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NOTICE OF ENTITLEMENT

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27181 Euclid Avenue, Cleveland, Ohio, 44132, United States of America
being authorised by the Applicant(s)/Nominated Person(s) in respect of an application entitled:

FOOD AND PHARMACEUTICAL COMPOSITIONS CONTAINING SHORT CHAIN
MONOUNSATURATED FATTY ACIDS AND METHODS OF USING

state the following:-

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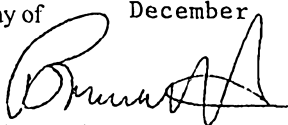
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DATED this 7th

day of December 19 92



(Signature)

Bruce Mavec , General Partner
(Name & Title)

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- (57) This invention encompasses compositions which are formulated to contain elevated levels of the MUFAs selected from the group composed of palmitoleic (hexadecenoic) acid (C16:1n-7) and its positional isomers C16:1n-6, C16:1n-5, C16:1n-4, and C16:1n-3, myristoleic (tetradecenoic) acid (C14:1n-5) and its positional isomers C14:1n-4 and C14:1n-3, and lauroleic (dodecenoic) acid (C12:1n-3), or their mixtures, whether as the free acids, salts, or esters thereof. Hereinafter, the above MUFAs are sometimes referred to as "short chain MUFAs" and the compositions in which they are contained are referred to as "DBD" compositions. The DBD compositions may comprise prepared foods, non-naturally occurring food components, food additives, or pharmaceuticals. The levels of short chain MUFAs in such compositions of matter are sufficiently high to produce beneficial improvements in the metabolic processing of lipids or glucose in animals to which these compositions of matter are regularly administered.

CLAIM

1. A formulated composition for administration to a human or an animal, said composition comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts

.../2

or esters of said monounsaturated fatty acids, and mixtures thereof, in a capsule for oral administration, in a prepared food or in a non-naturally occurring edible oil, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal upon systematic administration, together with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.

6. A method of improving the metabolic processing of lipids in a human or animal requiring said improvement, which method comprises increasing the plasma HDL/LDL cholesterol ratio of said human or animal compared to the HDL/LDL cholesterol ratio obtained when a composition containing a C12, C14, C16 or C18 saturated fatty acid is administered to a human or an animal.

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(54) Title: FOOD AND PHARMACEUTICAL COMPOSITIONS CONTAINING SHORT CHAIN MONOUNSATURAT- ED FATTY ACIDS AND METHODS OF USING (57) Abstract Food and pharmaceutical compositions are disclosed which contain amounts of short chain monounsaturated fatty acids or their derivatives sufficient to increase the content of the fatty acids within the tissues when said compositions are administered and to substantially improve the metabolic processing of lipids within animals.		

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FOOD AND PHARMACEUTICAL COMPOSITIONS CONTAINING
SHORT CHAIN MONOUNSATURATED FATTY ACIDS AND
METHODS OF USING

Field of the Invention

The present invention pertains to food and pharmaceutical compositions which contain amounts of short chain monounsaturated fatty acids or their derivatives sufficient to increase the content of such fatty acids within the tissues of the organism to which the said compositions are administered.

Background of the Invention

The chemical identity and quantity of fatty acids present in the diet of humans and other animals are known to have profound health consequences. Although popular attention to the harmful effects of dietary lipids has focused mainly on cholesterol, the biochemical properties of the fatty acids and their triglycerides have great significance in the development of degenerative conditions such as atherosclerosis and heart disease. A brief review of lipid uptake and processing is helpful in understanding the interconnections among dietary fatty acid composition,

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liver function, lipid metabolism and the development of atherosclerosis.

A. Biochemistry of Lipoproteins

The liver has a central role in the storage, synthesis, and metabolic transformations of lipids. One major function of the liver is to package triglycerides and cholesterol, which are insoluble in plasma, into particles called lipoproteins which can be carried in the bloodstream. The liver both secretes lipoproteins and also reabsorbs them after they have exchanged their lipid loads with peripheral tissues.

Four major classes of lipoproteins are known. All have an "oildrop" core of neutral lipid (triglyceride and/or cholesteryl esters) surrounded by an amphiphilic surface layer of phospholipids, cholesterol, and apolipoproteins. The larger the "oildrop" core, the less dense is the lipoprotein particle. In decreasing order of size, the four classes are:

1.) Chylomicrons, which are secreted by the small intestine rather than the liver, and consist mostly of triglycerides absorbed from dietary fat;

2.) very low density lipoproteins (VLDLs), which are secreted by the liver and contain mostly triglycerides;

3.) low density lipoproteins (LDLs), which are generated in the liver from VLDL remnants, and

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contain mostly cholesteryl esters rather than triglycerides; and

4.) high density lipoproteins (HDLs), which are secreted by the liver as phospholipid-rich discoidal particles, but which develop a lipid core by scavenging cholesterol from peripheral tissues.

1. Chylomicrons

Chylomicrons persist in plasma for only a brief time after a fatty meal. They are metabolized in the capillary beds of muscle and adipose tissue by the enzyme lipoprotein lipase (LpL) which is bound to the surface of the endothelial cells lining the capillaries. LpL hydrolyzes the triglyceride core of the chylomicrons, releasing free fatty acids. Some of the fatty acids immediately enter the adjacent muscle and adipose cells, while others are carried in the plasma bound to the circulating protein albumin. A large fraction (approximately one third) of the albumin-bound free fatty acids are taken up by the liver, about another third by skeletal muscles, and the remainder by other tissues, especially the myocardium. See Schiff, Diseases of the Liver, Ch. 22, "Fatty Liver," p. 824. In consequence, the fatty acids accumulated by heart, liver, and adipose tissue at least partially reflect the composition of dietary fats.

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The chylomicron remnants left after lipolysis of the triglyceride core retain cholesteryl esters derived from dietary cholesterol. The chylomicron remnants are removed from the circulation by the liver, which thereby becomes a repository for dietarily derived cholesterol and esters thereof.

2. Very Low Density Lipoproteins

The liver cells, which have accumulated the fatty acids liberated from the diet by chylomicron lipolysis, resecret these fatty acids in the form of triglycerides within VLDLs. Almost all the triglycerides found in the bloodstream more than a few hours after eating are present in VLDLs; hence a measurement of fasting plasma triglyceride concentrations is an indirect measurement of VLDL as well. As secreted by the liver, VLDLs are composed mostly of triglycerides. As is observed for chylomicrons, the triglycerides of VLDLs are lipolyzed by LpL, producing much smaller particles referred to as VLDL remnants. The intermediate density particles and VLDL remnants, produced by partial or extensive lipolysis of VLDLs, can accept cholesteryl esters from HDLs. Therefore over time these lipolyzed VLDLs become enriched in cholesterol. The liver removes some of the VLDL remnants from circulation, while others are converted by the liver into LDLs.

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3. Low Density Lipoproteins and Atherosclerosis

The resulting LDLs differ from their VLDL precursors in two important respects: first, they are much smaller particles; and secondly, they contain almost exclusively cholesteryl esters rather than triglycerides. These differences make LDL the major source of the esterified cholesterol which is a prominent component of atherosclerotic plaques formed within the arterial walls. The LDL particles are small enough to pass between the endothelial cells lining the arteries and they thereby may penetrate into the arterial wall. LDLs appear to bind to the cells and connective tissues of the artery wall at specific susceptible sites. Frequently these bound LDL are engulfed by immune system scavenger cells called macrophages. Macrophages engorged with cholesteryl ester-laden LDL particles ("foam cells") constitute a key intermediate in atherosclerotic plaque formation. Although smooth muscle cells begin to proliferate around such a lesion site, and connective tissue is often elaborated within it, the cholesterol deposit with its associated foam cells forms the central core of the developing plaque.

In addition, atheromas may become calcified. The calcium deposits are in the form of "insoluble" calcium salts of fatty acids, similar to the soap scum which is produced by the reaction between "hard"

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(Ca⁺²-rich) water and fatty acid anions. The fatty acid components are predominantly the high-melting, long chain saturated palmitate (C16:0) and stearate (C18:0). Polyunsaturated fatty acid components may be present as well. These are capable of undergoing cross-linking reactions to produce large, covalently bound complexes.

Atherosclerosis weakens the arterial wall and narrows the flow path of blood within the vessels. Atherosclerotic lesions frequently appear in particular in the coronary arteries, producing coronary heart disease. As the plaque increases in size, the coronary arteries may become completely blocked; when that occurs, the heart muscles are deprived of oxygen from the blood and the victim suffers a "heart attack", or myocardial infarction.

The risk of coronary heart disease increases dramatically as the plasma concentration of LDL cholesterol increases. Consequently, development of methods for lowering LDL cholesterol levels has become a major focus of medical research. The straightforward approach of reducing dietary cholesterol intake suffers from two limitations. The first is that cholesterol is present in all animal fats, and many Americans are unwilling to sacrifice their preferred diet. The second is that the liver and other tissues

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synthesize cholesterol de novo if the dietary supply is inadequate.

Cholesterol is an essential component of cellular membranes as well as a necessary precursor of metabolically important compounds such as bile acids and steroids. Cells obtain their necessary complement of cholesterol by taking up LDL particles through a specialized LDL receptor. The activity of the LDL receptors varies according to the cells' need for more cholesterol. Both peripheral cells and liver cells take up LDL through the receptor mechanism. However, unlike other cells, liver cells can both secrete and metabolically transform cholesterol, thereby removing it from the body. Thus when LDL receptor activity is low, the plasma LDL cholesterol level may be expected to rise, because LDL particles are not being removed from circulation as quickly as they are produced from VLDL remnants. This effect is accentuated by the fact that the liver removes VLDL remnants from circulation via the same LDL receptor; when LDL receptor activity is low, a smaller fraction of VLDL remnants is degraded, and consequently more remnants are converted into LDL particles instead. LDL receptor down-regulation thus decreases LDL clearance at the same time that the rate of LDL particle generation is increased. The result of this dual mechanism is that

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cholesterol levels climb markedly when LDL receptor activity decreases.

4. High Density Lipoproteins and Atherosclerosis

5 Whereas LDLs are a source of supply for cholesterol and have harmful atherogenic effects, HDLs scavenge excess cholesterol from peripheral tissues and protect against atherosclerosis. A high plasma HDL level is a negative risk factor for coronary heart
10 disease, and is therefore considered highly beneficial. The liver secretes nascent HDL in the form of flat discoidal particles in which the major lipid class is phospholipids such as phosphatidylcholine. These phospholipids are composed of two fatty acid
15 glyceryl esters, with a phosphate ester in the third position on the glycerol backbone. Excess cholesterol present in other cells is transferred to HDL. Over time an HDL particle develops a core of cholesteryl esters and assumes a more spherical shape. The
20 accumulation of cholesteryl esters within HDL requires the assistance of the plasma enzyme lecithin: cholesterol acyl transferase (LCAT), which esterifies cholesterol scavenged from peripheral cells. HDLs eventually transfer their cholesteryl ester
25 accumulation to intermediate density particles or VLDL remnants produced by lipolysis of VLDL. As indicated earlier, the cholesteryl ester enriched VLDL remnants

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are either taken up by liver cells or converted into LDLs. If taken up by liver cells, the VLDL cholesterol may be metabolically transformed into bile acids or secreted directly into bile. If incorporated into
5 LDLs, this cholesterol will become available for reuse by peripheral cells. Thus the HDLs complete the cycling of cholesterol between liver and peripheral cells, and contribute to the elimination of cholesterol from the body via secretion into the bile.

10 Another possible benefit from HDL may be the removal of cholesterol from pre-existing atheromas, thereby shrinking or reversing atherosclerotic plaques. This effect has not been directly demonstrated, but indirect evidence of atheroma reversibility in the presence of elevated HDL levels supports
15 this conclusion. In any case, elevated HDL levels have been shown clinically to protect against coronary heart disease and other atherosclerosis related diseases. See, e.g., Cotran, Kumar and Robbins,
20 Robbins Pathological Basis of Disease (4th ed. 1989).

B. Lipid Metabolism and Pathology

As the preceding discussion indicates, the liver plays a central role in integrating dietary lipid consumption with circulating lipid levels and
25 metabolic needs for lipid. Excessive dietary lipid and caloric intake can cause abnormal and possibly pre-pathological accumulation of triglyceride in the

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liver. The rate of liver triglyceride accumulation is affected by the plasma levels of insulin and glucose as well as of free fatty acid components; in turn, fatty acid utilization by the liver affects both
5 glucose and insulin levels. Obesity, with its concomitant high rate of release of free fatty acids into the circulation, is a major predisposing factor in the development of insulin-resistant (type II or "adult-onset") diabetes. These relationships between high
10 plasma fatty acid levels and fat accumulation in the liver, and between lipid utilization and incipient diabetes are highly relevant to the present discoveries.

Increased levels of free fatty acids in
15 serum reflect active lipolysis, either within adipocytes to reduce peripheral fat deposits, or of plasma VLDL and chylomicrons by LpL. High dietary fat consumption naturally leads to an elevated rate of lipolysis of the chylomicrons generated from such
20 dietary fat. Large amounts of triglyceride stored in adipose tissue also result in an enhanced rate of lipolysis within adipocytes. The released free fatty acids are used as a substitute for glucose as an energy source, especially by liver cells. Consequently serum glucose levels become elevated, since
25 cells do not take up as much glucose. The pancreas then secretes more insulin in order to increase the

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rate of glucose uptake. After a prolonged period of exposure to excessive insulin secretion, cells develop the insulin resistance characteristic of type II diabetes. Circulating fatty acids thus provide a link
5 between obesity, dietary fat, and the onset of insulin resistant diabetes.

When dietary fat consumption and/or adipose mass is high, the influx of free fatty acids into the liver exceeds its capacity to either metabolize these
10 fats for energy or to secrete them as new VLDL. Consequently, triglycerides begin to accumulate in vesicles within the hepatocytes. The numerous intracellular triglyceride vesicles eventually may coalesce to produce fat globules visible by light
15 microscopy. These large intracellular fat globules may produce hepatocyte injury or dysfunction and may even progress to macroscopically visible fatty streaks, a condition often referred to as "fatty liver." See Schiff, Diseases of the Liver, supra.

