean pup weight was significantly higher in rats fed CLA. CLA uptake into maternal and fetal tissues was significant. After 20 days of CLA treatment to pregnant females (*i.e.*, during gestation), the CLA content of maternal liver, muscle, and mammary gland was increased 2,100, 1,900, and 2,300%, respectively, over control levels. CLA content of the fetal liver was increased by 2,200% over control levels, while maternal milk levels of CLA were 2,600% higher than control levels.

In the second experiment, the concentration of CLA in maternal milk increased in proportion to the dietary level of CLA. Supplementation with 0.25 or 0.5% CLA throughout gestation and

lactation or throughout lactation alone increased CLA content of breast milk by 2,200, 4,800, and 4,300%, respectively. Supplementation with CLA had no significant effects on litter size, pup development and survival rate, or maternal food consumption. Pup body weights were significantly higher on day 10 of lactation in the 0.5% CLA group and in pups fed 0.25 and 0.5% CLA for 8 to 10 weeks compared to controls. There was no significant difference between groups in food consumption; however, feed efficiency was significantly increased in male pups fed 0.5% CLA, and in female pups fed 0.25 and 0.5% CLA. The NOAEL for this study was 0.5% CLA or 500 mg/kg body weight/day, the highest level fed.

Poulos *et al* (2001) investigated the effect of CLA fed to Sprague-Dawley rats on body weight gain and body composition. Pregnant dams were fed either a basal diet ( $n=11$ ) or the basal diet supplemented with 0.5% CLA ( $n=12$ ) from gestation day 7 until lactation day 21 (weaning). Litters were standardized to 3 pups/sex within 24 hours of birth. At weaning, one male and one female pup from each litter were euthanized and inguinal fat pads were removed for fatty acid analysis. Of the remaining pups in each litter, 2 pups (1 per sex) were fed the control diet, while 2 pups (1 per sex) were fed the CLA diet until 11 weeks of age. Maternal body weights, liver weights, and food consumption, and litter size and weights of whole litters were unaffected by CLA treatment. At weaning, female pups of dams fed CLA were significantly heavier than control female pups. Male pup weights were unaffected by CLA treatment at weaning, although male pups exposed to CLA during gestation, lactation, and until 11 weeks of age were reported to be the largest, fastest growing, and most feed efficient of all groups (significance not reported). Liver weights of male and female pups also were unaffected by CLA treatment. The NOAEL for this study was 0.5% CLA or 500 mg/kg body weight/day, the only level fed.

#### 7.3.6.2 Studies in Pigs

Bee (2000) examined the effects of CLA on piglet growth and tissue composition. Swiss Large White sows were fed a diet supplemented with 2% linoleic acid-enriched oil (containing 65.79% linoleic acid) ( $n=4$ ) or 2% CLA-enriched oil (containing 58.9% CLA, of which 70% was represented by a 50:50 mixture of *c9,t11* and *t10,c12* isomers) (approximately 450 mg CLA-enriched oil/kg body weight/day) ( $n=6$ ) at the time of mating and throughout pregnancy and lactation. After 35 days of rearing, piglets from 2 randomly chosen sows were assigned to a starter diet supplemented with linoleic acid or CLA for an additional period of 35 days. Body weight of each piglet was recorded at birth, weaning and after 35 days fed the supplemented starter diet (*i.e.*, post-weaning period). There were no significant differences between groups in piglet body weight at birth and weaning. Piglets exposed to CLA during gestation had significantly higher total feed intake, body weight gain, and final body weights, regardless of whether they were fed diets containing linoleic acid or CLA after weaning. No adverse effects were reported.

### 7.3.7 Summary (Preclinical Studies)

The available data from animals indicate that dietary administration of CLA is generally well tolerated and without adverse effects. Subchronic and chronic studies conducted in rats demonstrate that CLA administered in the diet up to a concentration of 5% (2,433 and 2,728 mg/kg body weight/day for males and females, respectively) for periods of 13 weeks to 18 months, produced no significant adverse effects (Scimeca, 1998; O'Hagan and Menzel, 2003; Park *et al.*, 2005). Although the administration of 15% CLA in the diet for 13 weeks induced a number of statistically significant changes in clinical chemistry parameters (ALP, ALAT, ASAT, SD) and organ weights (liver, spleen, adrenal glands, and pancreas) in males and females, they were generally reversible after a 4-week recovery period and were not dose-dependent (O'Hagan and Menzel, 2003). Increased relative liver weights, accompanied by hepatocellular hypertrophy and vacuolation also were reported in rats fed 15% CLA in the diet; however, the authors postulated that these effects may be the result of an adaptive response to the consumption of high levels of CLA. Despite the fact that these effects were not considered adverse, O'Hagan and Menzel (2003), using a self-described conservative approach, reported the NOAEL to be 5% CLA (as Clarinol™ G-80) in the diet, which was equivalent to 2,433 and 2,728 mg/kg body weight/day for males and females, respectively. Conventional (2-year) carcinogenicity studies were not available; however, Park *et al.* (2005) conducted an 18-month study in which the tumor incidence in rats fed diets containing 1.5% CLA was reported not to be different from that of controls. *In vitro* assays designed to investigate the potential of CLA to induce mutagenicity in 5 different *S. typhimurium* strains or to induce chromosomal aberrations in human lymphocyte cell cultures demonstrated no mutagenic or genotoxic potential (O'Hagan and Menzel, 2003).

Reproductive and developmental toxicity studies in rats and pigs also demonstrated a lack of adverse effects on maternal food consumption and body weight, litter size, or offspring growth and development following exposure to CLA (0.25 to 2% in the diet) throughout gestation, lactation, and/or during a post-weaning period (Chin *et al.*, 1994, Bee, 2000; Poulos *et al.*, 2001). Pup growth, as evidenced by increased body weights, was reported to be enhanced in rats fed 0.25 and 0.5% CLA (Chin *et al.*, 1994). Chin *et al.* (1994) reported significant uptake of CLA in maternal mammary gland tissue and milk; however, this was not accompanied by any adverse effects. The uptake of CLA into maternal milk is further discussed in Appendix C, Section C.3.

The results of the 13-week study in rats by O'Hagan and Menzel (2003) provide a NOAEL of 2,433 and 2,728 mg/kg body weight/day for males and females, respectively.

**Table 7.3-1 Summary of Subchronic, Chronic, and Reproductive and Developmental Toxicity Studies on CLA\***

Species (Strain)	Study Duration	Route of Administration	Treatment	Equivalent Dose of CLA (mg/kg bw/d)	Reported Effects <sup>a, b</sup>	Reference
<b>Subchronic and Chronic Toxicity Studies</b>						
Rat (strain and sex not specified)	Not specified	Oral	50,000 ppm CLA-ME provided as beadlets	5,000	No significant adverse effects. No further study details were available.	Berven <i>et al</i> , 2002 Unpublished
Rat [Wistar outbred (CrI: (WI)WU BR)] (20/sex/group)	13 weeks	Diet	0 (low-fat and high-fat control groups), 1, 5, or 15% Clarinol™ G-80 (containing 79% CLA)	0, 480, 2,433, or 7,200 (males); 0, 054, 2,728, or 8,200 (females)	No significant effects on survival, ophthalmoscopic examinations, urinalysis, hematological parameters. Food consumption and body weight were significantly lower in males and females of the high-dose group between days 7 and 14, which was attributed to unpalatability of the diet. Significant treatment-related effects (relative to one or both control groups) were primarily observed in the highest dose group (unless otherwise indicated below), and included the following: <b>Males:</b> ↑ ALP <sup>c</sup> , ALAT <sup>c</sup> , ASAT <sup>c</sup> , plasma cholesterol <sup>c</sup> plasma albumin <sup>c</sup> ↓ blood glucose <sup>c</sup> (weeks 8, 13); ↑ blood insulin <sup>c</sup> (weeks 4 and 8) ↑ absolute liver weights <sup>c</sup> , relative liver weights <sup>c</sup> (mid- and high-dose males), absolute and relative <sup>c</sup> spleen weights, and relative adrenal weights <sup>c</sup> Hepatocellular vacuolation (high-fat control group and low-dose group males) <b>Females:</b> ↑ ALP <sup>c</sup> , ALAT <sup>c</sup> , ASAT, SD, plasma triglycerides <sup>c</sup> , plasma albumin <sup>c</sup> ↑ blood insulin <sup>c</sup> (weeks 8 and 13) ↑ relative liver weights, relative spleen weights, relative adrenal weights <sup>c</sup> , and relative pancreas weights Hepatocellular hypertrophy (12/20 females) (present in 2/10 animals at end of 4-week recovery period) The NOAEL was reported to be 5% Clarinol™ G-80.	O'Hagan and Menzel, 2003

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Table 7.3-1 Summary of Subchronic, Chronic, and Reproductive and Developmental Toxicity Studies on CLA*						
Species (Strain)	Study Duration	Route of Administration	Treatment	Equivalent Dose of CLA (mg/kg bw/d)	Reported Effects <sup>a, b</sup>	Reference
Rat (male Fischer 344) (20/group)	36 weeks	Diet	0 or 1.5% CLA <sup>d</sup>	0 or 1,218.5	No significant compound-related effects in any parameter tested. The NOAEL was considered to be 1.218.5 mg/kg body weight/day	Scimeca, 1998
Rat (male Fischer 344) (n=10 or 11/group)	18 months	Diet	0 or 1% (85% of 50:50 mix of c9,t11 and t10,c12 isomers)	0 or 1,000	No significant effects on body weight gain, survival rate, water consumption, body fat composition, body water composition, or organ weights. CLA-fed rats had significantly reduced food consumption, significantly lower blood glucose levels, and significantly higher mean corpuscular volume compared to control rats. There were no significant compound-related differences in other clinical chemistry or hematological parameters, nor were there any differences observed following histopathological examinations.	Park <i>et al.</i> , 2005
Dog (strain not specified)	Not specified	Oral	50,000 ppm as CLA-ME or CLA-EE provided as beadlets	1,250	No significant effects were reported following consumption of CLA-ME. Liver impairment was reported following consumption of CLA-EE; however, this was not accompanied by any histopathological lesions. No further details were available.	Berven <i>et al.</i> , 2002 Unpublished
Pig (strain not specified)	Not specified	Diet	0, 0.48, or 0.95% (60% CLA isomer content)	0, 100, or 200	No significant effects.	Cook <i>et al.</i> , 1998
Reproductive and Developmental Toxicity Studies						
Rats (strain not specified)	Not specified	Oral	Details not provided	1,000	No significant effects. No further details were available.	Berven <i>et al.</i> , 2002 Unpublished
Rat (Fischer 344)	Throughout gestation and lactation (dams)	Diet	0 or 0.5% CLA	0 or 500	No significant effects on litter size, fetal body weights, fetal liver and brain weights, or gross evidence of fetal abnormality. Significantly higher mean pup weight in CLA-fed rats. Significant uptake of CLA into maternal liver, muscle, mammary gland, and milk and fetal liver.	Chin <i>et al.</i> , 1994

**Table 7.3-1 Summary of Subchronic, Chronic, and Reproductive and Developmental Toxicity Studies on CLA\***

Species (Strain)	Study Duration	Route of Administration	Treatment	Equivalent Dose of CLA (mg/kg bw/d)	Reported Effects <sup>a, b</sup>	Reference
Rat (Fischer 344)	Throughout gestation and lactation (dams); up to 10 weeks of age (pups)	Diet	0, 0.25 or 0.5% <sup>a</sup> CLA	0, 250, or 500	No significant effects on litter size, pup development and survival rate, and maternal food consumption. Significantly higher pup body weights on day 10 of lactation in the 0.5% CLA group and in pups fed 0.25 and 0.5% CLA for 8 to 10 weeks compared to controls. No significant difference between groups in food consumption; however, feed efficiency was significantly increased in male pups fed 0.5% CLA, and in female pups fed 0.25 and 0.5% CLA.	Chin <i>et al.</i> , 1994
Rat (Sprague-Dawley)	GD 7 to LD 21 (weaning)	Diet	0 or 0.5% CLA	0 or 500	No significant effects on maternal body weights, liver weights, and food consumption, and litter size and weights of whole litters. CLA-supplemented diet significantly enhanced pup growth compared to control diet.	Poulos <i>et al.</i> , 2001
Pig (Swiss Large White)	Throughout gestation and lactation (sows), up to 35 days of age (piglets)	Diet	2% linoleic acid-enriched oil or 2% CLA-enriched oil	450	No significant developmental effects. CLA exposure during gestation resulted in higher feed intake, body weight gain, and final body weight of piglets.	Bee, 2000

\* Studies considered to be pivotal are presented in **bold**

ALAT = alanine aminotransferase, ALP = alkaline phosphatase, ASAT = aspartate aminotransferase, CLA-EE = conjugated linoleic acid ethyl ester, CLA-ME = conjugated linoleic acid methyl ester; GD = gestation day; LD = lactation day; NOAEL = no-observed-adverse-effect level, SD = sorbitol dehydrogenase

<sup>a</sup> numbers in [ ] correspond to the dose(s) at which the reported effects were observed

<sup>b</sup> unless stated otherwise, all reported effects are relative to control group(s)

<sup>c</sup> These effects were reported to be reversible following a 4-week recovery period

<sup>d</sup> CLA preparation containing 85.5% of a 50:50 mix of c9,t11- or t9,c11 and t10,c12 isomers, 4.3% other CLA isomers, 7.1% linoleic acid, and 3.1% unknown constituents

<sup>e</sup> 0.5% CLA in the diet was provided to 2 groups: one group consumed CLA throughout gestation and lactation, and one group consumed CLA throughout lactation only

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## 7.4 Additional *In Vitro* and Animal Studies

Additional *in vitro* and animal studies have evaluated the effects of CLA on cardiovascular disease risk, insulin sensitivity, maternal milk fat, and biomarkers of oxidation. These are predominantly investigative studies of the mechanisms of action of CLA and their relevance to the effects observed in clinical studies. Because human data (reviewed in Section 7.5) are weighed more heavily than pre-clinical data for purposes of evaluation of safety, these pre-clinical data are not discussed in detail here but are provided in Appendix C. In addition, as previously noted, studies of CLA isomers other than the 50:50 mixture are accorded significantly lower weight.

To summarize these pre-clinical data:

- In terms of cardiovascular risk biomarkers it has been shown that CLA provides no significantly increased risk. Numerous studies have shown inter-species variation in cardiovascular risk markers, demonstrating that caution must be exercised when attempting to extrapolate to humans. The mouse, in particular, is very sensitive to the effects of CLA and is apparently not able to cope with the changes in fat metabolism induced by large relative doses. There is no evidence that hepatic lipid accumulation due to supplementation with CLA observed in experimental mice is of toxicological significance. Furthermore, the majority of data also demonstrate positive effects of CLA on inflammatory markers
- With regard to insulin resistance, in concurrence with the effects on adipose tissue, the mouse model was demonstrated to be the most sensitive species. Adipose tissue is almost completely ablated in the mouse following CLA intervention due to apoptosis resulting from decreased glucose uptake in the adipose tissue. Decreased glucose uptake is a result of inhibition of GLUT4 by predominantly the t10,c12 CLA isomer at the nuclear regulatory level. Blood glucose is then shunted to the liver and induces hepatic lipogenesis in order to deal with the higher amount of glucose that is further transformed into serum triglycerides. These effects have been demonstrated to be transient (Wargent *et al.*, 2005).
- Observations of reduced maternal milk fat in animal models, predominantly ruminants, are of minimal significance in relation to humans who rely to a lesser degree on *de-novo* fatty acid synthesis for milk fat secretion.

## **7.5 Clinical Studies**

### **7.5.1 Introduction/Overview**

The purpose of this Section is to critically evaluate clinical trials in which parameters relevant to safety were evaluated and to draw conclusions regarding the safety of CLA-Rich Oil (50:50 mixture) that is the subject of this report.

A summary of the clinical studies on CLA is provided in Tables 7.5.1-1 to 7 5.1-4. The clinical studies are divided according to the CLA mixtures or isomers administered in the particular study. Following the Tables, a comprehensive discussion of safety is presented, followed by a discussion of specific issues relating to cardiovascular disease risk, glucose and insulin sensitivity, and maternal milk fat depression. A final summary is presented in Section 7.5.7.

The primary focus of this discussion will be on the safety of the 50:50 CLA mixtures since this is the composition of the products under consideration and are therefore most relevant to the current notification. Nonetheless, studies with single isomers or the four isomer mixture have been included to show completeness in this body of work and also point to the safety of low doses of mixtures that include the 50:50 mixture as well as other isomers. Additionally, two human studies have investigated the effects of ingesting CLA-containing foods on various health parameters and one human study examined CLA intake from foods; these are summarized here. Where possible, all data reported were compared to population reference values as reported by Tietz (1995) (see Attachment 3), and were described to be "within population range" (WPR), if applicable.

**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
Høye <i>et al.</i> , 2004	Open label	12 healthy male volunteers	8.86 g CLA (4.40 g <i>n</i> 10, <i>c</i> 12 + 4.46 g <i>c</i> 9, <i>n</i> 11) in triglyceride form or 11.15 g CLA (5.65 g <i>n</i> 10, <i>c</i> 12 + 5.70 g <i>c</i> 9, <i>n</i> 11) in free fatty acid form. Subjects consumed a single dose of each preparation, with a 9- to 14-day washout period between doses.	Comparison of bioavailability	Peak serum concentration was reached within 2 to 8 hours. The free fatty acid form of CLA was 1.15 to 1.19 times more bioavailable than the triglyceride form, while <i>c</i> 9, <i>n</i> 11 CLA was 1.25 to 1.27 times more bioavailable than <i>n</i> 10, <i>c</i> 12 CLA	The authors noted no adverse events following CLA treatment.
Lambert <i>et al.</i> , 2007	Double-blind, randomized, placebo-controlled	64 regularly exercising men and women, aged 21 to 45 years	3.9 g/day of Clannol™ A-60, containing 65.9% of 50:50 CLA mixture in FFA form or placebo (olive oil) for 12 weeks	Body composition, long-term safety (clinical chemistry)	Significant decrease in body fat levels in women. No significant difference in plasma glucose levels. Significantly lower plasma insulin concentrations in women. Insulin sensitivity, as measured by the increment in glucose concentrations <i>versus</i> the increment in insulin concentrations and by the insulin sensitivity index (ISI) and Quantitative Insulin Sensitivity Check Index (QUICKI) models were not affected by treatment. Insulin resistance as measured using the Homeostasis Model Assessment (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, healthy subjects. No treatment related effects were found on total cholesterol, LDL-cholesterol, and HDL-cholesterol.	Occurrence of adverse effects, including headaches, flatulence, skin irritation, and flu/cold, did not differ between groups.

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
Gaullier et al., 2004	Double-blind, randomized, placebo-controlled	180 healthy, overweight volunteers (31 males, 149 females) (Data from the 157 subjects who completed the study were included in the results)	3.4 g/day 50:50 CLA mixture (1.7 g/day c9,t11 and 1.7 g/day t10,c12) in free fatty acid (FFA) or glyceride (TG) or placebo (olive oil) for 52 weeks	Body composition, long-term safety (clinical chemistry)	<p>Slight, but statistically significant increase in Lp(a) [321.0 +/- 390 mg/L to 346.8 +/- 448 mg/L for the TG form, 244.1 +/- 267.0 mg/L to 284.0 +/- 292.0 mg/L for the FFA form. Values WPR<sup>a</sup> (22.0-573.0 mg/L)]</p> <p>Statistically significant within-group decrease in LDL-C (3.6 to 3.5 mmol/L): these were also WPR. They also reported a minor, but statistically significant within-group decrease in HDL-cholesterol with 3.4g/day CLA supplementation (1.5 to 1.4 mmol/L: mean WPR range=0.72-2.25 mmol/L).</p> <p>Glycosylated hemoglobin was also noted to increase in both the CLA and placebo groups, suggesting this effect was not treatment-related.</p> <p>All other markers of safety (fasting glucose, fasting insulin, aspartate aminotransferase, alanine aminotransferase, total cholesterol, systolic and diastolic blood pressure, heart rate) were unaffected by CLA treatment.</p>	<p>68% of subjects in both CLA groups and the placebo group reported adverse events, with reports similar for all groups</p> <p>10 subjects total withdrew from the study due to gastrointestinal or musculoskeletal complaints. These included nausea, diarrhea, and abdominal discomfort. The investigators attributed only the gastrointestinal events to the test substances (including placebo). Three other subjects withdrew from the study due to unrelated complications such as uterine prolapse and accidents. All adverse events were evenly distributed among the 3 study arms (including placebo).</p>

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
Gaullier et al., 2005	Open-label one-year follow-up study	134 of the original 157 healthy, overweight volunteers from Gaullier et al., 2004 (24 males, 110 females)	3.4 g/day 50:50 CLA mixture (1.7 g/day c9,t11 and 1.7 g/day t10,c12) in TG form for one additional year (104 weeks total)	Body composition, long-term safety (clinical chemistry)	<p>No change in hemoglobin, bilirubin, potassium, chloride, sodium, creatinine, erythrocytes, TSH, thyroxin, or IGF-1. Systolic and diastolic blood pressure and heart rate remained WPR.</p> <p>Total serum cholesterol was reduced by the group that initially received the free fatty acid form of CLA in the first year (5.39 to 5.16 mmol/L baseline to month 24: mean WPR=3.37-7.15 mmol/L), although this was also WPR, and of potential benefit.</p> <p>HDL-C was reduced in 4 participants out of 93 subjects on one of 2 CLA treatments (4.3%) (n=1 for FFA CLA group; n=3 for TG CLA group). These individuals had "decreases in HDL cholesterol to a level below population range at 24 months compared to 0 months." However, mean values for HDL-C were still WPR (1.51 to 1.42 mmol/L: mean WPR range=0.72-2.28 mmol/L), even when taking into account the standard deviation from the mean, the lower limit of these values are still WPR.</p> <p>Serum insulin levels were significantly higher in the TG CLA group at 24 months (75 to 90.6 pmol/L), however, this 2 year mean (90.6 pmol/L) is still WPR (&lt;118pmol/L) and thus is not considered a safety risk.</p> <p>Total white blood cells were elevated from baseline values in both CLA groups as well as the placebo group</p>	<p>Adverse events were reported by 50% of year 2 trial participants, compared to 68% of participants within the first year of the trial. One hundred twenty four adverse events were reported and of those, only 7 were considered to be treatment related: these included primarily gastrointestinal complaints.</p> <p>Primarily GI complaints</p>

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\*

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
					<p>(FFA form 6.03 to 6.63 x 10<sup>9</sup>/L versus TG form: 5.30 to 6.19 x 10<sup>9</sup>/L: mean WPR range=4.50-11.0 x 10<sup>9</sup>/L): again, these values are WPR.</p> <p>Serum leptin decreased significantly in all groups: this is a physiological event as it reflects body fat metabolism changes and is not considered a clinical biomarker, nor are there established normal values, rather leptin is an experimental marker of fat metabolism.</p>	
Whigham <i>et al.</i> , 2004	Randomized, placebo controlled	63 obese volunteers (46 females, 17 males)	Subjects were placed on a very low calorie diet until they achieved 20% reduction in body weight. 6.0 g/day of a 50:50 CLA mixture (3.0 g/day c9,t11 and 3.0 g/day t10,c12) or placebo control (sunflower oil) were administered during weight loss and throughout the 12-month period	Weight regain, safety parameters	<p>Insulin, glucose, alkaline phosphatase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were unaffected by CLA treatment and remained WPR for this 52-week study with the exception of a statistically significant spike in glucose levels in CLA-consuming volunteers at week 2. However, this rise was still WPR and normalized at the next time point and thereafter for the remainder of the study.</p> <p>At week 28 (the beginning of the open label phase of the trial) CLA-consuming volunteers were reported to have significantly higher serum triglyceride (160 versus 154 mg/dL, at baseline and week 28, respectively: mean WPR range=39-327 mg/dL) levels. This level was maintained at week 52 compared to the placebo group. However, it should be noted that the placebo group was lower at baseline and week 12, making the statistical comparison appear significant.</p>	<p>Ten volunteers randomized to the CLA treatment withdrew from the study (4 at baseline; 2 at week 2; 3 at week 4 and 1 at week 8). The most common reason for withdrawal was that the volunteers were too busy to comply with study protocol. The only adverse event reported in the CLA group of those who withdrew (10 out of 63) was a skin rash that investigators did not deem related to the test product.</p> <p>Self-reported symptoms included skin rash, depression and</p>

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
					<p>When the data were compared as an analysis of change in measures between major time points, the CLA group had a significantly smaller increase in cholesterol levels from weeks 12 to 28 (<math>19.2 \pm 4.8</math> vs. <math>33.1 \pm 5.3</math>, <math>p = 0.054</math>) than the placebo group. The control group had a significantly greater rise in HDL from weeks 12 to 28 (<math>10.3 \pm 2.4</math> vs. <math>2.1 \pm 2.1</math> mg/dl, <math>p = 0.01</math>), but then had a decrease from weeks 28 to 52 while the CLA group increased HDL levels during that same time (<math>8.3 \pm 2.4</math> vs. <math>1.5 \pm 2.1</math>, <math>p = 0.003</math>). All other changes between major time points were not significantly different and WPR.</p> <p>Given these differences, the biological significance of this endpoint is questionable and may reflect the issue of multiple comparisons and unadjusted (Bonferroni adjustment) p values.<sup>b</sup></p> <p>White blood cell levels were also significantly increased at week 28 (<math>6.16</math> to <math>6.55 \times 10^3</math> cells/<math>\mu\text{L}^c</math>), which is WPR.</p>	irritability, hair loss and infection, which were reported to occur at lower incidences in the CLA group compared to placebo.
Larsen et al., 2006	Randomized, placebo-controlled parallel design	122 overweight or obese volunteers	3.4 g/day 50:50 CLA mixture or placebo (olive oil) as a control after a very low calorie diet for 52 weeks	Safety, insulin resistance, body composition	No significant differences in any blood parameters were noted, except for an increase in leukocytes in the CLA group. Values remained within reference values.	Of the total of 563 adverse events that were reported, 34 were considered to be related to treatment (including placebo), but no differences between groups existed.

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\*

Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Thom <i>et al.</i> , 2001	Randomized, placebo-controlled double-blind	20 healthy subjects BMI ~ 23 kg/m <sup>2</sup> in both treatment and control groups	3 g/day 50:50 CLA mixture (0.54 g/day c9,t11, 0.54 g/day t10,c12) for 12 weeks	Body fat and weight changes	Body fat was significantly reduced in the CLA group after 4, 8, and 12 weeks compared to baseline results and placebo group. Reduction in BMI or body weight for both groups was not statistically significant during the treatment period.	No serious adverse events were noted and no participants withdrew from the study due to adverse events. There were 2 reports of transient gastrointestinal disturbances within the first week of consumption (one in the placebo group and one in the CLA group). Both subjects reported adaptation with continuous treatment.
Mougios <i>et al.</i> , 2001	Randomized, double-blind, placebo controlled	22 overweight adults (13 males, 9 females)	0.7 g/d followed by 1.4 g/day 50:50 CLA mixture or placebo control (soybean oil) for 8 weeks	Body composition, blood lipids	Triglycerides, total cholesterol, creatinine kinase and cortisol levels remained unchanged. There was a statistically significant decrease in serum HDL levels (baseline=1.42 +/- 0.29; week 4=1.26 +/- 0.30; week 8=1.25 +/- 0.32 mmol/L, however the change was still WPR and poses no safety risk. WPR for mixed gender and age varies between 0.70 and 2.28 mmol/L)	No adverse events were reported by any of the participants.
Blankson <i>et al.</i> , 2000	Double-blind, randomized	60 overweight volunteers (20 males, 40 females)	1.7, 3.4, 5.1, or 6.8 g/day 50:50 CLA mixture or placebo control (olive oil) for 12 weeks. The CLA mixture contained 32.5% of the c9,t11 isomer and 32.5% of the t10,c12 isomer.	Body composition, blood lipids, liver and kidney function	All CLA treatment groups (1.7-6.8 g/day doses) exhibited significant within-group (from baseline) reductions in total cholesterol, LDL-C, HDL-C, and serum creatinine and platelets; however, they were not outside the range for the population. Serum potassium levels were also significantly increased. Authors give only mean changes in these parameters and do not report actual values. A significant reduction in creatine	Sixty percent of subjects reported adverse events, although there was no difference in frequency between the placebo and CLA groups. Eight volunteers withdrew from the study (7 CLA group and 1 placebo

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
					phosphokinase was seen in the volunteers consuming 6.8 g/day. However, the authors stated "none of these changes were considered clinically important". Vital signs such as heart rate and blood pressure were unaffected by CLA administration at any level.	group) due to adverse events. The major complaints were gastrointestinal symptoms and were reported to disappear with continuous treatment  Subjects reported no negative effects at any level of CLA on quality of life indices such as sleep, appetite, mood, stress, and working capacity measured using a visual analog scale (VAS) assessment method.
Berven <i>et al.</i> , 2000	Randomized, double-blind, placebo controlled	60 overweight or obese male and female volunteers (BMI 27.5 to 39)	3.4 g/day CLA (1.75 g c9,t11 and 1.75 g t10,c12) or placebo (olive oil) for 12 weeks	Body composition, blood lipids, liver function	Mean bilirubin levels decreased and were statistically significantly altered when subjects in the CLA group were compared to themselves at baseline (p=0.01), although a drop from 10 to 9 µmol/L is still well within the midpoint range for the population range for these subjects (5d-60y: 5-21 µmol/L) CLA treatment had no effect on a number of safety parameters including: blood lipid panels, hemoglobin, erythrocytes and total white blood cells, platelets, liver enzymes, blood electrolytes (calcium, potassium, chloride and sodium), and various markers of whole body macronutrient metabolism (e.g., creatinine and lipase).  No significant changes in serum total cholesterol, LDL or HDL-cholesterol, Lp(a)	There was no significant difference between treatment-related adverse effects, which included diarrhea, gastritis, bad oral smell, and perspiration, between CLA and placebo groups.

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\*

Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
					levels or serum triglycerides. All parameters remained WPR throughout this study. No significant CLA-induced changes in vital signs (systolic and diastolic blood pressure, heart rate) were noted.	
Basu <i>et al.</i> , 2000a; Risérus <i>et al.</i> , 2001	Randomized, double-blind, placebo controlled	24 subjects with abdominal obesity (BMI >32)	4.2 g/day 50:50 CLA mixture (1.24 g c9,t11 and 1.24 g t10,c12) or placebo (olive oil) for 4 weeks	Body composition, blood lipids, lipid peroxidation	No significant effects of CLA mixture on total cholesterol triglycerides, LDL-cholesterol, HDL-cholesterol, fasting glucose and insulin levels, free fatty acids and blood pressure were noted  Authors noted an increase in isoprostanes.	Increased lipid peroxidation was reported. There were no treatment-related adverse effects.
Smedman and Vessby, 2001, Basu <i>et al.</i> , 2000b	Randomized, double-blind, placebo controlled	53 healthy subjects (27 males, 26 females)	4.2 g/day 50:50 CLA mixture (1.24 g c9,t11 and 1.24 g t10,c12) or placebo (olive oil) for 12 weeks	Body composition, blood lipids	No significant difference in physiological parameters such as total cholesterol, HDL and LDL-cholesterol, serum triglycerides, VLDL-cholesterol, non-esterified fatty acids (NEFA), Apolipoproteins A1, Apo (a), blood glucose, plasma insulin and plasminogen activating factor-1 (PAF-1) were noted in the CLA group. A significant decrease in LDL-cholesterol in the control group was seen.  They also reported a small but significant increase in Apolipoprotein B (ApoB) 5.77 to 6.24 g/L (mean WPR range=0.46 to 1.74 g/L +/- +2 Standard Deviations), although they did not indicate this was of clinical relevance to their overall study.  Supplementation with CLA 50:50 mixtures increased urinary 8-iso-prostaglandin F <sub>2α</sub> , (morning and 24 hour collection), and increased 15-keto-dihydro-prostaglandin F <sub>2α</sub> , but had no effect of p-MDA or s-α-tocopherol levels, markers of oxidation and antioxidant status	Increased lipid peroxidation was reported. There was no other significant difference between treatment-related adverse effects between CLA and placebo groups

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\*

Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Smedman <i>et al.</i> , 2005	Randomized, double-blind, placebo controlled	53 healthy subjects	4.2 g/day 50:50 CLA mixture (1.24 g c9,t11 and 1.24 g t10,c12 t10,c12) or placebo (olive oil) for 12 weeks	Markers of inflammation	There was a statistically significant rise in CRP levels between control and CLA groups. However, it should be noted that the control group began the study with a median CRP level of 1.76 mg/L and the CLA group began the study with a median C reactive protein (CRP) level of 3.27 mg/L. The authors did not indicate whether baseline values were statistically different, thus the relevance of these results are questionable. No other inflammatory marker [i.e., TNF- $\alpha$ , TNF- $\alpha$ 1 & 2 receptors and vascular cell adhesion molecule-1 (VCAM-1)] changed significantly.	Not reported
Syvetsen <i>et al.</i> , 2006	Randomized, double-blind, placebo-controlled parallel design	83 healthy, overweight volunteers (18 males and 65 females) aged 18 to 65 years with BMI values ranging from 28 to 32 kg/m <sup>2</sup>  A sub-population of 41 healthy, overweight volunteers (13 males, 28 females) aged 27 to 64 years entered a euglycemic hyperinsulinemic clamp study <sup>d</sup>	3.4 g/day CLA or placebo (olive oil) provided as 6 capsules per day for 6 months	Body composition, insulin/glucose levels	No significant differences were noted in body composition, glycosylated hemoglobin, c-peptide, adiponectin, leptin, glucose uptake, glucose uptake to insulin concentration ratio, fasting glucose and insulin, HOMA and QUICKI values in the clamp study population or main study population.	While adverse events were studied, none were mentioned in the study paper.

**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Watras <i>et al.</i> , 2007	Randomized, double-blind, placebo controlled	40 healthy overweight (BMI between 25 and 30 kg/m <sup>2</sup> ) men and women between the ages of 18 and 44 years	4g/day of 78% active CLA isomers of safflower oil (3.2 g/day CLA) or placebo (safflower oil) provided as 4 capsules a day for 6 months	Body composition, blood chemistry, insulin resistance, and inflammatory biomarkers	<p>Significant decrease was noted in fat-mass weight and percentage compared to placebo group and baseline results of CLA group.</p> <p>Significantly decreased body weight and BMI values compared to baseline results of CLA group were noted</p> <p>Significantly increased resting metabolic rate and significantly reduced physical activity values compared to CLA baseline values were noted.</p> <p>No significant differences in any of the liver function, cardiovascular, or inflammation markers.</p> <p>No significant differences in glucose concentration, insulin concentration, insulin resistance as evaluated by HOMA, or leptin concentrations compared to placebo were noted.</p>	Decrease in frequency of reported depression and irritability in CLA group compared to placebo was noted; however, due to the small number of observations, the authors reported that the results are likely to be meaningless.
Smedman <i>et al.</i> , 2004; Basu <i>et al.</i> , 2000a	Randomized, open-label.	60 adult volunteers (25 males, 35 females) (healthy and overweight, BMI 18 to 36)	COX-2 inhibitor (12 mg/day rofecoxib), alpha-tocopherol, or neither for 6 weeks. The last 4 weeks, they were given 3.5g/day 50:50 CLA mixture, 4 g/day of a CLA treatment (3.6 g/day of t10,c12 isomer) as control	Lipid peroxidation 2 weeks pre-treatment with vitamin E or COX-2 inhibitor followed by CLA. CLA alone was placebo	Both CLA treatments induced increases in 8-iso-PGF-2 and 15-keto-dihydro-PGF2 $\alpha$ , the largest increase being seen in the pure isomer group (t10,c12 CLA). They also noted that the COX-2 inhibitor suppressed the rise in 15-keto-dihydro-PGF2 $\alpha$ , after t10,c12 CLA consumption, but not the mixed isomer CLA.	Lipid peroxidation increased dose dependently.

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures*						
Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
Noone <i>et al.</i> , 2002; Nugent <i>et al.</i> , 2005	Randomized, double-blind, placebo controlled	51 healthy, lean adult volunteers (18 males, 33 females)	3 g/day 50:50 CLA mixture (1.5 g c9,t11 and 1.5 g t10, c12), 80:20 CLA blend, or placebo (linoleic acid) for 8 weeks	Immuno-modulation, lipid metabolism	<p>No changes were found in plasma lipid, glucose, or insulin levels</p> <p>Supplementation with the 80:20 CLA isomer blend significantly (<math>p \leq 0.05</math>) enhanced PHA-induced lymphocyte proliferation. CLA decreased basal interleukin (IL)-2 secretion (<math>p \leq 0.01</math>) and increased PHA-induced IL-2 and tumor necrosis factor alpha (TNF-<math>\alpha</math>) production (<math>p \leq 0.01</math>). However, these effects were not solely attributable to CLA as similar results were observed with linoleic acid. CLA supplementation had no significant effect on peripheral blood mononuclear cells IL-4 production, or on serum-soluble intercellular adhesion molecule-1 (sICAM-1) or plasma prostaglandin E2 (PGE2) or leukotriene B4 (LTB4) concentrations</p>	No report of any adverse events associated with CLA consumption
Albers <i>et al.</i> , 2003	Randomized, reference-controlled, double-blind trial.	73 Hepatitis-B-vaccine challenged patients	3g/day 50:50 CLA mixture (0.85 g c9,t11 and 0.85 g t10,c12) or placebo control for 12 weeks	Immune function	<p>Blood pressure, blood lipid panels, liver enzymes, blood glucose and serum insulin levels were measured: no changes were observed after CLA administration.</p> <p>Systolic and diastolic blood pressure, fasting serum lipids and the blood chemistry parameters (including liver enzymes and glucose) were WPR, did not differ between groups and were not affected by the intervention, apart from a slight increase in HDL cholesterol in the placebo group, but not in CLA groups (<math>P=0.019</math>).</p> <p>These included TNF-<math>\alpha</math>, IL1-<math>\beta</math>, IFN-<math>\gamma</math>, IL-2, IL-4, and PGE-2 in an <i>ex vivo</i> model before and after treatment. These markers typically indicate immune stimulation, or lack thereof, although many also indicate</p>	No differences in adverse events were observed between the study treatment groups.

**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
					inflammatory status. The authors noted no adverse changes in these markers.	
Kamphuis <i>et al.</i> , 2003a,b	Randomized, double-blind, placebo controlled	54 healthy, overweight subjects (26 males, 28 females)	1.8 or 3.6 g/day 50:50 CLA mixture or placebo (oleic acid) for 13 weeks after 3 weeks on a very low calorie diet (VLCD)	Weight regain, body weight, satiety, fullness and appetite	No effect on was seen fasting blood glucose, insulin, substrate oxidation, triglycendes and free fatty acids at either CLA dose level.	The incidence of adverse events was low and did not differ between placebo and the 2 CLA treatments.
Eyjolfson <i>et al.</i> , 2004	Placebo-controlled	16 young, sedentary, overweight adults (4 males, 12 females)	4 g/day 50:50 CLA mixture for 8 weeks	Insulin sensitivity	The 50.50 CLA mixture did not adversely influence these parameters, in fact, insulin sensitivity indices were improved after 8 weeks in the CLA group. It should be noted that here was high variability within these groups, likely due to the low number of volunteers.	Not reported
Moloney <i>et al.</i> , 2004	Randomized, double-blind	32 type 2 diabetic, overweight volunteers (sex not reported)	3g/day 50.50 CLA mixture (1.2 g c9,t11, 1.2 g t10,c12) for 8 weeks	Insulin sensitivity, blood lipids	Increased fasting glucose levels by 6.3% in this population after an oral glucose tolerance test (7.34 to 7.80 mmol/L in the CLA group; mean WPR range=venous draw <6.7mmol/L; capillary draw <7.8 mmol/L) No effects on postprandial glucose, fasting or postprandial insulin, or C-peptide levels, area under the postprandial curve (AUC), incremental area under the postprandial curve (IAUC) Reduction in the insulin sensitivity index as measured by HOMA (2.81 to 3.35 for the CLA group; no clinical lab normal values for HOMA). Despite this, the levels of HbA1c did not change. Furthermore, CLA did not adversely influence other safety markers such as C-reactive protein or interleukin-6, both markers of long-term CVD risk Total HDL-cholesterol concentrations increased significantly and the ratio of LDL to HDL was decreased significantly. ApoB levels increased.	Not reported

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
					Fasting serum cholesterol, triglycerides and VLDL cholesterol did not significantly change.	
Masters <i>et al.</i> , 2002	Double-blind cross-over study	9 healthy lactating women	1.2 g CLA 50:50 and 0.3 g other fatty acids or 1.5 g olive oil daily. Total dose of administered CLA was 1.107 g/day  Each intervention lasted 5 days, with a 7-day washout period between treatments	Infant milk consumption, milk fatty acid content, and dietary CLA consumption levels	Plasma fatty acid profiles were unaffected by CLA administration, but plasma c9,t11-CLA and t10,c12-CLA were increased compared to the placebo. Although total milk fat content was reduced by 25%, CLA supplementation had no overall effect on total infant milk consumption	Not reported
Belury <i>et al.</i> , 2003	Randomized, double-blind placebo controlled	22 adult volunteers with non-insulin dependent diabetes mellitus	6 g/day of a 50:50 CLA mixture (2.24 g c9,t11, 2.37 g t10,c12) or placebo for 8 weeks	Fasting insulin and glucose, leptin	A decrease in glucose levels in 81% of the volunteers consuming CLA 50:50 mixture was noted. Leptin levels decreased	Authors did not report on any other safety-related biomarkers.
Atkinson, 1999	Randomized, double-blind placebo controlled	80 overweight or obese volunteers (sex not reported) (BMI 27 to 40)	2.7 g/day 50:50 CLA mixture (0.81 g c9,t11, 0.81 g t10,c12) or placebo for 26 weeks	Body composition, blood lipids	No significant changes were noted in either body weight or body fat after 6 months. A sub-population of the study population gained lean body mass as revealed by <i>post-hoc</i> analyses, where twice as many subjects in the CLA group gained lean body mass compared to placebo; however, the CLA group lost body fat on average, whereas placebo group gained body fat.	No adverse effects were noted for the 50:50 CLA mixture on any safety parameters  1 subject in the CLA group exhibited significant edema and weight gain for several weeks, which resolved after cessation of CLA. The subject restarted CLA consumption, without the knowledge of the investigators and

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures*						
Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
						reported no further problems over the last 3 months of the study, approximately.
Petridou <i>et al.</i> , 2003	Randomized double-blind crossover design.	16 healthy nonobese sedentary women	2.1g/day of a 50:50 mixture of CLA or placebo for 45 days	Body composition, blood lipids, leptin	No significant differences were found in body fat or serum leptin, TAG, total cholesterol, HDL-cholesterol, and alanine aminotransferase between CLA and placebo. The period of 2 wk after the end of CLA supplementation was sufficient for its washout from serum lipids. These data indicate that supplementation with 2.1 g of CLA daily for 45 d increased its levels in blood but had no effect on cardiovascular safety parameters in these nonobese women.	No adverse effects reportedly occurred. One subject withdrew from the study due to acute illness (influenza) at the request of the subject's physician.
Doyle <i>et al.</i> , 2005	Randomized, double-blind placebo controlled	60 healthy male volunteers	3 g/day 50:50 of a 50:50 CLA mixture or control (palm and soybean oil) for 8 weeks	Bone metabolism	Markers related to bone metabolism such as urinary and serum type I collagen cross-linked N telopeptides, urinary creatinine, urinary and serum calcium levels, serum osteocalcin and bone specific alkaline phosphatase as well as serum vitamin D status did not change significantly over the course of this trial	The authors noted no adverse events following CLA treatment.
Song <i>et al.</i> , 2005	Randomized, double-blind, placebo-controlled	28 healthy adult volunteers	3g/day CLA 50:50 mixture (1.2 g c9,t11, 1.2 g t10,c12) or placebo (sunflower oil) for 12 weeks.	Immune parameters	Plasma total cholesterol was not affected by CLA supplementation but the placebo elicited a small but significant decrease at week 12. HDL-cholesterol was decreased slightly but significantly by the CLA supplement but not the reference oil, while LDL-cholesterol was not altered by either supplement. The decrease in HDL-C was still WPR. Plasma triglyceride levels were unaffected  Plasma glucose and insulin levels were not affected by CLA. Plasma levels of IgA and IgM were significantly increased while IgE,	No adverse events

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures*						
Reference	Study Design	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
					<p>a marker of inflammation, was decreased. CRP, a marker of inflammation, was measured and was cited as not changing with CLA treatment. In addition, pro-inflammatory cytokines such as TNF-<math>\alpha</math> and IL-6 were decreased while IL-1<math>\beta</math> was significantly increased in the CLA group indicating positive changes in biomarker. Expression of adhesion molecules (ICAM-1 and L-selectin) was not affected.</p> <p>Authors reported all clinical values as percent change and made the judgment that these changes were not unsafe. As a result, it was not possible to compare the values to the population range table</p>	
Taylor <i>et al</i> , 2006	Randomized, double-blind, placebo-controlled, matched pair design.	40 healthy overweight white males	4.5 g/day 50:50 CLA mixture or placebo (olive oil) for 12 weeks	Body fat, lipid, insulin, and oxidative parameters	<p>Limb fat thickness as assessed by the caliper method was significantly decreased after CLA supplementation, but torso fat, liver and spleen sizes did not differ between treatments. The authors noted that plasma F2-isoprostanes increased after CLA administration [-36 <math>\pm</math> 95 placebo versus 94 <math>\pm</math> 200 pg/mL placebo, p=0.042]. This study was marked by extreme variability in samples and safety concerns regarding such changes are questionable. Furthermore, other markers of inflammation, such as TNF-<math>\alpha</math> and adiponectin were not significantly different from the placebo. Finally, plasma CRP, insulin, glucose and HOMA calculations levels were unaffected by CLA administration. Likewise, no significant differences in total, LDL or HDL cholesterol or serum triglycerides were found after CLA administration.</p>	None reported

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
Pinkoski <i>et al</i> , 2006	Randomized, placebo-controlled	76 healthy, male and female resistance-trained subjects	5 g/day CLA or placebo (36.1% c9,t11 and 36.3% t10,c12 isomer) for 7 weeks while resistance training 3 days/week. 17 subjects crossed over to the opposite group for an additional 7 weeks	Body composition, strength, myofibrillar and bone degradation, resting metabolic rate, resting substrate utilization	Subjects in CLA group had greater increases in lean tissue mass, greater losses of fat mass, and a smaller increase in markers of catabolism [myofibrillar degradation as measured by 3-methylhistidine (3MH) and bone resorption as measured by cross-linked N-telopeptides (NTx)] compared to placebo. Crossover subjects had minimal changes in body composition, but smaller increase in 3MH and NTx while on CLA versus placebo.	No serious adverse events occurred. Eight subjects in the CLA group and 7 in the placebo group reported adverse events that investigators considered related to supplementation (including placebo, with no significant difference between groups. Two of the 8 subjects in the CLA group and 4/7 in the placebo group reported multiple adverse events. Adverse events in the CLA group included upset stomach/ indigestion, heartburn/ reflux and nausea. Adverse events from the placebo group included upset stomach, diarrhea, loss of appetite, bloating, and constipation. Adverse events were considered to be mild to moderate and were transient.  Similar mild to moderate adverse

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Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures*						
Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
						events were reported during crossover, with no significant difference between groups. Four CLA subjects reported adverse effects (upset stomach, diarrhea) while 2 placebo subjects reported bloating, constipation upset stomach, and diarrhea.
Gaullier <i>et al.</i> , 2007	Randomized, double-blind, placebo-controlled	83 overweight and obese subjects (BMI: 28-32 kg/m <sup>2</sup> )	3.4 g/day of a 50:50 CLA mixture or placebo (olive oil) for 6 months	Body composition, inflammatory markers, blood lipids, Insulin sensitivity	<p>CLA decreased significantly BFM at month 3 (<math>\Delta = -0.9\%</math>, <math>P = 0.016</math>) and at month 6 (<math>\Delta = -3.4\%</math>, <math>P = 0.043</math>) compared to placebo. The reduction in fat mass was located mostly in the legs (<math>\Delta = -0.8</math> kg, <math>P &lt; 0.001</math>), and in women (<math>\Delta = -1.3</math> kg, <math>P = 0.046</math>) with BMI over 30 (<math>\Delta = -1.9</math> kg, <math>P = 0.011</math>), compared to placebo. Waist/hip ratio decreased significantly (<math>P = 0.043</math>) compared to placebo. LBM increased (<math>\Delta = +0.5</math> kg, <math>P = 0.049</math>) within the CLA group. Bone mineral mass was not affected (<math>P = 0.70</math>). All changes were independent of diet and/or physical exercise. Proinflammatory cytokines were unaltered by CLA except CRP (<math>\Delta = +1.52</math> mg/l, <math>P = 0.011</math>) that remained WPR. All other safety parameters including blood lipids and diabetogenic markers were not affected.</p> <p>Comparison of the blood lipids between the groups demonstrated no significant differences in Lp(a) (<math>P = 0.97</math>), total cholesterol (<math>P = 0.32</math>), HDL cholesterol (<math>P = 0.28</math>), LDL cholesterol (<math>P = 0.19</math>),</p>	Adverse events did not differ between groups.