20 C. Dietary Lipid Structure and Pathology

Unsaturated fatty acids have a vinylic or carbon-carbon double bond at one or more positions along the acyl hydrocarbon chain. Hereinafter the structure of the fatty acids will be characterized by
25 notations such as Cx:yn-a. Cx indicates that the fatty acyl group contains x carbon atoms; y indicates the number of carbon-carbon double bonds in the acyl

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chain; and n-a indicates that the most distal double bond terminates on the "a"th carbon counting from the terminal methyl end. The naturally occurring fatty acids are almost exclusively in the cis configuration, and all further references to unsaturated fatty acids will indicate the cis isomer unless explicitly stated otherwise.

One established approach to reducing plasma cholesterol levels is to consume a large proportion of dietary triglycerides as polyunsaturated fatty acid (PUFA) derivatives. The most widely occurring dietary PUFA is linoleic acid (C18:2n-6, or 9,12-octadecadienoic acid), which constitutes more than half of the fatty acid triglycerides of corn, soy, and safflower vegetable oils. The cholesterol lowering ability of PUFAs is believed to result from increased LDL receptor activity. See Spady & Dietschy, 82 Proc. Nat. Acad. Sci. USA 4576 (1985). This well established lowering of plasma LDL cholesterol concentration when PUFAs are substituted for dietary saturated fatty acids provides the rationale for the widespread substitution of a variety of vegetable oils for animal fats in cooking and food formulations. The American Heart Association in its Phase I and Phase II Recommended Diets has approved the use of PUFAs as part of a large scale dietary modification for the purpose of lowering cholesterol levels in the general population.

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See, e.g., S.M. Grundy, Disorders of Lipids and Lipoproteins, in Internal Medicine, Stein, ed. 2035,2046 (2nd ed. 1987).

However, PUFAs have significant deleterious health consequences as well as beneficial ones. Several negative effects of PUFAs may be ascribed to their increased rate of reaction via free-radical mechanisms. See, e.g., B. Halliwell and J. Gutteridge, "Lipid Peroxidation," Ch. 4 in Free Radicals in Biology and Medicine, (2d ed. 1989).

PUFAs usually have two vinylic groups separated by a methylene carbon as is exemplified by the 9,12 diene structure of linoleic acid. The bridging methylene carbon (e.g., C11 of linoleic acid) is activated towards free-radical substitution reactions by both of the adjacent vinylic groups. Consequently this methylene carbon reacts 10 to 20 times more readily in free radical reactions than does a methylene adjacent to only a single vinyl group. Their susceptibility to peroxidation and cross-linking reactions implicates PUFAs in several undesirable processes such as tissue aging and tumorigenesis. PUFAs have been implicated in increasing the incidence of human bowel cancer, in suppression of the immune system, in increasing the risk of cholesterol gallstones, and in promoting the oxidation of LDL trapped within the arterial wall.

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Moreover, PUFAs lower the level of beneficial HDL cholesterol as well as the level of harmful LDL cholesterol. Since high HDL levels protect against atherosclerosis, the HDL-lowering effect of PUFAs could make them more rather than less atherogenic than saturated fatty acids.

Because of these drawbacks of PUFAs, some investigators have advocated the use of monounsaturated fatty acids (MUFAs). In particular, oleic acid (C18:1n-9) has been suggested as a nonatherogenic substitute for PUFAs. Oleic acid is a major component of olive oil, and some epidemiological evidence suggests that Mediterranean populations with high olive oil consumption have a reduced incidence of atherosclerosis and associated heart disease. Controlled human feeding studies, in which oleic acid was compared versus linoleic acid or saturated fats, reveal that oleic acid lowers serum LDL cholesterol about as well as does linoleic acid. However, unlike linoleic acid, oleic acid caused no reduction in HDL cholesterol levels. Based on this reported HDL sparing property of oleic acid, some researchers have urged that oleic acid should become a major source of dietary fat, whereas linoleic acid should be restricted to modest intakes. See S.M. Grundy, "Monounsaturated Fatty Acids and Cholesterol Metabolism:

Implications for Dietary Recommendations," 119 J. Nutrition 529-533 (1989).

Medicinal properties also have been asserted for a particular type of C16:1 MUFA. Iwamura, et al. in U.S. Patent 4,239,756 have disclosed the use of a compound which is a positional and geometric isomer of palmitoleic acid in a method for treating diabetes or improving lipid metabolism. The method involves oral or parenteral administration of alpha, beta-unsaturated fatty acids of the structure $H_3C-(CH_2)_n-CH=CH-COOH$, where $n=10, 12, 14$, or 16 . The fatty acids disclosed are therefore C14:1n-12, C16:1n-14, C18:1n-16, and C20:1n-18.

Summary of the Invention

10 According to a first embodiment of the invention there is provided a formulated composition for administration to a human or an animal, said composition comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, in a capsule for oral administration, in 15 a prepared food or in a non-naturally occurring edible oil, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal upon systematic administration, together with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.

20 According to a second embodiment of the invention there is provided a formulated composition for administration to a human or an animal comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acids, and 25 mixtures thereof, wherein said monounsaturated fatty acids constitute at least about 1.2 percent by weight of the fatty acids present in said formulated composition, and the ratio by weight of the content of at least one of the said monounsaturated fatty acids to the combined content of all C20:x and C22:y fatty acids, where $x = 0$ to 5 and $y = 0$ to 6 , is at least about 1:1, together with a pharmaceutically or veterinarily acceptable carrier, 30 diluent, excipient and/or adjuvant.

According to a third embodiment of the invention there is provided a formulated composition for administration to a human or an animal comprising at least one lipid species containing the short chain monounsaturated fatty acid C16:1n-7, or salts or esters thereof, wherein said C16:1n-7 constitutes at least about 5 percent by weight of the fatty acids present in said formulated composition and wherein the ratio by weight of said C16:1n-7 content to the combined content of all C20:x and C22:y fatty acids, where $x = 0$ to 5 and $y = 0$ to 6 , is at least about 8:1, together with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.

According to a fourth embodiment of the invention there is provided a formulated composition for administration to a human or an animal, said composition comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal upon systematic administration, together with a pharmaceutically or veterinarily acceptable carrier, diluent excipient and/or adjuvant.

According to a fifth embodiment of the invention there is provided a method of improving the metabolic processing of lipids in a human or animal requiring said improvement, which method comprises increasing the plasma HDL/LDL cholesterol ratio of said human or animal compared to the HDL/LDL cholesterol ratio obtained when a composition containing a C18 unsaturated fatty acid is administered to said human or an animal.

According to a sixth embodiment of the invention there is provided a method of improving the metabolic processing of lipids in a human or animal requiring said improvement, which method comprises increasing the plasma HDL/LDL cholesterol ratio of said human or animal compared to the HDL/LDL cholesterol ratio obtained when a composition containing a C12, C14, C16 or C18 saturated fatty acid is administered to a human or an animal.

According to a seventh embodiment of the invention there is provided a method of improving the metabolic processing of lipids in a human or an animal comprising systematically administering to a human or an animal a composition comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal.

According to the eighth embodiment of the invention there is provided a method of improving the metabolic processing of lipids in a human or an animal comprising formulating a composition for administration to said human or animal comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acid, and mixtures thereof, wherein said monounsaturated fatty acids constitute at least about 1.2 percent by weight of the fatty acids present in said formulated composition, and the ratio by weight of the content of at least one of the said monounsaturated fatty acids to the combined content of all C20:x and C22:y fatty acids, where x = 0 to 5 and y = 0 to 6, is

at least about 1:1, and systematically administering said composition to said human or animal to improve the metabolic processing of lipids within said human or animal.

According to a ninth embodiment of the invention there is provided a method of improving the metabolic processing of lipids in a human or an animal comprising
5 formulating a composition for administration to said human or animal comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal upon
10 systematic administration; and systematically administering said composition to said human or animal to improve the metabolic processing of lipids within said human or animal.

This invention encompasses compositions which are formulated to contain elevated levels of the MUFAs selected from the group composed of palmitoleic (hexadecenoic)
15 acid (C16:1n-7) and its positional isomers C16:1n-6, C16:1n-5, C16:1n-4, and C16:1n-3, myristoleic (tetradecenoic) acid (C14:1n-5) and its positional isomers C14:1n-4 and C14:1n-3, and lauroleic (dodecenoic) acid (C12:1n-3), or their mixtures, whether as the free acids, salts, or esters thereof. Hereinafter, the above MUFAs are sometimes referred to as "short chain MUFAs" and the compositions in which they are contained are referred
20 to as "DBD" compositions. The DBD compositions may comprise



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prepared foods, non-naturally occurring food components, food additives, or pharmaceuticals. The levels of snort chain MUFAs in such compositions of matter are sufficiently high to produce beneficial improvements in the metabolic processing of lipids or glucose in animals to which these compositions of matter are regularly administered.

Beneficial improvements in the metabolic processing of lipids which are achieved by this invention are evidenced by different effects in various tissues. Generally, the metabolic processing of lipids may include any or all steps in the metabolic pathways: these include in part lipid uptake from dietary sources, hydrolysis, esterification of fatty acids to produce other lipid species, packaging of lipids into lipoproteins, lipid transport, lipid storage in tissues, lipid or lipoprotein cellular uptake, lipid synthesis, enzymatic modification and catabolism, and pathological lipid deposition in arteries, liver or other sites.

In the liver, the inventive compositions have been found to prevent or lessen fatty deposits. Such deposits occur in animals fed a high fat or high carbohydrate (which includes high alcohol) diet, and also in animals exposed to liver toxins such as halogenated hydrocarbons. In the experiments supporting the present invention, fatty deposits were

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evident upon microscopic examination of liver tissue from animals administered high fat olive oil or saturated fat dietary compositions, but surprisingly were absent in animals administered an equally high fat dietary composition containing elevated amounts of short chain MUFA triglycerides.

In the heart, the inventive compositions have been found to increase the level of palmitoleic acid or its esters, and unexpectedly to lower the level of saturated fatty acids or their esters within the heart tissue. As a previous study has demonstrated (63 Amer. J. Cardiology 269, 1989, see infra), increased palmitoleic acid and lowered saturated fatty acid levels correlate with a protective effect against ventricular arrhythmias. Previous studies have not demonstrated how the said levels of palmitoleic and saturated fatty acids within heart tissue may be favorably manipulated, however. The present invention achieves both improvements by administering a composition containing elevated amounts of short chain MUFA triglycerides.

In the blood, the inventive compositions have been found to lower the plasma LDL concentration, compared to the plasma LDL concentration obtained when the short chain MUFAs are replaced in the diet either with saturated fatty acids or with unsaturated fatty acids which are not short chain MUFAs. The inventive

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compositions also elevate the concentration of plasma HDL compared to the plasma HDL concentration obtained when the short chain MUFAs are replaced in the diet with saturated fatty acids. Lowering the level of plasma LDL and increasing the level of HDL within the circulation, and increasing the HDL/LDL ratio are beneficial in preventing atherosclerosis and coronary heart disease.

In adipose tissue, the inventive compositions have been found to lower the triglyceride content per unit weight of adipose tissue. This necessarily means that other tissue components, such as proteins, carbohydrates, nucleic acids and cellular water which are associated with cellular metabolic functions, are increased; and that the proportion of each adipocyte which is occupied by triglyceride is lowered. The decreased intracellular triglyceride deposits indicate that triglyceride is hydrolyzed more readily from adipose tissue when animals are administered a dietary composition containing elevated amounts of short chain MUFA triglycerides.

Beneficial improvements in the metabolic processing of glucose are achieved by this invention by lowering serum glucose concentrations which are abnormally elevated in insulin-resistant animals. Insulin-resistant elevated glucose concentrations occur in animals which are obese and have an excessive

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caloric intake. In humans, this condition is known as type II diabetes. In the experiments supporting the present invention, we have demonstrated that serum glucose levels can be normalized even in obese animals having excessive caloric intake, by administering a dietary composition containing elevated amounts of short chain MUFA triglycerides.

The compositions of the present invention are "formulated" in the sense that the fatty acid content of the food or pharmaceutical composition is manipulated or adjusted to provide a sufficient amount of the short chain MUFAs. A "sufficient amount" of the short chain MUFAs in any given composition is determined in relation to the total amount of the said short chain MUFAs required for regular administration in order to produce the particularly desired beneficial improvement.

This invention contemplates that the said formulated compositions should be regularly or systematically administered in order to produce beneficial improvement. The stated short chain MUFAs are incorporated into the structural lipids of the animal to which they are administered. If the short chain MUFAs are administered in low amounts or at long intervals, they will not become a significant proportion of the fatty acids present in these structural lipids. In

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consequence the beneficial improvements cannot be achieved.

Detailed Description of the Invention

A. Theory of the Invention

5 While we do not wish to be bound by theory, we believe that a brief presentation of the hypotheses which led us to perform these investigations will be helpful in understanding the empirical results which we have obtained.

10 We have hypothesized that the melting characteristics of fatty acids and their derivatives are crucial in determining whether they will be atherogenic or nonatherogenic. Solid-to-fluid phase transition temperatures are an indicator of the
15 viscosity properties of the fatty acyl chains. The atherogenic saturated fats, such as palmitic (C16:0) and stearic (C18:0) triglycerides, are solids at both room (about 25°C) and physiological (about 37°C) temperatures. Triglycerides having the same length
20 acyl chains but with one or more unsaturated (vinyllic) bonds are liquids at such temperatures. Naturally occurring triglycerides are mixtures of various lipid species and contain esters of several fatty acids which may differ in both acyl chain length and degree
25 of saturation. The relative proportions of high and low melting fatty acyl groups determine the melting behavior of the mixture. For example, lard (from

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pigs), tallow (from cattle) and mutton tallow (from sheep) are solid fats at room temperature, and between 40 and 50% of their acyl groups are saturated C16:0 and C18:0. The MUFA oleic acid (C18:1) constitutes about 40 to 50% of their acyl content and much of the remainder is the PUFA linoleic acid (C18:2). By contrast, most vegetable oils, which are liquids at room temperature, have only 10-20% of palmitic and stearic acid esters, with the remainder mostly unsaturated oleic and linoleic acid esters. The lower atherogenicity of these vegetable oils thus roughly correlates with their low melting temperatures. Unfortunately, whereas polyunsaturation permits vegetable oils to achieve greater fluidity, lower melting temperatures, and lower atherogenicity, it also makes them susceptible to undesirable cross-linking and peroxidation reactions causing the formation of insidious polymers.

The rank order of melting temperatures of triglycerides in general is reflected in the melting temperatures of the corresponding phospholipids, free fatty acids and acid salts derived from them. The melting temperature of the free fatty acids liberated from a triglyceride source by hydrolysis is frequently used to characterize the triglyceride and is known as the "titer." Titer decreases as the fatty acid structure goes from saturated to unsaturated, and also

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as the acyl chain length becomes shorter. The position of the vinylic bond is also an important determinant of the melting temperature of unsaturated fatty acids: titer decreases as the position of the double bond moves away from the carboxyl end of the molecule.

Similarly, the melting behavior of phospholipids reflects the melting trends of their constituent fatty acids. For example, the solid to fluid phase transition temperature of the physiologically important phospholipid dipalmitoylphosphatidylcholine (DPPC) is about 41.5°C, meaning that bilayer membranes made solely from DPPC are in the solid phase at physiological temperature. When the central acyl group is replaced with oleic acid (C18:1) to make palmitoyloleoylphosphatidylcholine (POPC), the phase transition temperature is lowered to near 0°C, meaning that a POPC membrane is very fluid at 37°C. Thus incorporation of an unsaturated fatty acid into a phospholipid produces a dramatic lowering of the rheological characteristics of the resulting membrane bilayers.

Although scientifically based claims of health benefits derived from dietary MUFAs previously have been asserted only for oleic acid, other mono-unsaturated fatty acids also occur naturally. The commonest are 11-eicosenoic acid (C20:1n-9) and 13-docosenoic acid (C22:1n-9), both of which are found

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in high levels in some oilseed plants such as jojoba and rapeseed. The shorter chain MUFA 9-palmitoleic acid (C16:1n-7) occurs as a minor component (ca. 2%) in olive and cottonseed oils and in trace amounts in a few other commercially available vegetable oils.

Palmitoleic acid occurs in somewhat higher amounts in animal fat triglycerides such as lard and tallow (up to 5%) and in still higher levels in some fish oils such as sardine oil. The next lower homologue, myristoleic (9-tetradecenoic) acid (C14:1n-5), occurs in minor amounts in animal fat and in butter. The even lower homologue, lauroleic (9-dodecenoic) acid (C12:1n-3), occurs rarely and in small amounts in natural sources.

Although the short chain MUFAs palmitoleic, tetradecenoic and dodecenoic acids have been known for many years, they have not been suggested as useful compounds for dietary modification. The advocates of oleic acid as a dietary replacement for PUFAs and saturated fats have not provided similar teachings for the utility of the shorter chain homologues such as palmitoleic acid. Little or no significance has been attributed by the medical or biochemical community to the presence of palmitoleic and myristoleic acids as important constituents of animal lipids.

One recent study did observe an unexpected negative correlation between palmitoleic acid concen-

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trations in the adipose tissue of patients who had suffered a recent myocardial infarction and their incidence of subsequent serious cardiac ventricular arrhythmias. Abraham, Riemersme, Wood, Efton and
5 Oliver, "Adipose Fatty Acid Composition and the Risk of Serious Ventricular Arrhythmias in Acute Myocardial Infarction," 63 Amer. J. Cardiology 269 (1989). These investigators had hypothesized that high tissue levels of PUFAs, and in particular linoleic acid, would
10 protect heart attack victims from developing life-threatening arrhythmias in their damaged hearts. Previous studies had shown that adipose tissue fatty acid composition is similar to that of heart muscle, and the investigators therefore analyzed the fatty
15 acid composition of biopsied adipose tissue from myocardial infarct patients as an indicator of the lipid composition of their heart muscle. These investigators did not find the predicted correlation between tissue linoleic acid levels and protection
20 against subsequent arrhythmias. However, they did observe that the patients who did not develop arrhythmias had significantly lower levels of saturated fatty acids and significantly higher levels of palmitoleic acid, compared with the arrhythmic group. The
25 authors suggested that "saturated fatty acids in cardiac membranes may lead to greater vulnerability to ventricular arrhythmias," but did not draw the corre-

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sponding inference that palmitoleic acid in cardiac membranes may be protective.

Based on our hypothesis concerning the beneficial role of lower melting and more fluid fatty acids, we believe that the correlation between high
5 palmitoleic acid levels and protection against arrhythmias is but one example of the general health benefits conferred by short chain MUFAs. The investigators who published the correlation failed to
10 appreciate that short chain MUFAs may be beneficial, apparently because the scientific literature does not teach this. Their failure to assert that high palmitoleic acid levels might be the cause of the observed cardiac protection is therefore quite compre-
15 hensible in view of the prevailing scientific neglect of short chain MUFAs.

Our hypothesis, that the beneficial effects of dietary PUFAs and MUFAs on health may be characterized by and understood in terms of the lower
20 melting temperatures, lower viscosities, and greater fluidity of the lipids which incorporate such fatty acids, has led us to postulate that the heretofore overlooked short chain MUFAs may confer health benefits comparable to or greater than those already
25 demonstrated for oleic acid (C18:1). As used herein, the term "short chain MUFAs" refers to monounsaturated fatty acids which are shorter than oleic acid

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(C18:1n-9), which is well known to provide health benefits. In particular, we have postulated that the short chain MUFAs palmitoleic (hexadecenoic) acid (C16:1n-7), myristoleic (tetradecenoic) acid (C14:1n-5), and lauroleic (dodecenoic) acid (C12:1n-3), and lipids incorporating them may be beneficial to health when administered as foods or pharmaceuticals. These C16:1n-7, C14:1n-5, and C12:1n-3 acids and their esters are even lower melting and less viscous than oleic acid due to their shorter chain length and favorably positioned unsaturated bonds. Moreover, we propose that positional isomers of these short chain MUFAs in which the vinylic bond is located even farther away from the carboxyl group will have similar or possibly greater utility. This postulate follows from the fact that monounsaturated fatty acid lipids melt at lower temperatures as the double bond is positioned more remotely from the carboxyl group. Thus C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-4, and C14:1n-3 are all predicted by our postulate to confer health benefits. In addition, all of the aforementioned C16:1, C14:1 and C12:1 fatty acids share with oleic acid the highly beneficial greater stability of monounsaturated compared to polyunsaturated alkyl structures.

The greater fluidity of the above short chain MUFAs may be expected to be advantageous in

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several ways. For one, the lipases which hydrolyze triglycerides to yield free fatty acids are known to require fluid rather than solid triglycerides as substrates. The greater fluidity of the short chain MUFAs and their triglycerides may permit lipoprotein lipase to hydrolyze chylomicrons and VLDL more efficiently when they are enriched in C16:1, C14:1, and C12:1. This might directly lower VLDL levels and ultimately lower LDL cholesterol levels. In addition, fatty acids might be released more readily from adipose tissue due to the enhanced activity of lipases on highly fluid C16:1, C14:1 and C12:1 triglycerides, thereby helping to reduce these fat deposits.

The rate of formation of cholesteryl esters by the enzyme LCAT is known to increase when the fatty acid being esterified is more fluid. See Emken, Biochemistry of Unsaturated Fatty Acid Isomers, 60 J. Amer. Oil Chem. Soc. 995, 1001 (1983) (cis-C18:1n-9 is esterified more rapidly than the higher melting trans-C18:1n-9, cis-C18:1n-6, and cis-C18:1n-5 isomers). This esterification step is rate-limiting in the transfer of cholesterol from peripheral cells to HDL and thence to VLDL remnants. Therefore high LCAT activity is important to the HDL-mediated removal of cholesterol from peripheral tissue. The even lower melting C16:1, C14:1, and C12:1 fatty acids may be expected in light of this invention to promote this

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activity and therefore contribute to the protective effect of HDL. The HDL cholesterol concentration in plasma therefore is also expected to increase due to administration of these short chain MUFAs.

5 Triglyceride contained within intracellular vesicles might also be processed and transported more readily when lower viscosity C16:1, C14:1, and C12:1 MUFAs comprise a significant fraction of the lipids. This might affect the rate of VLDL formation within
10 liver cells, and therefore lessen fatty accumulation and deposits within the liver.

Membranes containing elevated proportions of C16:1, C14:1, and C12:1 phospholipids would be more fluid than normal membranes composed of more saturated
15 and longer acyl chain fatty acid esters. This might necessitate greater use of cholesterol by cells, since one functional role of cholesterol when incorporated into phospholipid bilayers is to rigidify excessively fluid membranes. Increased cellular uptake of LDL to
20 satisfy the cells' enhanced cholesterol requirement in membrane formation would be expected to lower LDL cholesterol levels in light of the teachings herein.

The C16:1n-(7, 6, 5, 4, or 3) and C14:1n-(5, 4, or 3) compounds of the present invention also are
25 very different from the alpha, beta-unsaturated fatty acids, C16:1n-14 and C14:1n-12, disclosed by the Iwamura patent. The alpha, beta-unsaturated fatty

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acids have chemical properties significantly different from those of the monounsaturated fatty acids of the present invention, because the double bond in the former compounds is conjugated with the carbonyl group. This double bond is therefore highly reactive toward both electrophilic and nucleophilic addition reactions, in contrast to the relatively much more stable isolated double bond of the compounds disclosed herein. The alpha, beta-unsaturated fatty acids are in fact transiently occurring intermediates in biological oxidative degradation of fatty acids via the "beta-oxidation" pathway. Thus upon administration they probably would be preferentially metabolized rather than incorporated into tissue. Also, the Iwamura patent does not specify the geometry of the double bond. However, the claimed compounds are isolated from mollusks. The naturally occurring alpha,beta-unsaturated fatty acids produced by the beta-oxidation pathway are trans rather than cis, suggesting that the Iwamura compounds are trans. The fatty acids isolated by Iwamura are also stated to be identical with synthetically produced material; consistent with this interpretation, the alpha, beta-unsaturated fatty acids produced by the usual organic syntheses are predominantly in the trans-configuration. By contrast, the MUFAs which are the subject matter of the present invention are in the cis

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configuration. Finally, alpha, beta-unsaturated fatty acids are high melting compounds both as free acids and as esters. This contrasts with the low melting properties of the unconjugated cis-MUFAs of this invention, in which at least seven saturated carbons intervene between the carbonyl and the cis-double bond. We have found that this marked difference in physical properties has great physiological significance, as the preceding discussion of the role of low melting fatty acids would indicate. The Iwamura patent also does not disclose a preferential benefit from the shorter chain C14:1n-12 and C16:1n-14 compounds in comparison with the longer chain C18:1n-16 and C20:1n-18 members of the series. By contrast, the present invention is directed exclusively to the lower melting short chain MUFAs.

B. Experimental

Based on these anticipated benefits, we undertook feeding experiments to demonstrate that animals incorporate high dietary levels of the short chain MUFA palmitoleic acid into their tissue lipids. The lipids of liver, plasma, heart and adipose tissue from rats fed diets enriched in palmitoleic acid triglycerides were analyzed to provide evidence that these tissue contain elevated levels of palmitoleic acid, compared to tissues from rats fed control diets. Standard clinical chemistry tests were performed to

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indicate the metabolic state of the rats fed the palmitoleic acid enriched versus the control diets. Finally, histologic analyses of liver tissues from control and palmitoleate fed rats were performed to assess the health of this central lipid processing organ.

1. Preparation of a Formulated Composition Containing Short Chain MUFAs

Although the present invention encompasses pharmaceutical compositions as well as dietary compositions for animal experiments, we chose to formulate a prepared food containing elevated amounts of the short chain MUFA palmitoleic acid (C16:1n-7). A feeding study has an advantage compared to a study requiring administration of a pharmaceutical, in that the experimental animals will self-administer the food composition. We chose to limit the study to the single short chain MUFA palmitoleic acid, rather than including groups fed the congeneric myristoleic (tetradecenoic) (C14:1n-5) and lauroleic (dodecenoic) (C12:1n-3) acids, to increase the likelihood of obtaining significant results while using a minimal number of experimental animals.

As the preceding discussion of theory indicates, a beneficial effect on lipid metabolism should be expressed when the proportion of short chain MUFAs incorporated into tissue lipids becomes large enough to affect the physiochemical properties, such

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as fluidity and melting temperature, of the tissue lipids. Therefore both the total amount of short chain MUFAs and their relative proportion in the diet are expected to be significant. In the present case,
5 palmitoleic acid constituted 21.8 mol % of the fatty acids present in the prepared food given to the experimental group of rats.

This molar percentage of palmitoleic acid is quite high compared to the amounts present in most
10 natural fats and oils: 13% for sardine oil, 5% for tallow, 3% for lard, 5% for butter, 0.5-2% for cottonseed and olive oils, and only trace amounts in almost all other commercially significant vegetable oils.
See "Composition and Constants of Natural Fats and
15 Oils" (Sherex).

The proportion of fat in the formulated diet was also relatively high: 48.4% of total calories came from fat. This is slightly higher than but comparable to the fat content of the typical American
20 diet, which derives 40-45% of its calories from fat.
See Guyton, Textbook of Medical Physiology (4th ed. 1971) at 802. Some representative foods and their percent calories from fat are listed in Table I
(calculated from data in J. Weihrauch, "Provisional
25 Table of the Fatty Acid and Cholesterol Content of Selected Foods," USDA Human Nutrition Information Services, 1984). Clearly a number of common dietary

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items contain about 48% or more of their calories as fat. Thus the diet administered to rats in the study is not so laden with fat that it is outside the range of what a great many Americans normally consume.