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**Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures\***

Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
					and triglycerides ( $P = 0.22$ ) levels. After 6 months with CLA supplementation there were also significant increases in Lp(a) levels in both CLA group ( $P = 0.017$ ) and placebo group ( $P = 0.020$ ), thus no conclusions as to the safety of CLA from the Lp(a) perspective can be drawn from this study. HDL cholesterol levels decreased in the CLA group ( $P = 0.030$ ) compared to baseline, but these changes remained within the population range.	
Raff <i>et al.</i> , 2006	Randomized, double-blind, placebo-controlled	Healthy young men (n=60) with mean BMI of 22.5 kg/m <sup>2</sup>	CLA-rich diet providing 4.7 g/d of a 50:50 mixture of CLA isomers or a placebo diet for 5 weeks	Blood pressure and endothelial function, measured by a volume-oscillometric technique	No significant effects on systolic or diastolic blood pressure or pulse pressure were noted. No significant effects on isobaric arterial elasticity were noted.	Not reported.
Steck <i>et al.</i> , 2007	Randomized, double-blind, placebo-controlled	Healthy obese non-smoking subjects (n=48; 13 male, 35 female) between 18-50 years, BMI 30 to 35	3.2 or 6.4 g/day of a 50:50 mixture of CLA isomers (Tonalin) compared to placebo (8 g safflower oil) for 12 weeks	Body composition (by DEXA) and anthropometric data, resting energy expenditure (REE) and respiratory quotient (RQ) measured by indirect calorimetry, lipid profile, CRP, IL-6 and standard clinical blood chemistry	Compared to baseline values, HDL-cholesterol significantly decreased in the placebo and 6.4 g CLA/day groups, while CRP, IL-6, WBC counts, and alkaline phosphatase increased, and hemoglobin, hematocrit, and sodium levels decreased, in the 6.4 g CLA/day group  Compared to placebo, the increases in CRP, IL-6, and WBC counts in the 6.4 g CLA/day group were significant. The authors reported that all changes remained WPR.  There were no significant changes in any measured parameter in the 3.2 g CLA/day group when compared to baseline or placebo	No adverse effects were determined to be related to CLA treatment
Nazare <i>et al.</i>	Randomized,	Healthy obese	3.76 g/day of CLA	Body	No significant effects on total and HDL	Not reported.

000070

Table 7.5.1-1 Summary of Clinical Studies Conducted with 50:50 CLA Mixtures*						
Reference	Study Design	Subjects	Treatment (Level/ Duration)	Endpoints Evaluated	Results	Adverse Effects
<i>al</i> , 2007	double-blind, placebo-controlled	subjects (n=44, 22 male, 22 female); mean age 28.9 years	50:50 mixture in yogurt, compared to placebo yogurt, for 14 weeks	composition, blood lipids, blood biochemistry.	cholesterol, triglycerides, blood glucose or insulin, plasma leptin, blood cell count, and liver enzyme levels. No significant changes in body composition	
López Román <i>et al</i> , 2007	Randomized, double-blind, placebo-controlled	Healthy obese subjects (n=31; 15 male, 16 female)	3 g/day of CLA 50:50 mixture in milk compared to milk containing no CLA for 4 months	Blood lipids, hematological and biochemical parameters	No significant effects on total, HDL, and LDL cholesterol or triglycerides. No significant effects on hematological and biochemical parameters, including blood glucose levels.	Not reported.

\* Studies considered to be pivotal are presented in **bold**

<sup>a</sup> WPR = within population range, as established in "A Clinical Guide to Laboratory Tests" (Tietz, 1995) (see Attachment 3)

<sup>b</sup> In this study, multiple comparisons were made from the same data set and statistical adjustments to account for these multiple comparisons were not mentioned by the authors (e.g., Bonferroni adjustment). Thus, any statistically significant changes are questionable from a safety standpoint and values are still WPR.

<sup>c</sup> Units for WBC were reported as cells/ $\mu$ L in the text of the publication, but as cells/ $m^3$  in all tables. It is assumed that the units reported in the tables were in error, and that the values cited in the text should be  $\times 10^3$ , as indicated above.

<sup>d</sup> In euglycemic insulin clamp the insulin plasma concentration is raised and maintained at a high level by continuous infusion of insulin. The plasma glucose level concentration is held constant at a basal level by a variable glucose infusion controlled by repeatedly blood sugar measurements. Under these steady-state conditions the glucose infusion rate equals the glucose uptake by all body tissues and is therefore a measure of tissue sensitivity to exogenous insulin.

ALT = alanine aminotransferase; AST = aspartate aminotransferase; BMI = body mass index; CLA = conjugated linoleic acid; CRP = C-reactive protein; FFA = free fatty acid; HDL-C = high-density lipoprotein cholesterol; Lp(a) = Liprotein a, LDL-C = low-density lipoprotein cholesterol; NEFA = non-esterified fatty acids; PAF = plasminogen activating factor, TG = triglyceride, TSH = thyroid-stimulating hormone, VAS = visual analog scale, VLDL = very low-density lipoprotein; WBC = white blood cell

000071

Table 7.5.1-2 Summary of Clinical Studies Using Four Isomer Preparations					
Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Lowery <i>et al.</i> , 1998	24 male body builders	7.2g/day for 6 weeks (CLA isomer composition not reported)	Liver enzymes (AST, ALT), bilirubin, $\gamma$ -glutamyltransferase, blood glucose and serum insulin and lipid levels	No significant effect of CLA multiple isomer mixtures was seen on any of the safety parameters evaluated, including total cholesterol LDL-C and HDL-C	Not reported
von Loeffelholz <i>et al.</i> , 2003	21 novice and experienced athletes	7 g CLA/day for 6 months (providing 420 mg <i>t</i> 8, <i>c</i> 10-, 581 mg <i>c</i> 9, <i>t</i> 11-, 553 mg <i>t</i> 10, <i>c</i> 12-; and 497 mg <i>c</i> 11, <i>t</i> 13-CLA daily)	Body composition, blood lipids.	Fasting serum total cholesterol LDL and HDL cholesterol and triglycerides were unchanged after 6 months of CLA supplementation in trained athletes and HDL-cholesterol and serum triglycerides were unchanged in novice volunteers. However, the novice group experienced an increase in fasting total cholesterol and LDL-cholesterol at 6 months when compared to baseline values. They noted that in novice athletes, total and LDL-cholesterol decreased from baseline to month 3, and increased from baseline to month 6. No placebo group was included for comparison, so it was unclear if effects were related to CLA.	Not reported
Kreider <i>et al.</i> , 2002	24 experienced, trained athletes	6 g/day 4-(+)-isomer CLA or placebo for 4 weeks (CLA isomer composition not reported).	Body composition, muscle strength, liver and lipid profiles, blood glucose and insulin levels, hemoglobin and hematocrit, and markers of metabolism such as creatinine	No changes on any parameter after 4-(+)-isomer CLA administration, including total cholesterol, LDL and HDL-C, triglycerides were noted.	There were no reports of side effects after CLA consumption

000072

**Table 7.5.1-2 Summary of Clinical Studies Using Four Isomer Preparations**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Medina <i>et al.</i> , 2000, Benito <i>et al.</i> , 2001a,b; Zambell <i>et al.</i> , 2000, 2001; Kelley <i>et al.</i> , 2000, 2001	17 healthy, lean female adult volunteers	3.9 g/day of a 4-(+)-isomer CLA preparation or placebo (sunflower oil) for 64 days (providing 881.4 mg t10, c12-, 920.4 mg c11, t13-; 686.4 mg c9, t11-; and 647.4 mg t8, c10-CLA daily).	Lipids, immune parameters, glucose and insulin levels, platelet function, blood coagulation, and fat oxidation	No significant differences were noted in these parameters after treatment.	Authors indicated no safety concerns or adverse events following CLA treatment.
Emken <i>et al.</i> , 2002	6 healthy adult women	Normal diets supplemented with 6 g/d of sunflower oil or 3.9 g/d of 4-(+)-isomer CLA for 63 days (providing 881.4 mg t10, c12-, 920.4 mg c11, t13-; 686.4 mg c9, t11-; and 647.4 mg t8, c10-CLA daily)	Fatty acid metabolism	CLA supplementation had no effect on the metabolism of the deuterium-labeled FA. These metabolic results were consistent with the general lack of a CLA diet effect on a variety of physiological responses previously reported for these women. In conclusion, no effect of dietary CLA was observed, absorption of CLA was less than that of c9-18:1, CLA positional isomers were metabolically different, and conversion of CLA isomers to desaturated and elongated metabolites was low.	Not reported

000073

Table 7.5.1-3 Summary of Clinical Studies with Single CLA Isomer					
Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Smedman <i>et al.</i> , 2004	60 healthy overweight volunteers	The control group in a 50:50 isomer trial received of 4 g/day (3.6 g/day <i>t10,c12</i> CLA isomer for 4 weeks	Lipid peroxidation	Both CLA treatments induced increases in 8-iso-PGF-2 and 15-keto-dihydro-PGF2 $\alpha$ , the largest increase being seen in the pure isomer group ( <i>t10,c12</i> CLA). They also noted that the COX-2 inhibitor suppressed the rise in 15-keto-dihydro-PGF2 $\alpha$ after <i>t10,c12</i> CLA consumption, but not the mixed isomer CLA.	Dose-dependent rise in lipid peroxidation products
Tricon <i>et al.</i> , 2004a,b	49 healthy male volunteers (BMI 18 to 25)	0.59, 1.19 or 2.38 g/day <i>c9,t11</i> CLA isomer or 0.63, 1.26, or 2.52g/day <i>t10,c12</i> CLA isomer for 6-months (6 month crossover, 2 month sequential dose with 6-week washout period)	Lipid metabolism	<p>Glucose and insulin levels were unaffected by these doses. Further, the <i>c9,t11</i> form decreased total cholesterol.HDL, while the <i>t10,c12</i> CLA isomer increased this parameter. Ratios of lipoproteins are not clinical markers and are thus not compared according to standard clinical limits</p> <p>Mean plasma triglycende concentrations were higher after supplementation with the <i>t10,c12</i> CLA isomer, independent of dose compared to the <i>c9,t11</i> isomer. In addition, significant effects on total cholesterol were seen with both isomers. the <i>c9,t11</i> CLA isomer decreased total cholesterol compared to baseline values while the <i>t10,c12</i> CLA isomer increased total cholesterol values compared to baseline values. No effect on plasma HDL cholesterol concentrations was observed with either isomer. As we have seen with other clinical biomarkers, these changes are statistically significant although the mean values still fall WPR and are not considered a safety risk. Furthermore, the lack of a control group in the Tricon study makes it impossible to conclude</p>	No adverse events were reported.

000074

Table 7.5.1-3 Summary of Clinical Studies with Single CLA Isomer					
Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
				that any effects observed for the 2 CLA isomers are truly different from no intervention TNF- $\alpha$ , IL-10, IL-6, IL-1 $\beta$ , and IL-8 levels were not significantly changed, nor were there any effects on CRP There was a dose-response with both isomers in the production of TNF- $\alpha$ and IL-1 $\beta$ . However, there was no significant isomer x dose interaction on the production of these cytokines, indicating no inflammatory issues	
Burdge <i>et al.</i> , 2004			Effects of each pure isomer on incorporation into plasma and cellular lipids	Both isomers were incorporated in a dose-dependent manner into plasma phosphatidylcholine (PC) and cholesteryl ester (CE) Only t10,c12 isomer was enriched in plasma nonesterified fatty acids. Both c9,t11 and t10,c12 CLA were incorporated linearly into PBMC total lipids The highest concentrations of c9,t11 and t10,c12 CLA in PBMC lipids were 3- to 4-fold lower than those in plasma PC and CE These data suggest that the level of intake is a major determinant of plasma and PBMC CLA content, although PBMCs appear to incorporate both CLA isomers less readily	Not reported

000075

**Table 7.5.1-3 Summary of Clinical Studies with Single CLA Isomer**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Burdge <i>et al.</i> , 2005	31 healthy men	Dairy products naturally enriched in c9,t11 CLA by modification of cattle feed to provide 1.43 g/day c9,t11 CLA intake in CLA-enriched group or 0.17 g/day c9,t11 CLA intake in control group for 6 weeks, with a 7-week washout between treatments	Effects of consumption of c9,t11 CLA on plasma and cellular lipids	Consuming the CLA-enriched dairy products increased the c9,t11 CLA concentration in plasma phosphatidylcholine, triacylglycerol and cholesteryl esters, and in peripheral blood mononuclear cells. c9,t11 CLA concentration in plasma lipids was lower after consuming the control products, which may reflect the 2-fold greater c9,t11 CLA content of the commercial products	Not reported.
Malpuech-Brugère <i>et al.</i> , 2004	90 male and female overweight	1.5 or 3g/day of c9,t11 or t10,c12 CLA for 18 weeks	Body composition	Eighty-two volunteers completed this study, most dropped out prior to CLA intervention (run-in period), one dropped out in each CLA t10,c12 group and one was dropped by the investigators due to non-compliance. They found no changes in glucose or insulin levels at any dose, nor did liver echography reveal changes in liver morphology among groups	Authors reported no treatment-related adverse events.
Thijssen <i>et al.</i> , 2005	25 healthy, overweight men and women (BMI 25 to 30)	All subjects consumed a drinkable dairy product containing 3 g of oil that was rich in oleic acid for 6 weeks. For the next 18 weeks, control group (n = 7) continued to use this product while 9 subjects each consumed products with 3 g of purified c9,t11 CLA or t10,c12 CLA for 18 weeks	Plasma incorporation plasma and cellular lipids	For each gram of c9,t11 CLA consumed, the proportion in plasma phospholipids increased by 0.26% and 0.20% for the t10,c12 CLA. The t10,c12 CLA isomer increased plasma TAG levels of conjugated 18:3, whereas c9,t11 CLA increased those of both conjugated 18:3 and 20:3. The authors concluded that incorporation of c9,t11 and t10,c12 CLA into plasma lipids reflects dietary intakes. No other safety parameters were measured.	Not reported.

000076

**Table 7.5.1-3 Summary of Clinical Studies with Single CLA Isomer**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Hasin <i>et al.</i> , 2005	12 lactating women  Because body fat may modify the effect of CLA on milk fat, women were also classified into "high body fat (>30% body fat)" and "low body fat (<30% body fat)" groups	750 mg/d of c9,t11-CLA, t10,c12-CLA, or control (olive oil) for 5 days, with 9-day washout periods between treatments	Milk fat	The authors noted that there was no interaction between treatment and body fat on milk fat, protein or lactose. Likewise, there were no independent effects of treatment or body fat on these variables. Treatment and body fat interacted to influence 14:1, 15:0, 16:1, and 17:0 (p<0.10). No independent effect of body fat was found on any of the milk fatty acids examined, except for c9,t11-CLA and t10,c12-CLA which increased during their respective treatments. These data suggest that neither t10,c12-CLA nor c9,t11-CLA, when consumed individually, decreases milk fat in humans.	Not reported
Naumann <i>et al.</i> , 2006	87 overweight volunteers with LDL phenotype B	A drinkable dairy product not enriched with CLA (placebo, n=34), the same dairy product enriched with 3 g c9,t11 CLA (n=34), or the dairy product enriched with 3 g t10,c12 CLA (n=19) for 13 weeks.	Lipid parameters Effects on plasma glucose and insulin concentrations and on clinical parameters were also examined	Median changes in the proportions of plasma small dense LDL were -2.0% in the control group and -0.1% in the c9,t11 CLA and t10,c12 CLA groups (p=0.981 for the differences between the groups). c9,t11 CLA or t10,c12 CLA did not affect serum concentrations of LDL and HDL cholesterol, or triacylglycerol. Plasma concentrations of glucose and insulin were also unaffected.	Not reported
Risérus <i>et al.</i> , 2004a	25 obese volunteers	3 g/day c9,t11 CLA isomer for 12 weeks	Insulin sensitivity, lipid peroxidation, and body composition	No significant changes in total cholesterol HDL cholesterol, triglycerides or free fatty acids were seen, but a significant decrease in insulin sensitivity and increase in markers of lipid peroxidation was noted	Decrease in insulin sensitivity and increase in markers of lipid peroxidation

000077

**Table 7.5.1-3 Summary of Clinical Studies with Single CLA Isomer**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Risérus <i>et al.</i> , 2002a,b, 2004b	57 obese men	1.95 g/day t10,c12 CLA or 3.4 g/day 50:50 CLA mixture to overweight males with Syndrome X for 12 weeks.	Blood glucose and insulin, insulin sensitivity, Lipid peroxidation, inflammatory markers	<p>The 50:50 CLA mixture lowered HDL-cholesterol. No other significant changes were noted except for a within-group change in VLDL-cholesterol with the t10,c12 isomer but not the 50:50 CLA or the placebo.</p> <p>No significant difference in blood glucose or serum insulin levels but noted a decrease in insulin sensitivity and an increase in lipid peroxidation in the group receiving the t10,c12 CLA isomer. No such affect was observed in the 50:50 treatment group confirming the data from the previous trials. No other inflammatory markers (IL-6, CRP, TNF-<math>\alpha</math>) differed significantly.</p>	Decrease in insulin sensitivity and an increase in lipid peroxidation in the group receiving the t10,c12 CLA isomer
Ramakers <i>et al.</i> , 2005	42 men and women with high risk of CHD (moderately overweight with LDL-phenotype B)	3 g/day of c9,t11 CLA or t10,c12 CLA for 13 weeks	Inflammatory markers (IL-6, IL-8, TNF- $\alpha$ , CRP)	<p>No significant effects on <i>ex vivo</i> lipopolysaccharide (LPS)-stimulated IL-6, IL-8, TNF-<math>\alpha</math> production, and whole blood and plasma CRP were noted.</p> <p>Both CLA isomers induced a specific inflammatory signature as evidenced by cytokine expression profile. The c9,t11 isomer was reported to show more activity in terms of the number of proteins regulated, which suggests enhanced immune function.</p>	Not reported.

000078

**Table 7.5.1-4 Summary of Clinical Studies with Naturally Occurring CLA (e.g., c9, t11 isomer)**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Ritzenthaler <i>et al.</i> , 2005	36 lactating women	A low-CLA containing cheese providing 160mg/day of the c9,t11 CLA isomer, a high-CLA cheese providing 346 mg/day the c9,t11 CLA isomer, or a control for 8 weeks	Milk fat and immune parameters, CVD risk factors	No change in plasma triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol or VLDL-cholesterol among groups were noted. They also measured lymphocyte subsets (T-helper cells, T-cytotoxic cells, B cells and natural killer cells at baseline, 4 and 8 weeks and found no treatment-related effects. Finally, they reported that although c9,t11 CLA content of breast milk was higher in the high CLA group, total milk fat yield, lactose, or protein levels were unaffected by either CLA level. This study shows that CLA in the form of the naturally occurring do not alter milk fat levels in lactating humans.	Not reported.
Desroches <i>et al.</i> , 2005	16 men Age 36.6 +/- 12.4 y; Body mass index 31.2 +/- 4.4 kg/m <sup>2</sup> ;	Subjects were fed each of the 2 experimental isoenergetic diets, providing 15% of energy as protein, 45% as carbohydrates, and 40% as lipids, of which >60% was derived from experimental fats, for 4 wk. The diets differed with respect to the inclusion of a modified butter naturally enriched with CLA (4.22 g CLA/100 g butter fat) by the addition of sunflower oil to the diet of dairy cows versus a control butter that was low in CLA (0.38 g CLA/100 g butter fat). An 8-wk washout period was included between treatments	Body composition, cholesterol	Consumption of the CLA diet induced a significantly ( $p < 0.05$ ) smaller reduction in plasma total cholesterol and in the ratio of total to HDL cholesterol (-0.02 mmol/L and -0.00, respectively) than did consumption of the control diet (-0.26 mmol/L and -0.34, respectively). The authors suggest that a 10-fold CLA enrichment of butter fat does not induce beneficial metabolic effects in overweight or obese men.	No safety concerns were reported in this study.

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**Table 7.5.1-4 Summary of Clinical Studies with Naturally Occurring CLA (e.g., c9, t11 isomer)**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Brownbill <i>et al.</i> , 2005	36 Caucasian, healthy, postmenopausal women, mean age 68.6 years	Dietary CLA	Bone mass density	Dietary intake of CLA (63.1 +/- 46.8 mg, mean +/- SD) was a significant predictor of Ward's triangle BMD (p = 0.040) in a multiple regression model containing years since menopause (18.5 +/- 8.4 y), lean tissue, energy intake (1691 +/- 382 kcal/day) dietary calcium (873 +/- 365 mg), protein (70.6 +/- 18.6 g), fat (57.9 +/- 23.9 g), zinc (19.2 +/- 13.6 mg), and current and past physical activity, with R <sup>2</sup> (adj) = 0.286. Subjects were also divided into groups below (Group 1) and above (Group 2) the median intake for CLA. Group 2 had higher BMD in the forearm, p = 0.042, and higher BMD in the hip, lumbar spine and whole body, however statistical significance was not reached. The authors indicated that these findings indicate dietary CLA may positively benefit BMD in postmenopausal women.	No safety concerns were reported in this study
Malpuech-Brugère <i>et al.</i> , 2004	90 male and female overweight volunteers	1.5 or 3 g/day of c9,t11 or t10,c12 CLA for 18 weeks	Body composition	Eighty-two volunteers completed this study; most dropped out prior to CLA intervention (run-in period), one dropped out in each CLA t10,c12 group and one was dropped by the investigators due to non-compliance. They found no changes in glucose or insulin levels at any dose, nor did liver echography reveal changes in liver morphology.	No treatment-related adverse events reported.

080000

**Table 7.5.1-4 Summary of Clinical Studies with Naturally Occurring CLA (e.g., c9, t11 isomer)**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Thijssen <i>et al.</i> , 2005	25 healthy, overweight men and women (BMI 25 to 30)	All subjects consumed a drinkable dairy product containing 3 g of oil that was rich in oleic acid for 6 weeks. For the next 18 weeks, control group (n = 7) continued to use this product while 9 subjects each consumed products with 3 g of purified c9,t11 CLA or t10,c12 CLA for 18 weeks	Plasma incorporation plasma and cellular lipids	For each gram of c9,t11 CLA consumed, the proportion in plasma phospholipids increased by 0.26% and 0.20% for the t10,c12 CLA. The t10,c12 CLA isomer increased plasma TAG levels of conjugated 18:3, whereas c9,t11 CLA increased those of both conjugated 18:3 and 20:3. The authors concluded that incorporation of c9,t11 and t10,c12 CLA into plasma lipids reflects dietary intakes. No other safety parameters were measured	Not reported.
Hasin <i>et al.</i> , 2005	12 lactating women  Because body fat may modify the effect of CLA on milk fat, women were also classified into "high body fat (>30% body fat)" and "low body fat (<30% body fat)" groups	750 mg/d of c9,t11-CLA, t10,c12-CLA, or control (olive oil) for 5 days, with 9-day washout periods between treatments	Milk fat	There was no interaction between CLA and body fat that produced any significant effects on milk fat, protein or lactose. Likewise, there were no independent effects of treatment or body fat on these variables. Treatment and body fat interacted to influence 14:1, 15:0, 16:1, and 17:0 (p<0.10). No independent effect of body fat was found on any of the milk fatty acids examined, except for c9,t11-CLA and t10,c12-CLA which increased during their respective treatments. These data suggest that neither t10,c12-CLA nor c9,t11-CLA, when consumed individually, decreases milk fat in humans.	Not reported

000081

**Table 7.5.1-4 Summary of Clinical Studies with Naturally Occurring CLA (e.g., c9, t11 isomer)**

Reference	Subjects	Treatment (Level/Duration)	Endpoints Evaluated	Results	Adverse Effects
Tricon <i>et al.</i> , 2006	32 healthy male volunteers, aged 34 to 60 years	1,421 mg/d of c9,t11-CLA or 1,508 mg/d total CLA during CLA phase of intervention or 151 mg/d of c9,t11-CLA or 168 mg/d total CLA during control phase of intervention for 6 weeks with a 7-week washout period between phases	Blood lipid profile, atherogenicity of LDL, and markers of inflammation and insulin resistance, body composition	<p>No significant effects were reported on body weight, plasma glucose, insulin, interleukin 6, soluble vascular cell adhesion molecule 1, soluble intercellular adhesion molecule 1, soluble E-selectin, or serum C-reactive protein concentrations, plasma triacylglycerols, total cholesterol, or noesterified fatty acid concentrations. The authors reported that the change in the ratio of LDL to HDL cholesterol was significantly different, but this is expected not to be of biological significance as the individual levels of HDL-cholesterol and LDL-cholesterol were within the acceptable range and did not significantly change within the study period</p> <p>Also, there was no significant effect on the homeostasis model for insulin resistance or the revised quantitative insulin sensitivity check index</p>	Not reported

000082

As highlighted in these tables, 52 human clinical trials have been conducted on over 2,000 human volunteers (resulting in 66 peer-reviewed publications) with doses ranging from 0.7 to 7.2 g/day for time periods ranging from 5 days (Masters *et al.*, 2002) to 2 years (Gaullier *et al.*, 2005).

Of these 66 publications, 38 utilized the 50:50 isomer mixture. Table 7.5.1-1 summarizes these intervention trials investigating doses from 0.7 to 6.8 g/day of the 50:50 mixture in over 1,700 human subjects for periods of 12 weeks to 2 years. The conclusions outlined in this dossier are based primarily on the studies conducted by Gaullier *et al.* (2004, 2005, 2007), Larsen *et al.* (2006), and Whigham *et al.* (2004). These studies provided CLA (50:50 mixture) at doses ranging from 3.4 g/day for up to 2 years and 6 g/day for up to 1 year and measured numerous endpoints related to safety, including blood lipids, blood glucose and insulin, blood liver enzyme levels and other clinical chemistry parameters, markers of inflammation, and blood pressure. These studies, which demonstrated no significant adverse effects, are therefore considered pivotal to the safety of CLA-Rich Oil. Other shorter-term studies (up to 6 months in duration) providing doses ranging from 0.7 to 6.8 g CLA/day also measured various parameters related to safety, including cardiovascular disease risk, glucose and insulin sensitivity, and maternal milk fat depression, and provide additional support for the safety of CLA-Rich Oil.

Eleven publications represent trials using 4-isomer mixtures. Summarized in Table 7.5.1-2, these 5 intervention trials range from doses of 0.7 g/day to 7.2 g/day for periods of 4 weeks to 6 months in 92 human subjects. No adverse events were reported.

Finally, 14 publications used purified-isomer mixtures. As shown in Table 7.5.1-3, this represents 11 intervention trials ranging in doses of 0.59 to 3.6 g/day for periods of 4 to 18 weeks in at least 400 individuals. Two of these studies did not publish any observations of adverse events. However, 3 studies (Tricon *et al.*, 2004a,b; Malpuech-Brugère *et al.*, 2004) raised issues relating to cardiovascular disease risk and insulin resistance.

Additionally, two intervention trials have been conducted using CLA occurring in foods in 52 volunteers for 8 weeks. These 2 trials resulted in 2 publications and reported no adverse events from CLA-enriched foods. One trial measured CLA intake from foods in 136 volunteers by collecting dietary information

Of the adverse events reported in clinical trials, the most frequent complaints were gastrointestinal in nature (Blankson *et al.*, 2000; Smedman and Vessby, 2001), a common complaint with high fat diets and with people taking dietary supplement capsules. Several studies also reported rapid adaptation to dietary CLA addition. Some investigators suggested that the gastrointestinal upset could be due to either the CLA oil itself or the large number of gelatin capsules administered in most of these trials [up to 12 in the case of Blankson *et al.* (2000)]. Other studies do not report such effects, including the study by Whigham *et al.* (2004) whereby 6 gelatin capsules containing CLA TG form were administered. Gastrointestinal

complaints were evenly distributed between treatment and placebo groups in many studies, suggesting these complaints were not treatment related. Furthermore in the 3-arm study by Gaullier *et al.* (2004), more gastrointestinal effects were reported in the free fatty acid group than in the placebo or CLA TG (triglyceride) group, suggesting that the free fatty acid form caused more GI upset compared with the more natural TG form commonly consumed in the diet. A single oral dosage of approximately 15 g of CLA-Rich Oil (containing up to approximately 9 g of CLA isomers) in bioavailability studies has revealed no adverse events. Additionally, post market surveillance data from Spain suggest that, of consumers of CLA-Rich Oil supplemented dairy and juice products, at up to 4 daily servings (6 g CLA-Rich Oil per day), only 2% of the 1,235 respondents noticed adverse effects. The most common adverse effect was diarrhea with 0.7% of the total number of interviews. Other adverse effects were nausea with 0.2% and dyspepsia with 0.2% (Anadón *et al.*, 2006).

Although some statistically significant changes in various hematological or clinical chemistry parameters were reported in some studies, these changes are not considered to be outside of population range or to represent changes of clinical or toxicological significance.

The following sections provide further detailed discussion of 3 issues that have been raised in certain studies of CLA, namely (1) cardiovascular disease risk; (2) insulin sensitivity; and (3) maternal milk fat.

Several experts in specialized fields provided advice relevant to assessing the safety of CLA relating to these three issues. The information provided by these experts is included as Appendix B.

Based on the totality of the clinical data, the weight of the evidence demonstrates that estimated consumption of 50:50 CLA isomers (CLA-Rich Oil) is safe. In particular, the pivotal studies report safe use of up to 6 g/day for up to 1 year (Larsen *et al.*, 2006 and Whigham *et al.*, 2004) and 3.4 g/day for up to 2 years (Gaullier *et al.*, 2004, 2005, 2007). Further, the weight of the evidence demonstrates no significant effects on cardiovascular parameters (lipid metabolism, markers of inflammation, and markers of oxidative stress), insulin sensitivity and glucose, and maternal milk fat.

### **7.5.2 Cardiovascular Disease**

The following section is divided into 2 sub-sections for clarity. The first sub-section addresses blood lipid parameters, while the second sub-section addresses inflammatory and anti-inflammatory markers related to cardiovascular health. The human clinical trials using the 50:50 CLA mixture will be presented first, followed by studies using the pure isomer and mixed isomer. The range of values for the population that were reviewed in the overall safety section will be referred to with respect to the overall safety for the 50:50 CLA mixtures and their impact on cardiovascular health .

### 7.5.2.1 Lipid Metabolism

#### Studies using 50:50 CLA Mixtures

In 21 human intervention studies from 4 to 104 weeks in duration with doses ranging from 0.7 to 6.8 g/day using several subpopulations (healthy, overweight, obese; athletes, or syndrome X patients), the majority of trials (n=14) reported no effect on lipid parameters, while 3 trials showed a beneficial effect and 4 trials showed statistically significant adverse changes in lipid parameters that remained within population range.

#### *LDL-Cholesterol*

Smedman and Vessby (2001) conducted a randomized, double-blind placebo controlled study in 53 healthy subjects consuming 4.2 g/day of CLA for 12 weeks. They reported a significant increase in LDL-cholesterol as compared to baseline, but not as compared to the olive oil control. This effect on LDL-cholesterol was not observed in 13 other clinical trials with longer interventions at higher doses and with larger populations suggesting that the effect is unique to this study and not an effect of CLA. Since this effect was not observed in any of the other trials, it is concluded that 50:50 mixtures of CLA had no adverse effect on LDL-cholesterol levels.

#### *Apo B*

Apo B is the primary apolipoprotein of low density lipoproteins (e.g., LDL cholesterol) and is responsible for transporting cholesterol to tissues. Apo B is involved in the pathogenesis of atherosclerosis, and several recent prospective studies have shown that ApoB is a better predictor of cardiovascular risk than LDL-cholesterol (LDL-C). The relationship between Apo B and cardiovascular risk has been quantified as follows:

<b>Apo B Level (g/L)</b>	<b>Risk of Coronary Artery Disease</b>
< 1.04	Low
1.04-1.21	Moderate
1.22-1.39	High
1.40	Very High

\* from Connelly *et al*, 1999

Smedman and Vessby (2001) and Moloney *et al.* (2004) were the only 2 clinical trials to measure ApoB levels. Smedman and Vessby (2001) reported significantly increased plasma ApoB concentrations from baseline and from the control group in subjects who consumed 4.2 g/day of a 50:50 CLA isomer mix over a period of weeks. Reported baseline values for ApoB were 100-fold higher for the CLA group; however, the authors reported that there were no statistically significant differences between groups in baseline parameters, suggesting that there

was an error in the reporting of the baseline values. LDL triglyceride concentrations decreased significantly within the control group, which resulted in a significant difference from the CLA group. The authors stated that "when all participants were included in the statistical analyses, there were no significant differences between the changes in serum lipid and apolipoprotein concentrations in the two groups"; however, the authors did not elaborate to provide any context to this statement. LDL cholesterol and total cholesterol also were reported to significantly increase within the CLA group from baseline; however, these changes were not significantly different from the control group. There were no significant changes in other lipid-related markers (HDL-cholesterol, LDL-HDL ratio, triglycerides, VLDL-cholesterol, or Apo A1).

Moloney *et al.* (2004) conducted a randomized double-blind clinical investigation in 32 type 2 diabetics with a test dose of 3 g/day and reported that, with the 50:50 mixture, VLDL ApoB levels increased after CLA administration. However, these values were still on the lower end of the population range, and significant only between groups at the very end of the study. In addition, ApoB levels in the control group were shown to decrease (using a mixture of palm and soya oil), which has previously been shown to lower cholesterol, suggesting that the placebo may not have been an optimal comparison for lipid parameters. Because of changes in both CLA and placebo groups, it is difficult to interpret any risk associated with CLA. It is noted that the Canadian Cardiovascular Society considers an Apo B level of <0.9 g/L (or <900 µg/mL) to be optimal in patients at high cardiovascular risk. Comparison of these values to those of the subjects in the Moloney *et al.* (2004) trial, who had levels between 39 and 49 µg/mL, indicates little cause for concern.

#### *HDL-Cholesterol*

Three studies (Mougios *et al.*, 2001; Blankson *et al.*, 2000; Song *et al.*, 2005) reported decreases in HDL-cholesterol. In a double-blind study, 2 groups of healthy volunteers received either supplements of a 1:1 mixture of the c9,t11 and t10,c12 CLA-isomers, or soybean oil (control) for a period of 8 weeks (Mougios *et al.*, 2001). The dose was 0.7 and 1.4 g/d for the first and the second 4 weeks, respectively. Blood was sampled at baseline and after 4 and 8 weeks for analysis of serum total and HDL cholesterol as well as triglycerides. The fatty acid composition of the different serum lipid classes was examined as well. The only significant change in the CLA groups was a decrease in HDL cholesterol in weeks 4 and 8. However, the change was significant within-group only and not when compared to the placebo group. The apparent decrease of HDL cholesterol may, therefore, not be a treatment-related effect, but the indirect consequence of a high baseline value. This interpretation is supported by the fact that the increased CLA dose, which was applied from week 4 to 8, did not affect HDL cholesterol levels. No significant differences were found between the two groups at the three sampling times. However, the number of subjects in this study was relatively small (CLA group: 10; placebo group: 12). This effect is not evident in pivotal trials with a much greater power per treatment group.

Blankson *et al.* (2000) conducted a double-blind, randomized, dose response trial on 60 human volunteers using 1.7, 3.4, 5.1, or 6.8 g/day 50:50 CLA mixture or 4.5 g/day olive oil placebo for 12 weeks. Statistically significant reductions in total cholesterol, HDL and LDL-cholesterol were reported in the CLA groups (within-group change from baseline); however, these changes were deemed by the authors not to be clinically important. The magnitude of risk in this study is difficult to evaluate since Blankson *et al.* (2000) only reported changes within the groups and not actual mean values. Furthermore, the authors only assessed the within-group changes from baseline, and did not compare the results for each CLA group to those of the placebo group. Data presented for the placebo group also demonstrate within-group changes in total cholesterol, HDL cholesterol, and LDL cholesterol, which were not significant from baseline within the placebo group. Nonetheless, without any between-group comparisons, it is not possible to adequately interpret these data

Song *et al.* (2005) investigated the effects of 3 g/day 50:50 CLA mixture compared to placebo (sunflower oil) on 28 healthy adult human volunteers for 12 weeks using a double-blind, randomized study design. They found that the CLA supplement did not affect plasma total cholesterol, LDL-cholesterol, or plasma triglyceride concentrations. HDL-cholesterol was decreased slightly but significantly by the CLA supplement but not the reference oil, while LDL-cholesterol was not altered by either supplement. The decrease in HDL-cholesterol was still within the population range.

The sample size per group for each of the three trials (Mougios *et al.*, 2001; Blankson *et al.*, 2000; Song *et al.*, 2005) was <11, much smaller in comparison to the other studies that found no effect on HDL cholesterol [(Gaulhier *et al.* (2004) (n=180), Kamphuis *et al.* (2003a,b) (n=54), Noone *et al.* (2002) (n=51), Moloney *et al.* (2004) (n=32) and Atkinson (1999) (n=80), Whigham *et al.* (2004) (n=60), Taylor *et al.* (2006) (n=40)] In addition, the HDL-cholesterol levels observed in all studies are within the population range (WPR), and greater than 40 mg/dL, the recommended level (in the U S.). Thus, the weight of evidence indicates that 50:50 CLA mixtures do not have a significant adverse effect on HDL cholesterol. Some studies have even suggested a potential benefit. For example, Moloney *et al.* (2004), as part of their study on the effects of 3 g/day 50:50 CLA mixtures on glucose and insulin parameters in type 2 diabetic volunteers, also measured blood lipid panels. They noted that after 8 weeks of CLA supplementation, total HDL-cholesterol concentrations increased significantly while fasting serum cholesterol, triglycerides and VLDL cholesterol were unchanged. According to an NIH Consensus Statement (1993), for every 0.03 mmol/L increase in HDL concentrations, there is a 2 to 3% reduction in coronary heart disease risk. Thus, the 8% increase in HDL-cholesterol seen in the CLA group could result in a 7.3 to 11% risk reduction in this population. This may be significant since increasing HDL-cholesterol promotes reverse cholesterol transport, a lipid metabolism phenomenon that is impaired in type 2 diabetics. Fibrinogen levels, an increase of which is associated with increased risk of coronary heart disease (CHD), were also lowered in

the CLA group. The authors suggest a clinical benefit of CLA 50:50 mixtures in type 2 diabetics with respect to lipid metabolism.

#### *Triglycerides (TG)*

In a 1-year, randomized, double-blind, placebo-controlled safety study of 6 g/day 50:50 CLA mixture, Whigham *et al.* (2004) found no significant changes in fasting serum total cholesterol or LDL-cholesterol values. Although the clinical trial was not designed to investigate changes in TG, at weeks 28 and 52, CLA subjects had significantly higher TG ( $154.7 \pm 11.4$  vs.  $114.9 \pm 12.3$  mg/dL,  $p \leq 0.02$ ). Despite the statistical significance of the changes, values remained within population range. In addition, the CLA subjects started with higher TG and white blood cell (WBC) counts at baseline (in both cases  $p = \text{NS}$  compared to the placebo), and the patterns of change with treatments were similar. Therefore, the differences at week 28 were felt not to be clinically meaningful. The changes that existed between the groups at baseline may be responsible for the significant difference between the groups at week 52.

Notably, other authors (Berven *et al.*, 2000; Mougios *et al.*, 2001; Smedman and Vessby, 2001; Song *et al.*, 2005; Larsen *et al.*, 2006; Taylor *et al.*, 2006; Gaullier *et al.*, 2007; López Román *et al.*, 2007; Nazare *et al.*, 2007) have reported that CLA treatment had no significant adverse effects on triglyceride values, with some (Noone *et al.*, 2002; Moloney *et al.*, 2004) studies showing a potential beneficial (lowering) effect.

#### *Lipoprotein (a) [Lp(a)]*

As part of their randomized, double blind, placebo-controlled study on the long-term safety of 3.4 g/day 50:50 CLA mixtures, Gaullier *et al.* (2004, 2005), noted a slight, but statistically significant increase in Lp(a) with both the FFA and TG forms of CLA when paired t-tests were run on these data at 12 and 24 months. It should be noted that the variances in Lp(a) levels exceeded the mean values and thus caution is warranted when interpreting these data. Although when these data are compared to the baseline values, they are statistically significantly different, the data are clearly within the population range.

Similarly, Gaullier *et al.* (2007) reported that after 6 months with CLA supplementation there were significant increases from baseline in Lp(a) levels in both CLA group ( $P \leq 0.017$ ) and placebo group ( $p \leq 0.020$ )

Lp(a) was measured in 2 other smaller studies, where it was shown to be unaffected by CLA. Blankson *et al.* (2000) conducted a double-blind, randomized, dose response trial on 60 human volunteers using 1.7, 3.4, 5.1, or 6.8 g/day 50:50 CLA mixture or 4.5 g/day olive oil placebo for 12 weeks. Regardless of the dose, Lp(a) levels were unaffected by CLA. Since the authors did not cite actual clinical means, but rather differences within each group, it is difficult to evaluate the significance of these findings. Likewise, Berven *et al.* (2000) administered 3.4 g/day CLA 50:50 mixture or a placebo control (4.5 g/day olive oil) to 60 overweight or obese human

volunteers for 12 weeks. They also measured blood lipids, and found no significant changes in Lp(a) levels or any other parameter, all values remained WPR.

The evidence therefore does not support a conclusion that 50:50 CLA mixtures have an adverse effect on Lp(a) levels in humans.