5

TABLE I

	<u>Food Items</u>	<u>% Calories From Fat</u>
	Peanut Butter	77%
	Ground Beef, cooked-well done	58%
	Bacon, fried crisp	77%
10	Chicken, dark meat, fried	54%
	Frankfurter, beef	83%
	Potatoes, french-fried	47%
	Pound Cake	55%
	Veal Cutlet	46%
15	Doughnut, yeast	58%
	Egg, hard boiled	65%
	Cheese, cheddar	73%
	Milk, whole (3.3% fat)	48%

The high fat levels of this experimental diet were formulated to insure that the experimental animals consumed a large quantity of palmitoleic acid. Naturally, in a prepared food formulated for nonexperimental purposes, and especially for human consumption, the proportion of total fat and also the molar percentage of palmitoleic acid may be considerably lower, consistent with this invention.

Macadamia nuts were chosen as a raw material from which to isolate palmitoleic acid triglycerides for this formulated dietary composition. Unlike any other commercial crop, macadamia nuts contain high levels of palmitoleic acid, which comprises in excess of 19% of the fatty acid composition of macadamia nut oil. In addition, the other fatty acids of macadamia

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nut oil are closely similar in both identity and quantity to those present in olive oil. This similarity was extremely fortuitous for implementing the rat feeding experiments, because olive oil provided an ideal positive control. Olive oil contains oleic acid as its major component and is already established as a beneficial dietary fat source. By comparing the palmitoleic acid enriched diet (hereinafter designated POL) against an oleic acid enriched diet (hereinafter designated OLO), we could conclude that any equivalent or superior beneficial effects on lipid metabolism indicate clear utility for palmitoleic acid.

As Table II indicates, the minor fatty acid components of OLO were quantitatively similar to those of POL. Moreover, the two provided similar total amounts of saturated FAs (12:0+14:0+16:0+18:0), of MUFAs (16:1+18:1) and of total combined unsaturated fatty acids (16:1+18:1+18:2+20:4). The striking difference between the olive oil and macadamia nut oil compositions was that the latter contained a much larger fraction of MUFA which was 16:1 rather than 18:1.

25

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TABLE II

Fatty Acid Composition (mol %)
Of POL, OLO and HF Diets

	<u>Fatty Acid</u>	<u>POL</u>	<u>OLO</u>	<u>HF</u>
5	12:0	0.24	0.00	57.83
	14:0	1.02	0.00	17.38
	16:0	11.58	12.40	8.08
	18:0	2.33	2.33	6.19
	16:1	21.76	0.74	0.00
10	18:1 (cis)	43.47	60.67	2.91
	18:1 (trans)	2.91	2.04	0.00
	18:2	15.42	21.83	7.61
	20:4	1.38	0.00	0.00
	cis-MUFAs	65.23	61.41	2.91
15	Total unsaturated FA	84.84	85.28	10.52
	Total saturated FA	15.17	14.73	89.48

Although macadamia nuts have been suggested as an oilseed crop (see Macfarlane and Harris, "Macadamia Nuts as an Edible Oil Source", Amer. Oil Chem. Soc. Monograph 1981, 103-108), we were unable to locate a commercial source of the oil. Consequently, we extracted the oil from ground macadamia nuts by solvent extraction with hexane, followed by solvent evaporation under reduced pressure.

Olive oil for formulating the oleic acid enriched comparative dietary composition was obtained from commercial sources. A second comparative dietary composition containing only saturated fatty acids was

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also developed. Coconut oil was completely hydrogenated to provide a totally saturated fat source. This was substituted for the macadamia nut or olive oils in the formulation recipe. The resulting formulated dietary composition differed substantially from the two other compositions in its quantitative fatty acid content as Table II clearly indicates. Fully three-fourths of the fatty acids present in the hydrogenated fat composition (hereinafter designated HF) are the short chain saturated lauric (dodecanoic) (C12:0) and myristic (tetradecanoic) (C14:0) acids. No MUFAs or PUFAs remained in the hydrogenated coconut oil; the small amounts present in the final HF composition were derived from added corn oil. This unusual fatty acid profile was chosen because the resulting tri-glycerides, even though they are fully saturated, are quite low melting due to the shortness of the acyl chains. Thus some beneficial effects on lipid metabolism may be produced by the HF diet despite its high saturation, as a consequence of the low melting short acyl chains. However, we anticipated that beneficial effects would not be as widespread or as pronounced as with the POL and OLO diets, in part because C12:0 and C14:0 are known to be subject to chain elongation reactions which can convert them to the higher melting and atherogenic C16:0 and C18:0 fatty acids.

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In formulating the dietary compositions for rat feeding studies, we combined 200g of macadamia nut oil, olive oil, or hydrogenated coconut oil with 50g of corn oil. The corn oil served as a source of the essential fatty acid linoleic acid (C18:2), which is present in low amounts in macadamia nut oil and olive oil. This oil mixture was blended with 250g of casein protein, 224.9g sucrose, 15g maltodextrin, 62.5g of cellulose fiber, 56.3g of mineral and vitamin mix, 3.8g of methionine, 2.5g of choline bitartrate, and 0.05g of ethoxyquin antioxidant. The remaining dietary components are standard in the AIN-76 diet widely used for rat maintenance feeding. The POL, OLO, and HF diets each contained 21.75% protein, 25.25% fat, and 38.63% carbohydrate, and provided 4.69kcal/g energy. As previously indicated, 48.4% of total calories in these diets came from fat, a high value but only slightly greater than the American dietary norm. Just under two-thirds of the fatty acids present in the macadamia and olive oil diets were monounsaturated (either C16:1n-7 or C18:1n-9).

2. Animal Feeding Study

The formulated dietary compositions described above were fed to three groups of 6 rats each for a period of 8 weeks. These formulated diets constituted the only food allowed the animals during this period. The rats were housed individually in

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wire-bottom cages. Food and water were available ad libitum.

Male Sprague-Dawley rats 4-5 weeks old and weighing initially about 105g were used for all three groups. The animals were weighed weekly. Their food intake was recorded three days per week.

After 8 weeks of feeding, the rats were sacrificed by decapitation. Blood was collected, heparinized, and centrifuged to harvest plasma, which was used for a variety of clinical tests. Liver, heart, and epididymal fat pads were examined in situ, removed, rinsed, and stored at -70°C until fatty acid analysis could be performed. A portion of fresh liver tissue was fixed for morphological examination to determine the general health of the organ and the extent of fatty deposition.

Fatty acid analyses were performed on samples of liver, fat pad, heart and plasma taken from each rat. The tissue samples were weighed and extracted using the Folch procedure (acidified chloroform/methanol). The organic layer was evaporated and the residue saponified in ethanolic KOH. Methyl derivatives of the fatty acids were formed. These were identified and quantitated by capillary gas chromatography using a flame ionization detector. Similar analyses were also performed on the formulated dietary compositions and the source oils.

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Plasma samples were further analyzed using standard clinical chemistry tests for triglycerides and total cholesterol, as rough indicators of VLDL and LDL levels; for plasma glucose as an indicator of diabetic or prediabetic conditions which might arise because of obesity and elevated plasma fatty acid levels; for uric acid, a degradation product of purine metabolism which is excreted in urine; for creatinine and blood urea nitrogen, terminal metabolites of proteins which are secreted continuously in urine and indicate renal function; for bilirubin, a breakdown product of hemoglobin which is removed from the blood by the liver, and which becomes elevated when liver function is impaired; for calcium ion, a regulator of membrane permeability and muscle contractility; for glutamate-pyruvate and glutamate-oxaloacetate transaminases, enzymes required for protein metabolism by liver cells and which are elevated in plasma when liver cell breakdown occurs; for alkaline phosphatase, an enzyme present in liver cells which is also elevated in plasma secondary to liver cell injury; for amylase, a carbohydrate digesting enzyme secreted by the pancreas and elevated in the blood when pancreatic cells are damaged; and for total protein, albumin, and albumin-to-globulin ratio, which are measures of protein secretion by the liver.

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All feeding, tissue examination, and chemical analysis procedures were performed under double blind conditions. At the conclusion of the experiments the identities of the three groups of rats were revealed to be as follows:

Group I - POL diet

Group II - HF diet

Group III - OLO diet

3. Results

The three groups were remarkably similar in the total weight of food consumed, the total body weight gain, and the ratio of weight gain to weight of food consumed (Table III).

TABLE III

Mean Food Consumption and Weight Gain of Rats

(data in grams)

	<u>Group</u>	<u>Day 0</u>	<u>Day 62</u>	<u>Weight Gain</u>	<u>Weight of Food Consumed</u>	<u>Ratio %</u>
20	POL	105	490	385	1018	37.7
	HF	105	476	371	1099	34.0
	OLO	104	497	393	1105	35.5

The average weight gain among the three groups differed by only 6%, while the weight of food consumed differed by about 7.9% between the highest and lowest groups. The percent ratio of weight gained to weight of food consumed is a rough indicator of the efficiency of utilization of the diet, and ranges from

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34.0% for the HF group to 37.7% for the POL group. The differences in weight gain among the three groups are insignificant, but the efficiency of utilization is slightly higher in the POL group than in the other two. Thus palmitoleic acid appears to be at least as nutritive as the fatty acids present in the two comparison groups in the study, and may be somewhat better absorbed.

The weight percent composition of fatty acids isolated from epididymal fat pad adipose tissue, liver, heart, and plasma are given in Table IV, along with the composition of the dietary fats for comparison.

TABLE IV

Mean Fatty Acid Content of Rat Tissues
And Corresponding Diet
(% by weight)

POL Group						
	<u>Fatty Acid</u>	<u>Diet</u>	<u>Fat Pad</u>	<u>Liver</u>	<u>Heart</u>	<u>Plasma (w/v)</u>
	C12:0	0.2	0.0	0.0	0.6	0.6
	C14:0	1.0	1.0	0.8	0.7	0.7
	C16:0	11.6	13.0	26.0	15.0	18.6
	C18:0	2.3	1.6	8.3	24.0	9.6
	C16:1	21.8	17.3	7.7	2.9	8.7
	C18:1	46.3	51.5	42.7	19.9	36.6
	C18:2	15.4	15.3	6.0	15.6	10.6
	C20:4	1.4	0.4	8.4	21.6	14.6

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HF Group

	C12:0	57.8	40.4	1.7	2.2	14.5
	C14:0	17.4	14.5	5.6	3.1	8.6
	C16:0	8.1	12.9	32.0	16.9	21.2
5	C18:0	6.2	2.8	13.8	26.9	13.1
	C16:1	0.0	3.0	3.7	0.7	2.4
	C18:1	2.9	13.0	19.4	8.4	12.4
	C18:2	7.6	13.3	14.0	25.2	16.1
	C20:4	0.0	0.0	9.9	16.6	11.8

10

OLO Group

	C12:0	0.0	0.0	0.0	0.3	0.4
	C14:0	0.0	0.7	0.7	0.5	0.6
	C16:0	12.4	14.5	27.7	16.9	21.2
	C18:0	2.3	1.5	8.8	25.6	9.6
15	C16:1	0.7	2.2	1.9	0.5	1.9
	C18:1	62.7	61.6	45.3	19.3	42.3
	C18:2	21.8	19.5	7.4	15.5	11.4
	C20:4	0.0	0.0	8.1	21.4	12.8

Several interesting features may be discerned from these data. First, the animals in all three dietary groups either synthesized or selectively concentrated C16:0 and C18:0, presumably in their livers, and incorporated those saturated fatty acids into the liver, heart and plasma in proportions higher than those of the diets. The POL diet animals incorporated lower proportions of C16:0 into these three tissues than did animals in the other diet groups, although

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the POL diet is intermediate in C16:0 content. The POL animals also incorporated less C18:0 into heart tissue than did the other groups, although the levels of C18:0 in liver and plasma were equal in the POL and OLO diet groups. The HF diet contained high levels of C14:0, which was nearly absent in the other two diets. The HF group animals incorporated C14:0 into all tissues at levels lower than those in the diet, with the lowest level obtained in the heart at 3.1%. The POL and OLO animals incorporated C14:0 into all tissues at levels of 1% or less. A similar pattern occurred with C12:0. These shorter chain saturated fatty acids did not accumulate in those tissues, perhaps because they were preferentially metabolized for energy or were converted into longer chain fatty acids.

The concentrations of fatty acids per gram of tissue for animals from the three dietary groups are given in Table V.

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TABLE VConcentrations of Fatty Acids in Rat Tissues
(ug/g)

POL Group					
	<u>Fatty Acid</u>	<u>Fat Pad</u>	<u>Heart</u>	<u>Liver</u>	<u>Plasma (w/v)</u>
5	C12:0	0.0	59.6	0.0	16.8
	C14:0	7.2	69.8	356.4	20.0
	C16:0	90.4	1615.7	11040.8	542.6
10	C18:0	10.9	2580.7	3537.8	280.5
	C16:1	120.3	313.5	3261.5	259.1
	C18:1	359.0	2142.9	18119.3	1068.9
	C18:2	106.2	1687.6	2558.7	308.5
	C20:4	2.9	2329.9	3566.5	426.9
15	Total	696.8	10799.6	42441.0	2918.3
	Saturated	108.5	4325.8	14935.0	859.9
	Monoun- saturated	479.3	2456.4	21380.8	1323.0
HF Group					
20	C12:0	384.1	254.6	563.8	488.1
	C14:0	138.2	359.6	1847.9	291.4
	C16:0	123.0	1965.3	10624.2	717.4
	C18:0	26.3	3129.4	4577.4	441.1
	C16:1	28.0	77.4	1224.0	81.3
25	C18:1	123.8	974.0	6457.3	418.8
	C18:2	126.9	2930.4	4657.8	543.5
	C20:4	0.4	1932.8	3285.3	396.9
	Total	951.0	11623.4	33237.7	3378.4
	Saturated	671.9	5708.9	17613.3	1938.0

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	Monoun- saturated	151.8	1051.4	7681.3	500.1
	OL Group				
	C12:0	0.0	35.9	0.0	14.9
5	C14:0	4.0	57.9	318.1	20.0
	C16:0	95.8	1871.2	11838.4	755.7
	C18:0	10.0	2834.3	3774.6	341.5
	C16:1	14.7	50.7	808.8	66.6
	C18:1	408.2	2136.2	19351.0	1510.1
10	C18:2	128.9	1718.8	3153.5	406.4
	C20:4	0.0	2372.4	3452.2	457.3
	Total	662.1	11077.3	42696.5	3572.4
	Saturated	109.8	4799.3	15931.1	1132.1
15	Monoun- saturated	422.9	2186.9	20159.8	1576.7

One unanticipated beneficial effect which is apparent from this pattern of fatty acid distribution is that the POL group animals have lower total levels of saturated fatty acids in their heart tissue (4.33 mg/g) than do either the OLO (4.80 mg/g) or HF (5.71 mg/g) groups. While this effect is only mildly surprising when the POL group is compared to the highly saturated HF group, it is quite surprising when POL is compared to the OLO group. Apparently heart tissue adjusts the relative concentrations of various fatty acids in a highly selective manner, perhaps to maintain a preferential level of membrane fluidity. Thus although the POL diet actually contained slightly

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greater total levels of saturated fatty acids than did the OLO diet, the resulting POL heart tissue unexpectedly contained lower total levels of saturated fatty acids. The magnitude of this lowering apparently is sufficient to confer cardiac protection. In the report published in Amer. J. Cardiology, supra, the arrhythmia-free group had 4.4% lower total saturated fatty acid content than the arrhythmic group. In our feeding study, the hearts of POL group rats contained total saturated fatty acids 9.8% lower than the OLO group and 24.2% lower than the HF group (saturated fatty acid).

The increase in palmitoleic acid levels in all tissues, including heart, due to the administration of palmitoleic acid enriched triglycerides is dramatic. These values are included in Tables IV and V. The fractional C16:1 content of heart tissue from POL rats (2.9%) increased nearly 600% compared to OLO rats (0.5%) and more than 400% compared to HF rats (0.7%). By comparison, in the Amer. J. Cardiology study the increase in C16:1 content in nonarrhythmic patients compared to arrhythmic patients was only about 11%. Therefore the observed increase in C16:1 content due to the POL diet is clearly of a magnitude sufficient to produce beneficial effects on the heart.

In all tissues examined, including liver, plasma, and adipose tissue as well as heart, the

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content of C16:1n-7 was much greater for animals fed the POL diet than in those fed either the OLO or HF diets. However, the relative proportions of the fatty acids differed among tissues. Selective incorporation of C16:1 clearly occurred. For each diet group, the fatty acid composition of the epididymal fat pad adipose tissue was more closely similar to that of the diet than was any other tissue examined. This finding is consistent with the fact that chylomicrons derived from dietary fat are a chief source of the fatty acids incorporated into adipose tissue. The POL group fat pad was greatly enriched in C16:1 compared to the other dietary groups.

Interestingly, the HF diet rats had a small percentage of C16:1 in their fat pads and other tissues even though the diet provided none. The OLO diet rats also had a greater proportion of C16:1 in the fat pads than was present in the diet. These findings indicate that the animals synthesize their own C16:1 when dietary sources are inadequate. In addition, they may selectively sequester C16:1 in tissues as needed. The average content of C16:1 found in a particular tissue when animals have been fed for several weeks on a diet essentially devoid of C16:1 therefore may be used to define a mean basal level of C16:1 in such tissue for animals of that species. As is apparent from Table IV, the mean basal level of

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C16:1 varies among tissues and certainly is expected to vary according to the species of animal analyzed.

In addition to changes in the proportions of fatty acids present in adipose tissue, the various diets caused changes in the total weight of fatty acid triglycerides present per unit weight of adipose tissue. The POL group rats averaged 697 mg total fatty acids/g fat pad tissue; the OLO group rats, 662 mg/g; and the HF group rats, 951 mg/g. Thus both monounsaturated diets produced lower triglyceride content per unit weight than did the saturated fat diet. This result is consistent with the fact that lipases require fluid triglyceride substrates: the saturated HF triglycerides would be hydrolyzed more slowly than the lower melting monounsaturate enriched triglycerides produced by the POL and OLO diets. These results indicate that a diet containing elevated amounts of short chain MUFAs is useful for reducing the triglyceride content of adipose tissue fatty deposits. This property of short chain MUFAs is expected to be beneficial in promoting fat mobilization to reduce adipose tissue deposits, when accompanied by restricted caloric intake.

In the liver, the POL group had more than twice as much C16:1 as did the HF group and four times as much as did the OLO group on a percent by weight basis. Both the POL and OLO groups accumulated more

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total fatty acids per unit weight of liver than did the HF group: 42.4 mg/g tissue for POL, 42.7 mg/g tissue for OLO, and only 33.2 mg/g tissue for HF. The lower fat content of HF livers suggested that these
5 livers might be healthier than the fattier livers of the POL and OLO diet groups. However, analyses for plasma levels of liver-related enzymes and metabolites, reported in Table VI, did not support this inference.

TABLE VI

Plasma Levels of Enzymes, Proteins and Metabolites
Indicative of Liver and Kidney Function

		<u>GPT</u>	<u>GOT</u>	<u>APH</u>	<u>TPR</u>	<u>ALB</u>	<u>A/G</u>	<u>BIL</u>	<u>URI</u>	<u>CRT</u>	<u>BUN</u>
		(unit/L)			(g/dL)			(mg/dL)			
5											
	POL	49	221	374	5.9	3.3	1.25	0.3	1.8	0.4	15
	HF	48	200	335	6.0	3.3	1.20	0.3	1.4	0.4	16
	OL0	53	195	340	6.1	3.5	1.35	0.2	1.5	0.4	16
	Normal Rat										
10	Upper	107	192	525	7.1	4.5	1.30	0.6	3.7	0.4	28
	Lower	21	64	100	5.5	3.1	0.7	0.1	1.1	0.1	9
	Mean	64	128	313	6.3	3.8	1.0	0.4	2.4	0.3	14

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These results indicate that levels of significant liver enzymes which may become elevated in plasma secondary to liver cell injury were not uniformly raised, as would be expected if cellular damage were responsible. For example, levels of glutamate-pyruvate transaminase (GPT) were low normal in all three groups compared to the historical norms for male rats in the clinical analyzer employed. This lowered level may reflect the fact that pyruvate is produced by glycolysis, and is therefore elevated when glucose is the primary nutrient being metabolized for energy. Conversely, pyruvate is present in lesser concentrations when lipids are the predominant energy source. GPT therefore is not needed as much when lipids are being metabolized rather than glucose. This rationale also may explain the observed elevation in the plasma levels of the liver enzyme glutamate-oxaloacetate transaminase (GOT). GOT levels were slightly above the normal range for all three groups, with the POL rats measuring about 10% higher than the OLO and HF rats. Along with other Krebs cycle intermediates, oxaloacetate concentrations are elevated when lipids are metabolized for energy, because lipid oxidation produces large amounts of acetyl Co-A which then enters the Krebs cycle. Since this acetyl Co-A load causes elevated oxaloacetate levels, the synthesis of GOT should be induced as well. The slightly

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higher GOT level among POL group rats therefore may reflect a higher rate of metabolism of lipids for energy in that group. A third plasma enzyme which may be spilled from the liver is alkaline phosphatase (APH). APH levels are slightly above the mean but well within the normal range for all three groups. Together, these plasma enzyme levels do not indicate that liver cells were pathologically stressed in any of the diet groups. They do suggest that the liver cells were metabolizing a greater amount of lipid rather than glucose for energy and that the POL group animals were somewhat more active in lipid metabolism than the other groups.

Another indicator of the functional health of the liver is the plasma protein concentration, and especially the albumin concentration. Synthesis and secretion of these plasma proteins is a major function of the liver. Both total protein (TPR) and albumin (ALB) values were within normal ranges for all three groups, indicating adequate liver function. The albumin-to-globulin (A/G) ratio was high normal in the POL and HF groups, and slightly above normal in the OLO group. Since albumin concentrations were low normal for all groups, this suggests that globulin levels also must have been low normal for POL and HF groups, and possibly below normal for the OLO group.

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Finally, plasma bilirubin (BIL) levels were normal in all groups, indicating no biliary obstruction or severe liver disease.

Liver tissue samples from all eighteen rats were examined under the microscope. Mediastinal tissues including liver were fixed in paraffin, sectioned, and stained with hematoxylin and eosin. Histological examination of the thoracic tissue revealed striking differences among the groups. Despite the high fat intake and measured fatty acid content of the POL group rats, their livers evidenced no lipid accumulation within the hepatocytes. The hepatocyte density, or number of cells per high-powered field of view (HPF) was normal, indicating compact and healthy cells. In addition, the adjacent extrahepatic mediastinal tissues showed no evidence of fat deposits. All six POL group animals exhibited these characteristics. These observations led the pathologist conducting the histological examination under double blind conditions to label the POL animals a "lean" group.

By contrast, the mediastinal tissues of HF diet animals exhibited numerous hepatocytic foci of high fat accumulation. Many fat cells were swollen with intracellular triglyceride deposits. In consequence, cell density (hepatocytes per HPF) was decreased compared to the POL group animals. Also,

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the extrahepatic mediastinal tissues showed some increased lipid accumulation compared to the POL group. The pathologist labelled the HF animals as a "moderate fat gain" group.

5 The tissues of the OLO group animals exhibited even higher lipid accumulation within hepatocytes and the beginning of frank cellular fatty changes. Fat accumulation was diffuse rather than focal as in the HF group. Hepatocyte density was low. In addition,
10 the extrahepatic mediastinal structures showed the greatest increase in accumulated lipids. The pathologist characterized the OLO animals as a "heavy fat gain" group.

 Together, these dramatic differences indicate
15 an important and unanticipated benefit from the presence of short chain MUFAs in the diet: they can prevent or reduce the development of fatty liver in animals exposed to conditions which promote fatty liver. In the present study, the high fat and excessive
20 calorie diet itself was the predisposing condition. Other known predisposing factors include exposure to chlorinated solvents or similar toxic chemicals, heavy alcohol ingestion, viral hepatitis, or exposure to certain metabolic inhibitors (e.g.,
25 orotic acid) which interfere with the hepatocytic intracellular processing and secretion of VLDL. All of these agents produce fatty liver by causing the

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rate of input of fatty acids into hepatocytes to exceed the rate of processing of fatty acids. Processing of fatty acids within the liver includes both energy-producing metabolism of fatty acids to acetyl

5 Co-A, and the synthesis of triglycerides and their secretion as VLDL. From the present data we cannot deduce whether the mechanism by which short chain MUFAs protect hepatocytes involves primarily improved fatty acid metabolism or enhanced VLDL secretion. One

10 mechanism underlying the protective effect of short chain MUFAs apparently is to lessen the tendency of accumulated triglycerides within vesicles to aggregate into large, visible deposits. The healthy livers of the POL group rats contained as much fatty acid per

15 gram of tissue as did the obviously fatty livers of the OLO rats. However, in the healthy POL animals the short chain MUFA C16:1 constituted a much larger fraction of the total fatty acid content. The more fluid lipids of the POL animals apparently remained in

20 small, physiologically functional vesicles, whereas the less fluid lipids of HF and OLO animals aggregated into large intracellular droplets. Since the dietarily derived short chain MUFA concentration in the liver increased sufficiently to alter the fluidity charac-

25 teristics of liver triglycerides in the protected animals, we anticipate that the beneficial effect of preventing or reducing the development of fatty liver

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will occur regardless of which specific short chain MUFA is employed.

In addition, the reduction in mediastinal fat deposits observed among POL diet animals is a significant beneficial effect. This may be related to the overall lowering of triglyceride content per unit weight of adipose tissue, as was observed in the epididymal fat pads and discussed previously. Mediastinal fat deposits reflect the occurrence of small patches of adipocytes throughout the thoracic and abdominal cavities. When these adipocytes become laden with triglycerides, the cells expand individually to accommodate the triglyceride deposit. Eventually the adipocyte patch swells sufficiently to become noticeable upon histological examination. Conversely, when triglyceride content is low, the adipocytes shrink individually and the adipocyte patch becomes inconspicuous. The fact that mediastinal fat deposits were not observed in the "lean" POL group rats necessarily indicates the low triglyceride content of the adipocytes. This effect therefore may be another manifestation of the previously discussed propensity of short chain MUFAs for reducing the triglyceride content per unit weight of adipose tissue. However, an additional implication from these shrunken mediastinal fat deposits is that the overall size of the adipose tissue had not increased due to adipocyte

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proliferation to compensate for the decreased tri-
glyceride concentration. Thus another unexpected
benefit of a diet containing high proportions of short
chain MUFAs is a reduction in the total size of fat
5 deposits.

A further area in which we have now demon-
strated beneficial effects involves the plasma lipo-
proteins. Results of analyses for plasma triglycer-
ides, total cholesterol, HDL cholesterol, residual
10 (VLDL + LDL) cholesterol, and HDL/LDL ratio are
presented in Table VII.

TABLE VII

Plasma Lipids

	Triglycerides (mg/dL)	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	VLDL +LDL Cholesterol (mg/dL)	HDL/LDL
5					
POL	240	83	65	18	3.9
HF	280	82	36	46	2.8
OLO	324	93	62	31	1.2
10 Normal Rat					
Upper	183	90			
Lower	17	60			
Mean	100	75			

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Several points stand out. First, the plasma tri-
glyceride levels are unusually elevated for all three
groups. This is not surprising given the high levels
of consumption of dietary fat. The triglyceride
5 content of plasma reflects mainly the contributions
from VLDL. The POL group had the lowest average
triglyceride levels, only slightly above the normal
range for rats. The OLO group had the highest levels,
nearly one-third higher than the POL group. The short
10 chain MUFA diet has an apparent advantage over both
the saturated fat and olive oil diets with respect to
plasma triglyceride levels.

The total cholesterol levels of all three
groups were somewhat above the mean but within the
15 normal range for rats. The three diets were derived
from vegetable oils and were essentially cholesterol
free, which may have helped to keep the plasma cho-
lesterol levels in check. Here again the POL diet
provided somewhat lower levels than did the OLO diet.
20 However, the real advantage of the POL diet becomes
apparent when HDL cholesterol levels are considered.
The POL diet animals had 5% greater mean HDL choles-
terol levels than did the OLO animals. This is quite
important, inasmuch as olive oil itself is reported to
25 spare HDL cholesterol levels. The POL diet also
produced much higher HDL cholesterol levels than did
the HF diet. Since HDLs protect against

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atherosclerosis, the high HDL levels engendered by the short chain MUFA diet are therefore, in light of this discovery, expected to be of great benefit for humans at risk for atherosclerotic vascular disease.