#### Studies Using Multiple CLA Isomer Mixtures

Five studies have been conducted in humans using multiple CLA mixtures that resulted in 11 publications. Of these 11 publications, 4 have reported on lipid parameters (3 individual studies and 2 reports from one trial). Only one of these studies that measured lipid parameters reported an adverse influence of CLA multiple isomer. von Loeffelholz *et al.* (2003) noted that in novice athletes, total and LDL-cholesterol decreased from baseline to month 3, and increased from baseline to month 6. However, the trial design was limited as it was not placebo-controlled and contained only 7 volunteers. Despite these limitations, the LDL-cholesterol levels remained within population range.

No other studies using the 4-isomer mixture produced clinically relevant or statistically significant effects on blood lipid parameters.

#### Studies Using Single CLA Isomers

Six studies have measured the effects of single isomers of CLA on lipid parameters. Tricon *et al.* (2004a,b) examined the effects of 3 doses of *c9,t11* CLA (0.59, 1.19, or 2.38 g/day) or *t10,c12* CLA (0.63, 1.26 or 2.52 g/day) on blood lipid panels in healthy men during an 8-week intervention period. They found opposing effects of the 2 isomers. For example, mean plasma triglyceride concentrations were higher after supplementation with the *t10,c12* CLA isomer, independent of dose compared to the *c9,t11* isomer. In addition, significant effects on total cholesterol were seen with both isomers: the *c9,t11* CLA isomer decreased total cholesterol compared to baseline values while the *t10,c12* CLA isomer increased total cholesterol values compared to baseline values. No effect on plasma HDL cholesterol concentrations was observed with either isomer. As seen with other clinical biomarkers, these changes are statistically significant although the mean values still fall within population ranges and are not considered a safety risk. Furthermore, the lack of a control group in the Tricon study makes it impossible to conclude that any effects observed for the 2 CLA isomers are different from no intervention. The lack of randomization of the dose assignment makes it impossible to determine the true nature of the observed (or the lack thereof) dose-response effect. Other studies (Risérus *et al.*, 2004c; Desroches *et al.*, 2005; Ritzenthaler *et al.*, 2005; Naumann *et al.*, 2006, Tricon *et al.*, 2006) reported significant changes in blood lipids that are outside the population ranges.

## Discussion

In addition to the considerations discussed above, it should be noted that adverse CVD lipid biomarker effects may be attributable to the fact that subjects were all free-living volunteers. Thus, lifestyle, diet, and seasonal variation during long-duration trials all may have impacted these changes, independent of treatment effect. Also, these effects are not supported by the animal studies discussed in Appendix C, Section C.1.

Conclusions about the effect of CLA on Lp(a) could not be drawn based on the available data. While two studies on Lp(a) reported statistically significant changes from baseline, the biological significance of the results of these studies remains to be established. Considering the weight of the evidence, these studies do not indicate an increased risk of CVD.

Information from an expert in the field, Dr. W. Virgil Brown, Professor of Medicine, Emory University School of Medicine, Atlanta Department of Veterans Affairs Medical Center, is provided in Appendix B.

The weight of the evidence from the most relevant human trials demonstrates that CLA does not adversely affect lipid parameters that may be associated with CVD risk

### 7.5.2.2 *Markers of Inflammation*

Derangements in cardiovascular health have been correlated to inflammation and changes in platelet surface chemistry that may influence plaque formation. Ten studies have measured some aspect of inflammatory or anti-inflammatory biomarkers related to 50:50 mixtures, as did one trial related to a 4 isomer mixture and two trials using pure isomers. The most common biomarkers used in these 11 studies attributed to cardioprotective or cardiovascular risk were IL-1 $\beta$ , TNF- $\alpha$ , C-reactive protein, IL-6, IL-2 and IL-10. It is prudent to also include the human studies that measured cytokines and other biomarkers of inflammation in these safety analyses.

Smedman *et al.* (2005) reviewed indicators of inflammation from the randomized, double-blind placebo controlled study in 53 healthy subjects consuming 4.2 g/day of CLA for 12 weeks that was conducted earlier by Smedman and Vessby (2001). They measured CRP, TNF- $\alpha$ , TNF- $\alpha$  1 and 2 receptors and vascular cell adhesion molecule-1 (VCAM-1), and found that there was a statistically significant rise in CRP levels between control and CLA groups. It should be noted that when the control group began the study the median CRP level was 1.76 mg/L, and that when the CLA group began the study the median CRP level was 3.27 mg/L. The authors did not indicate whether baseline values were statistically different, thus the relevance of these results are questionable. No other inflammatory marker changed significantly. Steck *et al.* (2007) investigated the efficacy and safety of supplementation of 3.2 and 6.4 g/day of CLA for a period of 12 weeks in obese subjects and reported no significant changes in inflammatory markers at the 3.2 g/day dose. In the 6.4 g/day group, CRP and IL-6 increased significantly from baseline; however, values remained within population range. The absolute values of IL-6

and CRP did not differ significantly between groups, and changes were almost completely attributable to different baseline values of the 3 groups. Six additional studies (Albers *et al.*, 2003; Gaullier *et al.*, 2004; Moloney *et al.*, 2004; Nugent *et al.*, 2005; Ritzenthaler *et al.*, 2005; Song *et al.*, 2005) all show no adverse effects on inflammatory markers such as TNF- $\alpha$ , IL-6 and IL-10. Collectively, these studies show that 50:50 CLA mixtures do not adversely influence inflammatory biomarkers of cardiovascular health, especially CRP. Thus, these data support the safety of CLA 50:50 mixtures on inflammatory markers of CVD.

Four studies have been conducted in humans using multiple CLA mixtures that resulted in 10 publications. Of these 10 publications, 7 have reported on cytokines and interleukins (specifically TNF- $\alpha$ , IL-2 and IL-1 $\beta$ ) parameters (7 reports emanate from one trial). Only the *t*10,*c*12 CLA isomer in the Risérus study (Risérus *et al.*, 2002a,b, 2004b) showed an increase in CRP levels, while the Moloney *et al.* (2004) trial showed a 15% (not significant) decrease in CRP levels that accompanied a significant decrease in fibrinogen levels. This suggests an isomer-specific effect of the *t*10,*c*12 since the same response was not apparent for the 50:50 mixture when used in the same study design. However, the Tricon study showed no effect at any dose of either isomer when given to healthy men. Taken together (and considering the fact that lipid levels were not adversely affected by CLA administration), this does not indicate a cardiovascular risk associated with inflammation or otherwise for CLA 50:50 mixtures and may indicate some benefit for the attenuation of thrombosis in type 2 diabetics [with the reduction of fibrinogen levels seen in the Moloney *et al.* (2004) trial] (Kelley *et al.*, 2000, 2001; Medina *et al.*, 2000; Zambell *et al.*, 2000, 2001; Benito *et al.*, 2001a). There were no significant differences in TNF- $\alpha$ , IL-1 $\beta$ , or IL-2 secretion in stimulated human white blood cell and, no changes in platelet function or cytokine production

In a separate study divided into 3 publications, Risérus and colleagues administered 1.95 g/day *t*10,*c*12 CLA or 3.4 g/day 50:50 CLA mixture to overweight males with metabolic syndrome for 12 weeks (Risérus *et al.*, 2002a,b, 2004b). They reported no significant difference in TNF- $\alpha$  or IL-6 after CLA administration. However, they noted a significant ( $p \leq 0.007$ ) increase in CRP levels after administration of the *t*10,*c*12 isomer, but not the 50:50 CLA mixture. These 50:50 CLA mixture results agree with the data from Song *et al.* (2005) and from Moloney *et al.* (2004), demonstrating no effect of CLA on CRP, suggesting that this may be an isomer specific effect related to the *t*10,*c*12 isomer when it is present in a purified form.

It is likely that the transient increase in insulin resistance caused by the 10, 12 isomer in the syndrome X population is linked to the increase in CRP. Risérus *et al.* (2002a,b, 2004b). It must be further noted that CRP levels may rise at the onset of an infection by several orders of magnitude, which may lead to a statistically significant increase for an entire group when only one individual is affected. Thus, single biomarker measurements must be considered cautiously and should be viewed in the context of the overall findings.

Tricon *et al.* (2004a,b) also investigated the effects of different doses of either c9,t11 or t10,c12 CLA isomers on various cytokines and inflammatory biomarkers. They randomized 49 healthy men into groups who subsequently consumed 1, 2, or 4 capsules containing 0.59, 1.19, or 2.38 g/day c9,t11 CLA or 0.63, 1.26, or 2.52 g/day t10,c12 CLA for consecutive 8-week periods. They measured TNF- $\alpha$ , IL-10, IL-6, IL-1 $\beta$ , and IL-8 levels, and found that there were no significant effects of either isomer on the production of these cytokines. However, there was a dose-response with both isomers in the production of TNF- $\alpha$  and IL-1 $\beta$ . There was no significant 'isomer x dose' interaction on the production of these cytokines, indicating an absence of inflammation. They also measured CRP and noted that none of the doses or isomers altered serum CRP levels. Similarly, Ramakers *et al.* (2005) reported no significant effects on *ex vivo* lipopolysaccharide (LPS)-stimulated IL-6, IL-8, TNF- $\alpha$  production, or whole blood and plasma CRP, in subjects with high risk of developing coronary heart disease who consumed 3 g/day of either c9,t11 CLA or t10,c12 CLA for 13 weeks.

## Discussion

Overall, the human studies using the 50:50 mixture show no effect on biomarkers of inflammation related to cardiovascular disease risk. Risérus *et al.* (2002a,b, 2004b) reported that single isomers showed that the t10,c12 CLA isomer increased CRP levels, but not the 50:50 CLA mixture. Tricon *et al.* (2004a,b) using healthy men showed no adverse effects of either isomer on CRP levels. All other parameters remained within population range for these biomarkers. The animal studies (Section 7.4) also support the conclusion of a lack of effect of 50:50 CLA mixtures on inflammatory biomarkers, except for the Yang *et al.* (2000) study on lupus mouse models. However, their follow-up study indicated a benefit of CLA to lupus mice as it increased lifespan and reduced cachexia. Overall, these *in vitro* and animal studies support the human clinical studies that show no overall negative effect from 50:50 CLA mixtures on inflammatory biomarkers related to cardiovascular risk.

Indirect supporting evidence of the safety of CLA mixtures with regard to CVD can be further assured by the results of the work by Wilson *et al.* (2005). They performed a prospective observational cohort study of 1,949 men and 2,497 women without CVD from the Framingham Heart Study. The investigators assessed traditional risk factors such as age, gender, smoking, lipid levels, and inflammatory biomarkers such as CRP. After adjusting for age and gender and multiple variables, they found that a rise in traditional risk factors and CRP levels indicated CVD risk, whereas elevated CRP levels alone provided no further prognostic information beyond traditional office examination risk factor assessment to predict future CVD events.

### 7.5.2.3 Markers of Oxidative Stress

Oxidative stress is a term commonly used to denote the imbalance between increased exposure to free radicals, principally derived from oxygen, and the anti-oxidative defense mechanisms of the body. Such an imbalance plays a pivotal role in many pathological conditions such as

cancer and cardiovascular disease. Lipids are a major target of free radical attack, which induces lipid peroxidation. Lipid peroxidation is a self-propagating phenomenon terminated by anti-oxidants.

A condition of oxidative stress or inflammatory stimuli can result in 2 different scenarios. First, free radicals generated during oxidative stress may attack esterified arachidonic acid (and other polyunsaturated fatty acids), eventually resulting in the formation of a range of esterified F<sub>2</sub>-isoprostanes, such as 8-iso-PGF<sub>2α</sub>. The F<sub>2</sub>-isoprostanes may then be released from the phospholipid through the action of phospholipases (Morrow *et al.*, 1992; Li *et al.*, 1999; Bessard *et al.*, 2001). Secondly, oxidative stress may trigger the activation of phospholipase 2, subsequently releasing arachidonic acid from the *sn*-2 position on the phospholipid. This may be followed by conversion to a series of potent eicosanoids through the action of cyclooxygenase or lipoxygenase forming a host of highly biologically active compounds (Klein *et al.*, 1997). Prostaglandins may subsequently be metabolized, forming 15-keto-dihydro-PGF<sub>2α</sub> and other metabolites (Granström and Samuelsson, 1971). F<sub>2</sub>-isoprostanes also may be metabolized by a number of metabolic pathways, including β-oxidation. β-Oxidation in the peroxisomes to form 2,3-diinor (DIN) is thought to be the preferred pathway (Iannone *et al.*, 2007, In Press).

Free radical induced peroxidation of membrane lipids can be very damaging because it may lead to alterations in the biophysical properties of membranes, such as fluidity and membrane-receptor function, which in turn may impair cellular function. Measurements of products of lipid peroxidation, including F<sub>2</sub>-isoprostanes, malondialdehyde, lipid hydroperoxides, conjugated dienes and protein carbonyls, have been commonly used to assess oxidative stress. These resulting products of lipid peroxidation can be measured in body fluids such as urine and blood. One of the indirect methods to measure lipid peroxidation is the detection of F<sub>2</sub>-isoprostanes, 8-iso-prostaglandin F<sub>2α</sub> and 15-keto-dihydro-prostaglandin F<sub>2α</sub>, enzymatic and non-enzymatic (free radical induced) products of arachidonic acid, respectively.

Increased levels of urinary isoprostanes have been detected in most diseases involving inflammation and oxidative stress. However, detectable levels of F<sub>2</sub>-isoprostanes have also been found in all normal animal and human biological fluids as well, indicating that there is a certain level of ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses, even in the normal state (Roberts and Morrow, 1997). Since these biomarkers are research markers, no clinical laboratory population ranges have been assigned. Further, no threshold has been established at which the level of F<sub>2</sub>-isoprostanes would indicate an increased oxidative stress.

Since human clinical trials using CLA have demonstrated increases in levels of isoprostanes, such a response appears to contradict the anti-inflammatory and anti-oxidant properties of CLA. It was therefore important to determine whether there was other evidence of oxidative stress triggered by CLA treatment or whether the increase of urinary isoprostanes may be explained

by mechanisms other than lipid peroxidation. Evidence is presented below to establish that the use of 50:50 CLA does not increase lipid peroxidation.

Most of the work on measuring F<sub>2</sub>-isoprostanes as markers of oxidative stress has used the electron capture negative ionization gas chromatography-mass spectrometry (GC-MS) method (Montuschi *et al.*, 2004). The other method that has been used is the radioimmunoassay method (University of Uppsala, Sweden). Two groups suggest that comparisons of these 2 methods are problematic since they do not correlate well and measure different classes of compounds (Proudfoot, *et al.*, 1999; Bessard *et al.*, 2001). In fact, Lawson *et al.* (1999) reported that up to 64 isomers in four structural classes can be generated by free radical attack on arachidonic acid. Thus, investigators such as Cracowski suggest that immunoassays should be considered as semi-quantitative indices of F<sub>2</sub>-isoprostanes rather than definitive indices of relative risk (Cracowski *et al.*, 2002). Some researchers have begun to use a newer (non-validated) rabbit polyclonal antibody, but the specificity of this immunoassay is questionable. Given these methodological discrepancies, it cannot be excluded that CLA, or one of its metabolites or catabolites is actually being measured instead of isoprostanes. The inherent variability and flux of isoprostane levels throughout the day is also an issue. Like many metabolites, the levels of isoprostanes can vary within one day and from day to day (up to 40%) and from events unrelated to treatment (such as smoking, pregnancy, consuming other dietary components or even changing altitude) (Dietrich *et al.*, 2002).

Thompson *et al.* (2005) reported on urinary isoprostane output levels and suggests that they vary considerably between days. To circumvent highly variable data and potential misinterpretation of results seen with single sampling and single-day multiple sampling methods, they have altered their sampling methodology to include sequential first-voids (*e.g.*, first urine collection of the day) for 3 consecutive days as pooled samples containing antioxidants to inhibit further oxidative generation. In a field of low oxidative stress interventions (*e.g.*, fruit and vegetable intake and oxidative markers), high levels of variability inherent in this biomarker questions the rigidity of sampling and interpreting data. Thus, isoprostanes should ideally be measured on several consecutive days to adequately determine the average isoprostane level in an individual. To date, such sampling methodology has not been utilized in the current CLA studies and so only limited conclusions may be drawn from these studies. Another major shortcoming of the method used in the CLA clinical trials is that only free F<sub>2</sub>-isoprostanes have been measured. It has been noted by several authors that the majority of F<sub>2</sub>-isoprostanes are esterified in phospholipids rather than in free form (Li *et al.*, 1999, Roberts and Morrow, 2000; Bessard *et al.*, 2001). Due to the possible interference of CLA with the release and metabolism of F<sub>2</sub>-isoprostanes, it is necessary to measure both free and esterified levels (in plasma) of F<sub>2</sub>-isoprostanes to adequately address whether CLA indeed increases the formation of F<sub>2</sub>-isoprostanes.

Finally, correct statistical methods and interpretation of results is critical to understanding the safety of CLA for human use. This will be discussed in detail in the text below.

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 addition to potential problems with methodology and statistical analysis, much evidence exists that the observed increase in isoprostanes is unlikely to be the result of oxidative stress in the case of CLA.

Several investigations (Basu *et al.*, 2000a,b; Risérus *et al.*, 2002b, 2004a,b; Smedman *et al.*, 2004, Taylor *et al.*, 2006) have measured urinary prostaglandin  $F_{2\alpha}$  isoprostanes after administration of the 50:50 CLA mixture or the CLA isomers using a newly developed radioimmunoassay for 8-iso-prostaglandin  $F_{2\alpha}$  and 15-keto-dihydro-prostaglandin. Doses ranges were from 3 to 4.2 g/day for 4 to 12 weeks and included healthy, overweight and Syndrome X patients. The variation was widespread with a range from 35 to 405% change over baseline. Given that the doses and durations of these studies did not greatly differ (*e.g.*, 3 to 4.2 g/day for 4 to 12 weeks), it may be concluded that methodological comparisons, sampling, and handling differences accounted for at least part of the large variation.

Basu *et al.* (2000b) reported that supplementation with CLA 50:50 mixtures increased urinary 8-iso-prostaglandin  $F_{2\alpha}$  (morning and 24-hour collection), and increased 15-keto-dihydro-prostaglandin  $F_{2\alpha}$ , but had no effect of *p*-MDA or *s*- $\alpha$ -tocopherol levels, markers of oxidation and antioxidant activity. It should be noted, however, that the baseline values appear to be higher for the CLA group compared to the placebo group, although the authors do not account for significant differences at baseline with any Chi-square values. It should also be noted that the plasma 8-iso-prostaglandin  $F_{2\alpha}$  analysis contained only 63% of the subject population due to hemolysis in the samples rendering them unusable for an radioimmunoassay and thus raising doubts about the quality and interpretation of data from the remaining samples that were analyzed. Furthermore, if antioxidants were not added to the samples, then auto-oxidation could have occurred after sampling (this was not detailed in the methods and is also a major issue for urine samples) The authors concluded that, since the 8-iso-prostaglandin  $F_{2\alpha}$  changes were much more pronounced than the 15-keto-dihydro-prostaglandin  $F_{2\alpha}$ , the CLA mixtures were likely to have a more direct effect on non-enzymatic lipid peroxidation pathways.

Risérus *et al.* (2002b) examined the effects of CLA on urinary isoprostanes in abdominally obese men with metabolic syndrome. Subjects received 3.4 g/day of a commercial CLA mixture (CLA 50:50 mix) and a CLA preparation containing mainly the *t*10,*c*12 CLA isomer or olive oil (control) for a period of 12 weeks in this double-blind, placebo-controlled trial. Urinary isoprostane levels and serum tocopherol levels were measured at baseline and at week 12 of the study using methods described by Basu *et al.* (2000a,b) studies. The investigators reported that the 50:50 CLA mixture elicited an increase in urinary 8-iso-prostaglandin  $F_{2\alpha}$  and 15-keto-

dihydro-prostaglandin  $F_{2\alpha}$  levels compared to the control, while the *t10,c12* CLA isomer elicited an even greater response. However, the authors did not report actual means, as in previous studies, but instead relied on changes from baseline in graphical representation and correlation coefficients. No other inflammatory biomarkers (IL-6, CRP, TNF- $\alpha$ ) were affected by CLA administration

Smedman *et al.* (2004) investigated the effect of CLA supplementation in 60 healthy overweight humans. The study investigated 2 different CLA preparations, one with the 50:50 CLA mixture and one with mainly the *t10,c12* CLA isomer. There was no placebo oil in this trial. The study also investigated the effects of  $\alpha$ -tocopherol and a cyclo-oxygenase-2 inhibitor on the effects of CLA and CLA *t10,c12* isomers. 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 CLA *t10,c12* group, suggesting an isomer-specific effect. The levels of isoprostanes were not decreased by supplementation with  $\alpha$ -tocopherol, which would be expected if the increases were due to oxidative stress (Rosen *et al.*, 1998; Jialal *et al.*, 2002). The levels of 15-keto-dihydro-PGF $_{2\alpha}$  were not affected by supplementation with a COX-2 inhibitor (rofecoxib), apart from a smaller increase in subjects receiving CLA *t10,c12* and the COX-2 inhibitor (Smedman *et al.*, 2004).

Taylor *et al.* (2006) studied the effects of 4.5 g/day 50:50 CLA mixture or olive oil placebo on fat, lipid, blood cell and oxidative parameters in 40 healthy overweight white men in a randomized, double-blind, matched pair experimental design. The authors noted that plasma F2-isoprostanes increased after CLA administration [-36  $\pm$  95 placebo *versus* 94  $\pm$  200 pg/mL CLA;  $p \leq 0.042$ ] However, given the extreme variability in these samples, it is difficult to draw any accurate conclusions from this study. Other markers of inflammation, such as TNF- $\alpha$  and adiponectin were not significantly different from the placebo. Thus the wide variation seen in the responses may either be due to methodology, variability, or inadequate consecutive sampling to decrease sampling "noise" of the CLA treatment. It is also not clear from the methodology what extraction method was used and whether exogenous anti-oxidants were spiked to the urine and blood samples. Lipids are rapidly oxidized and if anti-oxidants are not added directly after sampling, isoprostanes might have been formed after sampling due to oxidation, thus being an artifact of the method

## Discussion

Of the 7 published human trials that measured oxidative markers such as isoprostanes, there is a demonstrated wide variation in changes both before and after CLA administration. All have demonstrated a significant increase in isoprostanes following CLA supplementation. If comparing the percent changes to the reported daily variation (40% as reported by Helmersson and Basu, 2001), the 15-keto form showed about half (3 out of 6 trials) that were generally within the normal variance for isoprostanes. Although the 8-iso form has much higher levels post-CLA administration, the trial by Risérus *et al.* (2004a), who administered 3 g/day of the

c9,t11 CLA isomer for 12 weeks showed a modest increase that was nearly identical to the average daily changes reported for isoprostanes in individuals receiving no dietary supplementation (Helmersson and Basu, 2001).

The question of whether increased F<sub>2</sub>-isoprostanes reflect a safety concern for CLA depends on whether the increase results from increased oxidative stress or a metabolic interaction. An association between isoprostanes and CVD risk remains to be conclusively established, since no clinical laboratory population range has been established at which the level of F<sub>2</sub>-isoprostanes indicates increased oxidative stress. Nevertheless, it is unlikely that CLA induces oxidative stress itself, because no biomarkers other than isoprostanes increased following CLA consumption.

Indeed, it is likely that CLA competes with F<sub>2</sub>-isoprostanes for the same metabolic pathway, as both undergo  $\beta$ -oxidation in the peroxisomes (Basu, 1998; Chiabrando *et al.*, 1999; S eb edio *et al.*, 2001; Banni *et al.*, 2004). A higher affinity for CLA would be reflected by an increase in free F<sub>2</sub>-isoprostanes levels, both in plasma and in urine. The increase in F<sub>2</sub>-isoprostane levels would be the result of a decrease in F<sub>2</sub>-isoprostane catabolism, rather than an increase in oxidative stress. This is further supported by the *in vitro* and *in vivo* results of Iannone *et al.* [2007-In press] in which the levels of the peroxisomal  $\beta$ -oxidation product of CLA, CD16:3, increased, and 2,3-dinor (DIN), the  $\beta$ -oxidation product of 8-iso-PGF<sub>2 $\alpha$</sub> , decreased, in the livers of rats fed CLA and treated with CCl<sub>4</sub>, a known pro-oxidant, and in human fibroblast cell cultures incubated with CLA and 8-iso-PGF<sub>2 $\alpha$</sub> . This data is discussed in more detail in Appendix C Sections C.1.3.1 and C.1.3.2.

The precursors of isoprostanes, arachidonic acid and prostaglandins, have been demonstrated in many studies to be decreased by CLA. In addition, treatment with the anti-oxidant tocopherol did not affect the levels of isoprostanes, which seems contradictory if oxidative stress caused the increase in isoprostanes. 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<sub>2</sub>-isoprostanes. Most likely, the increased availability of F<sub>2</sub>-isoprostanes appears to be the result of an inhibition of their metabolism by competition with CLA.

Accordingly, based on the weight of the evidence, the observed effects on F<sub>2</sub>-isoprostane levels do not represent a harmful effect of CLA preparation under its intended conditions of use.

Information from experts in the field, Prof. Giovanni Davi, Universita' Degli Studi Di Chieti "G D'annunzio", Centre of Excellence on Aging; and Prof. Dr J urgen Schrezenmeir; is provided in Appendix B.

#### 7.5.2.4 *Endothelial Function*

A small number of studies have evaluated the potential effects of 50:50 CLA isomer mixtures on endothelial function. Taylor *et al.* (2006) reported impaired endothelial function, as

demonstrated by a significant decrease in brachial artery flow-mediated dilatation, measured using the wrist cuff technique, in healthy overweight men who consumed 4.5 g/day of a 50:50 CLA mixture for a period of 12 weeks. This study was marked by extreme variability in samples thus preventing any reliable conclusions to be drawn. In contrast, Raff *et al.* (2006) reported no significant effects on arterial elasticity, measured by a volume-oscillometric technique, in healthy lean men who consumed 4.7 g/day of a 50:50 CLA isomer mix for a period of 5 weeks. Similarly, in a longer-term study in which healthy overweight subjects consumed 3.2 g/day of a 50:50 CLA isomer mix for 6 months, Watras *et al.* (2007) reported that soluble vascular cell adhesion molecule (sVCAM-1), a plasma biomarker of endothelial dysfunction, significantly decreased in the CLA group when compared to the placebo group, indicating no adverse effects on endothelial function.

In a double-blind, placebo controlled study 42 men with symptoms of metabolic syndrome were randomly assigned to one of four treatments: safflower oil, olive oil, heated safflower oil, or CLA mix. Endothelial function, measured as finger pulse by EndoPat (an FDA-cleared method to determine endothelial function) was unaffected with CLA nor did the atherosclerotic marker (adhesion molecules) change. Some secondary endpoints, such as LDL-cholesterol and blood pressure, improved significantly during CLA supplementation, compared to placebo groups (Pfeuffer *et al.*, 2007).

## Discussion

Of the 4 studies that measured endothelial function, 3 studies demonstrated no significant adverse effects at doses of 3.2 to 4.7 mg CLA 50:50 mix/day (Raff *et al.*, 2006; Pfeuffer *et al.*, 2007; Watras *et al.*, 2007) and the 4<sup>th</sup> study is marked by variability in methodologies. The results of these studies demonstrate that CLA 50:50 isomer mixtures do not impair endothelial function.

### 7.5.3 Insulin Sensitivity and Glucose Metabolism

Several human studies with CLA have measured glucose and insulin parameters. These studies vary in length of time, dose, population, number of volunteers and form of CLA administered. Most of these studies rely on fasting serum glucose and insulin measures as biomarkers of metabolic change. From a clinical safety perspective, fasting serum levels are the initial diagnostic tool to assess derangements in glucose and insulin metabolism. The Homeostasis Model Assessment (HOMA) model is also used to assess insulin sensitivity in 6 of the human clinical trials that will be discussed below.

Given the inherent variation (*i.e.*, reliability) of methods, coupled with the available data on glucose and insulin parameters in these publications, fasting glucose and insulin levels will be noted in the following text when measured but will not be considered pivotal to the safety of a 50:50 CLA mixtures with respect to insulin sensitivity. Rather, the trials that report oral glucose

tolerance and clamp techniques will be used to validate the safety of the 50:50 mixtures with respect to the issue of insulin sensitivity.

Out of 33 clinical trials (resulting in 38 publications) relating to the 50:50 CLA mixtures, 20 measured, and reported in the peer-reviewed literature, some aspect of insulin and/or glucose measurements. Of the 5 trials (resulting in 8 publications) from the multiple-isomer studies, 5 have reported effects on the glucose and insulin. Of the 5 pure isomer studies (resulting in 9 publications), 4 have investigated effects on glucose and insulin.

In 20 intervention studies, glucose and insulin have been measured using predominantly fasting levels, oral glucose tolerance test (OGTT) was utilized in four studies, three studies utilized a euglycemic clamp. The majority of these studies were conducted using the 50:50 CLA isomer composition. Three trials investigated four CLA isomer preparations and found no effect. Further, four trials used pure isomers to investigate effects on glucose and insulin metabolism and only 2 demonstrated significant effects. As this Section demonstrates, any changes in insulin or glucose levels with the 50:50 mixture were within population ranges.

At least 17 studies showed that 50:50 mixtures of CLA had no effect on insulin and glucose measures (Blankson *et al.*, 2000; Risérus *et al.*, 2001; Smedman and Vessby, 2001; Noone *et al.*, 2002; Albers *et al.*, 2003; Kamphius *et al.*, 2003a; Gaullier *et al.*, 2004, 2005, 2007, Whigham *et al.*, 2004; Song *et al.*, 2005; Syvertsen *et al.*, 2006; Taylor *et al.*, 2006; Lambert *et al.*, 2007; López Román *et al.*, 2007; Nazare *et al.*, 2007; Watras *et al.*, 2007) Two studies showed an improvement in insulin sensitivity (Belury *et al.*, 2003; Eyjolfson *et al.*, 2004), and only one publication noted a decrease in insulin sensitivity in human volunteers (Moloney *et al.*, 2004).

In a separate study divided into 3 publications (Risérus *et al.*, 2002a,b, 2004b), Risérus and colleagues administered 1.95 g/day t10,c12 CLA or 3.4 g/day 50:50 CLA mixture or placebo to overweight males with Syndrome X for 12 weeks. They reported no significant difference in blood glucose or serum insulin levels or insulin sensitivity as measured by hyperinsulinemic euglycemic clamp in the group receiving the 50.50 CLA compared with the placebo (note also that findings were different for the pure isomer but this is discussed in the pure isomer Section). As the clamp procedure is considered the gold standard for insulin resistance, this confirms the finding of other studies using methods of fasting levels or OGTT demonstrating no effect of the 50:50 mixture on insulin resistance.

Syvertsen *et al.* (2006) studied the effects of 50:50 CLA mixtures or an olive oil placebo in 83 subjects (BMI: 28-32 kg/m<sup>2</sup>) in a double blind, placebo-controlled trial. Subjects were randomized into two groups supplemented with either 3.4 g/day CLA or placebo for 6 months. Glucose uptake was measured by euglycemic insulin clamp in a total of 49 volunteers from the main study population (with 37 of these subjects completing the study). With the euglycemic insulin clamp the insulin plasma concentration is raised and maintained at a high level by

continuous infusion of insulin. The plasma glucose level concentration is held constant at a basal level by a variable glucose infusion controlled by repeated blood sugar measurements. Under these steady-state conditions the glucose infusion rate equals the glucose uptake by all body tissues and is therefore a measure of tissue sensitivity to exogenous insulin. There were no significant differences from baseline or between groups in glucose uptake or glucose uptake to insulin concentration ratio during the clamp procedure. HOMA and quantitative insulin sensitivity check index (QUICKI) values and fasting glucose, fasting insulin, glycohemoglobin, c-peptide, adiponectin, and leptin levels for the clamp population also did not differ significantly from baseline or between groups. Likewise, HOMA and QUICKI values measured in the total study population were not significantly different. These findings confirm the results of Riserus *et al.* (2002a,b, 2004b) that the 50:50 mixture does not affect insulin sensitivity.

### *Discussion*

In summary, there are numerous publications that have addressed the effect of 50:50 mixtures of CLA on glucose and insulin parameters and have reported no adverse effect and there is only one study that reported an adverse effect (Moloney *et al.*, 2004).

Three studies using the clamp method, considered to be the most reliable, have also demonstrated no adverse effects on glucose and insulin after periods of up to 6 months of CLA consumption at a dose of 4 g/day.

Collectively, the human studies, with support from the animal and *in vitro* studies, indicate that 50:50 CLA mixtures do not adversely affect glucose and insulin parameters at levels ranging from 1.7 to 6.8 g/day for periods of time between 1 and 24 months. Furthermore, 50:50 CLA mixtures do not negatively affect insulin sensitivity in healthy, overweight, obese, or sedentary individuals. However, pure CLA isomers may transiently affect insulin sensitivity in syndrome X as indicated by short-term studies. Thus, the data show that CLA 50:50 mixtures pose no long-term risk of insulin resistance in humans. Functional adaptation to CLA in the diet and increased in blood glucose results in compensatory insulin action that normalizes within 10 weeks. This also reflects the response reported by Whigham *et al.* (2004), human study where an initial rise was seen at 2 weeks, which rapidly disappeared thereafter.

Information from experts in the field, George Steiner, M.D., F.R.C.P.(C) Emeritus Professor of Medicine and former Head, Division of Endocrinology, University of Toronto and Toronto General Hospital, and Prof. Dr Jürgen Schrezenmeir, Head of the Institute for Physiology and Biochemistry of Nutrition, Federal Research Centre for Nutrition and Food, Germany, and Professor of the Johannes Gutenberg University Mainz, Germany; is provided in Appendix B.

#### **7.5.4 Maternal Milk Fat**

Several studies have investigated the effects of feeding dairy products containing naturally occurring CLA to lactating women and measuring the occurrence of CLA in breast milk (e.g.,

Fogerty *et al.*, 1988; Park *et al.*, 1999). Collectively, these studies indicate that consuming dairy products (which contain naturally occurring CLA) results in measurable increases of the CLA content of breast milk and adipose tissue in human volunteers without altering total milk fat levels in humans. Studies have also investigated whether addition of 50:50 CLA mixtures or purified c9,t11 or t10,c12 CLA isomers would similarly impact milk fat CLA levels or amounts of total milk fat.

Masters *et al.* (2002) found that maternal consumption of a CLA 50:50 mixture decreased milk fat in humans. Nine healthy lactating women took part in a 17-day study consisting of 3 periods: intervention I (5 days), washout (7 days), and intervention II (5 days). During the intervention period, subjects consumed supplements containing 1.2 g CLA 50:50 and 0.3 g other fatty acids or 1.5 g olive oil daily. Total dose of administered CLA was 1.107 g/day. Infant milk consumption, milk fatty acid content, and dietary CLA consumption levels were assessed. Baseline dietary levels of CLA were found to be similar between placebo and treatment groups prior to intervention. Total milk fat content was reduced by 25% compared to placebo: Post-treatment levels of milk fat (%) were approximately 3% in the olive oil group and 2.3% in the CLA group. The authors concluded that CLA 50:50 mixture supplementation had no overall effect on infant breast-milk consumption.

While some of these data represent a statistically significant change between placebo and treatment groups, this change may not be biologically relevant to the safety of CLA mixtures for four reasons: Firstly, the observations for the placebo group are at the very lower end of the population range for average milk fat content of human breast milk of 3 to 5%, especially taking into consideration that subjects were recruited at at least 1 month post-partum and taking into account that the fat content of breast milk increases with the time of nursing (Jenness, 1979). Secondly, U.S. infant formula requirements permit a fat level of between 2 and 4.2%, as fed (based on 3.3 to 6 g/100 kcal and assuming 60 kcal/g) (U.S. FDA, 2007c). Thirdly, the observations are within the normal inter- and intra-individual milk fat composition resulting from several factors which have been well-established as playing a role in milk fat variation besides dietary manipulation. These include right vs. left breast volume differences, fore-milk (pre-breast-feeding) vs. hind-milk (post-breast-feeding) sampling, diurnal variation in milk fat production, and stage of lactation (Mitoulas *et al.*, 2002). Mitoulas *et al.* (2002) found that differences in milk fat production varied as much as 2 to 8% in the same human volunteers without dietary intervention. They studied the effects of breast volume, time of feeding, and total volume expressed per feeding in 17 human volunteers and found that all these factors contribute to significant differences in milk fat percentage variation. Within this established framework and accepted variation, the change in the CLA-fed group in Masters *et al.* (2002) is still well within the population range. Fourthly and finally, a crossover design was used in this study which means that the dietary interventions were assessed at different stages of lactation. An expert opinion (Appendix B) suggests that not only the experimental design, but the number of subjects does not allow for a conclusive result.

**000101**

Recent work by Mosley *et al.* (2007) demonstrated that feeding up to 4 g/day 50:50 CLA mixture has no adverse effects on milk fat. Twelve lactating women were randomized to a 3x3 factorial design with 3 treatments: control, 2 or 4 g/day CLA 50:50 mixture. Treatments were administered for 5 days and included 9-day washouts between treatments. CLA had no significant effects on milk yield or milk fat percentages, milk protein, lactose, or cholesterol. Another study (Masters *et al.*, 2002) showed that administering doses 2-fold greater than those used by Mosley *et al.* (2007) resulted in no MFD or any adverse effects. Again, an expert opinion (Appendix B) suggests that the experimental design does not allow for a conclusive result.

Ritzenthaler *et al.* (2005) examined the effects of CLA consumption in the form of cheese as a delivery food on milk fat and immune parameters as well as CVD risk factors in humans. They randomized 36 lactating women to one of three treatments in this 8-week intervention: a low-CLA cheese providing 160 mg/day of the *c9,t11* CLA isomer, a high-CLA cheese providing 346 mg/day the *c9,t11* CLA isomer, and a control group. They reported that although *c9,t11* CLA content of breast milk was higher in the high CLA group, total milk fat yield, lactose, or protein levels were unaffected by either CLA treatment. This study showed that CLA in the form of the naturally occurring isomers (predominantly *c9,t11*, as well as *t10,c12*, and *t9,t11/t10,t12*) do not alter milk fat levels in lactating humans.

Hasin *et al.* (2005) conducted a human trial to examine the safety effects of individual isomers of CLA (the *c9,t11* and the *t10,c12*). Twelve lactating women were subjected to 3 different treatments over time in a latin-square designed trial: 750 mg/day *c9,t11* CLA, 750 mg/day *t10,c12* CLA, or olive oil as a placebo, for 5 days with 9-day washout periods between each treatment. Neither the *c9,t11* nor the *t10,c12* CLA isomers significantly altered milk fat in humans.

Mosley *et al.* (2005) also found a portion of dietary *trans*-11 octadecanoic acid (*trans*-vaccenic acid, TVA) can be endogenously converted to the *c9,t11* CLA isomer and excreted in the expressed breast milk of postpartum women. Overall, apart from one study (Masters *et al.*, 2002), these results indicate that CLA 50:50 mixtures and individual CLA isomers have no effect in lactating women in doses ranging between 0.75 to 4.0 g/day.

### *Discussion*

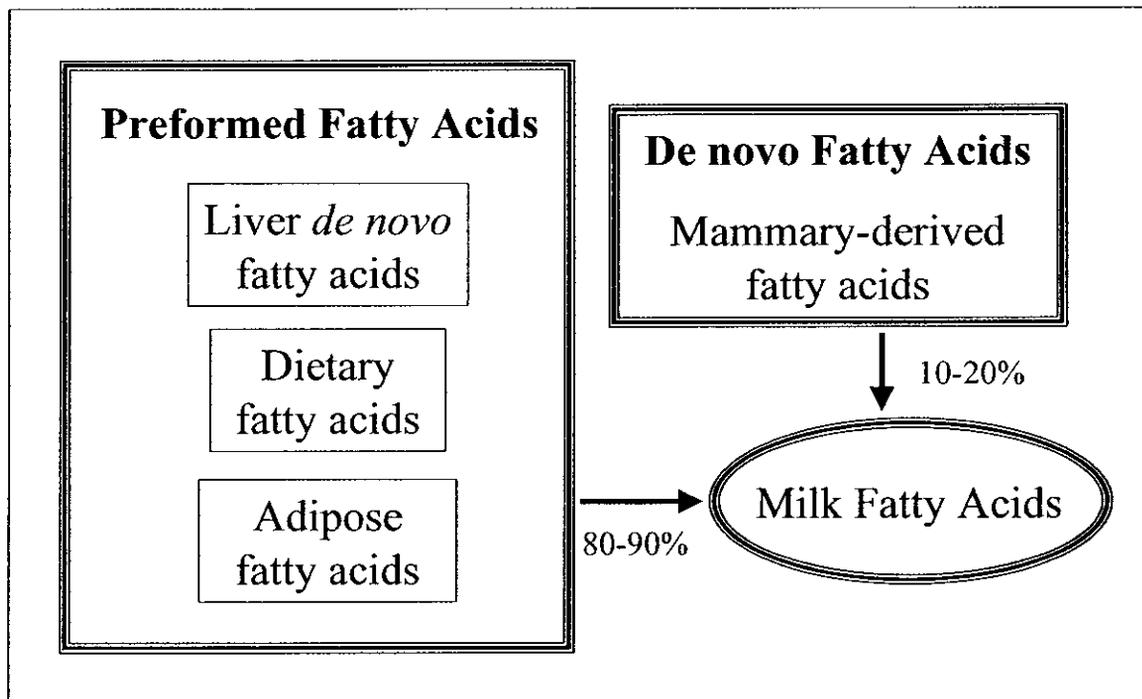
Numerous naturally occurring dietary and biological phenomena can alter milk fat production in humans. With the exception of Masters *et al.* (2002) no significant effect on milk fat deposition was reported in these human studies. The changes in milk fat percentage after CLA treatment in the Masters *et al.* (2002) study do not fall outside the range of acceptable milk fat levels. These results are in contrast to the animal studies described earlier, which have clearly demonstrated milk fat deposition after CLA administration, prompting initial investigation in humans.

000102

In animals the contribution of the fatty acids derived from *de novo* synthesis in milk fat is relatively higher than in humans and CLA (t10,c12) seems to specifically inhibit *de novo* synthesis in animals

Figure 7.5.4-1 shows the contribution of different lipid pools to maternal milk fat in humans. Species differences in the physiology and biochemistry of lipogenesis between ruminants, rodents, and humans exist. For example, ruminants gain most of their energy for milk production from carbohydrate sources and efficiently convert carbohydrates to fats, thus *de novo* fatty acid synthesis. Conversely, in humans, very low activity of citrate lyase glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and Nicotinamide adenine dinucleotide phosphate (NADP)-malate dehydrogenase help to explain the low level of lipogenesis found in humans (Leitch and Jones, 1993). Thus, the predominant sources of lipids for human milk fat are dietary sources and adipose tissue reserves.

**Figure 7.5.4-1 Contribution of Lipid Pools to Maternal Milk Fat**



In addition to differences in lipogenic rates between species, milk fatty acid composition differences also vary between species. Human milk contains mainly long chain saturated fatty acids, (predominantly palmitic acid) and unsaturated fatty acids that are derived by uptake of fatty acids from plasma, while medium chain fatty acids represent only about 13% of human milk fatty acids (Hachey *et al.*, 1989). Rodent milk contains high amounts of the medium chain fatty acids. Fernando-Warnakulasuriya *et al.* (1981) calculated that approximately 35% of total

**000103**

milk fatty acids are derived from *de novo* lipogenesis in the rat mammary gland. Similarly, ruminant milk is also very high in medium chain and short chain fatty acids, which together represent about 50% of cow's milk fatty acids (Schroeder *et al* , 2003).

These species differences demonstrate that inhibition of *de novo* fatty acid synthesis by CLA is of much greater significance in rodents and cows than in humans, since *de novo* fatty acid synthesis in humans is of much lower significance to milk fatty acid secretion (Bee, 2000)

The phenomenon of milk fat depression has been widely reported in the dairy industry, and followed up in studies on other animal models. However, human milk synthesis has been demonstrated to be different in fat source accretion than other animal milk synthesis systems. It is acknowledged that the body of work on milk fat depression after CLA administration is limited and the early study (Masters *et al.*, 2002) pointed to a safety concern for humans. However, the newer data by Hasin *et al.* (2005) by this same research group is in contrast to that of Masters *et al.* (2002) and showed no apparent risk, as evidenced by the absence of adverse effects of CLA on milk fat levels during lactation.

The partitioning and metabolic effects of CLA on milk fatty acid distribution in humans are not unique. Most fatty acids administered have measurable effects on alterations in maternal milk fat composition. In fact, Francois *et al.* (1998) demonstrated that a 10-week administration of various lipid treatments [e.g., either menhaden (20 g bi-weekly); herring (7 g/bi-weekly); safflower oil (40 g/bi-weekly), canola oil (40 g/bi-weekly); coconut oil ( 40 g/bi-weekly); or cocoa butter (40 g/bi-weekly)] resulted in a reduction in total polyunsaturated fatty acids (PUFA) , total monounsaturated fatty acids (MUFA), and total saturated fatty acids (SFA) in milk of women consuming such lipid diets. The investigators concluded that these dietary-induced changes in fatty acid composition were not biologically significant.

In summary, the effect of CLA on milk fat production has been studied in cows, rodents and pigs, but these data cannot be relied on as evidence of the effect of CLA on lipogenesis in humans due to a different mechanism of mobilization of fat stores during lactation in humans. One study in humans showed reduction of milk fat associated with CLA, whereas a more recent study from the same authors using the same protocol except for the use of higher doses and a larger cohort showed no effect. Based on the limited available data, there is no evidence to suggest that the consumption of CLA-containing foods by lactating women affects milk fat levels beyond the range of normal biological variation. Based on the weight of the evidence, CLA (50:50 mixture) would not be harmful with respect to effects on milk fat levels.