5 The fraction of total plasma cholesterol which is not in the HDL pool is necessarily divided between VLDLs and LDLs. When the total and HDL cholesterol results are combined, it is apparent that the POL diet also produced lower levels of (VLDL +
10 LDL). Although the cholesterol contents of VLDL and LDL pools were not determined directly, the tri-glyceride values indicate that the VLDL levels of the POL group animals are about 25% lower than those of the OLO group. By contrast, the residual (VLDL + LDL)
15 cholesterol levels of the POL group are 44% lower than those of the OLO group. Thus the lower level of VLDL observed for the POL diet cannot explain the entire difference in residual cholesterol values between the POL and OLO diets. Therefore the POL diet must have
20 produced lower LDL cholesterol levels as well. This interpretation is strongly bolstered by the fact that LDL cholesterol levels are normally much higher than VLDL cholesterol, and would be expected to represent most of the combined residual (VLDL & LDL) choles-
25 terol.

Furthermore, an independent lipoprotein determination confirmed the LDL cholesterol lowering

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effect of the short chain MUFA diet compared to either the saturated fat or olive oil diets. Plasma samples were subjected to electrophoresis on cellulose acetate strips, then stained with Fat Red 7B dye to visualize the lipoprotein bands. The strips were quantitated by optical scanner to give the relative percentages of each of the lipoproteins HDL, VLDL, and LDL. These percentages are based upon the amount of dye absorbed rather than upon the cholesterol content of the fractions; hence the numerical proportions of each lipoprotein component in a given sample based on absorbed dye content are quite different from the proportions calculated from cholesterol content. However, comparisons between samples are quite meaningful. The HDL/LDL ratio calculated by electrophoresis was 3.9 for the POL group, 2.8 for the HF group, and only 1.2 for the OLO group. This directly demonstrates that the HDL/LDL ratio was increased by the POL diet. In addition, these results indicate that LDL levels were decreased by the POL diet, since the increase in the HDL/LDL ratio is too large to be explained solely by the 5% increase in HDL cholesterol between the POL and OLO groups.

Yet another beneficial effect of the diet containing the DBD composition which has been demonstrated by these experiments is on plasma glucose levels. These are given in Table VIII.

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TABLE VIII

Plasma Glucose Concentration and Amylase Activity

	<u>Group</u>	<u>Glucose (mg/dL)</u>	<u>Amylase (units/L)</u>
	POL	138	279
5	HF	135	284
	OLO	150	332
	<u>Normal Rat</u>		
	Upper	197	410
	Lower	98	102
10	Mean	143	256

The plasma glucose concentrations of all three groups were within the normal range. However, the POL and HF groups gave values 5% and 3% respectively below the normal mean, whereas the OLO group gave values 5% higher than the normal mean. These differences are modest but statistically significant. Moreover, the increased glucose levels observed in the OLO group are ominous in light of the risk of type II diabetes among obese animals consuming a high fat diet. The fact that the plasma glucose levels of the OLO animals had begun to rise after only eight weeks on the high fat diet strongly indicates that they were becoming incipiently diabetic. This inference is supported by the elevation in plasma amylase concentration observed among the OLO animals. Amylase is a complex carbohydrate metabolizing enzyme normally secreted by the pancreas into the small intestine; increased amounts

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in plasma indicate stressing of or damage to pancreatic cells. The pancreas is also stressed by elevated plasma glucose levels, which require that organ to secrete greater amounts of insulin. The animals fed the OLO diet had a plasma amylase activity 30% higher than the normal means and 19% higher than the animals given the POL diet. These elevated plasma amylase concentrations suggest that the OLO animals experienced some pancreatic stress secondary to their prolonged increase in circulating glucose. The near normal plasma glucose and amylase levels observed for the POL animals demonstrate that they developed no pre-diabetic indicators despite their high fat diet and concomitant obesity. These surprising results support a role for administration of short chain MUFAs to control or prevent type II diabetes.

C. Preparation of oil enriched in short chain MUFAs

Although the oil extracted from macadamia nuts was convenient for animal studies because of its fortuitous similarity to the olive oil used as a control, a formulated oil produced by other means is desirable for many applications. We have produced such oils from animal fat sources by a combination of processing steps. The resulting DBD compositions are distinguishable from the naturally occurring precursors by having greater proportions of selected short

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chain MUFAs and lower proportions of long chain fatty acids.

Several animal fats contain short chain MUFAs in sufficiently high proportions to make them good starting materials for formulating DBD compositions. Chicken and turkey fats, beef tallow, and neatsfoot oil triglycerides contain C16:1n-7 in amounts of about 4-6% by weight. Some fish oils such as sardine and menhaden may contain as much as 10-16% C16:1n-7. Whale oil is reported to contain above 13% C16:1n-7, and the now unavailable sperm whale oil contained up to 26%. However, these fats and oils as rendered from the natural sources contain undesirably large relative proportions of the long chain fatty acids of the series C20:x and above. The more saturated and higher melting members C20:0, C20:1, C22:0, and C22:1 have been reported to contribute to the high atherogenicity of peanut oil, a phenomenon comprehensible in light of the teachings of this patent. See F. Manganaro, et al., 16 Lipids 508 (1981). The polyunsaturated and lower melting members C20:2, C20:3, C20:4, C20:5, C22:2, C22:3, C22:4, C22:5, and C22:6 are non-atherogenic or even cardioprotective, but are highly sensitive to free radical oxidation and cross linking reactions because of their polyunsaturation.

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The sole source of a dietary vegetable oil which contains appreciable amounts of C16:1n-7 is macadamia nuts. The two species, integrifolia and tetrafolia, contain C16:1n-7 in amounts ranging from 16 to 25% (w/w) of the fatty acids in the oil. However, both also contain about 4% C20 fatty acids.

Similarly, some natural fats and oils are acceptable starting materials from which to manufacture DBD, that is, an oil enriched in the other selected short chain MUFAs. For example, tallow contains about 0.5% C14:1n-5. It also contains about 1% or more C20 to C22 fatty acids. Butterfat contains very large proportions, up to 3%, of C14:1n-5. However, butterfat has other undesirable lipid components due to the large fraction of C4 to C10 fatty acids. These are metabolized by a quite different pathway from the C12 and longer fatty acids. Butterfat also contains greater than 2% C20 fatty acids. We have produced a DBD composition in which C14:1n-5 comprises greater than 1% of the fatty acids and the C4 to C10 and C20 to C22 fatty acids are present in much lower levels than in butter or tallow.

Example 1. Preparation of C16:1n-7 enriched oil

A mixture of lard and tallow triglycerides was used as starting material. The oil was chilled at 4°C for a period of about 14 days to accomplish crystallization of higher melting triglyceride

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components. The triglyceride mixture was then filtered at 4°C through a 200 mesh filter of cloth or paper to remove the solid fat crystals. The filtrate was then chilled to -9.5°C to induce further crystallization and was refiltered at that temperature. The resulting oil was then distilled at a reduced pressure of about 8 microns Hg and a temperature of about 222°C. The first approximately 2% of distillate was collected separately. The subsequently ensuing distillate was collected into 19 fractions of about 5% each. Over the course of distillation the pressure decreased to about 3 microns. The fatty acid profiles of selected fractions were determined by gas chromatographic analysis. These fatty acid compositions for the distilled triglycerides, for their cold treated filtrates, and for comparable starting material triglycerides are given in Table IX.

TABLE IX

Fatty Acid Compositions of Starting Materials, Cold Treatment Filtrate,
And Selected Fractions of Distilled Triglycerides
(% by weight)

5	Fatty Acid	Starting Material*		-9.5°C Filtrate	Distillate Fractions				
		Tallow	Lard		2	4	7	14	17
	C8:0				0.02				
	C10:0				0.02				
	C12:0	0.9	0.2	0.05	0.10	0.1	0.1	0.02	0.0
10	C14:0	3.7	1.3	2.7	4.3	3.8	3.2	1.9	1.3
	C16:0	24.9	23.8	18.0	20.2	20.2	18.4	16.3	13.7
	C18:0	18.9	13.5	3.8	3.2	3.3	3.7	3.9	4.5
	C14:1	1.0	--	0.7	1.2	1.0	1.3	0.3	0.3
	C16:1	4.2	2.7	12.9	15.9	13.9	14.3	11.0	8.9
15	C18:1	36.0	41.2	48.2	42.3	45.5	44.7	50.2	52.2
	C18:2	3.1	10.2	12.1	11.5	11.0	11.7	14.3	15.7
	C18:3	0.6	1.0	1.1	0.7	0.9	1.7	1.5	2.3
	C20 and above	~2.0	~2.0	~1.0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5

* Literature Values: See J. B. Reeves and J. L. Weihrauch, "Composition of Foods: Fats and Oils" Agriculture Handbook No. 8-4, U.S.D.A. (June, 1979 ed.); see also Sherex, "Composition and Constants of Natural Fats and Oils."

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These data indicate that the proportion of C16:1 was increased by as much as four to five fold compared to the starting tallow and lard triglycerides. At the same time, the proportion of C20 and C22 fatty acid containing triglycerides fell below 1%. The ratio of C16:1 to C20 and above fatty acids increased from about 2 for the starting material fats to as much as 31 for fraction 2. The proportion of C14:1 increased from no more than about 1% in the starting materials to 1.3% in fraction 7. The ratio of C14:1 to C20 and above fatty acids increased from about 1.7 in the lard and tallow starting material to as much as 2.6 in the distillate. The content of C8:0 + C10:0 was only about 0.04% in the distilled fractions, compared to about 4.5% in butterfat.

The distilled triglycerides contained low levels of free fatty acids and were suitable for dietary use. Inasmuch as the inventive composition, DBD, was derived from natural fat sources, a variety of chemically distinct lipid species were present in the distilled oil. For example, the C16:1n-7 fatty acid might form an ester with any of the three alcoholic hydroxyl groups of the glycerol backbone, and the other two positions might be occupied by any fatty acid moiety. The inventive composition is not limited to any particular triglyceride, nor is it confined only to triglyceride esters. Monoalcohol

esters such as ethyl esters are also suitable compounds for administration to animals. This invention requires only that the amount of short chain MUFA, regardless of the particular lipid species into which it is incorporated, be sufficiently great.

Example 2: The Effect of Dietary Supplementation with Macadamia Nuts on Serum Lipid Levels in Humans

Macadamia nuts are known to contain a high proportion of mono-unsaturated fatty acids. The nuts are approximately 75% oil by weight. A study was conducted to determine the effect of dietary supplementation with macadamia nuts on serum lipid values in humans.

There have been relatively few formal studies of the inclusion of significant amounts of macadamia nuts in the diet. The study reported here was utilized to examine palatability, acceptability and potential side effects, including weight gain, as well as to estimate the effect of regular macadamia nut ingestion on lipid levels in humans.

Methods

Healthy male or female subjects between the ages of 30 and 75 years and having a serum cholesterol of 200 mg/dl within the last year were considered eligible for the study. Participants were recruited from a variety of occupational settings including hospitals, university departments, state government offices and other miscellaneous sites through the use of flyers, posters and written announcements at worksites. All potential subjects were screened in person or by telephone for possible exclusions. Exclusions included history of allergy to macadamia nuts or any tree-grown nut, angina pectoris, peripheral vascular insufficiency, diabetes mellitus, gout, active peptic ulcer, liver or pancreatic disease, inflammatory bowel disease or other malabsorption syndrome, auto-immune disease such as rheumatoid arthritis or systemic lupus erythematosus, a seizure disorder, skin cancer, heart attack or coronary artery bypass surgery within the previous 6 months, previous stroke, transient ischemic attacks or surgery on the carotid arteries. In addition individuals taking prescription medication to lower cholesterol or triglycerides, antihypertensive medications, or estrogen preparations were excluded. Those individuals thought to have qualified after a telephone interview and who did not have documentation of a serum cholesterol of 200 mg% or more in the previous year were brought in for an initial screening serum cholesterol measurement. Those qualifying were scheduled for an initial clinic visit, usually one to two weeks later. At this randomization visit participants were given informed consent, and each subject underwent a lifestyle evaluation, a medical evaluation, and baseline laboratory evaluation. The lifestyle evaluation included quantitative food frequency to obtain a history of their usual diet and a 24 hour dietary recall, an alcohol and tobacco use history, and an exercise history. The medical

evaluation included a medical history, and measurement of height, weight, pulse rate, and blood pressure. The baseline laboratory evaluation consisted of an automated blood count and differential, fasting blood sugar, hemoglobin A1C, fasting serum triglycerides, total cholesterol, and HDL cholesterol, and a screening panel including liver function tests, uric acid, and other basic parameters. Blood specimens were processed within two hours and the specimens for the lipid determinations were frozen at minus 70°C and shipped as a single batch to a central research laboratory. Other specimens were picked up within 4 hours for immediate transport to a commercial laboratory (Nichols).

Subjects were randomly assigned to one of three treatment groups during the randomization examination using numerically sequenced opaque, sealed envelopes opened in the participants presence after explaining the process of random selection to the participant. The three treatment groups were High dose - 90 grams (3.2 oz) of macadamia nuts each day (600 calories); Group 2 - 45 grams (1.8 oz) of macadamia nuts each day (300 calories); and Group 3 - the control group who were asked not to consume any macadamia nuts during the experimental period. Randomization was carried out in blocks of 6 and stratified by sex.

At the randomization visit a 2 week supply of macadamia nuts was provided without cost to the participant. The nuts for each day were packaged individually for convenience and control of intake. At the randomization visit, utilizing the 24 hour dietary recall as a reference, a study dietician provided 10 to 20 minutes of dietary guidance regarding appropriate substitutions for the calories and fats provided each day by the macadamia nuts, based on a prudent diet for their height and weight and exercise level. Those participants assigned to the control group received dietary also received counselling regarding a prudent diet but were asked not to change their diet other than asking that macadamia nuts not be consumed.

The participants were asked to contact the project weekly by telephone to report their weight, dietary compliance and any problems. Those not reporting were contacted by the project coordinator. At 14 days after the randomization visit each participant was asked to return to the clinic where a repeat 24 hour dietary recall was taken as well as a weight, pulse rate and blood pressure. Reports of perceived side effects were recorded. Blood samples were drawn for repeat blood count and differential, serum cholesterol, fasting blood sugar, and SMA 20. Any subject reporting unacceptable side effects was given the choice of lowering their daily intake or discontinuing the macadamia nuts. At the end of the visit a second two week supply of macadamia nuts was distributed to those randomized to the macadamia nut groups.

At 28 days from the randomization visit, each participant was asked to return to the clinic for a repeat of the baseline dietary food frequency by a dietician and a questionnaire regarding the acceptability of the regimen. Reports of side effects will be recorded and a weight, pulse rate and blood pressure were measured. Blood samples will be drawn for

fasting blood sugar, hemoglobin A1C, UA, fasting serum triglycerides, total cholesterol, cholesterol, and HDL cholesterol and an SMA 20. All subjects were asked to stop consuming macadamia nuts at this point.

Approximately 2 weeks after terminating the intervention final dietary, medical and lifestyle histories, as well as the weight, pulse rate and blood pressure were collected for each subject at a final visit. Blood samples were again drawn for fasting blood sugar, UA, fasting serum triglycerides, total cholesterol, HDL cholesterol, and an SMA20. The participants were not advised of the results of laboratory tests during the study period unless medical attention was needed. The technicians and dieticians carrying out the examination were also blinded to these results.

Results

A total of 74 participants were randomized into the three treatment groups over a period of three weeks. There were no significant differences in any variable between the randomization groups (Table 1). Two subjects were terminated from the study due to medical problems uncovered in the initial trial visit. One was subsequently diagnosed as having diabetes mellitus and placed on therapy. A second subject found to have elevated blood pressure was placed on antihypertensive therapy by his primary physician. Both of these individuals were originally randomized to the control group (Group 3). An additional individual who completed the trial was excluded from the analyses due to laboratory problems with the measurement of his lipids due to an extremely elevated plasma triglyceride level. He was from Group 1.

Reported compliance was excellent. Twenty of those in the macadamia nut groups reported missing some daily doses. In only eight instances of were more than one or two days during the one month period missed. One each missed 4,5 and 7, 11, 15, and 18 days. Two missed a total of 20 days. All of those missing 7 or more days were eating less than the randomized amounts on advice from study personnel due to side effects. A total of 17 subjects reported side effects to eating the nuts. All of these were gastrointestinal. In 14 cases (7 in each group) the side effects were classified as minimal or mild. In almost all of these individuals the GI problems resolved with time. Generally these were increased gas or looser stools. The three individuals with more pronounced side effects were in the high dose (90 grams) group. There were loose stools and abdominal discomfort. All but one of those responded to a reduced daily intake and continued to eat nuts daily. Only one subject discontinued eating the study macadamia nuts completely due to GI side effects. No other significant side effects were noted. No changes in the white blood cell count, hepatic enzymes or other biochemical parameters followed were noted over the trial period. Thus there were no other side effects to the regular ingestion of macadamia nuts either by history or biochemical screen.

All subjects who remained in the trial were seen for all scheduled visits or within one to three days of the scheduled time in rare cases. All subjects report being fasting at

all visits. Palatability of the nuts was judged as good or excellent by most subjects. Most of the complaints were regarding our use of unsalted nuts. Two individuals in the high dose group stated the nuts were unpalatable and 3 others thought they were moderately unpalatable (1 in the high dose group and one two in the low dose group). Few of the
 5 subjects had difficulty eating the nuts daily.

As shown in Table Two, total daily caloric intake decreased slightly in the low dose group, increased by 250 calories in the high dose group and decreased by over 450 calories in the control group. The total fat intake increased significantly in the two macadamia groups, with a total increase of 25 grams per day in the low dose group and
 10 48 grams in the high dose group. It decreased significantly in the control group (21 grams/day). At the end of the one month intervention period this amounted to 47% of calories from fats in the low dose group and nearly 50% of calories in the high dose group as compared to 32% in the control group. Saturated fatty acid intake rose modestly in both intervention groups and mono-unsaturated fatty acid intake increased significantly
 15 (48 grams per day in the high dose group and 24 grams per day in the low dose group), whereas both decreased significantly in the control group. The cholesterol intake decreased in both macadamia nut groups and in the control group. In the macadamia groups this was most likely due to substitution of macadamia nuts for cholesterol rich fatty foods in the diet whereas in the control group it represented a more general
 20 reduction in intake. In the control group there were significant decreases in the intake of all fats in the diet. This was probably secondary to self imposed changes in the diet of these individuals due to the increased attention to their serum cholesterol and diet in the project. Such effects are not unusual in short term diet studies but exceeded expectations here, probably due to the recruiting of a highly motivated group in general.

25 As shown in Figure 1 there were no significant changes in body weight in any of the three groups although the low dose macadamia group had a slight trend towards increasing weight. No significant changes in smoking or physical activity were noted in any group during the trial period.

There were also no significant changes in systolic or diastolic blood pressure in any
 30 of the groups during the trial period (Figures 2 & 3).

As seen in Figure 4 there was an appearance of a slight trend toward an increase in mean total serum cholesterol in all three groups over the trial period. These trends were similar in both nut consumption groups and the control group and there was no significant difference between the groups. The mean fasting triglyceride values were quite variable
 35 by visit and by group (Figure 5). The high dose group had a significant decrease over the first three visits while the low dose group had a significant increase. The control group values did not show overall change over the whole trial period but were much higher than baseline at two weeks and down to baseline values by four weeks. Mean HDL cholesterol showed no significant trend in any group over the trial period (Figure 6).

As seen in Figure 7 there was a trend toward a decrease in calculated LDL values in all groups. Again there was no significant difference between groups. Shown in Figure 8 are the mean fasting glucose values by group and visit. There were no significant changes in the glucose levels over the trial period.

5

Discussion

It is apparent from the results of this pilot study that a number of the standardly held views regarding the consumption of Macadamia nuts were not substantiated. These might be termed the conventional wisdom regarding macadamia nuts. They include the following:

- 10 **1. Conventional wisdom:** Gastrointestinal side effects limit the ability of individuals to eat relatively large quantities of macadamia nuts on a regular basis.

Pilot study: while a few individuals had such problems, most tolerated the nuts well and enjoyed their experience in the study.

- 2. Conventional wisdom:** Individuals who eat macadamia nuts will gain weight.

- 15 **Pilot study:** There was no significant weight change in either macadamia nut group. Even with a relatively minor amount of dietary advice and large increases in the proportion of their calories coming from fats individuals were able to control the caloric content of their diets sufficiently to avoid significant weight change.

- 3. Conventional wisdom:** Eating large amounts of macadamia nuts regularly will
20 result in raising the blood cholesterol.

Pilot study: There was no significant difference between the macadamia nut groups and the control group in the changes in blood cholesterol or other lipids over the time period of the study. This is despite an increase in the percentage of total calories coming from fats to almost 50% in both macadamia nut groups.

25

Conclusions

The regular ingestion of Macadamia nuts proved safe and enjoyable for most individuals. Eating relatively large quantities of the nuts did not produce significant differential effects on body weight, cholesterol, triglycerides, HDL or LDL despite very significant increases in the % of calories derived from fats in the diet of individuals eating
30 macadamia nuts. The reduction in caloric intake in the control group, probably secondary to those individuals being sensitized to their diets by entry into the study, is an unfortunate confounder in the study. It probably makes the findings even more significant, however. Further studies of the effects of macadamia nuts and palmetoleic acid on blood lipids in which the dietary intake can be more closely monitored and
35 controlled are indicated. The current study provides adequate evidence that the high monounsaturated fatty acid content of macadamia nuts may make them a much healthier food than they are widely perceived.

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Table 1
MACADAMIA DIET STUDY
Baseline Comparison of Groups
mean (S.D.)

VARIABLE	GROUP 1	GROUP 2	GROUP 3	p value
n	25	25	21	
% Men	52%	56%	43%	
Age (years)	44.8 (13.09)	45.91 (11.63)	47.25 (11.79)	N.S.
Weight (Kg)	70.96 (14.71)	67.46 (12.94)	71.10 (21.80)	N.S.
BMI (Kg/m ²)	26.50 (5.24)	24.55 (3.35)	25.70 (5.53)	N.S.
PAI ¹	31.27 (3.97)	34.48 (9.91)	32.18 (7.78)	N.S.
Alcohol (ml/day)	1.64 (2.51)	1.94 (3.29)	2.49 (6.01)	N.S.
Cigarettes/day	1.60 (4.73)	2.60 (6.56)	2.52 (6.91)	N.S.
Cholesterol (mg/dl)	214.8 (28.2)	206.3 (36.9)	217.9 (29.8)	N.S.
Triglycerides (mg/dl)	162.5 (86.7)	140.6 (61.7)	134.3 (92.8)	N.S.
HDL cholesterol (mg/dl)	46.0 (11.4)	46.5 (11.1)	49.5 (9.8)	N.S.
LDL cholesterol (mg/dl)	151.8 (25.4)	150.6 (34.5)	157.0 (26.5)	N.S.
FBS (mg/dl) ²	102.3 (10.8)	101.8 (11.0)	101.2 (18.2)	N.S.
Hgb A ₁ C (%)	4.57 (0.62)	4.55 (0.41)	4.53 (0.52)	N.S.

Legend: ¹ Physical Activity Index; ² Fasting blood sugar

Table 2 - Daily Dietary Intake

	Total Calories	Percent Calories From Fats	Total Fat (gms)	Saturated Fat (gms)	Mono- unsat. Fat (gms)	Cholesterol (mgs)
	base-1 mo.	base-1 mo.	base-1 mo.	base-1 mo.	base-1 mo.	base-1 mo.
Low Dose	2216-2179	35.7-46.7	88-113	26-28	31-55	277-224
High Dose	2442-2692	36.8-49.5	100-148	30-33	34-55	303-243
Control	2006-1559	34.5-32.3	77-56	23-17	25-18	234-178

Example 3: Electrophoretic Determination of Lipoproteins in the Honolulu Study

Semi-Quantitation of HDL (ALPHA₁) and LDL (BETA) Levels

Rationale:

The values for HDL and LDL moieties in human plasma are reflective if not
 5 predictive of the tendency of the patient to develop coronary artery disease: HDL tends to
 inhibit the deposition of fatty materials in the vessels, while LDL promotes such
 deposition; this is the generally accepted view. As a result, screening procedures for
 HDL and LDL have been developed and are widely practiced in generally categorizing
 the "at risk" status of a patient.

10 Among others, an important criterion for a screening type test is affordability. The
 method used in the Honolulu Study (Example 2) to determine LDL, a prime predictor of
 atherosclerotic tendency, employs a calculation based on total cholesterol, HDL and
 triglyceride levels. The value for LDL by this protocol is, then, a "calculated value" as
 opposed to an "analyzed value."

15 Calculation: $LDL = Total\ Cholesterol - HDL - Triglyceride/5$

While this is an acceptable calculation for mass screening, it may not always offer
 the precision and accuracy required for comparison of group-to-group variations in a
 controlled study such as the Honolulu Study. This equation assumes a "constant"
 relationship between LDL and Triglycerides. While for rough screening purposes this
 20 assumption may be allowable, the dietary intervention with high levels of C16:1 and the
 greatly lowered fat intake of the control group compared to the highdose group in the
 Honolulu Study suggested that a more definitive analytical method in the form of
 electrophoresis might yield more meaningful, "apples-to-apples" values for these critical
 parameters.

25 The increased mutual solubility properties of C16:1 were striking in the Case Study;
 the lipid moieties were "freed-up" and transported more readily. Therefore, the
 triglyceride levels of the High Dose group in the Honolulu Study are probably not typical;
 the validity of the "standard formula" in this study is less than certain.

Electrophoresis allows the visualization of the lipoproteins as well as their
 30 semi-quantitation by integration of the peaks generated after a colorimetric tracing is
 performed. This represents a direct "analytical value" of both HDL and LDL, and
 eliminates the use of the "triglyceride factor" of the other method.

Electrophoresis is a much more expensive and time-consuming protocol.
 Nevertheless, it is routinely employed even for large-scale screening by certain workers

because of its reproducibility, accuracy and results similar to those obtained by the ultimate method for lipoprotein characterization, ultracentrifugation. (Ibid.)

Protocol:

Frozen EDTA plasma samples from the Study were received from Honolulu. These were
5 separated into Control, Low Dose and High Dose groups. Ten specimens from the Control and ten from the High Dose groups were selected at random. The corresponding specimens (baseline or end) were then paired up with the initially chosen specimens. A total of 40 specimens was submitted for analysis using the same specimen numbers as originally assigned in the Honolulu Study. That is:

10	Control Group Pairs,	High Dose Group Pairs,
	10 Baseline	10 Baseline
	10 End (30 Days)	10 End (30 Days)

King James Laboratories, Cleveland, Ohio, a lab specializing in lipid analysis, was chosen to perform the analyses. Aliquots of each specimen were submitted in the thawed
15 state for the testing; the original numbers were maintained.

Agarose gel "strips" were employed as the substrate. There was a "Standard Plasma" of known LP composition run on each strip in addition to seven (7) specimens. The inclusion of the standard allows for corrections in variations inherent in the strip such as agarose thickness, degree of hydration, temperature variations, current fluctuation,
20 changes in stain and other strip-to-strip and run-to-run variations. Corrections are made to the mean values of the Standard Plasma. This assures the existence of a level playing field when it comes time to compare and interpret the results.

Data Presentation

Results were organized according to Control Group and High Dose Group, by Subject
25 Number, in ascending order. See the attached "LipoTech Electrophoretic Data" sheet. The top value in each set is the "Baseline" value; the second result in the adjacent column is the "End" or 30-day value.

Correction factors for strip-to-strip variation are found in the heading. The raw data values from the integration are factored by these values to yield the "corrected values"
30 reported in tabular form.