Information from an expert in the field, Sheila Innis, Ph.D., Professor in the Department of Pediatrics at the University of British Columbia, is provided in Appendix B.

### 7.5.5 Summary with respect to Clinical Studies

Based on the totality of the clinical data, the weight of the evidence demonstrates that estimated consumption of 50:50 CLA isomers (CLA-Rich Oil) is safe. In particular, the pivotal studies report safe use of up to 6 g/day for up to 1 year (Larsen *et al.*, 2006 and Whigham *et al.*, 2004) and 3.4 g/day for up to 2 years (Gaullier *et al.*, 2004, 2005, 2007). Further, the weight of the evidence demonstrates no significant effects on cardiovascular parameters (lipid metabolism, markers of inflammation, and markers of oxidative stress), insulin sensitivity and glucose, and maternal milk fat.

### 7.6 Summary and Conclusions

A large number of published studies – including traditional toxicology studies and extensive human trials – have assessed the safety of CLA (50:50 mixture).

- Numerous clinical trials have evaluated the effects of the 50:50 mixture and a number of other isomers on similar parameters. A comprehensive review of the clinical data has demonstrated that consumption of 50:50 CLA isomers (CLA-Rich Oil) at levels up to 6 g/day for up to 1 year (Whigham *et al.*, 2004; Larsen *et al.*, 2006) and 3.4 g/day for up to 2 years (Gaullier *et al.*, 2004, 2005, 2007) is safe and has no significant effects on cardiovascular parameters (lipid metabolism, markers of inflammation, and markers of oxidative stress), insulin sensitivity and glucose, and milk fat deposition. For these “pivotal” studies, the levels of consumption represent the maximum dose consumed, rather than the absolute safety endpoints. A single oral dose of approximately 15 g of CLA-Rich Oil (containing up to approximately 9 g of CLA isomers) in bioavailability studies has revealed no adverse events.
- Preclinical data have demonstrated an absence of significant toxicological, mutagenic, or reproductive and developmental effects.
- The metabolism of CLA has been widely studied and reported, and follows the standard pathway of dietary triglycerides.

Accordingly, the weight of the evidence strongly supports that this ingredient is safe at the levels used in the pivotal human studies.

As discussed in section 6.0, consumption is estimated at 3.0 g CLA per day for consumers who intentionally seek CLA-containing foods, recognizing that short-term consumption may be higher (e.g., three servings per day would provide 4.5 g CLA) but that long-term consumption at this level is unlikely. For consumers who consume the target foods based on historical intake patterns and who do not intentionally seek CLA, consumption is estimated based on 90th percentile intake at 2.33 g/day CLA, ranging up to 3.0 g/day for adult males. Estimated intake by children aged 3-11, eaters only, at the 90th percentile, is 1.95 g CLA per day. The studied

levels of safe use discussed above indicate that these ranges of consumption estimates are safe.

## **8.0 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) (U.S. FDA, 1997). There must be a “consensus among qualified experts about the safety of the substance for its intended use.” 62 Fed. Reg. 18940 (U.S. FDA, 1997). This section summarizes why there is a basis for concluding that there is a consensus among qualified experts that there is reasonable certainty that CLA-Rich Oil will not be harmful under the intended conditions of use.

### **8.1 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 CLA-Rich Oil and related compounds in humans and animals, and also refers to unpublished corroborative information.

### **8.2. 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 the Companies unanimously concluded that the use of CLA-Rich Oil, 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 CLA-Rich Oil 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 CLA-Rich Oil is GRAS 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, the Companies conclude 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.

### 8.3 GRAS Determination

Based on the information summarized in this dossier, the Companies determine that CLA-Rich Oil, intended for use in certain foods as described herein, 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 (April 17, 1997).

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Appendix A

**Appendix A**

**ESTIMATED DAILY INTAKE OF CONJUGATED LINOLEIC  
ACID (CLA) FROM CLA-RICH OIL BY THE U.S. POPULATION  
FROM INTENDED FOOD USES**

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# ESTIMATED DAILY INTAKE OF CONJUGATED LINOLEIC ACID (CLA) FROM CLA-RICH OIL BY THE U.S. POPULATION FROM INTENDED FOOD USES

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# ESTIMATED DAILY INTAKE OF CONJUGATED LINOLEIC ACID (CLA) FROM CLA-RICH OIL BY THE U.S. POPULATION FROM INTENDED FOOD USES

## A-1.0 INTRODUCTION

Cantox Health Sciences International (Cantox) has completed an estimate of the consumption of CLA from CLA-Rich Oil by the U.S. population for use in certain specified beverages and beverage bases (soy milk, meal replacement beverages), grain and pasta products (meal replacement bars), milk and milk products (flavored milk, milk, yogurt), and processed fruits and fruit juices (fruit juices) (a list of specified foods is provided in Appendix A-C).

Estimates for the intake of CLA were based on these intended food uses and use-levels in conjunction with food consumption data included in the United States Department of Agriculture's (USDA) 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996) and the 1998 Supplemental Children's Survey (CSFII 1998) (USDA, 2000). Calculations for the mean and 90<sup>th</sup> percentile all-person and eaters-only intakes, and percent consuming were performed for each of the individual intended food uses of CLA. Similar calculations were used to determine the estimated total intake of CLA from all intended food uses combined. In both cases, the per person and per kilogram body weight intakes were reported for the following population groups:

- children, ages 3 to 11,
- female teenagers, ages 12 to 19;
- male teenagers, ages 12 to 19;
- female adults, ages 20 and up,
- male adults, ages 20 and up; and
- total population (all population and gender groups combined)

## A-2.0 FOOD CONSUMPTION SURVEY DATA

### A-2.1 Survey Description

Nationwide dietary intake data for the years 2001-2002 are now available for public use; however, only Day 1 interview data are included in the present release. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Anderson, 1988). Because two 24-hour dietary recalls administered on 2

non-consecutive days (Day 1 and Day 2) are available from the CSFII 1994-1996, 1998 surveys, these data were used to generate estimates for the current intake analysis.

USDA CSFII 1994-1996 provides food consumption data on persons of all ages, whereas, CSFII 1998 is limited to children from birth through 9 years of age. Combined, these surveys provide the most appropriate data for evaluating food use and food consumption patterns in the United States, containing 4 years of data on individuals selected *via* stratified, multistage area probability sampling of American households within all 50 states.

CSFII 1994-1996, 1998 survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Data were collected in-person, a minimum of 3 days apart, on different days of the week, to achieve the desired degree of statistical independence. CSFII 1994-1996 contains 2-day dietary food consumption data for more than 15,000 individuals of all ages, and 1-day data for 16,103 individuals. CSFII 1998 contributes data from an additional 5,559 children, birth through 9 years of age, to data reported for 4,253 children of the same ages within CSFII 1994-1996. The overall CSFII 1994-1996, 1998 response rate for individuals selected for participation in the survey was 81.5 and 77.5% for Day 1 and Day 2, respectively.

In addition to collecting information on the types and quantities of foods being consumed, CSFII 1994-1996, 1998 collected physiological and demographic information from individual participants in the survey, such as sex, age, self-reported height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. USDA sample weights were developed and incorporated with CSFII 1994-1996, 1998 to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (USDA, 2000).

## **A-2.2 Statistical Methods**

Consumption data from individual dietary records, detailing food items ingested by each survey participant on each of the 2 survey days, were collated by computer and used to generate estimates for the intake of CLA from all intended food uses by the U.S. population. Estimates for the daily intake of CLA from all intended food uses represent projected 2-day averages for each individual from Day 1 and Day 2 of CSFII 1994-96, 1998 data. These average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated using ratio estimation and non-parametric techniques, respectively, incorporating USDA survey weights in order to provide representative intakes for the entire U.S. population. All-person intake refers to the estimated intake of CLA averaged over all individuals surveyed, regardless of whether they consumed food products in which CLA-Rich Oil is used, and therefore includes "zero" consumers (those who reported no

intake of food products that may contain CLA during the 2 survey days) Eaters-only intake refers to the estimated intake of CLA by those individuals consuming food products in which CLA-Rich Oil is intended for use, hence the 'eaters-only' designation. Individuals were considered users if they consumed 1 or more food products in which CLA-Rich Oil is intended for use on either Day 1 or Day 2 of the survey.

### **A-2.3 Sample Size Criteria**

Mean or percentile intake estimates based on small sample sizes or with high variability relative to the mean [assessed using the coefficient of variation (CV)] may be less statistically reliable than estimates based on adequate sample sizes or low variability relative to the mean (LSRO, 1995). Data presented herein for the estimated daily intake of CLA follow the guidelines proposed by the Human Nutrition Information Service/National Center for Health Statistics Analytic Working Group for evaluating the reliability of statistical estimates adopted in the "Third Report on Nutrition Monitoring in the United States", whereby an estimated mean may be unreliable if the CV is equal to or greater than 30% (LSRO, 1995). The CV is the ratio of the estimated standard error of the mean to the estimated mean, expressed as a percentage (LSRO, 1995). Therefore, for the estimated intakes of CLA presented herein, values were considered statistically unreliable if the CV was equal to or greater than 30%. These values were not considered when assessing the relative contribution of specific food uses to total CLA consumption and are marked with an asterisk.

### **A-3.0 FOOD USAGE DATA**

The individual intended food uses and use-levels for CLA-Rich Oil employed in the current intake analysis are summarized in Table A-3-1. Food codes representative of each food use were chosen from the CSFII 1994-1996, 1998 (USDA, 2000) and grouped in food use categories according to Title 21, Section §170.3 of the *Code of Federal Regulations* (CFR, 2006). Product-specific adjustment factors were developed based on data provided in the standard recipe file for the CSFII 1994-1996, 1998 survey (USDA, 2000). All food codes included in the current intake assessment are listed in Appendix A-C.

**Table A-3-1 Summary of the Individual Intended Food Uses and Use-Levels for CLA in the U.S.**

Food Category	Intended Food Use	RACC* (g or mL)	CLA Level (g/serving)	Use Level (%)
Beverages and Beverage Bases	Specific Soy Milk Beverages	240	1.5	0.625
	Specific Meal Replacement Beverages	240	1.5	0.625
Grain Products and Pasta	Meal Replacement Bars	40	1.5	3.75
Milk and Milk Products	Specific Flavored Milk Products	240	1.5	0.625
	Milk (Filled)	240	1.5	0.625
	Specific Yogurt Products	225	1.5	0.667
Processed Fruits and Fruit Juices	Specific Fruit Juice Products	240	1.5	0.625

\* RACC – Reference Amounts Customarily Consumed Per Eating Occasion (21 CFR §101.12) When a range of values is reported for an intended food use, particular foods within that food use may differ with respect to their RACC

## A-4.0 FOOD SURVEY RESULTS

Estimates for the total daily intakes of CLA from all intended food uses are provided in Tables A-4.1-1 and A-4.1-2. Estimates for the daily intake of CLA from individual food uses in the U.S. are summarized in Tables A-1 to A-7 and B-1 to B-7 of Appendices A-A and A-B, respectively. Tables A-A-1 to A-A-7 provide estimates for the daily intake of CLA per person (mg/day), whereas Tables A-B-1 to A-B-7 provide estimates for the daily intake of CLA on a per kilogram body weight basis (mg/kg body weight/day)

### A-4.1 Estimated Daily Intake of CLA from All Intended Food Uses

The estimated total intake of CLA from all intended food uses in the U.S. by population group is summarized in Table A-4.1-1. Table A-4.1-2 presents this data on a per kilogram body weight basis.

Consumption of foods in which CLA-Rich Oil is intended for use by the total U.S. population is estimated to result in mean all-person and eaters-only intakes of CLA of 0.30 g/person/day (5.96 mg/kg body weight/day) and 1.22 g/person/day (24.41 mg/kg body weight/day), respectively (Tables A-4.1-1 and A-4.1-2) (assuming that all such foods are formulated with the maximum level of CLA-Rich Oil, which is a highly conservative assumption). The corresponding 90<sup>th</sup> percentile all-person and eaters-only intake is 1.04 g/person/day (19.97 mg/kg body weight/day) and 2.33 g/person/day (49.65 mg/kg body weight/day), respectively.

On an individual population basis, the greatest mean eaters-only intake of CLA on an absolute basis was estimated in male adults, with a value of 1.46 g/person/day (17.76 mg/kg body

weight/day). Children had the lowest mean eaters-only intake of CLA on an absolute basis, at 1 03 g/person/day. On a body weight basis, mean eaters-only intake of CLA was highest in children, with an estimated intake of 40 96 mg/kg body weight/day. The lowest mean eaters-only intake on a per kilogram body weight basis was estimated in male adults, with a value of 17 76 mg/kg body weight/day (Table A-4 2-2)

**Table A-4.1-1 Summary of the Estimated Daily Intake of CLA from All Intended Food Categories in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)**

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		Eaters-Only Consumption	
				Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
Child	3-11	31 4	1,982	0 36	1 17	1 03	1 95
Female Teenager	12-19	23 5	165	0 30	1 05	1 25	2 22
Male Teenager	12-19	25 0	174	0 33	1 16	1 28	2 67
Female Adult	20 and Up	23 9	1,092	0 29	1 07	1 16	2 13
Male Adult	20 and Up	19 7	935	0 29	0 97	1 46	3 00
Total Population	All Ages	24 3	5,002	0 30	1 04	1 22	2 33

When heavy consumers (90<sup>th</sup> percentile) were assessed, eaters-only intake was estimated to be highest in male adults (3 00 g/person/ day). The lowest 90<sup>th</sup> percentile eaters-only intake was in children at 1 95 g/person/day on an absolute basis (Table A-4 1-1). On a body weight basis, children were estimated to have the greatest eaters-only 90<sup>th</sup> percentile intake of CLA at 84 22 mg/kg body weight/day (Table A-4 1-2). The lowest 90<sup>th</sup> percentile intake of CLA on a body weight basis for eaters-only intake was in female adults (33.73 mg/kg body weight/day).

**Table A-4.1-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of CLA from All Intended Food Categories in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)**

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		Eaters-Only Consumption	
				Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
Child	3-11	31 4	1,982	14 15	45 48	40 96	84 22
Female Teenager	12-19	23 5	165	5 47	20 92	22 53	40 08
Male Teenager	12-19	25 0	174	5 38	19 27	20 76	39 32
Female Adult	20 and Up	23 9	1,092	4 50	16 04	17 87	33 73
Male Adult	20 and Up	19 7	935	3 55	11 67	17 76	36 53
Total Population	All Ages	24 3	5,002	5 96	19 97	24 41	49 65

#### **A-4.1.1 All-Person Intakes**

Estimates for the mean and 90<sup>th</sup> percentile daily intakes of CLA from each individual intended food category are summarized in Tables A-A-1 to A-A-7 and A-B-1 to A-B-7 on a g/day and mg/kg body weight/day basis, respectively. Tables A-A-7 and A-B-7 summarize the estimates for the mean all-person intakes of CLA by the total population (all ages) from each of the individual food uses on a g/person/day and mg/kg body weight/day basis, respectively. The total U.S. population was identified as being significant consumers of yogurt (9.60% users), fruit juice (7.00%), and flavored milk (6.34%).

Consumption of fruit juice accounted for the highest estimated mean all-person intakes of CLA, however due to the higher number of users, yogurt made the most significant contribution to the mean all-person intakes of CLA, at 0.07 g/person/day (1.45 mg/kg body weight/day). A significant impact on the mean all-person intakes of CLA was made from the consumption of fruit juice (0.08 g/person/day), and flavored milk (0.07 g/person/day). On a body weight basis, mean all-person intakes for flavored milk was 1.64 mg/kg body weight/day, for yogurt was 1.45 mg/kg body weight/day, and for fruit juice was 1.39 mg/kg body weight/day. Soy milk and filled milk had a negligible impact on the all-person intake estimates of CLA.

Of the individual population groups, the consumption of flavored milk made the most significant contribution to the mean all-person intake estimates of CLA (Tables A-A-1 to A-A-6 and Tables A-B-1 to A-B-6). The highest mean all-person intakes of CLA, on an absolute basis, were estimated in children consuming flavored milk, at 0.17 g/person/day. On a body weight basis, consumption of flavored milk led to the highest mean all-person intake of CLA in children (6.37 mg/kg body weight/day).

#### **A-4.1.2 Eaters-Only Intakes**

Tables A-7 and B-7 also summarize the estimates for the mean eaters-only intakes of CLA by the total population (all ages) from each of the individual food uses on a g/person/day and mg/kg body weight/day, respectively. Similar to the results observed for the all-person intakes, taking into account the number of users, yogurts were estimated as making the greatest contribution to potential CLA intake. Although meal replacement beverages accounted for the highest estimated mean and 90<sup>th</sup> percentile intakes of CLA, the number of consumers were much less than that of yogurt. The mean and 90<sup>th</sup> percentile eaters-only intakes of CLA for yogurts were 0.78 and 1.51 g/person/day, respectively (15.98 and 31.19 mg/kg body weight/day, respectively). Of the other food categories with a significant number of users in the total population, consumption of flavored milk and fruit juice also made significant contributions to the estimates for the mean (1.17 and 1.01 g/person/day, respectively) and 90<sup>th</sup> percentile (1.95 and 1.76 g/person/day, respectively) eaters-only intakes of CLA by the total population.

On an individual population group basis, the consumption of flavored milk made the most significant contribution to the eaters-only estimated intakes of CLA (Tables A-A-1 to A-A-6 and Tables A-B-1 to A-B-6). Adjusting for the number of users, male teenagers consuming flavored milk were estimated to have the highest mean and 90<sup>th</sup> percentile eaters-only intake of CLA of 1.17 and 2.34 g/person/day (18.71 and 34.38 mg/kg body weight/day). On a per kilogram body weight basis, children consuming soy milk were estimated with the highest reliable mean and 90<sup>th</sup> percentile eaters-only intakes of CLA at 93.87 and 223.71 mg/kg body weight/day, respectively.

The estimated intakes of CLA were considered statistically unreliable if the CV was equal to or greater than 30%. Assessing the CV for eaters-only intake estimates found the intake for meal replacement beverages to be statistically unreliable in the female teenager population group. Soy milks were statistically unreliable in the female and male teenager population groups. Soy milk also had a low number of users in most population groups resulting in higher CV values. The filled milk category contained no users thus there was no intake of CLA from this category.

## **A-5.0 CONCLUSIONS**

Consumption data and information pertaining to the individual intended food uses of CLA-Rich Oil were used to estimate the all-person and eaters-only intakes of CLA for specific demographic groups and for the total U.S. population. This type of intake methodology is generally considered to be 'worst case' as a result of several conservative assumptions made in the consumption estimates. For example, it is assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are consumed relatively infrequently.

In summary, on an eaters-only basis, the mean intake of CLA by the total U.S. population from all intended food uses was estimated to be 1.22 g/person/day or 24.41 mg/kg body weight/day. The heavy consumer (90<sup>th</sup> percentile) eaters-only intake of CLA by the total U.S. population from all intended food uses was estimated to be 2.33 g/person/day or 49.65 mg/kg body weight/day.

## **A-6.0 REFERENCES**

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**APPENDIX A-A**

**Estimated Daily Intake of CLA from CLA-Rich Oil from Individual  
Intended Food Uses by Different Population Groups Within the United States**

**Table A-A-1 Estimated Daily Intake of CLA from Individual Intended Food Uses by Children Aged 3 to 11 Years Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.27	17	< 0.01*	na	1.86	3.25
Specific Meal Replacement Beverages	0.40	25	0.01	na	1.53	3.70
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	5.76	363	0.06	na	0.87	1.58
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	11.85	747	0.17	0.78	1.10	2.15
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	11.33	714	0.06	na	0.57	0.98
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.39	466	0.06	na	0.76	3.53

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

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**Table A-A-2 Estimated Daily Intake of CLA from Individual Intended Food Uses by Female Teenagers Aged 12 to 19 Years Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.14	1	< 0.01*	na	0.77*	0.77*
Specific Meal Replacement Beverages	0.71	5	0.04*	na	5.55*	24.00*
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	5.13	36	0.05	na	0.88	1.22
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	7.69	54	0.09	na	1.12	1.56
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	6.13	43	0.04	na	0.71	1.21
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.69	54	0.08	na	1.01	1.56

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

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**Table A-A-3 Estimated Daily Intake of CLA from Individual Intended Food Uses by Male Teenagers Aged 12 to 19 Years Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.14	1	< 0.01*	na	1.15*	1.15*
Specific Meal Replacement Beverages	0.57	4	0.02*	na	2.48	3.70
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	4.89	34	0.05	na	1.01	1.61
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	11.35	79	0.14	0.78	1.17	2.34
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	4.45	31	0.04	na	0.91	1.67
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.04	49	0.08	na	1.08	1.56

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

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**Table A-A-4 Estimated Daily Intake of CLA from Individual Intended Food Uses by Female Adults Aged 20 and Over Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.26	12	< 0.01*	na	0.67	1.15
Specific Meal Replacement Beverages	1.66	76	0.04	na	2.22	3.70
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	2.93	134	0.03	na	0.96	1.61
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	3.74	171	0.05	na	1.15	1.95
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	11.15	510	0.10	0.38	0.82	1.55
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.76	355	0.07	na	0.92	1.74

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

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**Table A-A-5 Estimated Daily Intake of CLA from Individual Intended Food Uses by Male Adults Aged 20 and Over Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.15	7	< 0.01*	na	1.24	2.30
Specific Meal Replacement Beverages	1.26	60	0.05	na	3.67	8.70
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	2.57	122	0.04	na	1.29	2.44
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	3.24	154	0.05	na	1.37	2.34
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	6.78	322	0.06	na	0.87	1.51
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.96	378	0.09	na	1.24	2.33

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

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**Table A-A-6 Estimated Daily Intake of CLA from Individual Intended Food Uses by the Total Population (All Ages) Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (g)	90 <sup>th</sup> Percentile (g)	Mean (g)	90 <sup>th</sup> Percentile (g)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.23	48	< 0.01	na	1.10	2.30
Specific Meal Replacement Beverages	0.85	175	0.04	na	2.79	5.80
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	3.90	804	0.04	na	1.02	1.70
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	6.34	1,307	0.07	na	1.17	1.95
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	9.60	1,978	0.07	na	0.78	1.51
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.00	1,442	0.08	na	1.01	1.76

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**APPENDIX A-B**

**Estimated Daily Per Kilogram Body Weight Intake of CLA from CLA-Rich Oil from  
Individual Intended Food Uses by Different Population Groups  
Within the United States**

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**Table A-B-1 Estimated Daily Per Kilogram Body Weight Intake of CLA from Individual Intended Food Uses by Children Aged 3 to 11 Years Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.27	17	0.19*	na	93.87	223.71
Specific Meal Replacement Beverages	0.40	25	0.26	na	54.89	96.90
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	5.76	363	2.37	na	35.17	63.09
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	11.85	747	6.37	25.28	40.41	78.74
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	11.33	714	2.54	2.10	24.97	43.83
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.39	466	2.43	na	32.48	56.83

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

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**Table A-B-2 Estimated Daily Per Kilogram Body Weight Intake of CLA from Individual Intended Food Uses by Female Teenagers Aged 12 to 19 Years Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.14	1	0.01*	na	16.04*	16.04*
Specific Meal Replacement Beverages	0.71	5	0.57*	na	73.74*	301.71*
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	5.13	36	0.93	na	15.61	25.11
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	7.69	54	1.77	na	22.05	37.64
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	6.13	43	0.75	na	12.36	20.98
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.69	54	1.43	na	19.33	37.89

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

000151

**Table A-B-3 Estimated Daily Per Kilogram Body Weight Intake of CLA from Individual Intended Food Uses by Male Teenagers Aged 12 to 19 Years Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.14	1	0.03*	na	18.66*	18.66*
Specific Meal Replacement Beverages	0.57	4	0.24*	na	37.46	42.84
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	4.89	34	0.91	na	18.68	37.95
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	11.35	79	2.29	9.98	18.71	34.38
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	4.45	31	0.74	na	14.96	22.23
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.04	49	1.17	na	16.59	29.01

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

000152

**Table A-B-4 Estimated Daily Per Kilogram Body Weight Intake of CLA from Individual Intended Food Uses by Female Adults Aged 20 and Over Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.26	12	0.03*	na	11.19	20.21
Specific Meal Replacement Beverages	1.66	76	0.61	na	32.21	62.86
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	2.93	134	0.54	na	15.27	30.53
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	3.74	171	0.69	na	17.55	29.38
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	11.15	510	1.49	5.24	12.86	23.85
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.76	355	1.15	na	14.47	28.65

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

000153

**Table A-B-5 Estimated Daily Per Kilogram Body Weight Intake of CLA from Individual Intended Food Uses by Male Adults Aged 20 and Over Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.15	7	0.04*	na	20.50	36.08
Specific Meal Replacement Beverages	1.26	60	0.54	na	43.09	98.36
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	2.57	122	0.49	na	15.40	28.01
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	3.24	154	0.58	na	17.02	32.23
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	6.78	322	0.73	na	10.42	20.94
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.96	378	1.17	na	15.37	32.10

\*Indicates an intake estimate that may not be statistically reliable, as the CV of the mean is equal to or greater than 30% (see Section A-2.3)

000154

**Table A-B-6 Estimated Daily Per Kilogram Body Weight Intake of CLA from Individual Intended Food Uses by the Total Population (All Ages) Within the United States (1994-1996, 1998 USDA CSFII Data)**

Food Use Category	% Users	Actual # of Total Users	All-Person Consumption		Eaters only Consumption	
			Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)	Mean (mg/kg)	90 <sup>th</sup> Percentile (mg/kg)
<u>Beverages and Beverage Bases</u>						
Specific Soy Milk Products	0.23	48	0.07	na	33.41	84.22
Specific Meal Replacement Beverages	0.85	175	0.51	na	39.80	76.04
<u>Grain and Pasta Products</u>						
Meal Replacement Bars	3.90	804	0.89	na	21.93	44.34
<u>Milk and Milk Products</u>						
Specific Flavored Milk Beverages	6.34	1,307	1.64	na	27.32	52.88
Milk (Filled)	0	0	na	na	na	na
Specific Yogurt Products	9.60	1,978	1.45	na	15.98	31.19
<u>Processed Fruits and Fruit Juices</u>						
Specific Fruit Juice Products	7.00	1,442	1.39	na	18.57	35.81

000155

**APPENDIX A-C**

**Representative USDA CSFII 1994-1996, 1998 Food Codes for All Intended  
Food Uses of CLA from CLA-Rich Oil in the United States**

**000156**

**Representative CSFII 1994-1996, 1998 Food Codes for All Intended  
Food Uses of CLA in the United States**

**Beverages and Beverage Bases****Soy milk**

[CLA] = 0.625 %

11310000 Milk, Imitation, Fluid, Soy Based  
11320000 Milk, Soy, Ready-to-Drink, Not Baby  
11330000 Milk, Soy, Dry, Reconstituted, Not Baby

**Meal Replacement Beverages**

[CLA] = 0.625 %

11611000 Instant breakfast, fluid, canned  
11612000 Instant breakfast, powder, milk added  
11613000 Instant bfast, pwdr, swt w/ lo cal swt, milk added  
11621000 Diet beverage, liquid, canned  
11622000 Diet beverage powder, milk added  
11623000 Meal supplement / replacement, prepared, rtd  
11631000 High calorie bev, canned or powdered, reconstituted  
11641000 Meal replacement, milk based, high protein, liquid  
11651010 Meal replacement, cambridge, reconst, all flavors  
11830940 Meal replacement, protein, milk based, fruit juice mix  
11832500 Meal replacement, protein type, milk base, w/sugar &art

**Adjusted Meal Replacement Beverage Content for 15 g Concentrate or Powder**

[CLA] = 10 %

11830800 Instant breakfast powder, not reconstituted  
11830810 Instant bfast, pwdr, swt w/ lo cal swt, not reconst  
11830850 High calorie milk beverage, powder, not reconst  
11830900 Protein supplement, milk based, dry powder  
11830950 Nutrient supp, milk based, powdered, not reconstituted  
11830960 Protein supp, milk base, sodium controlled, powder  
11830970 Meal replacement, protein type, milk base, powder  
11830980 Protein supp, milk base, powder (incl sustacal)  
11830990 Nutrient supp, milk base, powder (incl sustagen)  
11831500 Nutrient supplement, milk base, high prot, not reconst  
11832000 Meal replacement, milk &soy base, powder, not reconst  
11835000 Meal replacement, cambridge, powder, not reconst  
11835100 Meal replacement, positrim drink mix, dry powder  
11835150 Dynatrim, meal replacement, powder  
11835200 Lose it (nanci), meal replacement, powder

**Grain Products and Pastas**

**Meal Replacement Bars**

[CLA] = 3.75 %

41435200	High protein bar cookie-like soy & milk base
53540000	Breakfast bar, nfs
53540100	Breakfast bar, cake like
53540200	Breakfast bar, cereal crust w/ fruit filling, lowfat
53540250	Breakfast bar, cereal crust w/ fruit filling, fat free
53540500	Breakfast bar, date, w/ yogurt coating
53541100	Breakfast bar, diet meal type
53541200	Meal replacement bar (incl slim fast bar)
53542100	Granola bar w/ oats, sugar, raisins, coconut
53542200	Granola bar, oats, fruit, nuts, lowfat
53542210	Granola bar, nonfat
53543100	Granola bar w/ peanuts, oats, sugar, wheat germ
53544100	Granola bar, w/ nougat
53544200	Granola bar, chocolate coated
53544210	Granola bar, w/ coconut, chocolate coated
53544220	Granola bar w/ nuts, chocolate coated
53544250	Granola bar, coated w/ nonchocolate coating
53544300	Granola bar, high fiber, yogurt coating, not choc
53544400	Granola bars, w/ rice cereal
53544450	Powerbar (fortified high energy bar)

**Milk and Milk Products**

**Flavored Milk**

[CLA] = 0.625 %

11541500	Milk shake, made w/ skim milk, chocolate
11541510	Milk shake, made w/ skim milk, not chocolate
11511200	Milk, chocolate, reduced fat milk based
11513100	Cocoa & sugar mixture, whole milk added
11513150	Cocoa & sugar mix, red fat milk added
11513200	Cocoa & sugar mixture, lowfat milk added
11513400	Chocolate syrup milk added, ns as to type of milk
11513500	Chocolate syrup, whole milk added
11513550	Chocolate syrup, red fat milk added
11513600	Chocolate syrup, lowfat milk added
11514100	Cocoa, sugar, & dry milk mixture, water added
11514300	Cocoa w/ nf dry milk, lo cal sweetener, water added
11514500	Cocoa w/ whey, lo cal sweetnr, fortifd, water added
11515100	Cocoa & sugar w/ milk, fortified, Puerto Rican
11516000	Cocoa, whey, lo cal sweetner mix, lowfat milk added
11519000	Milk beverage, not chocolate, w/ whole milk
11519050	Milk, not chocolate, whole milk based
11519100	Milk beverage, beads, whole milk added
11520000	Milk, malted, unfortified, flavor ns

11521000	Milk, malted, unfortified, chocolate flavor
11522000	Milk, malted, unfortified, natural flavor
11525000	Milk, malted, fortified, natural flavor (incl ovaltine)
11526000	Milk, malted, fortified, chocolate (incl ovaltine)
11527000	Milk, malted, fortified, (incl ovaltine)
11541000	Milk shake, ns as to flavor or type
11551050	Milk fruit drink (incl licuado)
11560000	Choc flavored drink, whey & milk based (incl yoo hoo)
11560020	Milk drink, whey & milk base, not choc (incl yoo hoo)
11561010	Cafe con leche prepared w/ sugar

**Milk**

[CLA] = 0.625 %

11114000	Milk, cow's, fluid, filled w/ veg oil, ns as to fat
11114100	Milk, cow's, fluid, filled w/ veg oil, whole
11114200	Milk, cow's, fluid, filled w/ veg oil, lowfat

**Yogurt**

[CLA] = 0.667 %

11411300	Yogurt, plain, nonfat milk
11423000	Yogurt, vanilla, lemon, coffee, nonfat milk
11424000	Yogurt, vanilla, lemon, coffee, nonfat milk, low cal sweet
11427000	Yogurt, chocolate, nonfat milk
11433000	Yogurt, fruit variety, nonfat milk
11460190	Yogurt, frozen, ns as to flavor, nonfat milk
11460200	Yogurt, frozen, chocolate, nonfat milk
11460300	Yogurt, frozen, not chocolate, nonfat milk
11460400	Yogurt, frz, chocolate, nonfat milk, w/ low cal sweet
11460410	Yogurt, frz, not choc, nonfat milk, w/ low cal sweet
11410000	Yogurt, ns as to type of milk/flavor
11411010	Yogurt, plain, ns as to type of milk
11411100	Yogurt, plain, whole milk
11411200	Yogurt, plain, lowfat milk
11420000	Yogurt, vanilla, lemon, coffee, ns as to milk type
11421000	Yogurt, vanilla, lemon, coffee, whole milk
11422000	Yogurt, vanilla, lemon, coffee, lowfat milk
11425000	Yogurt, chocolate, ns as to type of milk
11426000	Yogurt, chocolate, whole milk
11430000	Yogurt, fruit variety, ns as to milk type
11431000	Yogurt, fruit variety, whole milk
11432000	Yogurt, fruit variety, lowfat milk
11433500	Yogurt, fruited, nonfat milk, low cal sweetener
11444000	Yogurt, fruit & nuts, ns as to type of milk
11445000	Yogurt, fruit & nuts, lowfat milk
11459990	Yogurt, frozen, ns as to flavor, ns to type of milk
11460000	Yogurt, frozen, not chocolate, type of milk ns
11460100	Yogurt, frozen, chocolate, type of milk ns
11460150	Yogurt, frozen, ns as to flavor, lowfat milk
11460160	Yogurt, frozen, chocolate, lowfat milk

11460170 Yogurt, frozen, not chocolate, lowfat milk  
11460250 Yogurt, frozen, not chocolate, w/ sorbet/sorbet coated  
11460420 Yogurt, frozen, ns as to flavor, whole milk  
11460430 Yogurt, frozen, chocolate, whole milk  
11460440 Yogurt, frozen, not chocolate, whole milk  
11461000 Yogurt, frozen, chocolate coated  
11461100 Yogurt, frozen, carob coated  
11461200 Yogurt, frozen, sandwich  
11461250 Yogurt, frozen, cone, chocolate  
11461260 Yogurt, frozen, cone, not chocolate  
11461270 Yogurt, frozen, cone, not chocolate, lowfat milk  
11461280 Yogurt, froz, cone, chocolate, lowfat milk

### Processed Fruits and Fruit Juices

#### **Fruit Juice**

[CLA] = 0.625 %

61200500 acerola juice  
61201000 Grapefruit juice, nfs  
61201010 Grapefruit juice, freshly squeezed  
61201020 Grapefruit juice, unsweetened, ns as to form  
61201220 Grapefruit juice, canned, bottled, carton, unsweet  
61201230 Grapefruit juice, canned, bottled, carton, w/ sugar  
61201240 Grapefruit juice, canned/bottle/carton, w/ low cal sweetener  
61210000 Orange juice, nfs  
61210010 Orange juice, freshly squeezed  
61210230 Orange juice, canned/bottled/carton, w/ sugar  
61210250 Orange juice, w/ calcium, can/bottle/carton, unsweetened  
61216000 Grapefruit & orange juice, nfs  
61216010 Grapefruit & orange juice, fresh  
61216220 Grapefruit & orange juice, canned, unsweetened  
61216230 Grapefruit & orange juice, canned, w/ sugar  
64100100 Fruit juice, nfs (include mixed fruit juices)  
64132010 Prune juice, ns as to added sweetener  
64132020 Prune juice, unsweetened  
64132030 Prune juice, w/ sugar  
64134000 Fruit smoothie drink, w/ fruit only  
74301100 Tomato juice  
74302000 Tomato juice cocktail  
74303000 Tomato & vegetable juice, mostly tomato

Appendix B

**APPENDIX B**  
**Expert Opinion Statements**

**000162**



**EMORY**  
UNIVERSITY  
SCHOOL OF  
MEDICINE

W. Aigil Brown, MD  
*Chair, Emory Center for Prevention of Metabolic  
 Cardiovascular Disease*  
 Department of Medicine  
 Emory Department of Veterans Affairs Medical Center

June 5, 2006

To whom it may concern

I have been asked to review the literature and a document prepared for submission to the FDA on the safety of conjugated linoleic acid (CLA). There is an extensive list of publications that have examined both efficacy in changing blood lipids, inflammatory markers, adipose tissue mass and indicators of glucose metabolism and insulin resistance in humans and animal models. Although there are suggestions of beneficial effects in these measures, the major question put to this reviewer was the existence of harm to those given these substances. CLA has been available in mixtures of the trans-10, cis-12 (t10, c12) and the cis-9, trans-11 (c9, t11) structures in many countries as a dietary supplement. Some studies have used preparations of these isomers as separate entities. This letter provides my summary opinion as to the weight of the evidence on the safety of these substances. I have not provided specific references since the formal submission contains a complete bibliography of this literature.

Thirty two (human studies) of CLA as the mixtures or as the individual isomers have been reported. These have involved cohorts ranging from 16 to 180 individual participants and doses of CLA have ranged from 0.35 to 7.2 grams per day. The duration of the studies has usually been in the four to twelve week range. However, the largest study was the longest, observing 180 volunteers for 52 weeks with a follow on of an additional 52 weeks in 157 of these individuals. In summary, no significant change was observed in lipoprotein levels. When change was observed, it was inconsistent and was often counter to the direction and magnitude of the same parameter in another comparable study. I believe that it is appropriate to say that there is no convincing evidence that CLA used in the doses tested in these studies has any significant effect on any lipoprotein parameter in humans.

Inflammatory markers were also examined in human studies including leukocyte count, IL-6, IL-8, TNF $\alpha$  and CRP. Only CRP showed some tendency to rise but this was significant in only one study. Two other studies found no change. CRP is known to be highly variable from visit to visit in human populations and only in a relatively large group would convincing data be generated. Basu's group have found elevations of urinary isoprostanes (8-iso-prostaglandin F2 $\alpha$  and 15-keto-dehydro-prostaglandin). These studies used monounsaturated fatty acids (olive oil) or no added supplements as the control intervention. Others have had no controls. An increase in these parameters from 50% to 400% have been reported. However, there has been no other measure that might indicate oxidative damage. Furthermore, the increase in these substances is not surprising since similar findings have been made with a variety of polyunsaturated fats given as oral supplements. Omega-3 and omega-6 fatty acids cause an increase in these and other evidence of oxidation of lipids including the concentrations of malonyldialdehyde modified proteins and lipoproteins. These latter fatty acids have generally been associated with reduced



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incidence of vascular disease and there is no evidence that CLA has a negative vascular effect. In retrospect, these studies would have been more instructive if the control groups had received comparable amounts of vegetable oils or omega-3 fish oils as the comparator. It is quite possible that there would have been little or no difference in the generation of these isoprostanes. The same studies indicating increased isoprostanes are unique in finding evidence of increased insulin resistance and plasma glucose. The problem with proper controls is also evident in this data and at present, it requires further study to determine if others can reproduce these results.

Animal studies, in contrast to human trials have found reductions in arteriosclerotic lesions. This has been most dramatic and consistent in rabbits but these studies suffer from the relatively large doses and the very abnormal lipid patterns produced by high cholesterol diets in this model. The relevance to human metabolism remains to be proven. On the other hand, studies in the hamster are larger in number and are relatively consistent in showing reduced LDL and increased HDL while reducing the fatty liver often seen in these animals on a high fat and cholesterol diet. Arterial lesions have been reported to be less than in controls. Again, the relevance to human metabolism is in question. Studies in different strains of mice and in those with genetic modifications in lipoprotein metabolism have been less consistent. Six studies in rats were reviewed and in general, CLA was found to reduce both triglycerides and LDL with out changing HDL compared to saturated fat in the control diets. No significant vascular findings have been reported. In none of these animal models has toxicity attributable to the CLA been evident.

The measure of a variety of inflammatory markers has been reported in various cell lines in vitro and in a series of animals including hamsters, mice, rats and swine. Most studies have found suppression of these measures. Reduction in animal models of inflammatory bowel disease and prolongation of the life span in mice with a lupus like syndrome have been described. Negative impact of CLA in these systems has not been described.

In summary, CLA in either the combined form or the individual isomers has been studied extensively in both human trials and in animal models seeking evidence of harm in metabolic and inflammatory systems without convincing evidence that there is such harm. At the doses used previously in other nationalities, CLA appears to be safe for human consumption.

Yours sincerely,

W. Virgil Brown, MD  
Charles Howard

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## CLA and lipid peroxidation.

Recently 5 studies in humans have reported an increase in prostaglandin  $F_{2\alpha}$ -isoprostanes after dietary CLA intake. Increased levels of isoprostanes have been detected in most diseases involving oxidative stress and inflammation and the increased levels reported after CLA intake in humans therefore appear to contradict the anti-inflammatory and anti-oxidant properties of CLA reported in the literature. To be able to evaluate the safety of CLA it is essential to determine whether there is indeed a real increase in isoprostanes and if so, is this increase due to oxidative stress triggered by CLA treatment or due to other mechanisms than lipid peroxidation.

To date, five human trials conducted all by the same group of investigators in Sweden, have reported an increase in prostaglandin  $F_{2\alpha}$  isoprostanes after CLA intake, measured by a newly developed radioimmunoassay for 8-iso-prostaglandin  $F_{2\alpha}$  and 15-keto-dihydro-prostaglandin (Basu et al., 2000a; Basu et al., 2000b; Riserus et al., 2002b; Smedman et al., 2004). In this assay, a rabbit polyclonal antiserum was developed and used. While the authors 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 markers 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 this group of investigators 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). Furthermore, because of the use of a new unvalidated rabbit polyclonal antibody, the specificity of this immunoassay is questionable. Therefore, at the moment it cannot be excluded that CLA, or one of its metabolites or catabolites was measured instead of isoprostanes.

A second methodological remark concerns the variability of isoprostane levels. It is known that the levels of isoprostanes can vary from day to day (up to 40% variation) and therefore isoprostanes should ideally be measured on several consecutive days to be able to adequately determine the average isoprostane level. It is not clear from the Basu et al, Riserus et al and Smedman et al papers whether such measurements were indeed carried out on consecutive days. It is also not clear from their papers what extraction method they used and whether they added anti-oxidants to the obtained urine and blood samples. Lipids are rapidly oxidized and so if they didn't add anti-

oxidants directly after sampling, isoprostanes might have been formed after sampling, and thus might be an artifact of their method. Thus, currently there is no convincing evidence that isoprostanes are increased after CLA intake.

If the increase in isoprostanes turns out to be real then the main question is if it reflects a harmful effect. This depends on whether the increase results from increased oxidative stress, or from the development of an inflammatory state. Although it is well recognized that a higher level of F<sub>2</sub>-isoprostanes is found in most human diseases (review Davi 2004), an increase does not necessarily imply increased oxidative stress or inflammation. 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<sub>2</sub>-isoprostanes indicates increased oxidative stress.

In the case of dietary intake of CLA, an increase in F<sub>2</sub>-isoprostanes is likely unrelated to oxidative stress as 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<sub>2</sub>-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. In addition, treatment with anti-oxidant (tocopherol) did not affect the levels of isoprostanes measured, which seems contradictory if oxidative stress was the cause of the increase in isoprostanes. 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<sub>2</sub>-isoprostanes. The most logical explanation for the increased availability of F<sub>2</sub>-isoprostanes appears to be the result of an inhibition of their metabolism by competition with CLA.

It is proven that both isoprostanes and CLA are metabolized via the same peroxisomal pathway and therefore CLA likely causes a decline in metabolism of already-formed F<sub>2</sub>-isoprostanes by competing with F<sub>2</sub>-isoprostanes for the same metabolic pathways. As a result, levels of isoprostanes appear higher in the presence of CLA because fewer F<sub>2</sub>-isoprostanes are metabolized into a different form. In fact, preliminary results from an ex vivo study shows that addition of CLA to human fibroblast leads to an increase in isoprostanes, whereas CLA does not lead to a relative isoprostane increase in fibroblasts obtained from peroxisome-deficient patients, indicating that CLA may indeed compete for the same peroxisomal pathway. That this effect is specific for CLA is proven by the fact that oleic acid, for instance, did not lead to an increase in isoprostanes in normal human or peroxisome-deficient fibroblasts (Banni, unpublished results).

Accordingly, based on the weight of the evidence, it is reasonably certain that the observed effects on F<sub>2</sub>-isoprostane levels do not represent a harmful effect of CLA preparation under its intended conditions of use.

Prof. Giovanni Davi

**000166**

## Nutrition

March 14, 2007

Daniel R. Dwyer  
KLEINFELD, KAPLAN and BECKER, LLP  
1140 Nineteenth Street, NW  
Washington, DC 20036  
*Tel 202 223 5120*  
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Dear Mr. Dwyer,

**Re: Safety of CLA: effects on milk fat depression in lactating women**

Conjugated linolenic acids (CLA) are naturally occurring isomers of the essential fatty acid linoleic acid that contain a conjugated rather than methylene interrupted double bond. CLA are present in the diet as components of dairy fats and in the tissue fats (meat) of ruminant animals. CLA are well absorbed and are present in tissue, plasma and breast milk lipids in amounts proportional to that in the diet. This letter addresses the issue of whether CLA from foods supplemented with this fatty acid is likely to have a significant effect on milk fat production in lactating women, assuming CLA intake of about 2.13 g/day (which is the 90<sup>th</sup> percentile upper intake estimate).

A number of studies have provided convincing data to show that CLA inhibit lipogenesis through down-regulating the expression of genes for regulatory enzymes of fatty acid and triglyceride synthesis. *Cis* omega 6 and omega 3 polyunsaturated fatty acids, in particular the n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) also reduce triglyceride synthesis through down-regulation of lipogenesis, involving regulation of transcription factors that control multiple genes for enzymes involved in lipogenesis (Clarke 2004, Jump 2002). Thus, inhibition of triglyceride synthesis with intakes of CLA in the range of 2-4 grams per day is consistent with the hypotriglyceridemic action of other dietary polyunsaturated fatty acids that are considered to be generally recognized as safe by the Food and Drug Administration.

The effects of CLA on milk fat production have been extensively studied in dairy cattle and rodents, and to a lesser extent in pigs, and demonstrate the potential for CLA to inhibit fatty acid synthesis in organs other than the liver, and the mechanisms through which this occurs. However, there are important differences between these animals and humans in the physiology and biochemistry of lipogenesis, the composition of milk fatty acids, and the importance of mammary gland lipogenesis to milk fat secretion. In addition, the data in pigs are too limited to provide a basis for any conclusion. As a result, these animal data cannot be relied on as evidence of any effect of CLA on lipogenesis in humans.

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In addition to the differences in fatty acid metabolism secondary to digestion and metabolism of dietary lipids in the rumen and its microflora, cattle and rodents typically consume diets in which the major energy source is carbohydrate and fat is low. Lipogenesis from carbohydrate derived carbons is high in these species, whereas lipogenesis is low in humans (Jones 1996, Leitch and Jones 1993). The low lipogenic activity of human adipose tissue is explained by very low activity of citrate lyase glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP-malate dehydrogenase; these enzymes can be induced by fasting-refeeding with a high carbohydrate diet, indicating that down regulation of adipose tissue lipogenesis in humans may reflect higher fat intakes (Patel et al. 1975, Swierczynski et al. 2000).

The fatty acid components of milk triglycerides are derived from two sources: by uptake of circulating plasma triglyceride fatty acids following hydrolysis by the mammary gland lipoprotein lipase (LPL), and by de novo lipogenesis from glucose in the mammary gland. The mammary gland, unlike other tissues, truncates de novo fatty acid synthesis at the level of 14:0 via the action of the mammary specific enzyme thioesterase II, rather than at palmitic acid (16:0) as in the liver (Nolin et al 1982, Thompson and Smith 1985); the activity of the mammary gland fatty acid synthesis pathway, however, is also lower in humans than rodents (Thompson and Smith 1985). Rat milk, unlike human milk contains high amounts of the medium fatty acids, representing about 35% of total milk fatty acids, these being derived by de novo lipogenesis in the rat mammary gland (Fernando-Warnakulasuriya et al 1981). Cows' milk is also very high in medium chain as well as short chain fatty acids, which together represent about 50% of cow milk fatty acids (Schroeder et al 2003). Human milk contains much higher proportions of the long chain saturated fatty acids, predominately palmitic acid and unsaturated fatty acids that are derived by uptake of fatty acids from plasma, while medium chain fatty acids represent only about 13% of human milk fatty acids (Hachey et al 1989). Diets containing high amounts of carbohydrate and very low in fat (5% energy) result in a small increase in the contribution of medium fatty acids to about 16% milk fatty acids in humans (Hachey et al 1989). Medium chain fatty acids are also relatively low in pig milk, similar to that in human milk. These species differences suggest that any inhibition of mammary gland fatty acid synthesis by CLA may be of much greater significance in rodents and cows than it would be if it occurred in humans, since mammary gland lipogenesis in humans is of much lower significance to milk fatty acid secretion. In addition, the ability to detect statistically significant effects of CLA on milk fatty acids in humans may be influenced by the background diet, in particular both the amount of dietary energy derived from fat versus carbohydrate and the intake of *cis* polyunsaturated fatty acids.

Importantly, there is no reliable evidence to suggest that CLA inhibits mammary gland fatty acid synthesis in humans. A recent study reported that ruminic acid (c9,t11-18:2) (ruminic acid; RA) in cheese at up to 346 mg RA/day did not affect milk fat, protein, or lactose concentrations (Ritzenthaler et al 2005). Other studies by the same group reported that 5 days supplementation in 9 lactating women with 1.5g CLA lowered milk fat to a mean of 2.25 g/dL compared to 3g/dL during supplementation with olive oil (Masters 2002). The average content of fat in milk for the group of women in this study is well below the usual averages found in studies on human milk of about 3.5-3.7g fat/dL, with the

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mean value below the lower limit of the normal range. It is unclear from this study whether some aspect of the milk fat collection or analysis resulted in fat loss, causing the group to appear abnormal; it is also unclear whether CLA had any negative effect as compared with olive oil (which may have had a positive effect) Mosley *et al.* (2005) reported that a CLA 50:50 mixture and individual CLA isomers show no effect on milk fat in lactating women in doses ranging between 0.75 to 4.0 g/day, although again the methodology used does not permit any firm conclusion to be drawn from these results.

Sincerely,

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# **REEVALUATION OF GAULLIER'S STUDY ON INSULIN SENSITIVITY**

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Jean-Michel Gaullier et al conducted a study on the long-time effects of conjugated linoleic acid (CLA) on body composition and safety in healthy overweight adults (Gaullier et al , 2004) 180 volunteers aged 18-65 with body mass indices (BMI) of 25-30 were supplemented for one year with either CLA-free fatty acid (CLA-FFA, n=61), CLA-triacylglycerol (CLA-TG, n=60), or placebo (olive oil, n=59) 157 volunteers finished the study

CLA preparations were 50/50 mixtures of *cis*-9, *trans*-11 (c9,t11) and *trans*-10, *cis*-12 (t10,c12) CLA isomers

The fatty acid (FA) composition of the CLA-FFA preparation was c9,t11 CLA 39%, t10,c12 CLA 41%, 16:0 FA 1.3%, 18:0 FA 2.3%, 18:1 FA 9.4%, 18:2 FA 0.7%, others 2.3%

The fatty acid composition of the CLA-TG preparation was c9,t11 CLA 38%, t10,c12 CLA 38%, 16:0 FA 2.7%, 18:0 FA 2.6%, 18:1 FA 10.6%, 18:2 FA 0.9%, others 3.3%

Supplementation with CLA, either as FFA or TG, for 12 months significantly lowered body fat mass (BFM) whereas BFM in the placebo group did not significantly change Accordingly, the alteration of lean body mass (LBM) was more pronounced in the CLA groups than in the placebo group Body weight and BMI showed similar differences. Daily caloric intake did not differ significantly between groups This suggests that the observed effects of CLA on body composition were independent of diet (Gaullier et al , 2004)

The fasting glucose levels did not significantly change during intervention in all groups and the alteration during intervention did not significantly differ between the three groups (Gaullier et al , 2004)

The HbA<sub>1c</sub> significantly increased in all groups. But the alterations during intervention did not differ between the groups (Gaullier et al , 2004)

There were no data given on insulin sensitivity. Insulin resistance is considered to be a risk factor and a crucial feature of the metabolic syndrome

In order to assess insulin sensitivity in Gaullier's long-term study, COGNIS GmbH & Co KG provided us with the crude data set of this study. We reanalyzed the data using the Homeostasis Model Assessment (HOMA-IR) index The HOMA-IR index is reflecting insulin sensitivity in normal and diabetic patients. It is calculated as fasting glucose (mmol/l) \* fasting insulin (µU/ml) / 22.5 The HOMA-IR index has been shown to correlate well both, with the gold-standard euglycemic hyperinsulinemic clamp and the minimal model method in diabetics and normal subjects (Fukushima et al , 1999, Mathews et al , 1985, Haffner et al , 1996, Bonora et al , 2000)

There were data of serum insulin and serum glucose from 147 study subjects (123 females and 24 males) The data set of these subjects was the basis of further statistics. Comparisons between treatment groups were performed by using ANOVA on ranks

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(Kruskal-Wallis-Test), and Mann-Whitney rank sum test. Intraindividual comparisons during intervention were performed by using Wilcoxon signed rank test

In the reevaluated subset the alterations of BMI during intervention differed significantly (ANOVA  $p=0.004$ ) between the intervention groups in all subjects as well as in the male and female subgroup (Fig 1, 2, 3) Whereas the BMI in the control group did not significantly increase, it decreased significantly in the CLA-FFA group ( $p=0.031$ ) and in the CLA-TG group ( $p<0.001$ ) In males the alterations were similar. In females only the BMI in the CLA-TG group significantly decreased. Evaluation of the differences of the alterations during intervention ( $\Delta$  BMI) by the Mann-Whitney test showed according results (Fig 1, 2, 3)

Neither CLA-TG nor CLA-FFA supplementation had a negative influence on insulin sensitivity measured by HOMA-IR index during the 12 month period

No differences were found in absolute values (Fig 4) as well in the intraindividual alterations during intervention between the CLA-TG, the CLA-FFA, and the placebo group (Fig 5)

In male subjects, CLA-TG decreased HOMA-IR index compared to placebo indicating an increase of insulin sensitivity (Fig. 6 and 7) This, however, should be interpreted with caution since there were only 9 male subjects in the placebo group and 7 in the CLA-TG group and since the CLA-TG group started with somewhat higher HOMA-IR-indices (n s ) (Fig 6)

In females no significant effect was seen (Fig 8 and 9).

Interestingly, in males the alteration of HOMA-IR during intervention correlated with the alteration of BMI (Fig 11), whereas this was not the case in females (Fig 12) This would indicate that males may benefit more from a reduction of BMI than females.

Other studies on the effect of CLA on insulin sensitivity were mainly short term studies with an observation period up to 3 months (Table 1) Because of this, they are less useful to estimate long-term benefits and risks of CLA intake Furthermore, providing data on insulin sensitivity differ in CLA composition. The CLA isomers c9,t11 and t10,c12 were shown to explicit differing effects (Risérus et al , 2002) and to interact with PPAR ligands in a complex way (Brown et al , 2001, Brown et al , 2003, Brown & McIntosh, 2003) A weakness of some studies was the choice of placebo preparation. In some studies olive oil was used which is known to have effects on metabolism that seem to be attributed to minor components (Perez-Jimenez, 2005) differing from safflower oil, which is the basis of CLA preparations

Medina et al (2000) showed a non significant increase of plasma insulin levels in 24 healthy women after 64 days supplementation with a CLA mixture compared to baseline Plasma glucose was unchanged In this study a CLA mixture with another composition of isomers

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was used (Table 1). Thus, it cannot be compared with studies on CLA mixtures with ~40% c9,t11 isomer and ~40% t10,c12 isomer (50:50)

Smedman and Vessby (2001) found during a 12 week intervention with c9,t11 t10,c12 (50:50) CLA mixture a borderline significant difference between the changes of fasting blood glucose in the CLA and placebo group that received olive oil ( $p=0.053$ ) The increase in the CLA group was not significant For plasma insulin no differences were seen (Smedman & Vessby, 2001)

The 8 week intervention in 51 healthy volunteers by Noone et al. showed no change in plasma glucose and plasma insulin by both, c9,t11 t10,c12 (50:50) CLA mixture and c9,t11 t10,c12 (80:20) CLA mixture throughout the study (Noone et al , 2002)

In the study by Risérus et al no effects of a CLA mixture (50:50) on insulin sensitivity and fasting glucose were observed after 12 weeks supplementation (Risérus et al , 2002)

A clinical trial with 21 volunteers by Belury et al. revealed a decrease of fasting glucose in type 2 diabetics after 8 weeks supplementation with a CLA mixture (Belury et al , 2003) In this study a comparable CLA-mixture was used and safflower oil was chosen as control (Belury et al , 2003).

Eyjolfson et al found a decrease in fasting insulin and an improvement in insulin sensitivity index (ISI) in young sedentary volunteers after 8 weeks supplementation with a CLA mixture, whereas there were no alterations in the placebo group. In this study also a comparable CLA-mixture was used and safflower oil was chosen as control (Eyjolfson et al , 2004)

The study by Moloney et al. (2004) on the influence of CLA on insulin sensitivity and lipoprotein metabolism in patients with type 2 diabetes mellitus showed a significantly reduced insulin sensitivity measured by HOMA-IR index in the CLA group after 8 weeks supplementation compared to the control group that received a blend of palm and soya bean oil Quantitative insulin sensitivity check index (QUICKI)<sup>1</sup> and HbA<sub>1c</sub> were not affected by CLA supplementation It is notable, that the responses to an oral glucose load differed between the groups at baseline and these differences were maintained at the end of the intervention There does not appear to be a significant effect of CLA at the end of intervention compared to baseline For that reason, these findings should be interpreted with care Furthermore, the blend of palm and soya bean oil may not be a good control group for CLA as the composition of fatty acids and possibly other components is different.

The only other long-term study on CLA to the best of our knowledge was conducted by Whigham and coworkers Supplementing Clarinol<sup>®</sup> (37.3% c9,t11, 37.6% t10,c12) or high oleic sunflower oil for 12 months they found no change in serum insulin, serum glucose or

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<sup>1</sup> QUICKI =  $1 / [\log \text{fasting insulin } (\mu\text{U/ml}) + \log \text{fasting glucose } (\text{mg/dL} = \text{mmol/L} * 18,182)]$  (Katz et al . 2000)

HOMA-IR index between groups at any time throughout the study. No subject with a normal baseline glucose developed glucose intolerance during the study (Whigham et al., 2004).

In summary, in two long-term intervention studies over 12 months (Gaullier et al. reevaluated here and Whigham et al., 2004) no unfavorable effect on insulin sensitivity was observed. In Gaullier's study even a significant increase was seen in males, this, however, needs to be confirmed in studies with a higher sample size.

Based on these long-term studies there does not seem to be a matter of concern from effects of CLA on insulin sensitivity.

Kiel, August 14<sup>th</sup> 2006

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Prof. Dr. Jürgen Schrezenmeir

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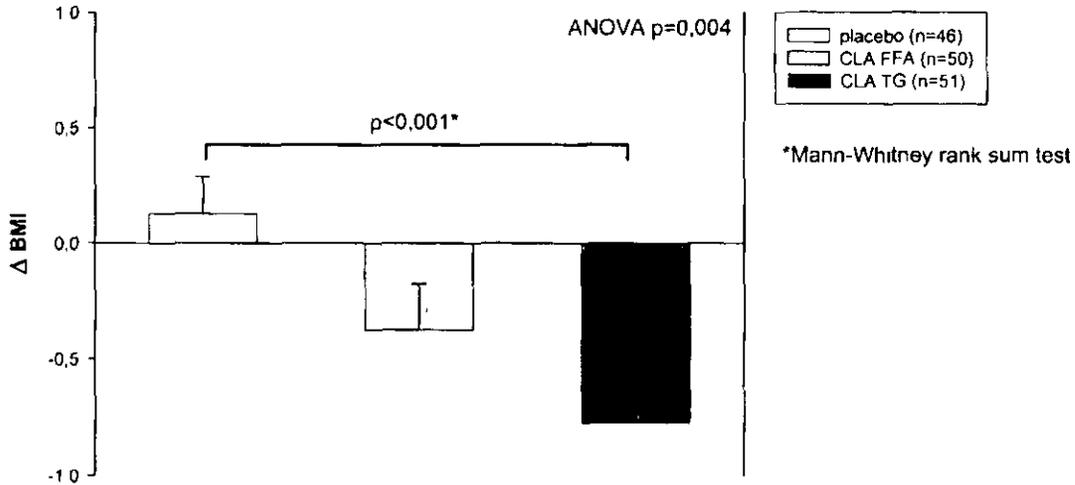
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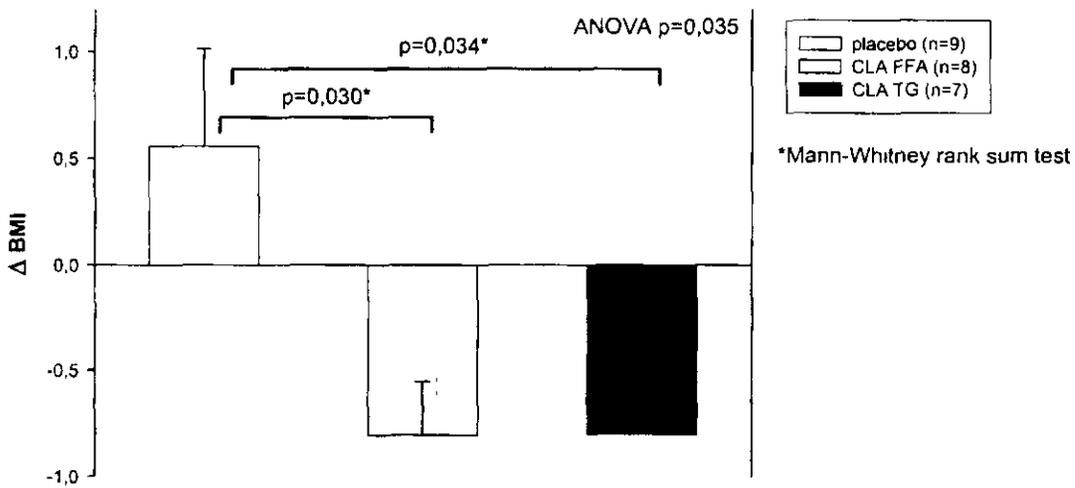
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**ANNEX**

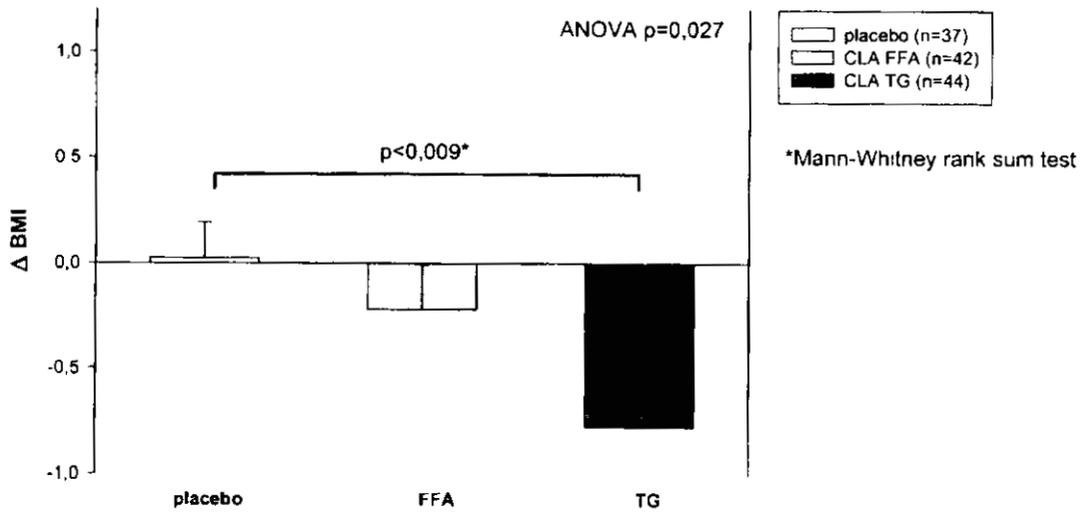


**Figure 1. Δ Body Mass Index (differences month 12 - month 0, mean + SEM).**  
Alterations during the study were significantly different between the CLA-TG and the placebo group

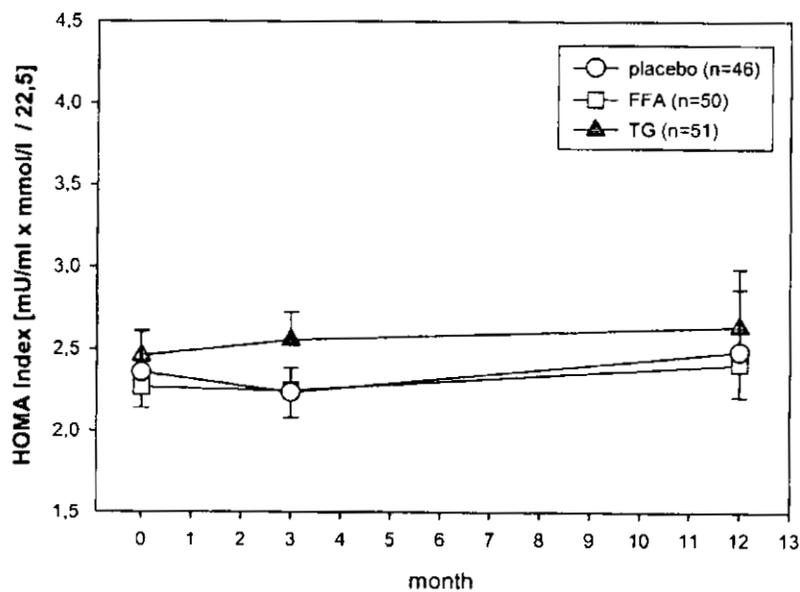


**Figure 2. Δ Body Mass Index in males (differences month 12 - month 0, mean + SEM).**  
Alterations during the study were significantly different between the CLA-TG and the placebo (olive oil group) as well as between the CLA-FFA and the placebo group

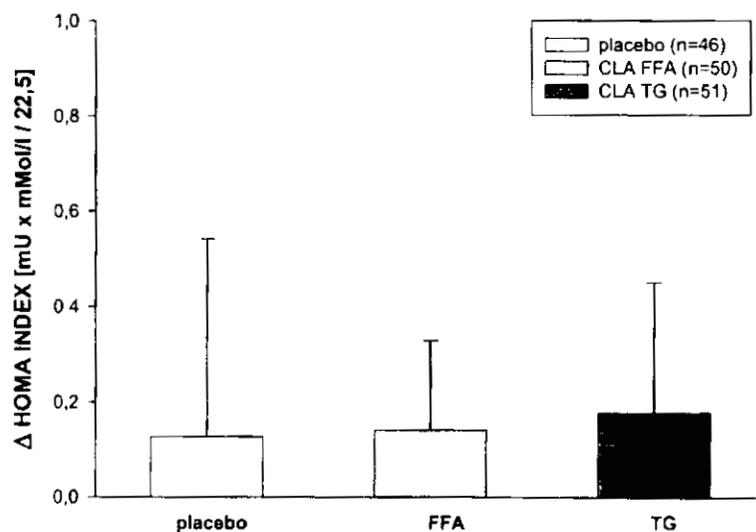
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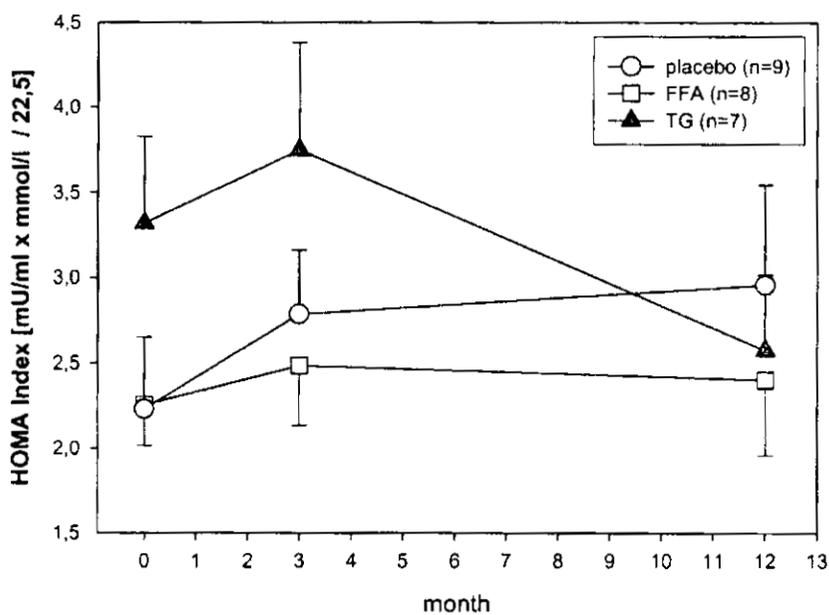
**Figure 3. Δ Body Mass Index in females (differences month 12 - month 0, mean + SEM).** Alterations during the study were significantly different between the CLA-TG and the placebo (olive oil group)



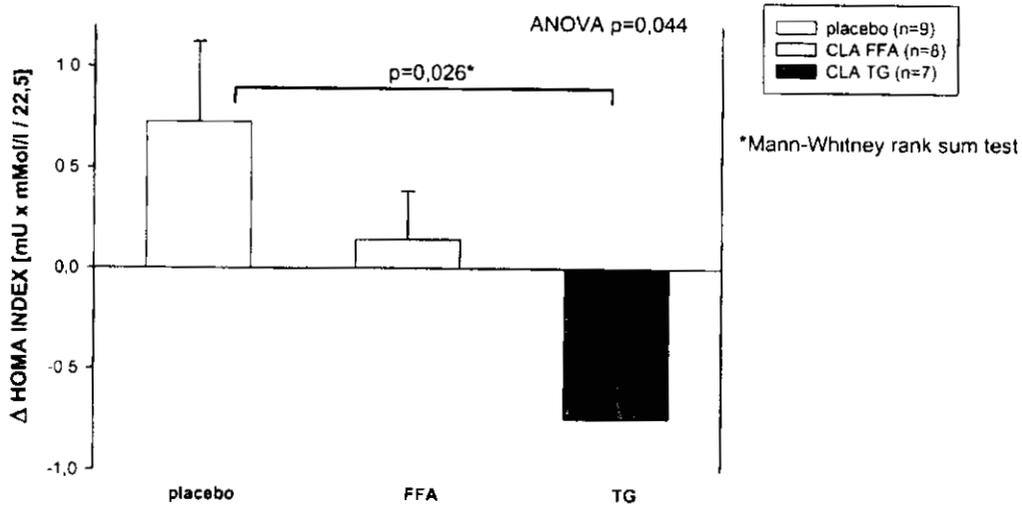
**Figure 4. HOMA indices (means + SEM) after 0, 3, and 12 months of treatment.** There were no significant differences between the CLA-FFA, CLA-TG, and placebo (olive oil) group at any time of the study



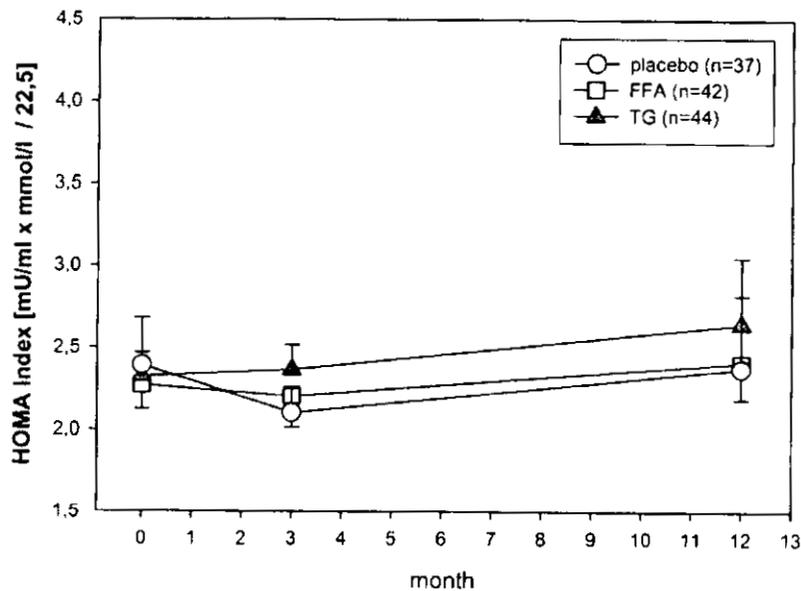
**Figure 5. Δ HOMA indices (differences month 12 - month 0, mean + SEM).**  
 There were no significant differences between the CLA-FFA, CLA-TG, and placebo (olive oil) group



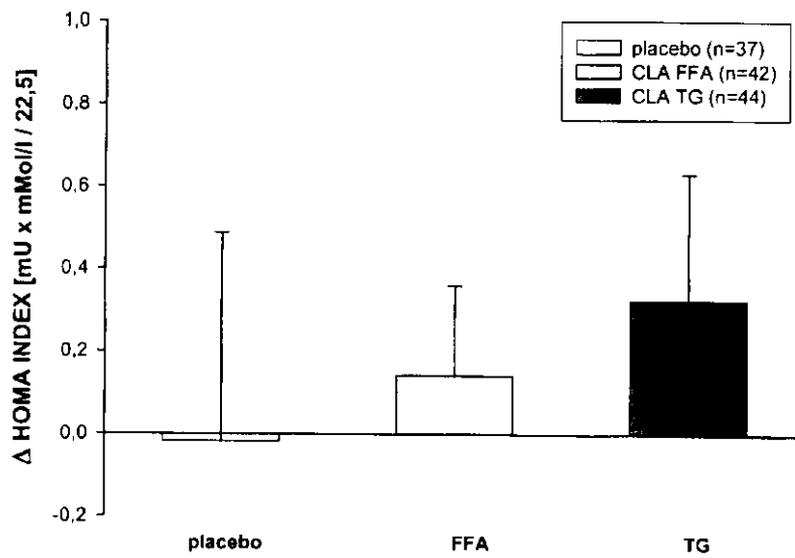
**Figure 6 HOMA indices in males (means + SEM) after 0, 3, and 12 months of treatment.**  
 There were no significant differences between the CLA-FFA, CLA-TG, and placebo (olive oil) group at any time of the study



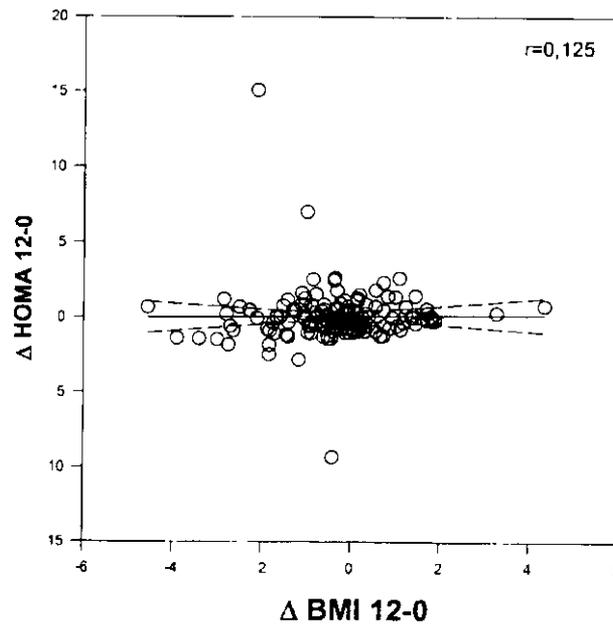
**Figure 7. Δ HOMA indices in males (differences month 12 - month 0, mean + SEM).**  
 Alterations during the study were significantly different between the CLA-TG and the placebo (olive oil) group



**Figure 8. HOMA indices in females (means + SEM) after 0, 3, and 12 months of treatment.**  
 There were no significant differences between the CLA-FFA, CLA-TG, and placebo (olive oil) group at any time of the study

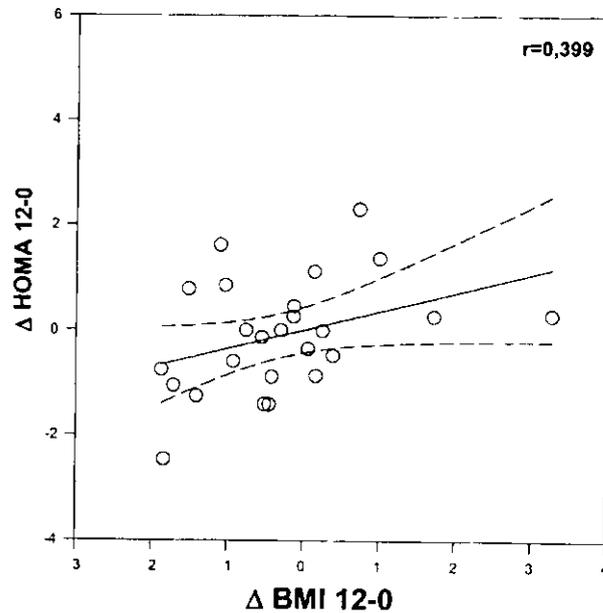


**Figure 9. Δ HOMA indices in females (differences month 12 - month 0, mean + SEM).**  
 There were no significant differences between the CLA-FFA, CLA-TG, and placebo (olive oil) group

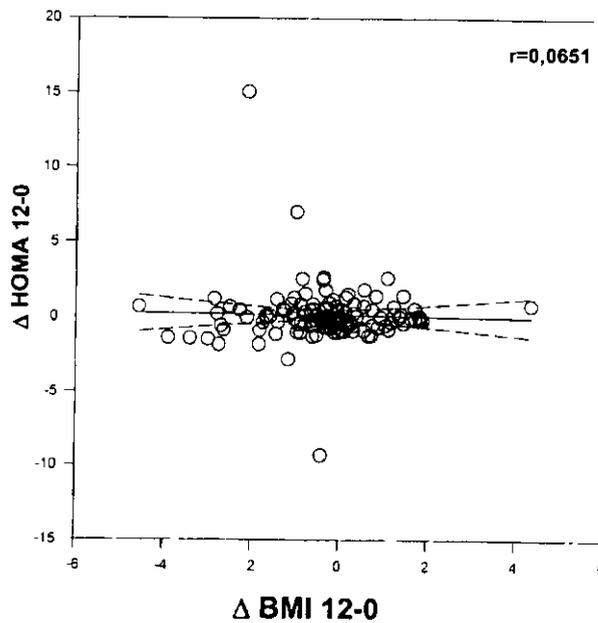


**Figure 10. Correlation of Δ HOMA with Δ BMI in all treatment groups (CLA-FFA, CLA-TG, and placebo, n=147).**  
 No correlation was found

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**Figure 11. Correlation of  $\Delta$  HOMA with  $\Delta$  BMI in males in all treatment groups (CLA-FFA, CLA-TG, and placebo, n=24).**  
 The pair(s) of variables with positive correlation coefficients and P values below 0,050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0,050, one variable tends to decrease while the other increases. For pairs with P values greater than 0,050, there is no significant relationship between the two variables.



**Figure 12. Correlation of  $\Delta$  HOMA with  $\Delta$  BMI in females in all treatment groups (CLA-FFA, CLA-TG, and placebo, n=147).**  
 No correlation was found

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**Table 1. Overview of studies on CLA and insulin sensitivity**

	test preparation	control	study population	objective	duration	result
Medina et al , 2000	t10,c12 22.6% c11,t13 17.6% c9,t11 16.6% t9,t11 7.7% t10,t12 7.7% other isomers 11.9%	sunflower oil	healthy women (n=24)	effects on circulating leptin concentrations and appetite	64 d	plasma insulin levels ↑ (n s ) plasma glucose ↔
Smedman & Vessby, 2001	c9,t11 t10,c12 (50/50) n=26	olive oil n=24	healthy volunteers	metabolic effects	12 w	serum glucose ↑ (n s ) plasma insulin ↔
Noone et al , 2002	c9,t11 t10,c12 (50/50) c9,t11 t10,c12 (80/20)	linoleic acid	healthy volunteers (n=51)	modulation of cardiovascular disease risk factors by CLA	8 w	plasma glucose ↔ plasma insulin ↔
Riserus et al , 2002	t10,c12 isomer (n=19)  CLA mixture (n=19)	most likely olive oil (n=19)	obese men with metabolic syndrome, HbA <sub>1c</sub> < 5%	effect of t10 c12 isomer or CLA mixture on insulin sensitivity, lipid metabolism, and body composition	12 w	insulin sensitivity ↓ fasting glucose ↑ fasting insulin ↑ HbA <sub>1c</sub> ↓  insulin sensitivity ↔ fasting glucose ↔
Belury et al , 2003	CLA mixture (n=11)	safflower oil (n=10)	type 2 diabetics without medications for glucose control	relationship of CLA to improvements in the management of type 2 diabetes mellitus	8 w	fasting glucose ↓
Eyjolfsson et al , 2004	CLA mixture c9,t11 35.5% t10,c12 36.8% (n=10)	safflower oil (n=6)	young sedentary volunteers	influence of CLA supplementation on insulin sensitivity	8 w	insulin sensitivity index ISI ↑ fasting insulin ↓
Malpuech-Brugère et al , 2004	1.5g or 3g c9,t11 isomer 1.5g or 3g t10,c12 isomer	high oleic sunflower oil	healthy overweight volunteers (n=81)	effects of CLA isomers on BFM	18 w	no effects in relation to insulin resistance
Riserus et al , 2004 a	c9,t11 isomer (n=13)	olive oil (n=12)	non-diabetic obese men with metabolic syndrome	effects of c9,t11 CLA on insulin sensitivity	3 m	insulin sensitivity ↓ fasting blood glucose ↔ serum insulin ↔
Riserus et al , 2004 b	t10,c12 isomer	?	non-diabetic obese men (n=57)	effects of t10,c12 CLA isomer on plasma proinsulin, C-peptide, and adiponectin	12 w	hyperproinsulinemia
Moloney et al , 2004	CLA mixture c9,t11 35.6% t10,c12 38.2% (n=16)	blend of palm and soya bean oil (n=16)	subjects with type 2 diabetes	insulin sensitivity and lipoprotein metabolism	8 w	insulin sensitivity ↓ fasting glucose ↑ HOMA-IR ↑ QUICKI ↔ HbA <sub>1c</sub> ↔
Tricon et al , 2004	c9,t11 isomer (increasing dosage) t10,c12 isomer (increasing dosage)	none	healthy men (n=49, cross over design)	influence of CLA isomers on body composition, bloodlipid profile, and markers of insulin resistance	8 w	plasma insulin ↔ HOMA-IR ↔ QUICKI ↔ plasma glucose ↑
Whigham et al , 2004	Clarnoi <sup>®</sup> c9,t11 37.3% t10,c12 37.6% (n=20)	high oleic sunflower oil (n=15)	healthy obese volunteers	safety profile of CLA over 12 months	12 m	serum insulin ↔ serum glucose ↔ HOMA-IR ↔

↓ decrease, ↑ increase, ↔ no alteration

**REVIEW ON  
CLA AND OXIDATIVE STRESS**

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### Metabolism of isoprostanes

The 15-F<sub>2t</sub>-Isoprostane (short names 15-F<sub>2t</sub>-IsoP or F<sub>2</sub>-IsoP, formerly named 8-iso-PGF<sub>2</sub>) and its major urinary metabolite 2,3-dinor-5,6-dihydro-8-iso-PGF<sub>2α</sub>, are considered to be the most sensitive and specific biomarkers of oxidant stress (lipid peroxidation) *in vivo*. Elevated levels are associated with a number of disorders like hypercholesterolemia, diabetes, obesity and with smoking (Morrow ATVB 05, Crakowski EurHJ 04, Davi et al CPLipids 2004). Increased concentrations are found in atherosclerotic plaques (Morrow ATVB 2005) and in urinary samples of patients with coronary heart disease (Schwedhelm et al Circ 2004).

Isoprostanes are structurally close to prostaglandins. There is a whole range of isoprostanes, with D,E and F-Rings, as well as isothromboxanes and isoketals (Crakowski and Ormezzano EurHJ 2004, Morrow and Roberts ProglR 1997). There are various isoforms within each group. D<sub>2</sub>/E<sub>2</sub>-isoprostanes can further undergo dehydration to form A<sub>2</sub>/I<sub>2</sub>-isoprostanes (Chen et al JBC 1999). IsoP A<sub>2</sub> readily adducts to glutathione (GSH) or albumin, to the same extent as PG A<sub>2</sub> (Chen et al JBC 1999). Interestingly, after adduction with GSH, the biological activity of IsoP A<sub>2</sub> is lost. Thus, this is likely a mechanism to detoxify these molecules. Such conjugated metabolites of IsoP A<sub>2</sub> are found in urine (Milne et al 2005).

While prostaglandins (PG) originate from enzymatic synthesis, with cyclooxygenase (COX) as a key enzyme, isoprostanes are generated non-enzymatically upon oxidative stress from the precursor arachidonic acid. Yet, there are exceptions to this rule. IsoP F<sub>2</sub> can be synthesized enzymatically (Watkins et al BJ 1999, Jourdan et al FASEB J 1999), and D<sub>2</sub> and E<sub>2</sub> prostaglandins can be synthesized via the isoprostane way to a considerable extent, i.e. with the corresponding D<sub>2</sub> and E<sub>2</sub> isoprostanes as intermediates (Gao et al JBC 2003). Enzymatic synthesis of prostaglandins occurs after the precursor fatty acids arachidonic acid (20:4ω6) or eicosapentaenoic acid (20:5ω3) has been released from a phospholipid molecule, while isoprostanes are formed *in situ*, i.e. while arachidonic or eicosapentaenoic acid are still at the sn2-position of a phospholipid molecule. Thus, isoprostanes exist in a "storage depot" and may be released later on. Isoprostane-containing phospholipids are remarkably kinked molecules. Therefore, it can be imagined that their presence in membranes does affect fluidity and integrity and may thus contribute to cellular dysfunction, and that release of free isoprostanes would be a measure to restore membrane integrity (Morrow PNAS 1992). Lipid peroxidation leads to loss of phospholipid asymmetry in plasma membranes, causing membrane vesiculation. Oxidized microvesicles may play a role in the initiation and amplification of chronic inflammatory processes. Whether isoprostane-containing phospholipids were involved is not clear (reviewed in Leitinger COL 2003). In all, their role in membranes has been surprisingly little addressed.

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CCl<sub>4</sub> intoxication is a well-characterized model of free radical-initiated damage, which causes lipid peroxidation. Administration of CCl<sub>4</sub> to rats increased F<sub>2</sub>-isoprostanes (Morrow et al. PNAS 1992) in liver tissues esterified on phospholipids, and later on appeared in plasma in its free form. IsoP D<sub>2</sub>/E<sub>2</sub> and IsoP A<sub>2</sub>/J<sub>2</sub> were likewise increased in livers of rats exposed to CCl<sub>4</sub> (Chen et al. JBC 1999). Jourdan et al. (FASEB J 1999) and others (reviewed in Jourdan et al. FASEB J 1999) showed that cytokines stimulate release of both IsoP F<sub>2</sub> and PG E<sub>2</sub> in various cell systems. Stimulation of both IsoP F<sub>2</sub> and PG E<sub>2</sub> was blocked by COX-2 inhibitors. Treatment of smooth muscle cells with the superoxide-producing enzyme xanthine oxidase also stimulated both IsoP F<sub>2</sub> and PG E<sub>2</sub> release, but in this case the COX-2 inhibitor prevented only PG E<sub>2</sub>, but not IsoP F<sub>2</sub> release (Jourdan et al. FASEB J 1999). In the presence of GSH, however, more IsoP F<sub>2</sub> than IsoP D<sub>2</sub>/E<sub>2</sub> are produced in peroxidizing rat liver microsomes as well as in livers of rats subjected to CCl<sub>4</sub>-treatment. In parallel, GSH favours synthesis of PG F<sub>2</sub> as compared to PG D<sub>2</sub>/E<sub>2</sub> (Morrow et al. ABB 1998). Marathe et al. (JLR 2001) confirmed that in livers of CCl<sub>4</sub>-treated rats predominantly IsoP F<sub>2</sub> are formed, as endogenous substances reduce the isoprostane endoperoxide intermediate to F-ring compounds. They confirmed that these IsoP F<sub>2</sub> are platelet activating factor (PAF) receptor agonists, that esterification to phospholipid is required for activity, but that activity was around 1,000-fold less than the natural ligand PAF, which was also produced.

Focus is usually on the measurement of F<sub>2</sub>-IsoP, because of its chemical stability. It has vasoconstricting properties and in model systems it stimulated mitogenesis, monocyte adhesion to endothelial cells and induced necrosis (Crakowski EurHJ 2004). Other isoprostanes are biologically active as well, the most potent ones being the isoprostanes from the E-series. Potency of 15-E<sub>2</sub>-IsoP as a vasoconstrictor is 10 times that of F<sub>2</sub>-IsoP (Crakowski EurHJ 2004). In fact, F<sub>2</sub>-IsoP has dual functions, depending on the biological setting. It may modulate vascular tone by a direct action on the thromboxane (TP) receptor to cause contraction. On the other hand it may cause dilatation via another receptor, leading to the release of nitric oxide (NO) (Jourdan et al. J Pharmacol 1997). It did inhibit arachidonic acid-induced platelet aggregation (Morrow et al. P 1992) and monocyte adhesion to microvascular endothelial cells via either the TP receptor or another independent mechanism (Kumar et al. FASEB J 2005). Distinct antiinflammatory properties were also observed for A<sub>2</sub>/J<sub>2</sub>-IsoP (Musiek et al. JBC 2005) and for IsoP F<sub>3</sub>, which can be formed from eicosapentaenoic acid in vivo (Gao et al. JBC 2006). All the observed biological activities are those of the free isoprostanes. In plasma, however, the majority of IsoP F<sub>2</sub> are in fact present in the esterified form (Moore CPLipids 2004).

One of the earliest steps in the development of the atherosclerotic lesion is the accumulation of monocyte/macrophages within the vessel wall, leading to formation of fatty streaks. Oxidized lipids present in minimally modified-low density lipoproteins (MM-LDL) contribute to this process by activating endothelial cells to express monocyte-specific adhesion molecules and chemoattractant

factors like monocyte chemotactic protein-1 (MCP-1) or interleukin-8 (IL-8). When a certain critical concentration of so-called seeding molecules including hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE) is reached, the nonenzymatic oxidation of LDL phospholipids begins. A series of biologically active, oxidized phospholipids are produced that mediate the cellular events leading to fatty streaks. The biologically most active molecules are oxidized derivatives of 1-palmitoyl-sn-2-arachidonyl-phosphatidylcholine (Ox-PAPC), namely 1-palmitoyl-2-(5-oxovaleroyl)-phosphatidylcholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), and 1-palmitoyl-2-(5,6-epoxyisoprostane E<sub>2</sub>)-sn-glycero-3-phosphatidylcholine (PEIPC) (see Navab et al. *COL* 2002 for more references). But also epoxy pentenone phospholipids (PECPC), dehydrated derivatives of PEIPC, are biologically active (Subbanagounder et al. *JBC* 2002). Isoketals are among the Ox-PAPC that modify apolipoprotein B in LDL, thus promoting atherogenic properties further (Brame et al. *JBC* 2004). The various Ox-PAPC have not identical effects. Chemical structure of POVPC and PGPC is very similar to platelet-activating-factor (PAF). Nevertheless, even the effects of POVPC and PGPC are not identical (Leitinger et al. *PNAS* 1999). PEIPC and PECPC initiate the hallmark event, the expression of MCP-1 and IL-8 on endothelial cells (Subbanagounder et al. *JBC* 2002), acting through the prostaglandin E<sub>2</sub> receptor subtype 2 (Moullisseaux et al. *Circ Res* 2006). Of note, oxidized LDL and Ox-PAPC induced COX expression and COX-2-dependent secretion of the proinflammatory eicosanoid PG E<sub>2</sub> from primary monocytes, but isoprostanes were not involved in this action (Pontsler *JBC* 2003). Ox-PAPC are found in atherosclerotic lesions.

Continued oxidative modification turns MM-LDL into Ox-LDL. During this process, Ox-PAPC are increasingly destroyed, yielding, amongst others, lysophosphatidylcholine (lyso-PC) and free isoprostanes. Lyso-PC is also vasoactive and stimulates smooth muscle cell proliferation, but clearly less than Ox-PAPC. Ox-LDL, as compared to MM-LDL, is thus less stimulatory, but becomes increasingly cytotoxic (Berliner et al. 1995). Isoprostanes as a whole (Marathe et al. *JLR* 2001) or specifically PEIPC and PECPC (Subbanagounder et al. *JBC* 2002) are only active as intact phospholipids, not as free (modified) fatty acids. The hydrolysis of Ox-PAPC is therefore considered a protective mechanism.

IsoP F<sub>2</sub> are also formed during LDL oxidation (Goupal et al. *FEBS L* 1994, Lynch et al. *JCInv* 1994, Proudfoot et al. 1995, Marathe et al. *JLR* 2001). During continued oxidation in vitro, esterified IsoP F<sub>2</sub> are lost and free IsoP F<sub>2</sub> increase (Lynch et al. *JCInv* 1994). The kinetics of formation of isoprostane F<sub>2</sub>-containing phosphatidylcholines and PAF-like substances is largely identical (Marathe et al. *JLR* 2001). But, as mentioned above, isoprostane-containing Ox-PAPC with E<sub>2</sub>- rather than F<sub>2</sub>-ring structure are the most potent ones, while F<sub>2</sub>-ring isoprostanes have only a weak effect (Marathe et al. *JLR* 2001). Of note, preincubation with oxidized LDL or Ox-PAPC stimulated synthesis of GSH in

cultured endothelial cells, and this made the cells resistant to oxidative stress imposed lateron (Moellering JBC 2002) Such a response suggests an adaptive mechanism The chemical structure of the Ox-PAPC was not determined in this study

#### **CLA effects on isoprostane metabolism**

An increase of urine IsoP F<sub>2</sub> levels was observed in humans (Riserus et al Circ 2002, Riserus et al AJCN 2004), as well as an increase of serum IsoP F<sub>2</sub> (Taylor et al ATVB 2006) In all these studies, olive oil was used as control fat An increase induced by the t10,c12 isomer as compared to sunflower oil as control was observed in mice The c9,t11-CLA had no effect. (Rajakangas et al JN 2003)

Cell culture (Ma et al Nutr Cancer 2002) and animal experiments (Turek et al JNB 1998, Li and Watkins Lipids 1998, Liu and Belury Cancer Letters 1998, Sugano et al Lipids 1998) fairly consistently showed that PG E<sub>2</sub> release or PG E<sub>2</sub> levels are decreased following CLA treatment PG E<sub>2</sub> synthesis was lower in PBMC isolated from pigs on a CLA diet (Changhua et al JN 2005) There was a concomitant increase in PG F<sub>2</sub> (Miller et al Lipids 2001) or PG F<sub>2</sub> and IsoP F<sub>2</sub> (Miller et al BJN 2003) in cultured cells In humans, no change in serum PG E<sub>2</sub> levels were observed in comparison to linoleic acid (Nugent et al EurJCN 2005) CLA as compared to sunflower oil did not alter the in vitro secretion of PG E<sub>2</sub> in PBMC stimulated with LPS (Kelley et al Lipids 2001), but there was a trend towards lower levels in another study (Albers et al EJCEN 2003) Reduced PG E<sub>2</sub> synthesis was also observed in Raw264 7 macrophages and in mouse lung in vivo, due to decreased COX expression Inhibition of NF-κB was one of the mechanisms for the reduced COX-2 expression The t10,c12-isomer was more active than c9,t11-CLA (Li et al JLR 2005) Both t10,c12-CLA and c9,t11-CLA exerted such an effect in normal and malignant mammary cells In this system a reduced expression of both COX-2 and the PG E<sub>2</sub> receptor subtype EP2 was observed (Wang et al Anticanc Res 2006) Reduction of PG E<sub>2</sub> production is considered beneficial, and it is yet not be examined whether a concomitantly increased production of IsoP F<sub>2</sub> would outweigh this beneficial effect

In vitro systems show that CLA affects several pathways through which IsoP may be regulated CLA suppressed PAF production in endothelial cells (Sneddon et al BBA 2006) This antiinflammatory effect could suppress monocyte adherence to the endothelium Furthermore, as t10, c12-CLA reduced the production of the 5-lipoxygenase metabolite of linoleic acid, 5-hydroxyicosatetraenoic acid (5-HETE) (Kim BBA 2005), it may be assumed that CLA inhibits lipoxygenase activity That effect was not seen with c9, t11-CLA or linoleic acid Reduction of lipoxygenase activity would transfer to a reduced number of precursors (“seeding molecules”) for IsoP synthesis and thus be protective CLA, like troglitazone, prevented the destabilisation of the ATP binding cassette transporter A1 (ABCA1) in macrophages, brought about by the saturated fatty acids palmitate and stearate (Wang et al JLR 2004)

ABCA1 transfers cholesterol and phospholipids to free apolipoprotein A1 or to HDL. This process is essential for the cells to get rid of excess cholesterol and also phospholipids, possibly also Ox-PAPC. This might ensure integrity of the cell membrane, but in turn it might also mean enhanced transfer of oxidized lipids onto lipoproteins, challenging the lipoprotein antioxidative systems. CLA is among the lipids that regulate glutathione peroxidase (GPx4), an antioxidant enzyme that directly reduces phospholipid hydroperoxides within membranes. CLA enhanced selenium-induced GPx4 mRNA levels, while enzyme activity was unaffected (Sneddon Atherosclerosis 2003). Diminished concentrations of these "seeding molecules" would protect lipoproteins in the microvasculature, and probably also membranes, to experience a more extensive oxidative modification. GPx4 activities were also induced in MCF-7 cells exposed to milk fat over 8 days (O'Shea et al. Anticancer Res 2000). In splenocytes of CLA-fed mice, concentration of GSH was increased, paralleled by diminished NF- $\kappa$ B activation. In vitro, 25  $\mu$ M CLA enhanced intracellular GSH concentration, but with 100  $\mu$ M this effect was reversed (Bergamo et al. JLR 2006, in press, online). As outlined before, GSH acts in a protective way, by inducing a shift from IsoP E<sub>2</sub>/D<sub>2</sub> to IsoP F<sub>2</sub>. Furthermore conjugates of IsoP A<sub>2</sub> (and probably also IsoP J<sub>2</sub>) with GSH are formed, rendering these molecules biologically inactive.

CLA increases plasma levels of  $\gamma$ -tocopherol in humans (Basu et al. FEBS L 2000). Increased levels of free retinol and a decreased ratio retinyl ester/retinol was found in animal tissues (Carta et al. PLEssFA 2002). This could be due to a mere shift of these vitamins between compartments, or release from storage depots, but at least it makes it more unlikely that CLA consumption is associated with an increased oxidative stress.

### Conclusions

At this point it is not entirely clear whether increased levels of free IsoP F<sub>2</sub> are due to an increased production of IsoP F<sub>2</sub> and thus a truly increased oxidative stress, or rather to an enhanced release of free IsoP from phospholipids, which would reflect an increased clearing function, or rather to a shift from more potent IsoP D<sub>2</sub>/E<sub>2</sub> to less potent IsoP F<sub>2</sub>. There are even findings which indicate an antioxidative action of CLA. Of course, several mechanisms may apply. Until this is clarified, one should be cautious to draw premature conclusions or even deduce a risk for the use of CLA. One should also pay attention to the oil chosen as control. Phenols and terpenes in olive oil may have an inhibitory effect on isoprostane formation of their own. Results from cell culture studies are to be regarded with caution unless physiologically reasonable concentrations of CLA were chosen, as effects may be dose-dependent (Bergamo et al. JLR 2006, in press).

Kiel, August 14, 2006

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Prof. Dr. Juergen Schrezenmeier

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OPINION RE: THE EFFECTS OF CLA ISOMER MIXTURES ON INSULIN SENSITIVITY .

GEORGE STEINER

I have been asked to comment on the question of conjugate linoleic acid (CLA) and insulin sensitivity. The basis for this lies in the disparate information that is available in the literature. That information covers both human and animal studies. The animal data may support, question or indicate investigations that are needed in humans. They may also suggest modes of action. However, until these questions are tested in humans, they will not help in deciding on safety with respect to insulin sensitivity in humans. Thus, in my mind, the human studies will weigh more heavily in considerations about this aspect of CLA use in humans.

In preparing my opinion, I have been supplied with an overall document prepared by the company and reprints of several articles that I have requested. In addition I have had a telephone conversation with Dr. O'Shea.

The company has asked me to comment on a mixture of CLA isomers, trans-10, cis-12 (t10,c12) and cis-9, trans-11 (c9,t11). Each isomer appears to have different effects on insulin sensitivity. In addition, the effects appear to differ in relation to the time for which the CLA is used. The vast majority of studies have used fasting insulin and glucose or an index derived from these values to reflect insulin sensitivity. The "gold standard" glucose-insulin clamp method has only been used in a few and the other widely accepted method, the frequent sampling intravenous glucose tolerance test has not been used in any. In assaying insulin levels one must be certain to use an assay for insulin that is specific. The studies that are considered have done so.

In those studies that have measured several time points it appears that any deterioration in insulin sensitivity that might be noted is seen at an early time point but not later. One example is Whigham *et al.* (2004) in which there was an apparent deterioration at two weeks but not later up to 1 year. However, it should be noted that there were 10 statistical comparisons made and only the two week data for glucose showed a  $p < 0.05$  difference. When multiple statistical examinations are made there is a 1/20 chance of a difference being significant by chance alone. Thus corrections for multiple comparisons should be made. This does not seem to have been done and so even the two week point would not be significant and one would argue that, in this study, there is no deterioration in glucose. at any time. This is further born out by the failure to find any change over time in insulin sensitivity.

A number of studies in humans with the mixture failed to show any effect on fasting insulin or glucose levels. Only three have measured insulin sensitivity itself. Moloney (2004) and Tricon (2004b) used the HOMA or QUICKI indices to reflect insulin sensitivity. As noted earlier these indices are is not the gold standard and are an index calculated from fasting insulin and glucose. Thus all of the studies to this point have inferred that there is no change in insulin sensitivity. However, in people without

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diabetes there is a good correlation between fasting levels of insulin and insulin sensitivity. That plus the number of studies done suggests that the mixture does not reduce insulin sensitivity. Pure isomers were studied by Malpeuch Brugere studies et al. In individuals with a BMI of approximately 27.5 (overweight), they found no change in insulin sensitivity as reflected by the concentrations of glucose and insulin. Tricon et al (2004b) examined the effects of pure isomers in individuals with an average BMI of approximately 24.5 (not overweight). However, their protocol admitted people with a BMI up to 34 so they might have had some overweight and even some obese members in the study group. Their publication did not include an examination of individuals categorized by their degree of obesity. They evaluated two parameters somewhat more reflective of insulin resistance: HOMA and QUICKI. Using these calculated estimates, they also found no effect of the pure isomers on insulin sensitivity. The confusion that can be raised with such indices is apparent in Moloney's study. That study examine the effects of a mixture of CLA isomers in men and women with diabetes. It used four different indices: HOMA (showed CLA to increase insulin sensitivity vs the control group); QUICKI (revealed no change in insulin sensitivity) and ISI and OGIS (both of which suggested a decrease in insulin sensitivity vs the control group). This confusion is compounded by the problems of interpreting insulin assays in those with diabetes. The one group that used clamp methodology to evaluate insulin sensitivity was that of Risérus (2002a, 2002b, 2004b – these are all the same study – and 2004c. they found that there was an increase in insulin resistance in those given t10,c12. They also were the only group to find that the c9,t11 increased insulin resistance in humans (Risérus 2004c). It should be noted that in the c9,t11 study the difference in insulin sensitivity was  $p < 0.05$ . Sixteen statistical comparisons were made and no apparent allowance was made for multiple comparisons in that study. In the study with t10,c12 there was no increase in insulin resistance when the mixed isomers were given. This supported other studies with the mixed isomers. Preliminary data from another clamp study in humans and found no change in sensitivity giving the mixed isomers to an overweight and obese population (Gaulhier et al.). Thus, although there may be some uncertainty about the impact of the pure isomers on insulin sensitivity all human studies agree that there is no effect of the CLA mixture on insulin sensitivity. Another interesting point of note is that Risérus' study with t10,c12 was done in those with the metabolic syndrome, their study with c9,t11 was conducted in obese individuals who might have had the metabolic syndrome. It is intriguing to speculate that insulin resistance with the pure CLA isomers is only seen in those who can not compensate adequately (i.e. those with the metabolic syndrome) and not in healthy or perhaps even diabetic individuals.

Some may ask whether the lack of effect of a CLA mixture on insulin sensitivity, in the face of an increase in resistance in those receiving t10,c12 might reflect the mixture providing a lower dose of the t10,12 isomer. However, that does not appear to be the case as there are studies with the single isomer in which doses similar to those that would be given in the mixture still produced an increase in insulin resistance. There is no readily apparent explanation of how a mixture would protect against the effects of t10,c12 on insulin sensitivity.

A number of animal studies, primarily in rats and mice but also in hamsters and pigs, have been consistent with the observations in humans. There was little effect on insulin sensitivity and, where it was seen, it was transient and generally occurred only with the pure t10,c12 isomer. These studies and those conducted in cell cultures have raised several possible explanations for any observed effects. They have suggested that a fatty liver and reduced adipose tissue mass occurs with CLA. They have raised the possibility of a number of mechanisms whereby this might occur: changes in PPAR, particularly PPAR-gamma; changes in TNF-alpha; changes in the glucose transporter, GLUT4, and changes in certain adipokines (adiponectin and leptin)

In summary, the most important information with respect to any safety effects on glucose/insulin metabolism is that obtained in the human studies. If anything only one group (Risarius) observed increases insulin resistance in humans. Where this was observed, it appeared to be when pure isomers were given alone and, if this is a true phenomenon, it remains to be explained. Administration of the mixture of CLA isomers does not increase insulin resistance.

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November 4, 2005

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# Appendix C

**Appendix C**

**Additional *In vitro* and Animal Studies**

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## APPENDIX C

### ADDITIONAL *IN VITRO* AND ANIMAL STUDIES

Within this appendix we provide a detailed review of additional *in-vitro* and exploratory animal studies on CLA and CLA-rich oils. As explained in the main dossier, because of the nature of how CLA is metabolized in humans compared to animals and because of the exploratory rather than safety focus of such studies in many cases, these data are not considered pivotal. They do in some cases provide further explanation of effects observed in human clinical studies and classic pre-clinical toxicity studies, and in such cases more specific reference is made to parts of this appendix in the main text. However, whilst we present this detailed review of the data, to show that the totality of the evidence in relation to the safety of CLA-rich oil has been considered, we present it as an appendix to allow the reviewer to focus with greater clarity on the pivotal data and the structure of the risk assessment that is presented in the main dossier.

#### C.1 Cardiovascular Disease

Animal studies evaluating cardiovascular disease risk factors are summarized in Table C 1.6.3-1 below.

##### C.1.2 Lipid Metabolism Parameters

##### C.1.2.1 *In Vitro* Studies

Pal *et al.* (2005) studied the effects of a mixture of CLA isomers (isomers not specified) on very low density lipoprotein/VLDL metabolism. The responses of HepG2 liver cells to a CLA mixture, a saturated fatty acid (palmitic acid), an n-6 fatty acid (linoleic acid), and a control were compared. It was demonstrated that apolipoprotein B100 (apoB100) and intracellular cholesterol levels were significantly decreased in cells exposed to CLA compared to control cells. The authors concluded that CLA reduces apoB100 production and secretion compared to saturated and polyunsaturated fatty acids, potentially by limiting the availability of free cholesterol (required for apoB100 production). A reduction in apoB100 production in the body would decrease the levels of VLDL and low density lipoprotein (LDL) and thus decrease the risk of developing cardiovascular disease.

##### C.1.2.2 Animal Studies

##### *Studies in Mice*

Several investigators have studied the effects of 50:50 CLAs mixture and the individual c9,t11 and t10,c12 CLA isomers on various aspects of lipid biology in mice. Wargent *et al.* (2005) observed a transient rise in triglycerides in mice fed CLA, which normalized after 3 weeks.

However, with the exception of this study, 50:50 CLA mixtures have been reported not to produce deleterious effects on lipid parameters in mice (Munday *et al.*, 1999; Hamura *et al.*, 2001a,b).

Several studies revealed that the *c9,t11* isomer either has no effect or significantly decreases serum triglycerides (Roche *et al.*, 2002; de Roos *et al.*, 2005; Degrace *et al.*, 2003) and that the *t10,c12* CLA isomer appears to have variable, strain-dependent effects on serum triglycerides by either lowering, elevating, or having no effect on serum lipids (Roche *et al.*, 2002; Degrace *et al.*, 2003; de Roos *et al.*, 2005).

#### *Studies in Rats*

Preclinical trials reported in Section 7.3.3 above, show that serum lipids were either unchanged or altered in a beneficial way regarding cardiovascular health [e.g., decreased plasma cholesterol in male rats as reported by O'Hagan and Menzel (2003)] in the rat model. The 50:50 mixture and the pure isomers showed no adverse effects to lipid parameters.

#### *Studies in Rabbits*

The rabbit has been used extensively to study cholesterol metabolism since they quickly develop atherosclerotic plaques when fed a cholesterol-rich diet. Lee *et al.* (1994) reported that a 50:50 mixture of CLA isomers (0.5 g CLA/rabbit/day) administration for 22 weeks reduced total and LDL cholesterol and triglycerides and reduced atherosclerosis. Similarly, Kritchevsky *et al.* (2000) reported that dietary levels as low as 0.1% CLA (43.29% *c9,t11* and 44.07% *t10,c12*) for 90 days inhibited atherogenesis, while levels of 1% caused 30% regression of established atherosclerosis. In another study, Kritchevsky *et al.* (2004) demonstrated that the 2 CLA isomers and the isomer mix were equally effective at reducing the severity of pre-existing atheromatous lesions in rabbits fed diets containing CLA for a period of 90 days. Corino *et al.* (2002) reported that a 50:50 mixture of CLA reduced triglycerides and total cholesterol in plasma, but serum leptin levels tended to increase

#### *Studies in Hamsters*

Numerous studies have investigated the effects of CLA on plaque formation, lipid metabolism, and cholesterol levels in hamsters. Studies in which hamsters were fed a proatherogenic diet demonstrated a decrease in early atherosclerosis and improved serum lipid parameters (Nicolosi *et al.*, 1997; Valeille *et al.*, 2004). Three studies that investigated the effects of a 50:50 CLA mixture demonstrated no consistent effects on lipid metabolism (Bouthegeourd *et al.*, 2002; Sher *et al.*, 2003; Valeille *et al.*, 2004)

Navarro *et al.* (2005) reported that the *t10,c12* isomer decreased hepatic cholesterol levels and Mitchell *et al.* (2005) reported an increase in high density lipoprotein (HDL) cholesterol. Sher *et al.* (2003) and Navarro *et al.* (2003) reported an increase in LDL-cholesterol and a decrease in

LDL-cholesterol, respectively. The c9,t11 CLA isomer also had variable effects on lipid parameters, either showing no effect (Bouthegeourd *et al.*, 2002; Navarro *et al.*, 2003; Zabala *et al.*, 2004; Macarulla *et al.*, 2005) or improved lipid parameters (Valeille *et al.*, 2004).

#### C.1.2.3 Summary

Based on the weight of the evidence, *in vitro* and animal feeding studies (different species) indicate either a benefit or no significant risk from the consumption of a CLA 50:50 isomer mixture.

### C 1.3 Markers of Oxidative Stress

#### C.1.3.1 *In Vitro* Studies

Flintoff-Dye and Omaye (2005) examined the effects of individual CLA isomers on LDL oxidation. The authors reported that CLA isomers were prooxidant at low concentrations, protective against oxidation at medium concentrations, and prooxidant again at high concentrations.

An *in vitro* study by Iannone *et al.* (2007) offers supporting data relating to the safety of CLA mixtures as they relate to oxidative stress. The authors noted that previous studies demonstrated that CLA increases plasma 8-iso-PGF<sub>2α</sub> levels and is metabolized in peroxisomes to form the metabolite CD16:2. In this study, skin fibroblasts were obtained from normal subjects (control) and patients affected by X-linked adrenoleukodystrophy (ALD), a neurodegenerative disorder in which peroxisomal beta-oxidation is impaired, resulting in the accumulation of very long chain fatty acids. Both cell cultures were incubated 8-iso-PGF<sub>2α</sub> alone or in combination with a 50:50 mixture of CLA isomers. The incorporation of 8-iso-PGF<sub>2α</sub> and its metabolite 2,3-diinor (DIN) into cell lipids was measured, as was the formation of CD 16:2. The authors reported that 8-iso-PGF<sub>2α</sub> was not well incorporated into the lipids of normal culture cells and was only present in the free form, which indicates that it was not incorporated into the fatty acids. DIN was detected in esterified form and was efficiently incorporated into cell lipids. In ALD fibroblasts, the formation of DIN was lower than that in the control cells. In the presence of CLA, the formation of DIN was significantly lower in both cell cultures, with a greater reduction in ALD cells. The formation of CD16:2 was significantly lower in ALD cells compared to normal cells; however, incubation with 8-iso-PGF<sub>2α</sub> did not significantly affect the formation of CD16:2. In addition, the t10,c12 CLA isomer was more efficiently metabolized to CD 16:2 than the c9,t11 isomer. These results suggest that CLA affects the metabolism of 8-iso-PGF<sub>2α</sub> in the peroxisomes, and thus competes for the same metabolic pathway. This mechanism provides a clear explanation for why an increase in isoprostanes is seen following CLA intervention. This coupled with the lack of effect on oxidative stress markers other than isoprostanes clearly shows that CLA does not cause oxidative stress (Iannone *et al.*, 2007).

### C.1.3.2 Animals Studies

Further to their *in vitro* study, Iannone *et al.* (2007) assessed isoprostane formation *in vivo* in male Sprague-Dawley rats. A total of 16 rats were fed diets containing 0 or 1% of a 50:50 mixture of CLA isomers for a period of 2 weeks. At the end of the feeding period, an unspecified number of control rats and rats fed CLA were administered carbon tetrachloride (CCl<sub>4</sub>), a known pro-oxidant and hepatotoxin, in mineral oil, while the remainder of the rats received an equal volume of mineral oil alone. Rats were then euthanized 4 hours after CCl<sub>4</sub> intoxication. The livers of each animal were then evaluated for the presence of each CLA isomer, CLA metabolites, levels of 8-iso-PGF<sub>2α</sub> and its metabolite, DIN. The metabolic products of CLA were incorporated into liver lipids, and incorporation of c9,t11 CLA, t10,c12 CLA and their metabolites CD16:2 and CD18:3 were significantly higher, and CD20:4 was lower, in the livers of rats treated with CLA + CCl<sub>4</sub> compared to CLA alone. Esterified 8-iso-PGF<sub>2α</sub> was significantly higher in CCl<sub>4</sub>-treated rats compared to rats not treated with CCl<sub>4</sub>. CLA consumption did not influence the levels of esterified 8-iso-PGF<sub>2α</sub> detected. Similar results were reported for arachidonic acid hydroperoxides. The formation of DIN, however, was reported to be significantly decreased in rats treated with CLA + CCl<sub>4</sub>. The decrease in DIN formation, together with the increase in CD16:2 in the livers of rats treated with CLA + CCl<sub>4</sub>, suggest that CLA competes for the same peroxisomal β-oxidation pathway that metabolizes 8-iso-PGF<sub>2α</sub> to DIN

Other *in vivo* studies into the effects of CLA on markers of oxidation have largely been confined to humans. These studies and the mechanisms involved are discussed in detail in Section 7.5.6 below.

### C.1.4 Hepatic Lipid Accumulation

#### C.1.4.1 In Vitro Studies

CLA has been shown to activate peroxisomal proliferator-activated receptor (PPAR)-α *in vitro* (Moya-Camarena *et al.*, 1999; Evans *et al.*, 2001). This mechanism has been proposed as being responsible for the effects observed *in vivo* predominantly in the mouse model (see Section C.1.4.2).

#### C.1.4.2 Animal Studies

##### *Studies in Mice*

Several studies demonstrated that feeding high concentrations of CLA to mice resulted in increased hepatic lipid accumulation (Belury and Kempa-Steczko, 1997; DeLany *et al.*, 1999; Tsuboyama-Kasaoka *et al.*, 2000; Clément *et al.*, 2002). Several hypotheses have been proposed that might explain this phenomenon (Belury and Kempa-Steczko, 1997; Moya-Camarena *et al.*, 1999; Clément *et al.*, 2002). These proposed mechanisms include (1)

activation of a PPAR (Moya-Camarena *et al.*, 1999), (2) increased plasma insulin and/or reduced leptin concentrations (Clément *et al.*, 2002), and (3) uptake of CLA into fat stores of the liver (Belury and Kempa-Steczko, 1997).

Activation of PPAR is known to induce the transcription of proteins involved in lipid metabolism transport and intracellular binding (Dreyer *et al.*, 1993; Kaikaus *et al.*, 1993; Keller *et al.*, 1993; Barbier *et al.*, 2002; Duplus and Forest, 2002). In support of this mechanism, CLA activates PPAR- $\gamma$  in mice *in vivo* and *in vitro* (Clément *et al.*, 2002; Takahashi *et al.*, 2002; Kang *et al.*, 2003; Wang and Tafuri, 2003). PPAR- $\gamma$  may be the important isoform because Clément *et al.* (2002) demonstrated that the *t*10,*c*12-CLA isomer induces the expression of PPAR- $\gamma$  target genes (*i.e.*, FAT/CD36 and ALBP). Further, Clément *et al.* (2002) demonstrated that the activation of PPARs responsible for hepatic lipid accumulation is an indirect effect, rather than a direct effect of CLA, which is further supported by *in vivo* data from Poirier *et al.* (2001), Peters *et al.* (2001), and by *in vivo* data on other polyunsaturated fatty acids (Yoshikawa *et al.*, 2002).

Hepatic lipid accumulation may be the result of increased plasma insulin and/or reduced leptin concentrations observed in mice fed CLA. For instance, Tsuboyama-Kasaoka *et al.* (2000) provided data demonstrating that hepatic lipid accumulation can be reversed by subcutaneous injection of leptin. Alternatively, insulin, rather than leptin, may be the mediator of hepatic lipid accumulation as proposed by Clément *et al.* (2002).

#### *Studies in Rats*

In rats, CLA has been shown to activate peroxisome-proliferator activated receptor *gamma* (PPAR- $\gamma$ ) *in vivo* (Houseknecht *et al.*, 1998). This phenomenon has been more extensively studied in the mouse model (Section C.1.4.2).

#### *Studies in Pigs*

CLA has also been shown to activate PPAR- $\gamma$  in pigs *in vivo* (Meadus *et al.*, 2002; Meadus, 2003).

#### C.1.4.3 Summary

Hepatic lipid accumulation is associated with certain pathologic states induced by xenobiotics that might result in liver injury. However, it is possible that hepatic lipid accumulation observed in mice is a species-specific phenomenon that is dependent on body fat turnover (*i.e.*, rodents have higher fat turnover than humans) (Pariza *et al.*, 2001). Studies in which mice were fed CLA did not demonstrate any pathological or reduced functional consequence due to hepatic lipid accumulation. There is no evidence that hepatic lipid accumulation due to dietary supplementation with CLA observed in experimental mice, rats or pigs is of toxicological significance. A further discussion of the relationship of hepatic lipid accumulation to insulin effects is discussed in Section C.1.2.2.

A clear link between the adipose tissue reducing effects of CLA and the observed liver lipid and insulin effects was demonstrated by Tsuboyama-Kasaoka *et al.* (2003). The authors reported that CLA treatment in combination with an increase of the dietary fat level to 34% resulted in a more moderate fat decrease in mice, and subsequent insulin resistance and hepatomegaly were no longer observed. These results demonstrated that the marked loss of functional adipose tissue rather than direct effects of CLA were responsible for the observed insulin resistance and hepatomegaly. The loss of functional adipose tissue resulted in a lack of lipogenic adipocytes with the capacity to take up more fat, increasing the lipogenic burden of non-adipose tissues such as muscle and liver, resulting in a decreased sensitivity to insulin action. Insulin resistance has been reported to be associated with elevated amounts of triglycerides in muscle tissues (Storlien *et al.*, 1991; Krssak *et al.*, 1999; Kelley and Goodpaster, 2001).

#### C.1.5 *Aortic Fat Deposition*

##### C.1.5.1 *In Vitro Studies*

No *in vitro* studies of significance to this report have been conducted on aortic fat deposition.

##### C.1.5.2 *Animal Studies*

###### *Studies in Mice*

Munday *et al.* (1999) has reported that CLA might result in fatty streak formation in mice. Female C57BL/6 mice were fed either a high-fat diet (control), high-fat diet supplemented with 2.5 g/kg diet (*i.e.*, 0.375 g/kg body weight) of 50:50 CLA along with 2.5 g/kg diet of linoleic acid, as well as other fat-containing components, or a high-fat diet supplemented with 5 g/kg diet (*i.e.*, 0.75 g/kg body weight) of CLA (no linoleic acid) for 15 weeks. Body weights, food intake, mean serum total cholesterol concentration and mean serum HDL cholesterol concentration were unaffected by CLA treatment. Mean serum HDL cholesterol to total cholesterol ratio and mean serum triglyceride concentrations were increased 26% and reduced 16%, respectively, in mice fed 0.75 g/kg CLA. Serum HDL cholesterol to total cholesterol ratio and mean serum triglyceride concentrations were unaffected in mice fed 0.375 g/kg CLA. Mean total aortic fatty streak area was increased 150% in mice fed 0.375 g/kg CLA and unaffected in mice fed the higher dose of 0.75 g/kg CLA.

Based on the plasma HDL cholesterol and triglyceride data, the investigators concluded that CLA treatment produced a lipoprotein profile that is indicative of reduced atherogenic potential. Fatty streak formation increased in CLA-treated mice, which, according to the investigators, might indicate a pro-fatty streak effect by CLA (Munday *et al.*, 1999). The authors suggested an immune mechanism may be involved in stimulation of atherogenesis.

### *Studies in Rabbits*

Lee *et al.* (1994) fed New Zealand White rabbits a semi-synthetic diet augmented with 50:50 CLA mixtures (0.5 g CLA/rabbit per day). By 12 weeks, total and LDL cholesterol and triglycerides were markedly lower in the CLA-fed group. The LDL cholesterol to HDL cholesterol ratio and total cholesterol to HDL cholesterol ratio also were significantly reduced in CLA-fed rabbits. Examination of the aortas of CLA-fed rabbits showed less atherosclerosis.

Kritchevsky *et al.* (2000) reported that at dietary levels as low as 0.1%, CLA (43.29% *c9,t11* and 44.07% *t10,c12*) inhibited atherogenesis in New Zealand White rabbits. For establishment of atherosclerosis, rabbits were fed a semipurified diet containing 0.1 to 0.2% cholesterol for 90 days. Administration of CLA for 90 days at a dietary level of 1% resulted in substantial (30%) regression of established atherosclerosis.

### *Studies in Hamsters*

Nicolosi *et al.* (1997) fed hamsters (strain not specified) with diets of up to 1.1% of a 50:50 CLA isomer mix for up to 11 weeks. Morphometric analysis of aortas revealed less early atherosclerosis in the conjugated linoleic acid and linoleic acid-fed hamsters compared to the control group.

#### C.1.5.3 Summary

The evidence is insufficient to conclusively determine that CLA induces fatty streaks in experimental animals because this effect is reported in only one study (Munday *et al.*, 1999). Actually, there are several other studies demonstrating an anti-fatty streak effect in experimental animals (Lee *et al.*, 1994; Nicolosi *et al.*, 1997; Kritchevsky, 2000). The association between hyperinsulinemia observed in experimental rodent models and the development of fatty streaks is speculative. Hence, based on this single report, the effect of CLA on inducing fatty streaks *in vivo* is equivocal (see Section 7.5.2 for a discussion of the effects in human trials).

#### C.1.6 Markers of Inflammation

##### C.1.6.1 *In Vitro* Studies

*In vitro* studies support the anti-inflammatory effects of CLA mixtures and isomers that decrease inflammatory markers such as TNF- $\alpha$  and some interleukins (ILs) related to inflammation. Furthermore, the down regulation of these inflammatory markers appears to also be regulated through the PPAR system. This concurs with the *in vitro* investigations in other sections of this dossier (e.g., blood lipids) showing regulation through the PPAR system.

## C.1.6.2 Animal Studies

### *Studies in Mice*

Mice have also been used as animal models to study the effect of CLA on inflammatory parameters.

CLA mixtures or isomers reduce inflammatory mediators in mice. Yang *et al.* (2000) and Yang and Cook (2003) reported that the 50:50 CLA mixture prolonged survival time in NZB/W F1 lupus mice, while Yang and Cook (2003) also reported reduced cachectic symptoms associated with lupus.

### *Studies in Rats*

Studies by Sisk *et al.* (2001) and Nagao *et al.* (2003a,b) report an attenuation or reduction in blood pressure and a reduction in TNF- $\alpha$  levels in 3 rat models (lean and obese Zucker rats, Otsuka Long-Evans Tokushima fatty rats, and Zucker diabetic fatty rats). These data support findings reported in mice (Section C.1.6.2).

### *Studies in Pigs*

Changhua *et al.* (2005) investigated the anti-inflammatory role of CLA in inflammation-challenged weaned pigs. Dietary supplementation with 2% CLA alleviated growth depression and prevented the elevations in production and mRNA expression of pro-inflammatory cytokines (*i.e.*, IL-6 and TNF- $\alpha$ ) induced by the lipopolysaccharide challenge. CLA enhanced the expression of IL-10 and PPAR- $\gamma$  in spleen and thymus.

To further elucidate the inhibitory effects and the mechanism of action of CLA on cytokine profiles (*e.g.*, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ), PBMCs were isolated from weaned pigs and cultured in media containing c9,t11 CLA and t10,c12 CLA. Each CLA isomer suppressed the production and expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , and enhanced PPAR- $\gamma$  activation and gene expression in cultured PBMCs. At the molecular level, the inhibitory actions of CLA on IL-1 $\beta$ , IL-6, and TNF- $\alpha$  are attributable mainly to t10,c12-CLA and the anti-inflammatory properties of CLA are mediated, at least in part, through a PPAR- $\gamma$ -dependent mechanism.

Hontecillas *et al.* (2002) investigated the anti-inflammatory actions and molecular mechanisms underlying the regulation of colonic health by CLA 50:50 isomer mix (1.33% CLA in the diet for 49 or 72 days). Inflammation of the colonic mucosa was triggered by challenging pigs fed either soybean oil-supplemented or CLA-supplemented diets with an enteric bacterial pathogen. Supplementation of CLA in the diet before the induction of colitis decreased mucosal damage; maintained interferon-gamma (IFN- $\gamma$ ) and IL-10 levels and lymphocyte subset distributions (*i.e.*, CD4+ and CD8+), similar to noninfected pigs; enhanced colonic expression of PPAR- $\gamma$ ; and

attenuated growth failure. The authors concluded that CLA fed prophylactically before the onset of enteric disease attenuated inflammatory lesion development and growth failure.

Bassaganya-Riera *et al.* (2003) showed that CLA (1.33% in the diet for 42 days) ameliorates viral disease in a viral challenge model of pigs infected with type-2 porcine circovirus (PCV2).

Cook *et al.* (1993) reported that CLA (0.5% in the diet for an unspecified duration) was effective in preventing the catabolic effect of immune stimulation of chickens.

#### C.1.6.3 Summary

Studies in animals demonstrate that CLA mixtures or individual isomers reduced inflammatory mediators, including TNF- $\alpha$  levels in rats (Sisk *et al.*, 2001; Nagao *et al.*, 2003a,b), decreased mRNA expression of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$  in pigs (Changhua *et al.*, 2005), and attenuated the development of inflammatory lesions in pigs (Hontecillas *et al.*, 2002). Yang *et al.* (2000) and Yang and Cook (2003) reported prolonged survival time in mice with lupus, while Yang and Cook (2003) also reported decreased cachectic symptoms associated with lupus. The majority of studies demonstrate the anti-inflammatory effects of CLA on inflammatory mediators.

**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
<b>Lipid metabolism</b>					
Mouse (Female C57BL/6 <i>lep<sup>ob</sup>/lep<sup>ob</sup></i> )	3 weeks	50.50 c9, t11- and t10, c12-CLA (Clannol A-60)	2,250	Significantly ↑ plasma TG	Wargent <i>et al.</i> , 2005
		50.50 c9, t11- and t10, c12-CLA (Clannol A-60)	3,750	Significantly ↑ plasma TG	
		50.50 c9, t11- and t10, c12-CLA (Clannol A-60); Clarinol A-60 enriched with 90% t10, c12-CLA; Clannol A-60 enriched with c9, t11-CLA	3,750	CLA enriched with 90% c9, t11-CLA significantly ↑ plasma TG in the beginning of the experiment, however, by the end of 3 weeks, ↑ was not statistically significant. CLA mixture and CLA mixture enriched with 90% t10, c12-CLA significantly ↑ plasma TG by the end of the experiment.	
	11 weeks	50.50 c9, t11- and t10, c12-CLA	1,500 (low) or 3,750 (high)	After 2 weeks, low-CLA significantly ↑ plasma TG; however, by the end of 11 weeks, no statistically significant ↑ in plasma TG was present.	
Mouse (Male C57BL-6 ob/ob)	4 weeks	c9, t11-CLA	6,645	Significantly ↓ serum TG and NEFA. No significant change in serum C.	Roche <i>et al.</i> , 2002
		t10, c12-CLA	6,885	No significant changes in serum TG, NEFA, or C	
Mouse (Male C57BL/6J)	12 weeks	c9, t11-CLA or t10, c12-CLA	1,500	Significantly ↓ plasma TG	Degrace <i>et al.</i> , 2003
Mouse (Male db/db)	12 weeks	50.50 c9, t11- and t10, c12-CLA	1,800	Significantly ↑ plasma FFA after 6 weeks of treatment, but significantly ↓ after 12 weeks. No significant change in plasma TG or total C.	Hamura <i>et al.</i> , 2001a
Mouse (Male apolipoprotein knockout)	12 weeks	c9, t11-CLA	10,500	Significantly ↓ plasma TG and NEFA.	de Roos <i>et al.</i> , 2005
		t10, c12-CLA		Significantly ↑ plasma TG and NEFA.	
Mouse (Female C57BL/6)	15 weeks	NR	2,500 (low) or 5,000 (high)	In low-CLA group, no significant changes in serum total C, HDL-C, HDL-C:total C, or TG. In high-CLA group, significantly ↑ serum HDL-C:total C and significantly ↓ serum TG. No significant changes in serum total C or HDL-C	Munday <i>et al.</i> , 1999

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**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (Male C57BLKS-Lepr <sup>ob</sup> /lepr <sup>ob</sup> )	23 weeks	50:50 c9, t11- and t10, c12-CLA	795 (low) or 1,800 (high)	Significantly ↓ plasma TG after 2, 4, and 6 weeks of treatment in the low-CLA group and after 4 and 6 weeks in the high-CLA group. At the end of the treatment period, no significant changes in plasma TG, total C, or FFA were observed.	Hamura <i>et al.</i> , 2001b
Rat (Male and female Wistar outbred [CrI.(WI)WU BR])	13 weeks	50:50 c9, t11- and t10, c12-CLA (Clarnoll G80)	1,500 (low), 7,500 (mid), or 22,500 (high)	Significantly ↓ plasma C in high-dose males after 4, 8, and 13 weeks of treatment, but significantly ↑ plasma C in mid-dose females after 4, 8, and 13 weeks of treatment. Significantly ↑ plasma TG in high-dose females after 4, 8, and 13 weeks of treatment and in mid-dose males after 13 weeks of treatment.	O'Hagan and Menzel, 2003
Rabbit (Male and female New Zealand White)	7 weeks	50:50 c9, t11- and t10, c12-CLA	75 (low) or 150 (high)	Significantly ↓ plasma total C and TG in both dose groups. No significant changes in plasma leptin or NEFA.	Corino <i>et al.</i> , 2002
Rabbit (Male New Zealand White)	13 weeks	50:50 c9, t11- and t10, c12-CLA	30 (low), 150 (mid), or 300 (high)	No significant changes in serum C, HDL-C, or TG.	Kritchevsky <i>et al.</i> , 2000
Rabbit (Male New Zealand White)	13 weeks	c9, t11-CLA	150	No significant changes in serum C or TG.	Kritchevsky <i>et al.</i> , 2004
		t10, c12-CLA			
		50:50 c9, t11- and t10, c12-CLA			
Rabbit (Male and female New Zealand White)	22 weeks	NR	1,875	Significantly ↓ plasma LDL-C and LDL-C:HDL-C from 12 to 22 weeks. No significant change in plasma total C, TG, or total C:HDL-C	Lee <i>et al.</i> , 1994
Hamster (Male)	4 weeks	50:50 c9, t11- and t10, c12-CLA	30 (low) 60 (mid), or 600 (high)	Significantly ↓ plasma total C and non-HDL-C in all dose groups, and plasma TG in the low- and mid-dose groups. No significant changes in plasma HDL-C.	Nicolosi <i>et al.</i> , 1997
Hamster (Male Syrian Golden)	6 weeks	c9, t11-CLA	60	No significant changes in hepatic TG content compared to LA supplemented control hamsters.	Zabala <i>et al.</i> , 2004
		t10, c12-CLA		Significantly ↓ hepatic TG content compared to LA supplemented control hamsters	

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**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Hamster (Male Syrian Golden)	6 weeks	c9, t11-CLA	220.8	No significant changes in serum total C, HDL-C, LDL-C, VLDL-C, or TG compared to LA supplemented control hamsters.	Navarro <i>et al.</i> , 2003
		t10, c12-CLA	239.3	Significantly ↓ serum total C and LDL-C, compared to LA supplemented control hamsters. No significant changes in serum HDL-C, VLDL-C, or TG.	
Hamster (Male Syrian Golden)	6 weeks	c9, t11-CLA	600	No significant changes in serum TG levels compared to LA supplemented control hamsters.	Macarulla <i>et al.</i> , 2005
		t10, c12-CLA		Significantly ↓ serum TG levels compared to LA supplemented control hamsters.	
Hamster (Male Syrian Golden)	6 weeks	t10, c12-CLA	600	Significantly ↑ serum total C, HDL-C and VLDL-C, but ↓ serum LDL-C.	Navarro <i>et al.</i> , 2005
Hamster (Male Syrian LPN)	8 weeks	c9, t11-CLA	720	Significantly ↑ plasma total C and HDL-C:LDL-C. No significant changes in plasma TG or phospholipids	Valiella <i>et al.</i> , 2004
		50:50 c9, t11- and t10, c12-CLA	1,440	Significantly ↑ plasma TG. No significant changes in plasma total C, HDL-C:LDL-C, or phospholipids.	
Hamster (Male Syrian LPN)	8 weeks	c9, t11-CLA	720	Significantly ↑ whole body TG	Bouthegeourd <i>et al.</i> , 2002
		50:50 c9, t11- and t10, c12-CLA	1,440	No significant changes in whole body TG.	
Hamster (Male Syrian)	12 weeks	50.50 c9, t11- and t10, c12-CLA	576	No significant changes in plasma total C or TG.	Sher <i>et al.</i> , 2003
	7 weeks		1,200	Significantly ↑ plasma TG, but did not significantly change plasma total C	
Hamster (Male outbred Syrian Golden CRL:LVG(SYR))	12 weeks	c9, t11-CLA	1,200	No significant changes in plasma total C, TG, VLDL-C, VLDL-TG, LDL-C, HDL-C, or non-HDL-C:HDL-C compared to LA supplemented control hamsters.	Mitchell <i>et al.</i> , 2005
		t10, c12-CLA			

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**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
<b>Markers of Oxidative Stress</b>					
Rat (male Sprague-Dawley)	2 weeks	50:50 c9, t11- and t10, c12-CLA	500	Significantly ↓ DIN in rats fed CLA and intoxicated with CCl <sub>4</sub> . No significant changes in esterified 8-iso-PGF <sub>2α</sub> , or arachidonic acid hydroperoxides compared to rats treated with CCl <sub>4</sub> alone.	Iannone <i>et al.</i> , In Press
<b>Hepatic Lipid Accumulation</b>					
Mouse (male C57bL/6J)	3 weeks	50:50 c9, t11- and t10, c12-CLA	3,000	Significantly ↓ serum TG and FFA, and mRNA expression of leptin and PPARγ in epididymal WAT. No significant changes in mRNA expressions on perirenal WAT	Takahashi <i>et al.</i> , 2002
Mouse (male ICR)				No significant changes in serum lipid levels, mRNA expressions of leptin or PPARγ in epididymal or perirenal WAT	
Mouse (Female 57BL/6J)	4 weeks	c9, t11-CLA	600	No significant changes in liver mass, hepatic lipid content, mRNA expression of PPARα, PPARβ/δ, PPARγ, FAT/CD36, ALBP, SREBP1a, SREBP1c, SREBP2, FAS, or PEPCK, or plasma leptin levels.	Clément <i>et al.</i> , 2002
		t10, c12-CLA		Significantly ↑ liver mass, hepatic lipid content, or mRNA expression of PPARγ, FAT/CD36, ALBP, and FAS. Significantly ↓ PEPCK and plasma leptin levels. No significant changes in mRNA expression of PPARα, PPARβ/δ, SREBP1c, or SREBP2.	

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**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (male C57BL/6n wild-type or PPAR $\alpha$ -null type)	4 weeks	50:50 c9, t11- and t10, c12-CLA	750	Significantly $\uparrow$ liver weight in both genotypes, but significantly $\downarrow$ serum TG and significantly $\downarrow$ fat in terms of body fat composition. Two-, 4-, and 6-fold increases in hepatic ACO, CYP4A1, and CPT-11 mRNA levels, respectively, among wild-type mice, compared to wild-type controls. Less than a 2-fold reduction of hepatic apolipoprotein CIII in wild-type mice, but not mutants. Modest inductions (1.5- to 4-fold) of hepatic SCAL, MCAD, LCAD, and VLCAD levels in both genotypes, 2- to 3-fold induction of UCP2 in both genotypes, and significant induction of FAS, S14, and SCD-1 in both genotypes compared to their respective controls.	Peters <i>et al.</i> , 2001
Mouse (Harlan Sprague-Dawley)	6 weeks	50:50 c9, t11- and t10, c12-CLA	750 (low), 1,500 (mid), or 2,250 (high)	Significantly and dose-dependently $\uparrow$ the lipid concentration in the liver.	Belury and Kempa-Steczko, 1997
Mouse (Male AKR/J)	6 weeks	50:50 c9, t11- and t10, c12-CLA	375, 750, 1,125, or 1,500	Significantly $\uparrow$ cytoplasmic vacuolation in liver and no significant change in lipid accumulation in group administered highest dose. No significant changes in any other dose groups.	DeLany <i>et al.</i> , 1999
	2 to 12 weeks		1,500	Significantly $\uparrow$ centrilobular fatty change in the liver.	
Mouse (Female C57BL/6J)	4 days to 8 months	50:50 c9, t11- and t10, c12-CLA	1,500	Significantly enlarged and very pale livers of CLA-fed mice suggesting deposition of fat. Histopathological examination revealed panlobular macrovesicular steatosis. Significantly $\downarrow$ mRNA expression of PPARY, FAS, and ACC. No significant changes in SREBP-1, ACS, or LPL.	Tsuboyama-Kasaoka <i>et al.</i> , 2000
Rat [male Zucker diabetic fatty (fa/fa, ZDF/GMI)]	2 weeks	50:50 c9, t11- and t10, c12-CLA	17,120	Significantly $\downarrow$ plasma FFA and significantly $\uparrow$ aP2 mRNA levels in epididymal fat.	Houseknecht <i>et al.</i> , 1998

000210

**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Rat (female Sprague-Dawley)	Up to 6 weeks	50:50 c9, t11- and t10, c12-CLA	500 (low), 1,000 (mid), and 1,500 (high)	Significantly ↓ hepatic lipid content in low-dose females, no significant change in mid-dose females, and significantly ↑ hepatic lipid content in high-dose females. Significant ↑ in L-FABP mRNA levels among low- and mid-dose rats, but no significant changes in ACO and CYP4A1 mRNA levels in any dose group.	Moya-Camarena <i>et al.</i> , 1999
Rat (male Sprague-Dawley)			1,500	Significantly ↑ ACO and L-FABP mRNA expressions. No significant changes in total hepatic lipid content or CYP4A1 mRNA expression.	
Pig (male, castrated, Landrace X Large White)	5 weeks	NR	471.4	Significantly ↑ percentage in lean fat, expressed as percentages/kg body weight and ↑ percentage of intramuscular fat, expressed as g of lipid/g of muscle on a dry matter basis. Significantly ↓ subcutaneous fat, expressed as a percentage/kg body weight. Significantly ↑ mRNA levels of GFAT, AFABP, and PPAR $\gamma$ in muscle. No significant changes in m-calpain, PPAR $\alpha$ , and ACO.	Meadus <i>et al.</i> , 2002
Pig (male, castrated, Landrace X Large White)	5 weeks	NR	471.4	Significantly ↑ mRNA levels of GFAT, AFABP, and PPAR $\gamma$ in muscle. No significant changes in m-calpain, PPAR $\alpha$ , and ACO.	Meadus, 2003
<b>Aortic Fat Deposition</b>					
Mouse (female C57BL/6)	15 weeks	NR	2,500 (low) or 5,000 (high)	Significantly ↑ total aortic fatty streak area in low-dose group. When data from both dose groups were combined, significantly ↑ aortic fatty streak development was observed. Intima of aortic sinus of all mice had lipid-containing lesions.	Munday <i>et al.</i> , 1999
Rabbit (male New Zealand White)	13 weeks	50:50 c9, t11- and t10, c12-CLA	30 (low), 150 (mid), or 300 (high)	Significantly ↓ atherosclerotic lesions in the thoracic aorta and aortic arch in the mid- and high-dose groups. No significant changes in the low-dose group.	Kritchevsky <i>et al.</i> , 2000

000211

**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Rabbit (male and female New Zealand White)	22 weeks	NR	1,875	No significant changes in thoracic and abdominal maximal thickness of plaque, or thoracic and abdominal plaque to wall volume ratio. Also, no significant changes in lipid deposition in thoracic or abdominal aorta	Lee <i>et al.</i> , 1994
Hamster (male)	4 weeks	50:50 c9, t11- and t10, c12-CLA	30 (low) 60 (mid), or 600 (high)	No significant changes in aortic fatty streak among individual groups; however, when results were pooled, a significant ↓ was observed.	Nicolosi <i>et al.</i> , 1997
<b>Markers of Inflammation</b>					
Mouse (female SLE-prone NZB/W F1)	Up to 44 weeks (lifetime)	50:50 c9, t11- and t10, c12-CLA	750	Significantly earlier onset of proteinuria, and development of anti-ss DNA and anti-ds DNA antibodies. Significantly longer survival post-onset of proteinuria.	Yang <i>et al.</i> , 2000
Mouse (female NZB/W F1)	NR (lifetime)	50 50 c9, t11- and t10, c12-CLA	750	Significantly ↑ survival after onset of proteinuria and lost significantly more weight	Yang and Cook, 2003
Rat (male Otsuka Long-Evans Tokushima fatty)	3 weeks	c9, t11-CLA	500	No significant changes in blood pressure, perirenal WAT, epididymal WAT, angiotensinogen, or leptin.	Nagao <i>et al.</i> , 2003a
		t10, c12-CLA		Significantly ↓ blood pressure, perirenal WAT, epididymal WAT, angiotensinogen, and leptin.	
Rat (female lean and obese Zucker)	8 weeks	50 50 c9, t11- and t10, c12-CLA	Lean: 300, Obese. 240	No significant changes in TNFα levels.	Sisk <i>et al.</i> , 2001
Rat [male Zucker diabetic fatty (fa/fa)]	8 weeks	50.50 c9, t11- and t10, c12-CLA	480	Significantly lower ↑ in systolic blood pressure.	Nagao <i>et al.</i> , 2003b

000212

**Table C.1.6.3-1 Summary of CLA Animal Feeding Studies on Cardiovascular Disease Risk Factors**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Pig (large White X Landrace)	4 weeks	50:50 c9, t11- and t10, c12-CLA	85.7	<p>Pigs not challenged with LPS: Significantly ↓ in IL-6. Significantly ↑ PPAR<math>\gamma</math> mRNA levels in the spleen. No significant changes in IL-1<math>\beta</math>, IL-6, TNF<math>\alpha</math>, or IL-10 mRNA levels in the spleen, or IL-1<math>\beta</math>, IL-6, TNF<math>\alpha</math>, IL-10, and PPAR<math>\gamma</math> mRNA levels in the thymus.</p> <p>Pigs challenged with LPS: Significantly ↓ IL-1<math>\beta</math>, IL-6, TNF<math>\alpha</math>, and PGE<sub>2</sub>. Significantly ↑ IL-10. Significantly ↓ IL-6 and TNF<math>\alpha</math> mRNA expression in the spleen and thymus. Significantly ↑ in IL-10 and PPAR<math>\gamma</math> mRNA expression in the spleen and thymus. No significant changes in IL-1<math>\beta</math> in either organ.</p>	Changhua <i>et al</i> , 2005
Pig (strain not identified)	9 weeks	50:50 c9, t11- and t10, c12-CLA	400 (unchallenged with PCV2)	<p>No significant changes in immune responses or relative proliferation index of CD8<sup>+</sup> T cells. Significantly ↑ percentage of CD8<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes and percentage of CD8<sup>+</sup>CD29<sup>low</sup> and CD8<sup>+</sup>CD45RC<sup>+</sup> PBMC. Significantly ↓ IL-18 mRNA expression and no significant changes in Bcl-xl, Bak, PPAR<math>\alpha</math>, PPAR<math>\gamma</math>, IL-12, and IL-2.</p>	Bassaganya-Riera <i>et al</i> , 2003
			393 (challenged with PCV2)	<p>Significantly ↑ percentage of CD8<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes, percentage of CD8<sup>+</sup>CD45RC<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> in PBMC, and relative proliferation index of CD8<sup>+</sup> T cells. Significantly ↓ IFN-<math>\gamma</math> production in CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells. Significantly prevented depletion of B cells and significantly ↑ mRNA expression of Bcl-xl, PPAR<math>\gamma</math>, and IL-2. Significantly ↓ PPAR<math>\alpha</math> and IL-2 mRNA expressions. No significant changes to Bak and IL-18 mRNA expressions.</p>	

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Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Pig (strain not identified)	Up to 11 weeks	50:50 c9, t11- and t10, c12-CLA	30	Unchallenged pigs with <i>Brachyspira hyodysenteriae</i> : No significant changes in colonic histology sections examining mucosal thickness and epithelial erosion. Also, no significant changes in CD markers. Challenged pigs with <i>B. hyodysenteriae</i> . Significantly ↓ colonic mucosal thickness and epithelial erosion. Significantly ↑ CD4 and CD8α levels. No significant changes in CD3 or TCRγδ. mRNA levels of IFN-γ and IL-10 were similar to unchallenged pigs.	Hontecillas <i>et al.</i> , 2002
Chicken	NR	50:50 c9, t11- and t10, c12-CLA	0.5% supplemented diet	Significantly prevented weight loss following immune stimulation	Cook <i>et al.</i> , 1993

ACC = acetyl CoA carboxylase, ACO = peroxisomal acyl-CoA oxidase, ACS = acyl-CoA synthetase; AFABP = adipocyte fatty acid binding protein ; ALBP = adipocyte lipid-binding protein; aP2 = adipocyte fatty acid binding protein; C = cholesterol; CCl<sub>4</sub> = carbon tetrachloride; CD = cluster of differentiation; CLA = conjugated linoleic acid; CPT= carnitine palmitoyl transferase; CYP4A1 = microsomal cytochrome P450 4A1; DIN = 2,3 diinor ; ds = double strand; FAT = fatty acid transporter; FAS = fatty acid synthase; FFA = free fatty acids, GFAT = glutamine-fructose aminotransferase; HDL-C = high density lipoprotein cholesterol; IFN = interferon; IL= interleukin; L-FABP = liver fatty acid binding protein; LA = linoleic acid; LCAD = mitochondrial long-chain acyl-CoA dehydrogenase, LDL-C = low density lipoprotein cholesterol; LPL = lipoprotein lipase; LPS = lipopolysacchande, MCAD = mitochondrial medium-chain acyl-CoA dehydrogenase, mRNA = messenger RNA; NEFA = non esterified fatty acid; NR = not reported, PBMC = peripheral blood mononuclear cell; PCV2 = type-2 porcine circovirus; PEPCK = phosphoenolpyruvate carboxykinase; PG = prostaglandin; PPAR = peroxisomal proliferator-activated receptor; S14 = spot 14, SCD= stearyl CoA desaturase; SCAL = mitochondrial short-chain acyl-CoA dehydrogenase, SREBP = sterol responsive element-binding protein; ss = single strand; TCR = T cell antigen receptor; TG = triglyceride; TNF = tumor necrosis factor; UCP = mitochondrial uncoupling proteins; VLCAD = mitochondrial very long-chain acyl-CoA dehydrogenase, VLDL-C = very low density lipoprotein cholesterol; WAT = white adipose tissue

<sup>1</sup> CLA administered in the diet, unless otherwise noted

<sup>2</sup> Treatment groups compared to respective control group, unless otherwise noted.

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## C.2 Insulin Sensitivity and Glucose Metabolism

In order to put the effects of high dietary levels of CLA on insulin in animal models into context, it is perhaps appropriate to provide a brief introduction to the biochemistry. Carbohydrate and fat metabolism are tightly controlled processes that are both influenced by insulin (Stryer, 1995). Following consumption of a meal, increased blood glucose stimulates insulin secretion, which stimulates glycogen synthesis and inhibits lipolysis and gluconeogenesis. As a result, blood glucose is converted to glycogen stores and fatty acid synthesis is stimulated in the liver, resulting in increased storage of triacylglycerols. In contrast, during a fasting state, insulin secretion is decreased, while glucagon secretion is increased, resulting in glycogen degradation to release glucose, which is readily available for glycolysis and subsequent metabolism in the citric acid cycle. Fatty acids in turn are metabolized by  $\beta$ -oxidation. In times of carbohydrate stress, fatty acids are converted to ketone bodies to provide fuel, and eventually the body shifts from carbohydrate metabolism to fatty acid metabolism as a source of energy.

Experiments focused on the effects of CLA on insulin and glucose metabolism have been conducted using both *in vitro* and animal models. *In vitro* data suggest that the *t10,c12* CLA isomer is involved in the regulation of fatty acid synthesis and the reduction of lipid in adipocytes; however, the relevance of the results from these studies is not clear. Studies in animal models show that adaptation and normalization of glucose and insulin levels occurs, supporting the hypothesis that CLA induces an adaptive effect, but does not produce deleterious effects. Initial studies in rodents showed a rise in fasting glucose levels after CLA administration and a decrease in insulin sensitivity. Subsequent investigation has demonstrated that this effect is transient in animals. Animals, particularly mice, are extremely sensitive to the effects of CLA on fat metabolism. Differences also exist between strains of the same species (*e.g.*, lean vs. obese strains of mice) that suggest extreme sensitivity to CLA. The available human data support the lower sensitivity to CLA with respect to body fat-reduction and changes in glucose metabolism. However, the apparent differences in sensitivity could be related to different doses; for example, mice received doses that were 30 times the human dose. The other possible explanation is that, as indicated above, mice have a high metabolic rate and therefore are highly sensitive to CLA.

### C.2.1 *In Vitro* Studies

Numerous *in vitro* studies suggest that the *t10,c12* CLA isomer is responsible for fat reduction (Brown and McIntosh 2003; Granlund *et al.*, 2003; Kang *et al.*, 2003; Kennedy *et al.*, 2005). Collectively, these *in vitro* studies show that the *t10,c12* CLA isomer inhibits PPAR- $\gamma$  (Granlund *et al.*, 2003; Kang *et al.*, 2003; Kennedy *et al.*, 2005), resulting in numerous downstream events that culminate in the down-regulation of genes related to insulin status. The suite of genes involved (*e.g.*, GLUT4, LPL, FAB) relate to an increase in insulin resistance in cell culture systems (Brown *et al.*, 2003; de Roos *et al.*, 2005). These changes in glucose and insulin metabolism also appear to be regulated by the increase in IL-6 seen after *t10,c12* CLA isomer

administration (Provo *et al.*, 2005), and potentially through the down-regulation of the IGF-receptor (Kim *et al.*, 2003). These alterations are seen in some animal species as transient effects.

### C.2.2 *Animal Studies*

Numerous animal studies in rodents and pigs have documented varying effects of both a 50:50 CLA mixtures and the *c9,t11* and *t10,c12* CLA isomers on insulin sensitivity and glucose tolerance. Studies in mice and hamsters indicate that CLA administration may promote insulin resistance but this is not shown in rat studies..

#### Studies in Mice

Poirier *et al.* (2005) administered a 1% isomeric mixture of CLA by gavage to C57BL/6J female mice (approximately 1,500 mg/kg body weight/day) for 2 to 28 days. It was reported that levels of leptin and adiponectin sharply decreased after 2 days of CLA feeding, although adipose tissue mass only decreased after day 6. Hyperinsulinemia developed at day 6 and worsened up to day 28, in parallel with increases in hepatic lipid content. Islet cells from CLA-fed mice displayed 3- to 4-fold increased rates of glucose-stimulated insulin secretion, both in the absence and presence of isobutyl methylxanthine or carbachol. The increased insulin-releasing capacity of islet cells from CLA-fed mice was apparently due to an increase in pancreatic beta cell mass and number. The authors suggested that CLA supplementation induced a reduction of leptinemia and adiponectinemia, followed by hyperinsulinemia due to the increased secretory capacity of pancreatic islets, leading to liver steatosis.

Ohashi *et al.* (2004) examined the plasma and mRNA expression levels of several adipocytokines thought to be involved in the regulation of insulin sensitivity in normal C57BL, mildly obese/diabetic KK and morbidly obese/diabetic KKAY mice. CLA oil, 0.5% (approximately 750 mg/kg body weight/day) and consisting of 30.5% *c9,t11*-CLA and 28.9% *t10,c12*-CLA was administered by gavage for 4 weeks. They reported an increase in liver weight with excess accumulation of triglyceride, and insulin resistance associated with hyperglycemia and hyperinsulinemia in the CLA groups compared to the placebo. Levels of leptin in white adipose tissue and plasma were higher in all mice, whereas adiponectin levels were higher only in C57BL (normal) mice. CLA-feeding decreased the levels of leptin, adiponectin, and resistin, especially in KK (mild obesity) and KKAY (morbid obesity) mice. In contrast, tumor necrosis factor-alpha (TNF $\alpha$ ) mRNA levels were higher in KK (mild obesity) and KKAY (morbid obesity) mice than in C57BL (normal) mice, were increased by CLA feeding. The authors concluded feeding CLA promotes insulin resistance in obese/diabetic mice compared to normal control mice by inverse regulation of leptin, adiponectin, and TNF $\alpha$ . Adipocytokines are known to either ameliorate or deteriorate insulin sensitivity. It should be noted that although this was a short-term study, normal mice were not adversely influenced by CLA addition to the diet, while

genetically modified mice such as the KK and KKay mouse models experienced decreased insulin sensitivity after CLA.

Bhattacharya *et al.* (2005) examined the effect of a low concentration of either safflower oil as control (0.5%) or mixed isomers of CLA (0.4% or approximately 600 mg/kg body weight/day) for 14 weeks along with treadmill exercise on body composition in male Balb/C mice fed a high-fat diet (20% corn oil) in a 2 x 2 factorial design. They reported that CLA consumption reduced fat mass ( $P < 0.001$ ) confirming the results of other studies, and change in fat mass decreased further ( $P < 0.001$ ) with CLA and exercise. This effect was accompanied by decreased serum leptin levels and lower leptin mRNA expression in peritoneal fat ( $P < 0.001$ ). Serum insulin, glucose, TNF $\alpha$ , and interleukin-6 were lower in CLA-fed mice than in controls ( $P < 0.05$ ). There was no increase in insulin resistance observed in this study. A study on male C57BL/6 mice was conducted to investigate whether torpor (state of inactivity) in rodent models was dependent on the inability of mice to use lipids as energy substrates (Bouthegourd *et al.*, 2004). Mice were fed a standard synthetic diet or a diet augmented with 1.0% of a 50:50 CLA free fatty acid mixture (approximately 1,500 mg/kg body weight/day) for 2 or 6 weeks. Plasma glucose, insulin or leptin levels were not affected by dietary CLA.

DeLany *et al.* (1999) investigated the effect of CLA on plasma leptin and insulin levels in male AKR/J mice fed a basal high-fat diet (control) or the basal high-fat diet supplemented with CLA (0, 0.25, 0.5, 0.75, or 1.0%) for 39 days. Plasma leptin and insulin concentrations were measured in fasted mice. In the dose-response study (*i.e.*, 0, 375, 750, 1,125, or 1,500 mg CLA/kg body weight/day for 39 days), plasma leptin concentrations were unaffected by CLA treatment. CLA consumption resulted in a hyperinsulinemic state in mice fed a high-fat diet and the highest dose of CLA (1,500 mg/kg body weight/day) at weeks 8 and 12 of the study, but not in mice fed lower doses (375, 750, or 1,125 mg CLA/kg body weight/day) of CLA. At low doses, plasma insulin appeared to increase with increases in dose, but the magnitude of change was not statistically significant from the control group. In the time-course study (*i.e.*, 1,500 mg/kg for 12 weeks), plasma leptin was reduced ~70% only at week 6 of treatment. However, plasma insulin concentration was increased ~150 and ~160% on weeks 8 and 12; respectively, compared to the control group. Plasma insulin concentrations were unaffected by CLA at the earlier time points. The investigators described the effect on plasma insulin as "*paradoxical*" because a reduction in adipose tissue weights is usually associated with decreased plasma insulin levels (Markovic *et al.*, 1998). Further, the investigators acknowledged that the AKR/J mouse strain has "*higher insulin levels and respond to a high-fat diet with higher insulin levels*" than SWR/J mice (Eberhart *et al.*, 1994; West *et al.*, 1995). The authors speculated that the biological significance of the increased plasma insulin concentration may lead to "*mild insulin resistance*." Importantly, neither tissue pathology nor reduced tissue function of any organ was reported in this obese mouse model treated with CLA.

West *et al.* (2000) investigated the effect of CLA (1% in the diet for 5 weeks) on metabolic rate, plasma growth hormone, insulin, and glucose levels in male AKR/J mice and reported no effect on plasma insulin concentration. The authors attributed the discrepancy in their findings between other studies by the lack of appropriate plasma sampling techniques. These investigators stated that the lack of appropriate fasting "...likely accounts for the large variation in insulin and glucose measures and obscures true differences in insulin levels between control and CLA-treated mice" (West *et al.*, 2000).

Tsuboyama-Kasaoka *et al.* (2000) investigated the effect of CLA on plasma leptin and insulin concentration in female C57BL/6J mice. Mice were fed a low-fat control (n=14) diet (containing safflower oil) or the low-fat diet supplemented with CLA (1% or 1,500 mg/kg body weight/day, n=14) for up to 8 months. Oral glucose tolerance testing conducted after 17 weeks of CLA supplementation revealed no difference in blood glucose levels compared to controls; however, insulin tolerance test, conducted after 9 weeks of CLA supplementation, was reported to demonstrate marked insulin resistance. After 5 months of CLA treatment, fasting and non-fasting plasma insulin concentrations were reported to be 4- and 8-fold higher, respectively, than control values, while plasma leptin concentrations were reduced by 49 and 79% in fasted and non-fasted CLA-treated mice, respectively. To determine whether the observed insulin resistance was due to leptin depletion, mice that had been fed 1% CLA in the diet for a period of 8 months were infused with leptin (5 µg/day for 12 days) *via* subcutaneous implanted mini-pumps or saline. In CLA-treated mice infused with saline, plasma leptin concentration remained low (<1 ng/mL), while plasma insulin concentration increased ~150%. In CLA-treated mice infused with leptin, plasma leptin concentration increased 900%, while plasma insulin concentration decreased 80%. Thus, increasing plasma leptin concentration resulted in reduced plasma insulin in CLA treated mice. The investigators concluded that CLA treatment of female C57BL/6J mice resulted in insulin resistance as demonstrated by the higher plasma insulin concentrations and absence of hypoglycemia observed in the insulin tolerance test. The investigators hypothesized that the mechanism of increased plasma insulin concentration may be due to the reduced plasma leptin concentration. However, the modulation of plasma insulin by leptin was demonstrated in only 2 mice. The main source for cytokines such as leptin is the adipose tissue and the leptin deficiency may have been due to the extreme adipose tissue reduction.

Clément *et al.* (2002) investigated the effect of CLA on plasma insulin and leptin concentrations in female C57BL/6J mice. Mice were fed a basal diet (control) or basal diet supplemented with c9,t11- or t10,c12-CLA isoforms (0.4% or 600 mg/kg body weight) for 4 weeks. Plasma leptin and insulin concentrations were unaffected by c9,t11-CLA treatment. However, plasma leptin concentration was reduced ~47% and plasma insulin concentration was increased ~900% in t10,c12-CLA treated mice. Neither c9,t11- nor t10,c12-CLA treatments altered blood glucose concentration. These data indicate that the t10,c12-CLA isoform, but not c9,t11-CLA, results in hyperinsulinemia in female mice. The investigators did not determine the dose-response

relationship for the hyperinsulinemic effect in these mice. Lowest and maximum doses that elicit hyperinsulinemia in non-diabetic mice remain unknown. Although hepatic lipid accumulation was associated with increases in plasma insulin concentration, effects on tissue pathology or function were not reported. The biological significance of these changes (*i.e.*, hepatic lipid accumulation and increased plasma insulin concentration) is unknown (Clément *et al.*, 2002).

Another mechanism for liver lipid accumulation by the *t10,c12* CLA isomer was suggested by Warren *et al.* (2003). Female C57BL/6N mice were fed 0.5% of the purified *t10,c12* CLA isomer or the *c9,t11* CLA isomer (approximately 750 mg/kg body weight/day) at the expense of corn oil for 8 weeks. Simultaneous with decreased adipose tissue and increased liver lipids and weights, the CLA *t10,c12* diet decreased transcription of leptin and adiponectin levels. Levels of peroxisome proliferator-activated receptor *alpha* (PPAR $\alpha$ ) mRNA in liver were decreased by the CLA *t10,c12* diet, but increased by the CLA *c9,t11* diet. Both diets increased acyl CoA oxidase levels, but CYP4A1, LPL and UCP2 levels were not affected. Levels of serum lipids and weights of heart, soleus muscle and spleen likewise were not affected. These data suggest that the increase in liver weight and lipids does not involve PPAR $\alpha$  activation. Instead, the effects of the CLA *t10,c12* diet appeared to be linked to the decreased transcription of leptin and adiponectin levels, possibly by increasing lipolysis and hepatic glucose production.

The decrease of adiponectin, another recently discovered cytokine from the adipose tissue, has also been implicated in the observed insulin effects of CLA. Adiponectin reduces hepatic glucose production, therefore, a reduction in adiponectin could increase hepatic glucose production that may be used for fatty acid synthesis (Warren *et al.*, 2003).

It is possible that several mechanisms involved with insulin resistance occur simultaneously in mice supplemented with CLA. The factors playing a role in these mechanisms are, in part, regulated by the adipose tissue. It is clear that the insulin resistance is linked to the significant decreases in adipose tissue and subsequent changes in metabolic regulators secreted from the adipocytes.

The conclusion from lean (*i.e.*, normal) mouse model studies is that some short-term studies show insulin resistance; longer term >41 days do not show insulin resistance. Furthermore, the occurrence of insulin resistance may depend on the metabolic state (diabetic or obese mice being more resistant than normal mice) (Ohashi *et al.*, 2004).

Lean and obese mice were very sensitive to the fat mass reducing properties of CLA. These effects were also associated with increased liver weights and insulin levels. However, after long-term supplementation with CLA, insulin levels decreased and insulin sensitivity was improved. Wargent *et al.* (2005) and Roche *et al.* (2002) reported the effects of feeding CLA preparations enriched with either *c9,t11* or *t10,c12* CLA isomers in leptin-deficient obese C57B1/6 *ob/ob* mice. Feeding CLA, specifically the *t10,c12* CLA isomer, caused a significant

decrease in adipose tissue weights. Both isomers caused increased liver weights. An initial increase in insulin resistance was also observed in mice fed the *t*10,*c*12 CLA isomer. However, in the long-term study with C57BL/6 *ob/ob* mice, this effect was almost reversed after treatment for 35 days (Wargent *et al.*, 2005). At the end of the study (11 weeks), insulin sensitivity was significantly improved (Wargent *et al.*, 2005). A similar beneficial effect on insulin sensitivity was found in a study in diabetic leptin receptor-deficient mice by Hamura *et al.* (2001a). Studies in obese mice demonstrate (1) an improvement in glucose and insulin levels after CLA administration (Hamura *et al.*, 2001a,b); (2) a transient rise in insulin levels followed by normalization (Hamura *et al.*, 2001b; Wargent *et al.*, 2005); and (3) a rise in insulin levels in short term trials (which may also have normalized if the trial was carried out for longer period of time). Human studies also report a transient rise, followed by normalization, of insulin and glucose patterns.

The results of the studies with lean and obese mice are fairly consistent in that in all studies dietary CLA treatment decreased adipose tissue and increased liver weight, which was often accompanied by liver lipid accumulation. In addition, dietary CLA elevated fasting insulin levels in lean mice, but no effects were found on plasma glucose levels. The effect on insulin was only seen in short-term interventions, but not in long-term interventions, suggesting transience of this effect.

Among the lean and obese mouse models, 2 studies reported on the effect of CLA on enzyme expression and activities of insulin and glucose related genes. Clément *et al.* (2002) showed that CLA isomers (both *c*9,*t*11 and *t*10,*c*12 forms) activated liver PPAR- $\gamma$  activity in lean mice. Similarly, Wargent *et al.* (2005) noted that the *t*10,*c*12 CLA isomer increased PPAR- $\gamma$  and a mediated reported gene activity and the authors asserted that CLA initially decreases insulin sensitivity, but that subsequently, (a time effect) that insulin sensitivity is increased in the *lep/ob* mouse model. Thus it may be concluded that activation of the PPAR system may contribute to improving insulin sensitivity in a time-dependent manner.

Further support for this hypothesis may be found in various in markers such as glucose transport. For example, a decrease of GLUT4 in adipose tissue may restrict glucose from entering the cells and as such may contribute to development of insulin resistance (de Roos *et al.*, 2005). In addition, TNF $\alpha$  exerts its effects on insulin levels *via* the induction of lipolysis, causing a rise in free fatty acids. Increased plasma free fatty acid levels inhibit glucose utilization (Randle cycle [Randle *et al.*, 1963]) and would trigger insulin secretion (Girard, 1997). TNF $\alpha$  also impairs tissue insulin sensitivity by interfering with the catalytic activity of the insulin receptor, transduction signaling pathway of insulin and the GLUT4 gene expression, thereby decreasing glucose transport (Hotamisligil, 2000; Cederberg and Enerback, 2003; Kim *et al.*, 2003).

Collectively, these mouse studies [especially Hamura *et al.* (2001a,b) and Wargent *et al.* (2005)] support the hypothesis of a transient effect of CLA mixtures on insulin and glucose sensitivity.

Both lean and obese mice are very sensitive to the fat mass reducing properties of a 50:50 CLA mixture, and especially to the *t10,c12* isomer. These effects were also associated with increased insulin levels. However, after long-term supplementation with a 50:50 CLA mixture, insulin levels decreased and insulin sensitivity was improved. This indicates a transient, time-sensitive effect of CLA isomers on insulin-related events. The effects in obese mice, as noted earlier, either reflect no effect, a transient effect, or an improvement in glucose tolerance. However, the apparent trend is an adaptive response which appears rooted in an alteration in gene expression *via* the PPAR- $\gamma$  system.

#### Studies in Rats

The effects of CLA on insulin sensitivity in different rat models seem to show that CLA increases insulin sensitivity, with only one exception showing a rise in insulin levels at a threshold dose of at least 5% CLA 50:50 mixtures (O'Hagan and Menzel, 2003).

As noted in Section 7.3.3.1, increased plasma insulin levels were observed in both males and females rats fed 15% 50:50 CLA mixture. 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.

In male rats fed 15% of the 50.50 CLA mixture, the data suggested a trend toward decreased glucose levels from week 8. At the end of the recovery phase, glucose levels in CLA-fed 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. Glucose levels were not measured in females after the recovery period since no effect was observed previously. It is uncertain whether the increased plasma insulin levels in males and females and the decreased glucose levels in the males were related to a physiological adaptation to high levels (15%) of a 50:50 CLA mixture. Such effects did not result in any adverse functional changes (*e.g.*, on blood glucose or in the pancreas in both sexes). Thus, levels in the range of 1 to 5% did not negatively influence insulin sensitivity, whereas only in the high level (15%) was there an increase in insulin levels compared to controls. This increase in insulin levels after the 15% CLA dose decreased during the recovery period, indicating that the CLA-induced rise in insulin levels was recoverable at cessation. It should be noted that this was a safety and toxicity study, and levels such as 5 or 15% in the human diet would never be achieved.

Stangl (2000) looked at the effects of 1, 3, or 5% CLA vs. a sunflower oil control in male Wistar rats for 5 weeks and found that glucose levels were unaffected in the 1 and 3% CLA mixtures, but were elevated at the 5% level compared to the control. These data are supported by the work of O'Hagan and Menzel (2003) who found that levels of the 50:50 CLA mixture at 15% raised serum glucose levels in rat models. Thus, there appears to be a threshold of tolerance in

these animal models that supports safety in humans since levels that are intended for human consumption do not reach these levels.

In contrast, other authors (e.g., Ryder *et al.*, 2001; Henriksen *et al.*, 2003; Nagao *et al.*, 2003b; Teachey *et al.*, 2003; Zhou *et al.*, 2005), reported beneficial metabolic responses in rat models, including enhanced glucose/insulin metabolism.

CLA induced decreases in adipose tissue are found in rats, but these effects were moderate compared to mice. One study reported a decrease in adipose tissue in lean rats, but an increase in obese rat (Sisk *et al.*, 2001). A suggested mechanism by which CLA decreased fat in lean rats but increased fat in obese rats was the normalized glucose tolerance paired with the hyperphagia of obese animals, resulting in more glucose availability as a substrate for an enlarged fat mass. This model of metabolic shifting could explain the transient change in insulin or glucose levels that normalizes with time.

CLA or CLA isomers do not elicit consistent responses in rat models. Studies with mice and rats are difficult to compare, due to the different models and assessed parameters. In addition, not many studies with rats have looked at the mode of action of CLA at the enzymatic level. However, it is clear that the effects of comparable levels of dietary CLA supplementation exert less drastic effects on adipose tissue, liver weight, and insulin and glucose levels in rats than in mice, which is likely due to the higher metabolic rate of mice. Such species differences have been pointed out in other publications (Pariza, 2004).

The effects of CLA on insulin and glucose levels in rats have been studied mostly in diabetic obese models such as the ZDF Zucker rat. These models are characterized by obesity, insulin resistance and impaired glucose tolerance. A number of studies reported an improvement of glucose tolerance and a reduction of insulin levels by CLA (Houseknecht *et al.*, 1998, Ryder *et al.*, 2001; Sisk *et al.*, 2001; Henriksen *et al.*, 2003; Zhou *et al.*, 2005), although part of this effect may be indirectly due to a reduced food intake (Ryder *et al.*, 2001). It was suggested that the mechanism of action for this effect is that CLA acts as an insulin sensitizer by activating PPAR $\gamma$  which is highly expressed in adipose tissue (Houseknecht *et al.*, 1998). Two studies reported an increase in glucose levels: (1) after 5 weeks of a 5% dose of a CLA mixture (Stangl, 2000) and (2) after 4 weeks of 1% dose of CLA FFA mixture in diabetic rats (Rahman *et al.*, 2001).

The improvement in glucose tolerance seen in most of these studies may also be a direct effect of the reduction in fat tissue, muscle triglyceride content or decreased oxidative stress (Henriksen *et al.*, 2003). The findings of improved glucose tolerance, insulin stimulated glucose transport and insulin stimulated glycogen synthase have been attributed to the  $\epsilon$ 10, $\epsilon$ 12 CLA isomer specifically (Ryder *et al.*, 2001; Henriksen *et al.*, 2003). In the non-insulin dependent diabetes mellitus model (*i.e.*, OLETF rats), no effect of CLA on insulin levels was reported (Rahman *et al.*, 2001).

Only a few studies with lean rat models have evaluated the effect of CLA on glucose metabolism (Houseknecht *et al.*, 1998; O'Hagan and Menzel, 2003). Although elevated glucose levels after CLA administration were reported, they still remained within normal ranges.

#### Studies in Hamsters

Bouthegourd *et al.* (2002) reported that the administration of a purified diet augmented with c9,t11 CLA isomer to 0.6% or CLA 50:50 to 1.2% in purified diets to male Syrian hamsters for a period of 6 weeks or 8 weeks resulted in significantly higher plasma glucose levels in the group receiving the CLA mixed isomers compared with the other groups. Plasma insulin levels did not differ significantly between the groups. The homeostatic model assessment (HOMA) for insulin resistance was calculated using the insulin and glucose values and insulin resistance was found to be significantly increased in the group receiving the CLA mixed isomers compared to the other groups.

#### Studies in Pigs

It should be noted that of the species that have been studied, the pig is the most closely related to humans, and in this model there is no evidence for negative effects on glucose and/or insulin metabolism.

Stangl *et al.* (1999) reported that the administration of basal diets containing 1.0% of a CLA preparation containing 34.6% c9,t11 CLA and 18.4% t10,c12 CLA for a period of 6 weeks to adult female pigs resulted in non-significant increases in plasma insulin concentrations. The authors suggested that stimulated insulin secretion might be caused by increased glucose oxidation, although glucose levels remained unaffected by CLA. Ramsay *et al.* (2001) found that the administration of 0.25, 0.5, 1.0, or 2.0% CLA preparations (25% of the c9,t11 CLA isomer and 35% of the t10,c12 CLA isomer) to male and female crossbred grower pigs (Yorkshire x Landrace) had no effect on serum glucose and insulin levels.

The effect of CLA treatment on plasma variables related to lipolysis and lipogenesis in growing pigs and their metabolic responses to the homeostatic signals, adrenalin, or insulin were investigated by Ostrowska *et al.* (2002). Sixteen female cross-bred (Large White x Landrace) pigs were randomly allocated to 4 treatment groups in a 2x2 factorial design, including a dietary fat (25 or 100 g/kg diet) group and a CLA-55 (0 or 10 g/kg diet) group for 8 days. CLA did not affect plasma glucose or insulin levels.

#### C.2.3 Summary

Numerous studies have been conducted in *in vitro* cell culture systems, rodent models, pigs and humans to evaluate insulin resistance due to CLA. A decrease in leptin levels has been reported in several published studies with CLA (DeLany *et al.*, 1999; Tsuboyama-Kasaoka *et al.*, 2000). However, due to the complex interactive regulation of leptin and insulin, it is not clear

whether a decrease in leptin levels in CLA treated lean mice is the result or cause of insulin resistance.

Most studies evaluating the effects of CLA on insulin resistance were conducted in rodents and humans. Responses to CLA are species-dependent (Pariza, 2004). Collectively, these studies, on the effects of CLA on various aspects of insulin and glucose biology, differed greatly among the animal models (species and strain, sex, age, and metabolic state) and experimental design (dose levels that are relevant to what we assert as safe for humans, CLA isomer compositions, diet and fat levels, control diets used, length of study, variety of parameters and their time of measurement). As a result, interpretation of data is very complicated. In addition, inherent to studies on dietary ingredients, the observed effects may be secondary to the effect on food intake and body fat stores, although most studies accounted for this.

Different modes of action of CLA on the decrease of adipose tissue have been suggested. In mice, CLA was reportedly causing a decrease in (a) adipose tissue weights, (b) fatty acid oxidation *via* PPAR $\alpha$ , (c) fat deposition in adipose tissue *via* the SREBP-1 or PPAR $\gamma$  pathway, and (d) an increase in lipolysis and apoptosis, all pathways that have been suggested as underlying mechanisms. Some researchers suggest that the increase in fatty acid oxidation enzymes may be due to modulation of eicosanoid production, since various eicosanoids are potent activators of PPARs. The mechanisms are not mutually exclusive and may therefore occur simultaneously (Belury, 2002; Belury *et al.*, 2003; Brown *et al.*, 2003).

Hyperinsulinemic effects of CLA have been found mainly in obese mice suggesting that these effects may be linked to the decrease in adipose tissue. The adipose tissue is the source for many factors that influence lipid metabolism, such as leptin, adiponectin, GLUT4, and TNF $\alpha$ . Indeed, leptin infusion in CLA-fed mice reduced insulin levels, and also reduced the increase in liver weight and vacuolization produced by CLA. The decrease in adipose tissue together with the down-regulation of GLUT4 may result in a compensatory increase in hepatic lipogenesis to metabolize glucose (Tsuboyama-Kasaoka *et al.*, 2000). The same researchers recently reported that CLA intake did not cause the extreme adipose tissue loss, increased hepatic lipids, and hyperinsulinemia in mice supplemented with a very high fat diet. This clearly indicated that the lipodystrophic effects are not caused by CLA directly but by the ablated or dysfunctional adipose tissue response (Tsuboyama-Kasaoka *et al.*, 2003).

Clément *et al.* (2002) suggests that the decrease in adipose tissue and the increase in liver weight and insulin levels are mainly due to effects of the t10,c12 CLA isomer. This is further supported by the study by Ryder *et al.* (2001) who found effects on insulin action, leptin and free fatty acid levels in rats fed a CLA 50:50 diet, but not in rats fed a diet enriched with the only c9,t11 isomer. CLA may thus deteriorate insulin-dependent glucose disposal due, in part, to an increase in lipolysis and fatty acid oxidation through the Randal-glucose-fatty acid cycle (Wargent *et al.*, 2005).

The hyperinsulinemia seen in the mouse is the result of the sensitivity of the mouse to CLA and its apparent inability to cope with the changes in fat metabolism induced by CLA administration. Indeed, this may be the result of the lower fat content of the mouse diets (approximately 10% fat), compared to the average human diet, which contains about 30% fat. Further, the loss of adipose tissue may involve genetic mechanisms and an adaptive response due to the metabolic switching of enzymes linked to glucose and lipid metabolism (Hamura *et al.*, 2001a,b; Wargent *et al.*, 2005).

From the *in vitro* and animal studies, a collective interpretation regarding CLA and insulin resistance in mice after CLA administration may be summarized with the following conclusions: (1) adipose tissue is almost completely ablated in the mouse following CLA intervention due to apoptosis resulting from decreased glucose uptake in the adipose tissue; (2) decreased glucose uptake as a result of inhibition of GLUT4 by predominantly the t10,c12 CLA isomer at the nuclear regulatory level; (3) blood glucose is shunted to the liver and induces hepatic lipogenesis in response to the higher amount of glucose that is further transformed into serum triglycerides.

In concurrence with the moderate effects on adipose tissue, CLA did not affect insulin levels, liver weights or hepatic lipid content in most studies in rats, hamsters, and pigs. A normalizing, physiological effect of CLA on glucose and insulin levels was reported in obese rat models (Houseknecht *et al.*, 1998; Ryder *et al.*, 2001; Sisk *et al.*, 2001). CLA may act as a PPAR $\gamma$  agonist in obese models (Houseknecht *et al.*, 1998) or exert its effect *via* the reduction in fat tissue, muscle triglyceride content or decreased oxidative stress (Henriksen *et al.*, 2003). Humans are clearly less sensitive than mice to the adipose tissue reducing effects of CLA. Human studies conducted with CLA have reported decreases in body fat mass in the range of 3.0 to 9.0%. In contrast, body fat mass in mice was almost completely ablated. Terpstra (2001) has calculated that the different effects of CLA between mice and humans can be explained for the greater part by the higher metabolic rate of mice. Studies on CLA in mice reported effects on liver and insulin levels as a result of the loss of functional adipose tissue and related deregulation of cytokines and fat distribution. Consumption of CLA supplements in humans did not result in such effects on adipose tissue. In conclusion, CLA mixtures in obese animal rodent models cause short term increases in insulin resistance. Some obese rodent models appear to be particularly susceptible to the CLA-induced reduction in adipose tissue stores. As a result of this loss in functional adipose tissue, the liver initially compensates and lipodystrophy occurs. However, as Hamura *et al.* (2001a,b) and Wargent *et al.* (2005) showed in some rodent strains, there is functional adaptation to CLA in the diet and the increased blood glucose results in compensatory insulin action that normalizes within 10 weeks (Section 7.5.4 discusses the results of clinical trials on insulin and glucose levels). Animal studies on insulin sensitivity and glucose metabolism are summarized in Table C.2.3-1 below.

**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (female C57BL)	4 weeks	50:50 c9, t11- and t10, c12-CLA	707.05	Significantly ↑ plasma glucose, plasma insulin, HOMA-R value, and mRNA expression of TNF $\alpha$ . Significantly ↓ plasma adiponectin and resistin. No significant changes in plasma leptin and TNF $\alpha$ , or mRNA expression of leptin adiponectin, and resistin. After insulin tolerance test, no significant changes in plasma glucose levels.	Ohashi <i>et al.</i> , 2004
Mouse (female KK)	4 weeks	50:50 c9, t11- and t10, c12-CLA	614.61	Significantly ↑ plasma insulin, HOMA-R and TNF $\alpha$ mRNA expression. Significantly ↓ plasma adiponectin and resistin, and mRNA expression of leptin and adiponectin. No significant changes in plasma glucose, leptin, TNF $\alpha$ , or mRNA expression of resistin	Ohashi <i>et al.</i> , 2004
Mouse (female KKAY)	4 weeks	50:50 c9, t11- and t10, c12-CLA	636.49	Significantly ↑ plasma glucose, plasma insulin, and HOMA-R value. Significantly ↓ plasma leptin, adiponectin, and resistin, or mRNA expression of leptin, adiponectin, and resistin. No significant changes in plasma TNF $\alpha$ or mRNA expression of TNF $\alpha$ . After insulin tolerance test, no significant changes in plasma glucose, however, significantly less integrated changes in plasma glucose were observed.	Ohashi <i>et al.</i> , 2004

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**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (female C57BL/6J)	4 weeks	50 50 c9, t11- and t10, c12-CLA	1,500	Significantly ↑ plasma insulin levels. No significant changes to blood glucose levels. Significantly ↓ adiponectin and plasma leptin levels by day 2 and 6, respectively. Significant positive correlation between hepatic lipid content and plasma insulin was observed. Pancreatic islet cells from CLA-fed mice displayed significantly ↑ (3- to 4-fold) rates of glucose-stimulated insulin secretion in the presence and absence of isobutyl methylxanthine or carbachol. Also, mice had significantly ↑ islet sizes, beta cell mass, beta cell density, and islet area/pancreas area.	Poirier <i>et al.</i> , 2005
Mouse (female C57BL/6J)	4 weeks	c9, t11-CLA	600	No significant changes in plasma leptin, plasma insulin or blood glucose	Clément <i>et al.</i> , 2002
		t10, c12-CLA		Significantly ↓ plasma leptin and significantly ↑ plasma insulin. No significant changes in blood glucose.	
Mouse (male C57BL-6 ob/ob)	4 weeks	c9, t11-CLA	6,645	No significant changes in blood glucose and insulin	Roche <i>et al.</i> , 2002
		t10, c12-CLA	6,885	Significantly ↑ serum glucose and insulin levels.	
Mouse (male AKR/J)	5 weeks	50:50 c9, t11- and t10, c12-CLA	1,500	No significant changes in plasma glucose or insulin levels, although the mean of insulin levels were almost 2-fold higher in the CLA group compared to the control group	West <i>et al.</i> , 2000

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**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (female C57BL/6 <i>lep<sup>ob</sup>/lep<sup>ob</sup></i> )	3 weeks	50:50 c9, t11- and t10, c12-CLA (Clannol A-60)	2,250	Significantly ↑ plasma insulin. No significant changes in blood glucose levels during the OGTT	Wargent <i>et al.</i> , 2005
		50:50 c9, t11- and t10, c12-CLA (Clannol A-60)	3,750	Significantly ↑ plasma insulin. Animals fed mixed CLA had significantly ↑ blood glucose levels during the OGTT.	
		50:50 c9, t11- and t10, c12-CLA (Clarinol A-60); Clannol A-60 enriched with 90% t10, c12-CLA; Clannol A-60 enriched with c9, t11-CLA	3,750	CLA mixture significantly ↑ plasma insulin on days 13 and 22. t10, c12-CLA significantly ↑ plasma insulin on day 13, 21, and 22. c9, t11-CLA ↑ plasma insulin on day 21. CLA mixture and t10, c12-CLA significantly ↑ blood glucose levels during the OGTT.	
	11 weeks	50:50 c9, t11- and t10, c12-CLA	1,500 (low) or 3,750 (high)	Both doses significantly ↑ plasma insulin and insulin sensitivity index on day 14 and day 35, but plasma insulin levels and insulin sensitivity index were significantly ↓ on day 70. By day 70, high-dose CLA significantly ↓ blood glucose levels during the OGTT, whereas, the low-dose CLA did not significantly change the blood glucose levels.	
Mouse (Male AKR/J)	6 weeks	50:50 c9, t11- and t10, c12-CLA	375, 750, 1,125, or 1,500	Dose-dependently ↑ plasma insulin, reaching statistical significance in the highest dose. No significant changes in plasma leptin or plasma glucose.	DeLany <i>et al.</i> , 1999
	2 to 12 weeks		1,500	Significantly ↓ plasma leptin concentration at week 6 only. Significantly ↑ plasma insulin concentration at weeks 8 and 12. No significant changes in plasma glucose levels.	

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**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (Male db/db)	12 weeks	50:50 c9, t11- and t10, c12-CLA	1,800	Significantly suppressed ↑ in blood glucose after glucose administration during OGTT and significantly ↓ AUC. Significantly ↑ insulin concentration reported during OGTT after 5 weeks of treatment, but significantly ↓ insulin levels after 11 weeks of treatment. No significant changes in blood glucose or insulin.	Hamura <i>et al.</i> , 2001a
Mouse (Male apolipoprotein knockout)	12 weeks	c9, t11-CLA	10,500	Significantly ↓ plasma glucose, plasma insulin, and HOMA-R. Significantly ↑ QUICKI.	De Roos <i>et al.</i> , 2005
		t10, c12-CLA		Significantly ↑ plasma glucose. Significantly ↓ QUICKI. No significant changes in plasma insulin or HOMA-R	
Mouse (male Balb/c)	14 weeks	50:50 c9, t11- and t10, c12-CLA	403 (sedentary) or 463 (exercised)	Sedentary mice: Significantly ↓ serum glucose, insulin, leptin, TNFα, and IL-6. Significantly ↓ mRNA expression of leptin. No significant changes in plasma adiponectin levels, or mRNA expression of adiponectin or TNFα. Exercised mice: Significantly ↓ serum glucose, insulin, leptin, and TNFα. Significantly ↓ mRNA expression of leptin and TNFα. No significant changes in serum adiponectin and IL-6, or mRNA expression of adiponectin. Also, compared to sedentary mice, exercised mice had significantly ↓ serum glucose and leptin, significantly ↑ serum TNFα, and significantly ↓ mRNA expression of leptin. No significant changes in serum insulin, adiponectin, and IL-6, or mRNA expression of adiponectin and TNFα.	Bhattacharya <i>et al.</i> , 2005

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**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (Male C57BLKS-Lepr <sup>db</sup> /lepr <sup>db</sup> )	23 weeks	50:50 c9, t11- and t10, c12-CLA	795 (low) or 1,800 (high)	No significant changes in blood glucose or insulin concentrations	Hamura <i>et al.</i> , 2001b
Mouse (Female 57BL/6J)	4 days to 8 months	50:50 c9, t11- and t10, c12-CLA	1,500	Significantly ↑ plasma insulin and significantly ↓ plasma leptin concentrations. No significant changes in blood glucose during OGTT. Significantly ↑ blood glucose during ITT. Continuous infusion with leptin ↑ plasma leptin concentrations, but ↓ plasma insulin concentrations, compared to saline infused animals. These results were not statistically analyzed.	Tsuboyama-Kasaoka <i>et al.</i> , 2000
Rat [male Zucker Diabetic Fatty (ZDF/GMI; fa/fa)]	2 weeks	c9, t11-CLA	750	Significantly hyperglycemic compared to baseline values. After oral glucose challenge, no significant changes in glucose tolerance were observed. No significant changes in plasma insulin or leptin.	Ryder <i>et al.</i> , 2001
		50:50 c9, t11- and t10, c12-CLA		No significant changes in glycemia. Significantly improved glucose tolerance after oral challenge with glucose. Significantly ↓ plasma insulin and leptin.	
Rat [male Zucker diabetic fatty (fa/fa; ZDF/GMI)]	2 weeks	50:50 c9, t11- and t10, c12-CLA	17,120	Significantly ↓ fed and fasting blood glucose levels were reported in fatty CLA group compared with fatty control. Significantly ↓ plasma insulin levels compared with fatty control. Significant ↓ in rise in glucose levels during OGTT.	Houseknecht <i>et al.</i> , 1998

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**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Rat [female obese Zucker (Hsd/ola.ZUCKER-fa)]	3 weeks	c9, t11-CLA	1,140 (gavage)	No significant changes in plasma glucose or insulin throughout the experimental period or during the OGTT. No significant changes in AUC for glucose and insulin, or glucose-insulin index.	Henriksen <i>et al</i> , 2003
		t10, c12-CLA	1,350 (gavage)	Significantly ↓ plasma glucose and insulin throughout the experimental period or during the OGTT. Significantly ↓ AUC for glucose and insulin during OGTT and glucose-insulin index. Significantly ↑ insulin-mediated glucose transport in epitrochlearis and soleus muscles.	
		50:50 c9, t11- and t10, c12-CLA	1,500 (gavage)		
Rat [female obese Zucker (Hsd/ola.ZUCKER-fa)]	3 weeks	50:50 c9, t11- and t10, c12-CLA	300 (gavage)	Significantly ↓ plasma glucose. No significant changes in plasma insulin. Significantly ↓ plasma glucose 30 min after glucose challenge with no significant changes in plasma insulin levels. Significantly ↓ glucose AUC during OGTT and glucose-insulin index, but no significant changes in insulin AUC during OGTT. Significantly ↑ insulin-mediated glucose transport in epitrochlearis muscle, but no significant changes in soleus muscle.	Teachey <i>et al</i> , 2003
			1,500 (gavage)	Significantly ↓ plasma insulin. No significant changes in plasma glucose. Significantly ↓ plasma glucose 30 min after glucose challenge. Significantly ↓ plasma insulin up to 90 min after oral glucose challenge. Significantly ↓ glucose AUC and insulin AUC during OGTT and glucose-insulin index. Significantly ↑ insulin-mediated glucose transport in epitrochlearis and soleus muscle.	

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Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism					
Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Rat [male Otsuka Long Evans Tokushima Fatty (OLETF)]	4 weeks	50:50 c9, t11- and t10, c12-CLA	1,000 (CLA TG mixture)	Significantly ↓ serum leptin levels. No significant changes in serum insulin or glucose.	Rahman <i>et al.</i> , 2001
			1,000 (CLA FFA mixture)	Significantly ↑ serum glucose. Significantly ↓ serum leptin. No significant changes in serum insulin.	
Rat (male SPF Sprague-Dawley)	5 weeks	50:50 c9, t11- and t10, c12-CLA	500 (low), 1,500 (mid), or 2,500 (high)	Significantly ↑ blood glucose levels in the high-dose. No significant changes in blood glucose levels in the low- and mid-dose groups.	Stangl, 2000
Rat (female lean and obese Zucker)	8 weeks	50:50 c9, t11- and t10, c12-CLA	Lean: 300; Obese: 240	No significant changes in insulin levels in obese or lean rats	Sisk <i>et al.</i> , 2001
Rat [male Zucker diabetic fatty (fa/fa)]	8 weeks	50:50 c9, t11- and t10, c12-CLA	480	Significantly ↓ plasma insulin and glucose levels, resulting in improved insulin sensitivity. Significantly ↑ plasma adiponectin and mRNA expression of adiponectin in WAT. No significant change in plasma leptin levels	Nagao <i>et al.</i> , 2003b
Rat (male and female Wistar outbred [CrI.(WI)WU BR])	13 weeks	50:50 c9, t11- and t10, c12-CLA (Clarinol G80)	1,500 (low), 7,500 (mid), or 22,500 (high)	Males: Significantly ↓ blood glucose in high-dose in week 13 and after recovery period. Significantly ↑ plasma insulin levels in high-dose at week 4; however the effect was statistically not significant by week 13.	O'Hagan and Menzel, 2003
				Females: Significantly ↑ plasma insulin levels at week 8 and 13. No significant changes in blood glucose and insulin levels during testing period or after recovery period.	
Rat (male Wistar)	NR	NR	375 (low), 750 (mid), and 1,500 (high)	Significantly ↓ serum insulin and glucose in high-dose group Significantly ↓ serum glucose in mid-dose group, but no significant changes in serum insulin. No significant changes in serum glucose or insulin in the low-dose group	Zhou <i>et al.</i> , 2005

**Table C.2.3-1 Summary of CLA Animal Feeding Studies on Insulin Sensitivity and Glucose Metabolism**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Hamster (male Syrian LPN)	8 weeks	c9, t11-CLA	720	No significant changes in plasma glucose, insulin, leptin, or HOMA-R.	Bouhagourd <i>et al.</i> , 2002
		50.50 c9, t11- and t10, c12-CLA	1,440	Significantly ↑ plasma glucose level and HOMA-R. No significant changes in plasma insulin or leptin.	
Pig (female Large White X Landrace)	8 days	NR	338.6 (low-fat, CLA) or 303.8 (high-fat, CLA)	No significant changes in plasma glucose or insulin levels. After challenging pigs with adrenaline or insulin, no significant changes in plasma glucose were observed.	Ostrowska <i>et al.</i> , 2002
Pig (female)	6 weeks	34.6% c9, t11-CLA and 18.4% t10, c12-CLA	241.9	No significant changes in circulating insulin or serum glucose.	Stangl <i>et al.</i> , 1999
Pigs (male and female Yorkshire X Landrace)	Up to 7 weeks	25% c9, t11-CLA and 35% t10, c12-CLA	204.5, 409.1, 818.2, or 1,636.4	No significant changes in blood insulin levels at any dose. Significant ↓ in blood glucose levels in pigs administered 818.2 mg/kg bw/d, but no significant changes in any other dose group were observed.	Ramsay <i>et al.</i> , 2001

AUC = area under curve, CLA = conjugated linoleic acid; HOMA-R = homeostasis model for insulin resistance; IL= interleukin; ITT = insulin tolerance test, mRNA = messenger ribonucleic acid; NR = not reported; NS = not significant, OGTT = oral glucose tolerance test; QUICKI = quantitative insulin sensitivity check index; TNF = tumor necrosis factor; WAT = white adipose tissue

<sup>1</sup> CLA administered in the diet, unless otherwise noted

<sup>2</sup> Treatment groups compared to respective control group, unless otherwise noted

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### C.3 Milk Fat Deposition (MFD) (Bovine)

Two main dietary alterations have been found to influence MFD in cattle: high carbohydrate/low fiber diets (e.g., high grain, low roughage) and high-oil diets consisting of oils high in polyunsaturated fatty acids (e.g., plant and fish oils) (Grummer, 1991). A low roughage diet alters ruminant bacterial bio-hydrogenation and appears to be a major factor in the induction of MFD. Likewise, a diet rich in plant or fish oils that provide unsaturated fatty acid precursors also appears to lower the threshold for MFD due to low roughage diets (Griinari and Bauman, 2001). A low roughage diet supplemented with plant or fish oils results in MFD *via* changes in ruminant bacteria enzyme processes.

While it is not the intent of this dossier to provide a thorough review of ruminant MFD, this Section will highlight pivotal studies relating to the mechanism(s) whereby CLA induces MFD in ruminants. The CLA isomer responsible for MFD has been reported by several research groups and will be discussed in this review

#### C.3.1 *In Vitro* Studies

No *in-vitro* studies of significance to this report have been conducted into milk fat deposition.

#### C.3.2 *Animal Studies*

##### Bovine/Ruminant Studies

In bovine animals, CLA is formed *via* the enzymatic biohydrogenation of linoleic acid by ruminant microorganisms (Lor and Herbein, 1998). The first isomerization step converts linoleic acid to the c9,t11 form, which is the principal isomer found in dairy foods (Fujimoto *et al.*, 1993).

Mackle *et al.* (2003) administered CLA at doses of 0, 20, 40, or 80 g/day CLA for 4 days by abomasal infusion techniques, thereby bypassing the bacterial biohydrogenation step that synthesizes *de novo* CLA from dietary fatty acids. Milk fat concentrations were decreased in proportion to the increase in CLA infusions (e.g., 36, 43, and 62%). They also noted an increase in milk yield in the 40 g/day infusion. Mackle *et al.* (2003) concluded that *de novo* fatty acid synthesis and desaturation were most profoundly affected by CLA infusion. This study showed that despite bypassing bio-hydrogenation steps, CLA can still impact milk fat yield in a ruminant model.

Peterson *et al.* (2003) reported the role of t10,c12 CLA in suppressing lipogenic enzymes and thus *de novo* synthesis of fat in milk resulting overall in less milk fat yield in ruminants. These findings are supported by the finding of by Lor and Herbein (2003), who reported a decrease in fatty acid synthase and desaturation after exogenous administration of the t10,c12 CLA isomer. They fed 4 Holstein cows diets with either high-oleic sunflower oil or high-linoleic safflower oil

and infused with either *c9,t11* or *t10,c12* CLA isomers. Although milk yield was unaffected by these treatments, milk fat in the *t10,c12*-infused samples decreased by 25% compared to the *c9,t11*-infused samples. Baumgard *et al.* (2000, 2002) have further elucidated that the *t10,c12* CLA isomer is responsible for MFD in ruminants. In a collection of works in abomasally infused dairy cows, they found that infusing the *t10,c12* CLA isomer results in a decrease in mRNA expression of numerous enzymes involved in *de novo* fatty acid synthesis.

While the effects of diet and CLA addition have consistent effects on milk fat depression in ruminants, the ruminant animal is difficult to compare to humans from a comparative physiological standpoint. However, they may be useful to help understand the underlying mechanisms of such effects, due to their long lactation period and large organ size. Such attributes allow for greater ease and accuracy of conducting biopsies and, therefore, of measuring biochemical effects. Ruminants have four stomachs and the bulk of fermentation occurs in these spaces. Conversely, monogastric animals such as pigs and some rodents provide a more similar gastric physiology to humans on which to model diet-induced changes in MFD after CLA administration. However, as the following Section will show, the rodent model also has shortcomings with respect to consistent biological responses.

#### Studies in Mice

Loor *et al.* (2003) examined litter growth and lipogenic enzyme activity in CD-1 mouse dams. They administered diets containing individual isomers (*c9,t11* or *t10,c12*) with or without *trans11:18;1* vs. a rapeseed oil control diet and found that the *t10,c12* CLA isomer reduced food intake and carcass fat compared to other treatments. Further, they noted the *t10,c12* CLA isomer reduced milk fat as well as the levels of delta-9 desaturase and elongase activity. They concluded that the *t10,c12* CLA isomer was likely responsible for a reduction in *de novo* fatty acid synthesis. This mouse model agrees with experimental outcomes in ruminants in that lipogenic enzyme activity is reduced after addition of the *t10,c12* CLA isomer to the diet. The *c9,t11* isomer, however, did not cause any change in milk fat. In a continuation of this study, the investigators also measured mRNA of enzymes involved in fatty acid synthesis. They noted that levels of acetyl CoA-carboxylase were reduced by both CLA isomers. Further, steroyl CoA desaturase 1 levels were reduced in mice receiving the *t10,c12* CLA isomer. They concluded that the *t10,c12* CLA isomer is a more potent inhibitor of mammary lipogenesis and enzymes of desaturation than is the *c9,t11* CLA isomer (Lin *et al.*, 2004).

#### Rat

The effect of CLA consumption on milk fat has been investigated in Fischer rats (Chin *et al.*, 1994). Although the milk fat content was found to decrease after CLA administration, supplementation with CLA did not affect litter size nor induce abnormalities in rats (Chin *et al.*, 1994). Actually, 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.

Ringseis *et al.* (2004) fed 2 groups of female Sprague-Dawley rats either 14.7 g/kg diet of CLA (735 mg/kg body weight/day) or sunflower oil control during growth, pregnancy, and lactation. The major findings in the CLA-fed group were significant decreases in fatty acid synthase activity, and lower plasma triglyceride concentrations. They also reported a decrease in the number of pups per litter, pup weights, and total litter weights in the CLA treated group. It should also be pointed out that the pups were not randomized and thus these findings cannot be attributed solely to CLA-effects but may contain artefacts of experimental design bias. However, the CLA preparation administered provided 54% CLA as lipid contribution to the diet, a total not representative of the amounts fed in other rodent trials (0.5 to 5%), nor would it be relevant to the percent CLA as contributed to total dietary fat in human studies. Importantly, this CLA preparation is not representative of the product under review in this dossier as it contained 22 isomers, and levels of the relevant isomers were very low (18.5% of *t*10,*c*12 CLA; 15.6% of *c*9,*t*11 CLA).

The effect of CLA on rodents with respect to MFD seem to be dependent upon amount fed and isomeric composition.

#### Studies in Pigs

Bee (2000) reported that polyunsaturated fatty acids such as CLA administered to pigs during lactation increased piglet growth rates. A CLA blend consisting of 6 major isomers was administered to 12 multiparous sows during gestation and lactation. All major CLA isomers were found in tissues and milk fat, compared to a linoleic acid control, similar to the findings of Bee (2000). They also estimated transfer efficiency of CLA into tissue and mature milk and found that the transfer efficiency of CLA isomers into tissue ranged from 41 to 52%, while the transfer efficiency into milk was between 55 to 69%. Furthermore, Bee (2000) reported that the *c*9,*t*11 CLA isomer had the highest rate of incorporation. Overall, they reported no adverse effects of CLA in pigs. This is contrary to the bovine literature and further points to the species-specific effects of CLA-based research results.

Poulos *et al.* (2004) examined the long-term effects of CLA administered at 0.83% (representing a concentration of 0.5% CLA at a 60% isomer purity) on neonatal growth and development compared to soy oil controls. Sows were treated from day 40 or 75 of gestation and through weaning on day 28. The authors noted that CLA did not change sow feed intake, body weight, back fat, or litter size and weight at birth. Although CLA was shown to decrease milk fat by 17%, and decrease growth up to day 14 in piglets on the CLA treatments (presumably due to a decrease in fat in sow's milk), overall growth rates at weaning were not different from control, suggesting that compensatory mechanisms are in place.

### C.3.3 Summary

Given the numerous perturbations that are possible in the diet, coupled with evidence in the ruminant that any plant or fish oil may provide unsaturated fatty acids that contribute to MFD when coupled with low roughage feed, it is not surprising that many other dietary fat sources induce MFD in rodent models and that the effect seen for CLA is by no means unique

These species differences demonstrate that inhibition of *de novo* fatty acid synthesis by CLA is of much greater significance in rodents and cows than in humans, since *de novo* fatty acid synthesis in humans is of much lower significance to milk fatty acid secretion (Bee, 2000).

Section 7.5.5 discusses the effect of CLA on human maternal milk fat deposition. Animal studies on milk fat deposition are summarized in Table C.3.3-1 below.

**Table C.3.3-1 Summary of CLA Animal Feeding Studies on Milk Fat Deposition**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Mouse (female)	12 days	c9, t11-CLA	1,500	No significant change in total fatty acid concentration in mammary tissue; however, significantly ↑ concentration of treatment isomer in mammary tissue. Significant ↓ in mammary mRNA abundance of SCD, ACC, and FAS, and activity of ACC. No significant changes in hepatic mRNA abundance and enzyme activity of ACC or FAS	Lin <i>et al.</i> , 2004
		t10, c12-CLA	1,500		
Mouse (CD-1 dams & litters)	12 days	c9, t11-CLA	1,500	No significant changes in fat weight in dams and litters, milk fat concentration in dams, or body fat content in litters.	Loor <i>et al.</i> , 2003
		t10, c12-CLA	1,500	Significantly ↓ milk fat concentration of the dams. Significantly ↓ body weight, carcass fat, protein, and ash weight in pups. Significantly ↓ levels of SCD and elongase activity in dams and pups. No significant changes in total FA of carcass and liver tissue of pups.	
Rat (Fischer dams and pups)	20 days	NR	For dams: 250 For pups: 500	No significant changes on litter sizes and fetal body weights, and no signs of fetal abnormalities. Significantly ↑ postnatal body weight gain in pups receiving CLA during gestation and lactation	Chin <i>et al.</i> , 1994
	Male pups weaned after 12 days, then fed treatment for 8 weeks	NR	Pups: 250, 500	Significantly ↑ total body weight gain in pups that continued CLA treated diet at both doses.	
	Female pups weaned after 12 days, then fed treatment for 10 weeks				

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**Table C.3.3-1 Summary of CLA Animal Feeding Studies on Milk Fat Deposition**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Rat (Sprague-Dawley)	13 weeks	50:50 c9, t11- and t10, c12-CLA	735	Significantly ↓ milk fat concentration, milk TG and significantly ↑ CLA isomers in milk. Significantly ↓ mRNA concentration of FAS and LPL, and the activity of FAS in mammary gland. Significantly ↓ number of pups per litter, pup weights and total litter weights; however experimental design may be bias since pups were not randomized and thus findings cannot be attributed solely to CLA-effects.	Ringseis <i>et al.</i> , 2004
Bovine (Holstein cows)	48 h	c9, t11-CLA	0.625 g/hr (abomasal infusion)	No significant changes in milk fat percentage and yield.	Loor and Herbein, 1998
		t10, c12-CLA		Significantly ↓ milk fat percentage and yield.	
Bovine (Holstein cows)	4 days	c9, t11-CLA	17 (abomasal infusion)	No significant changes in milk fat yield, percentage of milk fat, or milk yield. Significantly ↑ c9, t11-CLA in milk fat.	Baumgard <i>et al.</i> , 2000
		t10, c12-CLA		Significantly ↓ milk fat percentage and yield. No significant changes in milk yield. Significantly ↑ t10, c12-CLA in milk fat. Significantly ↓ milk FA C <sub>6,0</sub> to C <sub>16,1</sub> (except C <sub>15,0</sub> ) compared with c9, t11 isomer.	
Bovine (Friesian cows)	4 days	50:50 c9, t11- and t10, c12-CLA	38 (low), 76 (mid), 153 (high) (abomasal infusion)	Significantly and dose-dependently ↓ milk fat concentration and milk urea concentration. Significantly ↑ total CLA concentration and individual CLA isomer concentration in the milk of all dose groups.	Mackie <i>et al.</i> , 2003
Bovine (Holstein cows)	5 days	t10, c12-CLA	23 (abomasal infusion)	Significantly ↓ milk fat percentage and milk FA. No significant changes in milk fat yield. Significantly ↑ milk fat content of t10, c12-CLA. Significantly ↓ the rate of lipogenesis, mRNA abundance for the genes encoding ACC, FAS, SCD, LPL, FABP, GPAT and AGPAT.	Baumgard <i>et al.</i> , 2002

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**Table C.3.3-1 Summary of CLA Animal Feeding Studies on Milk Fat Deposition**

Strain	Duration	CLA Isomer	Dose (mg/kg bw/d) <sup>1</sup>	Effect <sup>2</sup>	Reference
Pig (Swiss Large White)	35 days	50:50 c9, t11- and t10, c12-CLA	360	Significantly ↑ piglet growth rates in animals nursed by mothers fed CLA compared with LA group. Significantly ↑ total saturated FA and significantly ↓ monosaturated FA in back fat tissue, omental fat and longissimus dorsi muscle in progeny of sows compared with LA group. No changes in mRNA abundance of FAS in adipose tissue of growing pigs.	Bee, 2000
Pig (sows)	40 or 75 days of gestation + 28 days lactation	50:50 c9, t11- and t10, c12-CLA	149	No significant changes in litter size and weight at birth; however, body weight of offspring significantly ↓ from day 7 to day 14. The decrease was not maintained post-weaning. No significant changes in relative organ weights of the offspring. Significantly ↓ total milk fat of sows.	Poulos <i>et al</i> , 2004

ACC = acetyl CoA carboxylase; AGPAT = acylglycerol phosphate acyltransferase, CLA = conjugated linoleic acid; FA = fatty acids; FABP = fatty acid binding protein, FAS = fatty acid synthase, GPAT = glycerol phosphate acyltransferase; LA = linoleic acid; LPL = lipoprotein lipase, NR = not reported; SCD = stearoyl CoA desaturase; TG = triglyceride

<sup>1</sup> CLA administered in the diet, unless otherwise noted.

<sup>2</sup> Treatment groups compared to respective control group, unless otherwise noted.

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## C.4 Peroxisomal Proliferation (PP)

Conjugated linoleic acid has been demonstrated to induce hepatic peroxisomal proliferation in experimental animals (Belury *et al.*, 1997; de Deckere *et al.*, 1999; Jones *et al.*, 1999; Moya-Camarena *et al.*, 1999). Peroxisomal proliferation may have toxicological significance because peroxisomal proliferators are considered non-genotoxic hepatocarcinogens in rodents (Roberts, 1999). Peroxisomal proliferation may not be relevant to the discussion of the potential effects of CLA (e.g., cardiovascular parameters, insulin sensitivity and glucose, milk fat deposition, and markers of oxidative stress), and is not discussed further in the discussion of clinical data (Section 7.5), it has been included in the sections below.

### C.4.1 *In Vitro* Studies

Xenobiotic-induced peroxisome proliferation is mediated by activation of PPAR- $\alpha$  (Lee *et al.*, 1995; Peters *et al.*, 1997). CLA has been shown to activate PPAR- $\alpha$  in *in vitro* trans-activation assays (Moya-Camarena *et al.*, 1999; Clément *et al.*, 2002). Moya-Camarena *et al.* (1999) reported that the c9,t11- isomer was the most effective isomer at inducing PPAR- $\alpha$  *in vitro*. Clément *et al.* (2002) reported that both t10,c12- and c9,t11- isomers activated PPAR- $\alpha$  *in vitro*.

### C.4.2 *Animal Studies*

#### Mouse

Belury *et al.* (1997) investigated the effect of CLA on several hepatic indicators of peroxisomal proliferation (*i.e.*, mRNA expression of acyl-CoA oxidase (ACO), cytochrome P4504A1 (CYP4A1), and liver fatty acid binding protein (FABP), as well as ornithine decarboxylase (ODC) activity) in female SENCAR mice. Dietary CLA induced accumulation of peroxisome-associated enzymes. Hepatic mRNA expression of ACO, CYP4A1 and FABP were dose dependently increased with the maximum occurring in mice fed 1.0% CLA. Although ACO, CYP4A1 and FABP mRNA levels were increased in the 1.5% CLA group compared to control, expression of these proteins was lower relative to the 1.0% CLA group. ODC activity was increased, relative to control, in mice fed 1.0% CLA (~700%) and 1.5% CLA (~800%) groups. However, this study has several limitations because only indirect measures of peroxisome proliferation were quantified, rather than evaluating peroxisome number by microscopy. Liver weights were not reported and histopathological analysis was not performed. It is unknown whether CLA increased hepatic lipid accumulation.

#### Rat

Jones *et al.* (1999) suggested that CLA does not induce peroxisome proliferation in the rat since no effect on PCoA oxidase activity, CAT activity or total cytochrome P450 content was reported. However the use of a CLA mixture with a low isomer content and lack of supporting data leaves

the possibility that the lack of an effect in this study could have been dose related (*i.e.*, the dose was insufficient to elicit a response).

Moya-Camarena *et al.* (1999) investigated the effect of CLA<sup>1</sup> on peroxisome proliferation in male and female Sprague-Dawley rats and found that in male rats fed CLA, increases in hepatic ACO and FABP mRNA are not a reliable indicator of peroxisome proliferation *in vivo*. However, hepatic FABP mRNA was unaffected in female rats fed 1,500 mg/kg CLA, and hepatic CYP4A1 was unaffected by CLA treatment in both male and female rats. Furthermore, histological examination of livers from male rats fed the highest dose of CLA (1,500 mg/kg), revealed no effect on the number of peroxisomes.

#### Hamster

Results from a study by de Deckere *et al.* (1999) indicate that CLA does not affect peroxisome proliferation indices in hamsters. The dose of CLA used in this study was high (6 g/kg/day), thus, the lack of response would not seem to be due to inappropriate dose selection.

#### Pig

Meadus (2003) reported that PPAR- $\alpha$  was not activated *in vivo* in CLA treated pigs.

#### C.4.3 Summary

Following critical evaluation of the available literature on species differences of peroxisomal proliferation, Roberts (1999) concluded that "...humans differ from rodents in their response to PPs and the weight of evidence supports the supposition that PPs do not pose a carcinogenic risk to humans."

#### C.5 Summary and Conclusions

Additional *in vitro* and animal studies have identified areas of further discussion in relation to the effects of CLA on cardiovascular disease; insulin sensitivity; maternal milk fat deposition and biomarkers of oxidation.

In terms of cardiovascular risk biomarkers it has been shown that CLA provides no increased risk. Numerous studies have shown inter-species variation in cardiovascular risk markers, demonstrating that caution must be exercised when attempting to extrapolate to humans. The mouse, in particular, is very sensitive to the effects of CLA and is apparently not able to cope with the changes in fat metabolism induced by large relative doses. There is no evidence that hepatic lipid accumulation due to supplementation with CLA observed in experimental mice is of

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<sup>1</sup> Isomers in administered CLA were t9,c11- and c9,t11- (43%), c10,t12- (45%), t9,t11- , t10,t12- , c9,c11- , c10,c12- (6%), linoleic acid (2%) and unidentified constituents (4%)

toxicological significance. Furthermore, the majority of data also demonstrate positive effects of CLA on inflammatory markers.

With regard to insulin resistance, in concurrence with the effects on adipose tissue, the mouse model was demonstrated to be the most sensitive species. Adipose tissue is almost completely ablated in the mouse following CLA intervention due to apoptosis resulting from decreased glucose uptake in the adipose tissue. Decreased glucose uptake is a result of inhibition of GLUT4 by predominantly the *t10,c12* CLA isomer at the nuclear regulatory level. Blood glucose is then shunted to the liver and induces hepatic lipogenesis in order to deal with the higher amount of glucose that is further transformed into serum triglycerides. These effects have been demonstrated to be transient (Wargent *et al.*, 2005).

Observations of reduced milk fat deposition in animal models, predominantly ruminants, are of minimal significance in relation to humans who rely to a lesser degree on *de-novo* fatty acid synthesis for milk fat secretion.

Attachment 1

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**Confidential Manufacturing Information**

**For Cognis GmbH**

**– See Confidential Information**

**Provided Separately –**

**000245**

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**ATTACHMENT 1**

**CONFIDENTIAL MANUFACTURING PROCESS FOR  
TONALIN® TG80**

***Prepared for:*** Cognis GmbH  
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40789 Monheim/Germany

***Prepared by:*** Cantox Health Sciences International  
2233 Argentia Road, Suite 308  
Mississauga, Ontario, Canada  
L5N 2X7

March 30, 2007

**000246**



**CONFIDENTIAL**







**CONFIDENTIAL**

**000252**



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Attachment 2

**Confidential Manufacturing Information**

**For Lipid Nutrition B.V.**

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**ATTACHMENT 2**  
**CONFIDENTIAL MANUFACTURING PROCESS FOR**  
**CLARINOL™ G-80**

***Prepared for:*** Lipid Nutrition  
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***Prepared by:*** Cantox Health Sciences International  
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Mississauga, Ontario, Canada  
L5N 2X7

March 30, 2007

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Attachment 3

## ATTACHMENT 3

### CONVENTIONAL CLINICAL REFERENCE RANGES

As discussed in the dossier entitled "GRAS Notification for Conjugated Linoleic Acid (CLA)-Rich Oil for Use in Certain Foods", a number of parameters evaluated in clinical studies on CLA were compared to clinically normal ranges as published by Tietz (1995). These values, excerpted from Tietz (1995), are presented in Table 1.

<b>TABLE 1 Conventional Clinical Reference Ranges with International Recommended Units for Safety Parameters Measured in Clinical Trials (Tietz, 1995)</b>		
<b>Endpoint</b>	<b>Range in USA</b>	<b>Alternate Unit Range</b>
C Reactive Protein	6.8-8.20 mg/dL (adult median=58mg/dL)	68-8200 µg/L (adult median=580 µg/L)
Cholesterol, total	30-34y M 138-254 mg/dL F 130-230 35-39y M 146-270 F 140-242 40-44y M 151-268 F 147-252 45-49y M 158-276 F 152-265  * <200 mg/dL Recommended 200-239 mg/dL Moderate Risk >240 mg/dL High Risk	30-34y M 3.57-6.58 mmol/L F 3.37-5.96 35-39y M 3.78-6.99 F 3.63-6.27 40-44y M 3.91-6.94 F 3.81-6.53 45-49y M 4.09-7.15 F 3.94-6.86
High-density Lipoprotein Cholesterol (HDL-C)	30-34y M 28-63 mg/dL F 36-77 35-39y M 29-62 F 34-82 40-44y M 27-67 F 34-88 45-49y M 30-64 F 34-87	30-34y M 0.72-1.63 mmol/L F 0.93-1.99 35-39y M 0.75-1.60 F 0.88-2.12 40-44y M 0.70-1.73 F 0.88-2.28 45-49y M 0.78-1.66 F 0.88-2.25
Low-density Lipoprotein Cholesterol (LDL-C)	30-34y M 78-185 mg/dL F 70-156 35-39y M 81-189 F 75-172 40-44y M 87-186 F 74-174 45-49y M 97-202 F 79-186	30-34y M 2.01-4.74 mmol/L F 1.79-4.00 35-39y M 2.08-4.85 F 1.92-4.41 40-44y M 2.23-4.77 F 1.90-4.46 45-49y M 2.49-5.18 F 1.95-4.77

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**TABLE 1 Conventional Clinical Reference Ranges with International Recommended Units for Safety Parameters Measured in Clinical Trials (Tietz, 1995)**

Endpoint	Range in USA	Alternate Unit Range
Serum Triglycerides	30-34y M 50-266 mg/dL F 39-150 35-39y M 54-321 F 40-176 40-44y M 55-320 F 45-191 45-49y M 58-327 F 46-214 *Range reflects 5-95 <sup>th</sup> percentile	30-34y M 0.56-3.01 mmol/L F 0.44-1.70 35-39y M 0.61-3.62 F 0.45-1.99 40-44y M 0.62-3.61 F 0.51-2.16 45-49y M 0.65-3.70 F 0.52-2.42 *Range reflects 5-95 <sup>th</sup> percentile
Fasting Serum Insulin	<17 µU/mL	<118 pmol/L
Alkaline Phosphatase	<u>Method dependent results</u> King-Armstrong Method: Adult 4.5-13 units/dL  Bowers McComb method: Male 25-100 U/L Female 25-100 U/L *conversion factor of x7.1	King-Armstrong Method Adult 32-92 U/L  Bowers McComb method: Male 0.43-1.70 Female 0.42-1.70
Aspartate Aminotransferase	<u>Method dependent results</u> Optimized Henry Method Adults 8-20 U/L  IFCC 37°C 24 mo-60y M 15-40 U/L F 13-35  SMAC 37°C M 25.6 +/- 19.5 (SD) F 20.4 +/- 7.8 (SD)	Optimized Henry Method Adults 0.14-0.34 µKat/L  IFCC 37°C 24 mo-60y M 0.26-0.68 F 0.22-0.60  SMAC 37°C M 0.44 +/- 0.33 (SD) F 0.35 +/- 0.13 (SD)
Alanine Aminotransferase	<u>Method dependent results</u> IFCC P-5'P, 37°C 12mo-60yM 10-40 U/L 12mo-60yF 7-35 U/L SMAC 37°C M 25.9 +/- 30.5 (SD) F 17.6 +/- 12.4 (SD)	IFCC P-5'P, 37°C 12mo-60yM 0.170-0.68 µKat/L 12mo-60yF 0.12-0.60 µKat/L SMAC 37°C M 0.44 +/- 0.52 (SD) F 0.30 +/- 0.21 (SD)
Hemoglobin, total	18-44y M 13.2-17.3 g/dL F 11.7-15.5 45-64y M 13.1-17.2 F 11.7-16.0 65-74y M 12.6-17.4 F 11.7-16.1	18-44y M 132-173 g/L F 117-155 45-64y M 131-172 F 117-160 65-74y M 126-174 F 117-161
Leukocyte Count (WBC count)	Adults. 4.5-11.0 x10 <sup>3</sup> cells/µl	Adults 4.5-11.0 x10 <sup>9</sup> cells/L
Erythrocyte Count	Number x 10 <sup>6</sup> cells/µl 18-44y M 4.3-5.7 F 3.8-5.1 45-64y M 4.2-5.6 F 3.8-5.3 65-74y M 3.8-5.8 F 3.8-5.2	Number x 10 <sup>12</sup> cells/L 18-44y M 4.3-5.7 F 3.8-5.1 45-64y M 4.2-5.6 F 3.8-5.3 65-74y M 3.8-5.8 F 3.8-5.2

**TABLE 1 Conventional Clinical Reference Ranges with International Recommended Units for Safety Parameters Measured in Clinical Trials (Tietz, 1995)**

Endpoint	Range in USA	Alternate Unit Range
Monocytes	Adults 0.22-0.95 x10 <sup>3</sup> cells/ $\mu$ L	Adults 0.22-0.95 x10 <sup>9</sup> cells/L
Neutrophils	<u>Segmented</u> Adults 1.5-6.7 x10 <sup>3</sup> cells/ $\mu$ L	<u>Segmented</u> Adults 1.5-6.7 x10 <sup>9</sup> cells/L
Basophils	Adults 0.0-0.15 x10 <sup>3</sup> cells/ $\mu$ L	Adults 0.0-0.15 x10 <sup>9</sup> cells/L
Platelet Count (Thrombocyte Count)	Adults 150-400 xE3/ $\mu$ l	Adults 150-400 xE9/L
Hematocrit	% Packed Ercs Volume 18-44y M 39-49 F 35-45 45-64y M 39-50 F 35-47 65-74y M 37-51 F 35-47	Volume Fraction 18-44y M 0.39-0.49 F 0.35-0.45 45-64y M 0.39-0.50 F 0.35-0.47 65-74y M 0.37-0.51 F 0.35-0.47
Lipoprotein (a)	Caucasians(5-95percentile) M 2.2-49.4 mg/dL F 2.1-57.3  Afro-americans M 4.6-71.8 F 4.4-75.0	Caucasians(5-95percentile) M 0.022-0.494 g/L F 0.021-0.573  Afro-americans M 0.046-0.718 F 0.044-0.750
IGF-1	Adults 135-449 ng/mL	Adults 135-449 $\mu$ g/L
Sodium, serum or plasma	Adults 136-185 mEq/L	Adults 132-146 mmol/L
Chloride, eerum	Adults 98-107 mEq/L	Adults 98-107 mmol/L
Potassium, serum	Adults 3.5-5.1 mEq/L	Adults 3.5-5.1 mmol/L
Bilirubin	Adults<60y 0.3-1.2 mg/dL	Adults<60y 5-21 $\mu$ mol/L
Creatinine, serum	Adult M 0.62-1.10 mg/dL F 0.45-0.75	Adult M 55-96 $\mu$ mol/L F 40-66
Creatinine, urine	Adult M 14-26 mg/dL F 11-20	Adult M 124-230 $\mu$ mol/L F 97-177
$\gamma$ -glutamyl transferase ( $\gamma$ GT)	American Monitor Parallel 16-60y M 7-47 U/L F 4-25  RIA 2.03-5.52 $\mu$ g/mL	American Monitor Parallel 16-60y M 0.12-0.80 $\mu$ Kat/L F 0.07-0.43  RIA 2.03-5.52 mg/L
Cortisol	Adult fluxes during day 0800h 5-23 $\mu$ g/dL 1600h 3-16 2000h (<50% 0800h)	Adult fluxes during day 0800h. 138-635 nmol/L 1600h 83-441 2000h (<50% 0800h)

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**TABLE 1 Conventional Clinical Reference Ranges with International Recommended Units for Safety Parameters Measured in Clinical Trials (Tietz, 1995)**

Endpoint	Range in USA	Alternate Unit Range
Lipase, serum (Triacylglycerol hydrolase)	Turbidim Method Adults 13-141 U/L >60y 0-320  Optimized Turbidim (BMD) Adults 20-60y 31-186 U/L >90y 26-267	Turbidim Method Adults 0.22-2.40 Kat/L >60y 0-5.13  Optimized Turbidim (BMD) Adults 20-60y 0.53-3.16 $\mu$ Kat/L >90y 0.44-4.54
Bilirubin, total	5d-60y 0.3-1.2 mg/dL 60-90y 0.2-1.1 >90 0.2-0.9	5d-60y 5-21 $\mu$ mol/L 60-90y 3-19 >90 3-15
Apolipoprotein A-I (ApoA-I)	20-29y M 81-153 mg/dL F 80-184 30-39y M 79-155 F 83-187 40-49y M 100-140 F 93-181 50-59y M 81-169 F 76-204 60-65y M 86-166 F 122-214	20-29y M 0.81-1.53 g/L F 0.80-1.84 30-39y M 0.79-1.55 F 0.83-1.87 40-49y M 1.00-1.40 F 0.93-1.81 50-59y M 0.81-1.69 F 0.76-2.04 60-65y M 0.86-1.66 F 1.22-2.14
Apolipoprotein B (ApoB)	Adults M 46-174 mg/dL +/-2SD F 46-142	Adults M 0.46-1.74 g/L +/-2SD F 0.46-1.42

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**SUBMISSION END**

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