HDL is found in the "Alpha" globulin segment of the strip, while LDL migrates with the "Beta" globulin; VLDL travels with the "pre-Beta" fraction, as noted. The strip number on which each specimen was tested is also included. (The Chylomicrons migrate only slightly, if at all, from the point of application. These specimens were centrifuged
35 before testing and this may well affect the accuracy of the "Chylo" values. Therefore, no more mention is made of these values.)

The group averages for HDL, VLDL and LDL are calculated.

Data Synopsis

Refer to "Synopsis of Results in % of Each Component" and "HDL/LDL Ratios" and "Atherogenic Indices".

Discussion of Results

5 Any discussion and interpretation of the results must be prefaced with a discussion of the Honolulu Study as reported in the Curb Report (Example 2). THE EFFECT OF DIETARY SUPPLEMENTATION WITH MACADAMIA NUTS ON SERUM LIPID LEVELS IN HUMANS by Curb et al.

Table 2 is presented in the Example 2. From these data at Baseline and End ("1
10 mo.", or 30 days from the start of dietary supplementation) the following observations are made:

1-The High Dose Group (HDG) consumed daily 250 Kcal more than at Baseline while the Control Group (CG) 447 Kcal less than at Baseline.

2-The HDG diet represented a 34.5% increase in calories from fat, while the CG diet
15 represented a 2.2% decrease in calories from fat. That is, the HDG consumed -150% more fat calories during the 30 day test period than the CG consumed.

3-As compared to the CG the HDG consumed on a gram basis:

a-260% more fat;

b-190% more saturated fat;

20 c-140% more cholesterol;

d-300% more unsaturated fat.

Referring now to the graph section of the report ...

1-There was no weight gain by either group. (Fig. 1)

Comment: Considering the fat rich (~50%) ~2700 Kcal diet of the HDG and the lean,
25 ~1500 Rcal diet of the CG, it is remarkable that the HDG did not gain weight and the CG did not lose weight.

2-Serum cholesterol remained essentially unchanged in both groups. (Fig. 4)

Comment: Again, it would be expected that, since the HDG consumed 1.4 times as
much cholesterol as the CG, the HDG would experience a rise in serum cholesterol;
30 however this did not occur.

3-The triglyceride (TG) level in the HDG rose by ~24% while the TG level in the CG
dropped ~7%. (Fig. 5)

4-The HDL levels remained constant in both groups. (Fig. 6)

5-Likewise, the LDL levels in the HDG remained const but the level dropped by ~9%
35 in the CG.

Comment: As discussed in "Rationale"--Paragraph 3, above, due to the atypical diet of the HDG, as evidenced here by an essentially constant LDL value despite huge fat intake and an expected rise in TG level, the incorporation of TG values in the "calculated LDL"

may well give somewhat spurious results. These data prompted the performance of the electrophoretic studies.

Electrophoretic Data

Exclusion of the "triglyceride factor" and direct quantitation of the lipoproteins reveal a
 5 number of effects consistent with improved lipid metabolism akin to the results found in the Case Study; refer to the above-cited "Synopsis of Results..."

1-HDL rose 8.8% in the HDG; HDL fell 6.1% in the CG.

2-VLDL, an important indicator of atherogenesis, Fose 70.8% in the CG; VLDL fell 2.4% in the HDG.

10 3-LDL rose 1.2 % in the CG; LDL fell 0.7% in the HDG.

4-HDL/LDL Ratio fell in the CG; it rose in the HDG.

5-Perhaps most indicatively, the "Atherogenic Index"³ increased in the CG (8% rise) but fell in the HDG by -9%!

Conclusion:

15 In this important human study, it is clear that DBD (our symbol for substances containing effective amounts of short chain monounsaturated fatty acids, such as the Macadamia nut source of this study, which contain palmitoleic acid, C_{16:1} isomer) is highly effective in improving the metabolic processing of lipids (even when compared to a "control group" that consumed remarkably fewer Calories, both total and of lipid origin)
 20 as reflected by no weight gain, enhanced levels of the preferred HDL, no rise in serum cholesterol and a decreased risk of atherosclerosis.

References

1-Kwiterovich, P.O., Jr. *Plasma Lipid and Lipoprotein Levels in Childhood*, from A. of the N.Y. Acad. of Sci., Volume 623, 1991, New York. p. 91.

25 2-Kupke, I.R., *Microstudies Used In Lipid Studies on Young Children and Their First-Degree Relatives.*, from Laboratory of Pediatric Clinical Chemistry and Lipid Research, Heinrich-Heine University, 1991, Germany, from A. of the N.Y. Acad. of Sci., Volume 623, 1991, New York. p. 168.

3-*Ibid.* p. 169.

LIPOTECH ELECTROPHORETIC DATA

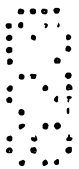
CONTROL STRIP	ALPHA	PRE-BETA	BETA	CHYLO
1	38.1	10.6	49.6	1.7
2	37.0	12.8	47.3	3.0
3	37.4	12.9	47.0	3.1
4	36.1	12.1	50.3	1.5
5	31.3	9.9	55.2	3.6
6	36.2	15.0	46.0	2.9
MEAN:	36.0	12.2	49.2	2.6

TEST STRIP CORRECTIONS	ALPHA	PRE-BETA	BETA	CHYLO
1	5.78% 0.9422	13.23% 0.8677	7.40% 0.926	-35.44% 1.3544
2	2.73% 0.9727	4.77% 0.9523	-3.93% 1.0393	13.92% 0.8608
3	3.84% 0.9616	5.59% 0.9551	-4.54% 1.0454	17.72% 0.8228
4	0.23% 0.9977	-0.95% 1.0095	2.17% 0.9783	-43.04% 1.4304
5	-13.10% 1.131	-18.96% 1.1896	12.12% 0.8788	36.71% 0.6329
6	0.51% 0.9949	22.78% 0.7722	-6.57% 1.0657	10.13% 0.8987

CONTROL SUB/SAMP	STRIP #	HDL ALPHA	VLDL PRE-BETA	LDL BETA	CHYLO
4-4	3	28.1	3.2	60.7	8.5
4-151	4	24.9	3.9	68.5	2.0
15-15	3	34.0	3.0	62.1	0.6
15-163	4	33.9	0.5	63.4	0.7
21-67	3	38.2	0.5	61.3	0.7
21-232	6	40.8	0.9	59.7	0.9
27-27	3	21.4	3.7	69.7	5.1
27-180	4	22.7	0.5	73.9	0.9
37-43	3	27.0	2.9	67.9	1.1
37-197	5	30.1	5.6	61.6	2.1
41-41	6	32.6	2.2	64.4	0.6
41-196	5	22.9	9.9	63.1	3.6
44-39	3	35.0	0.5	61.2	1.6
44-195	5	32.0	3.4	61.0	3.6
60-39	6	27.7	2.8	68.1	1.8
60-219	5	22.2	6.9	68.7	1.8
70-79	6	27.3	2.9	65.0	4.2
70-236	4	27.6	3.4	67.2	0.8
77-68	4	31.5	2.2	63.2	0.9
77-223	5	27.1	5.9	64.1	1.6
		HDL	VLDL	LDL	CHYLO
VG., BASE/END:		30.3 28.4	2.4 4.1	64.4 65.1	2.5 1.8

HIGH DOSE SUB/SAMP	STRIP #	HDL ALPHA	VLDL PRE-BETA	LDL BETA	CHYLO
76-97	2	33.7	1.4	65.1	1.7
76-243	6	35.5	1.2	60.3	2.4
75-96	2	37.2	1.4	58.4	0.4
75-242	6	32.1	3.3	57.2	3.1
72-77	2	22.9	3.8	74.4	0.5
72-202	5	22.4	5.6	65.7	1.0
54-51	1	42.2	4.1	52.3	3.1
54-230	6	39.8	1.8	56.5	1.8
46-45	1	23.9	2.9	69.6	1.9
46-194	5	25.0	3.5	65.3	3.6
35-35	1	39.3	1.2	57.1	2.8
35-188	4	34.6	0.5	61.8	1.6
26-26	1	29.5	2.8	64.3	2.0
26-174	2	34.4	3.1	60.1	1.0
18-18	1	35.0	4.9	54.7	4.5
18-169	2	34.4	2.4	61.8	0.4
16-16	1	32.6	0.8	64.3	2.3
16-161	2	32.1	0.5	64.6	2.5
10-10	1	34.0	4.4	59.5	2.9
10-155	2	32.4	5.2	61.8	1.1

	HDL		VLDL		LDL		CHYLO	
1/G., BASE/END: 1G d@s0B	29.7	32.3	2.8	2.7	62.0	61.5	2.0	1.9



* * * * *

SYNOPSIS OF RESULTS IN % OF EACH COMPONENT

	CONTROL HDL	HIGH DOSE HDL	CONTROL VLDL	HIGH DOSE VLDL	CONTROL LDL	HIGH DOSE LDL
BASELINE	30.3	29.7	2.4	2.8	64.4	62.0
28 DAYS	28.4	32.3	4.1	2.7	65.1	61.5
CHANGE:	-1.9	2.6	1.7	-0.1	0.8	-0.5
%CHANGE	-6.1%	8.8%	70.8%	-2.4%	1.2%	-0.7%

69m

HDL/LDL RATIOS and "ATHEROGENIC INDICES"

	CONTROL (HDL/LDL)	HIGH DOSE
BASELINE RATIOS:	0.47	0.48
28 DAYS	0.44	0.52
	(LDL/HDL)	
BASELINE INDICES:	2.12	2.09
28 DAYS	2.29	1.91
CHANGE:	0.17	-0.18
	(INCREASE)	(DECREASE)

NORMAL RANGE:

0.7 to 2.74

(NOTE: THE HIGHER THE VALUE, THE
GREATER THE RISK OF ATHEROSCLEROSIS)

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The claims defining the invention are as follows:

1. A formulated composition for administration to a human or an animal, said composition comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, C16:1n-6, 5 C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, in a capsule for oral administration, in a prepared food or in a non-naturally occurring edible oil, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal upon systematic administration, together 10 with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.
2. A formulated composition for administration to a human or an animal comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, 15 C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, wherein said monounsaturated fatty acids constitute at least about 1.2 percent by weight of the fatty acids present in said formulated composition, and the ratio by weight of the content of at least one of the said monounsaturated fatty acids to the combined content of all C20:x and C22:y fatty acids, 20 where x = 0 to 5 and y = 0 to 6, is at least about 1:1, together with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.
3. A formulated composition for administration to a human or an animal comprising at least one lipid species containing the short chain monounsaturated fatty acid C16:1n-7, or salts or esters thereof, wherein said C16:1n-7 constitutes at least about 5 percent by 25 weight of the fatty acids present in said formulated composition and wherein the ratio by weight of said C16:1n-7 content to the combined content of all C20:x and C22:y fatty acids, where x = 0 to 5 and y = 0 to 6, is at least about 8:1, together with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.
4. The composition of claim 2 or claim 3, wherein the said composition is an edible 30 oil.
5. A method of improving the metabolic processing of lipids in a human or animal requiring said improvement, which method comprises increasing the plasma HDL/LDL cholesterol ratio of said human or animal compared to the HDL/LDL cholesterol ratio obtained when a composition containing a C18 unsaturated fatty acid is administered to 35 said human or an animal.
6. A method of improving the metabolic processing of lipids in a human or animal requiring said improvement, which method comprises increasing the plasma HDL/LDL cholesterol ratio of said human or animal compared to the HDL/LDL cholesterol ratio

obtained when a composition containing a C12, C14, C16 or C18 saturated fatty acid is administered to a human or an animal.

7. A method of improving the metabolic processing of lipids in a human or an animal comprising systematically administering to a human or an animal a composition
5 comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, said lipid species present in said composition in amounts sufficient to improve the metabolic processing of lipids within
10 said human or animal.

8. The method of claim 7 conducted for preventing or lessening fatty deposits within the liver of said human or animal.

9. The method of claim 7 conducted for increasing the level of any said C16 monounsaturated fatty acid or its esters within the heart tissue of said human or animal.

15 10. The method of claim 7 conducted for lowering the level of saturated fatty acids or their esters within the heart tissue of said human or animal.

11. The method of claim 7 conducted for lowering the triglyceride content per unit weight of adipose tissue.

12. A method of claim 7 conducted for lowering the serum glucose concentration of a
20 human or an animal having insulin-resistant elevated serum glucose concentrations.

13. The method of any one of claims 7 to 12, further comprising first providing said lipid species in a pharmaceutically acceptable carrier or prepared food for oral or parenteral administration.

14. The method of claim 7 conducted for increasing the plasma HDL cholesterol
25 concentration compared to the HDL cholesterol concentration obtained when a composition containing a C18 unsaturated fatty acid is administered to a human or an animal.

....
15. The method of claim 7 conducted for increasing the plasma HDL cholesterol concentration compared to the HDL cholesterol concentration obtained when a
30 composition containing a C12, C14, C16 or C18 saturated fatty acid is administered to a human or an animal.

16. The method of claim 7 conducted for decreasing the plasma LDL cholesterol concentration compared to the LDL cholesterol concentration obtained when a composition containing a C12, C14, C16 or C18 saturated fatty acid is administered to a
35 human or an animal.

17. A method of improving the metabolic processing of lipids in a human or an animal comprising formulating a composition for administration to said human or animal comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-6, C16:1n-5, C16:1n-4,

C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salt, or esters of said monounsaturated fatty acid, and mixtures thereof, wherein said monounsaturated fatty acids constitute at least about 1.2 percent by weight of the fatty acids present in said formulated composition, and the ratio by weight of the content of at least one of the said
 5 monounsaturated fatty acids to the combined content of all C20:x and C22:y fatty acids, where $x = 0$ to 5 and $y = 0$ to 6, is at least about 1:1, and systematically administering said composition to said human or animal to improve the metabolic processing of lipids within said human or animal.

18. The method of claim 17, wherein said formulated composition comprises said
 10 C16:1n-7 acid, or salts or esters thereof, wherein said C16:1n-7 acid constitutes at least about 5 percent by weight of the fatty acids present in said formulated composition and wherein the ratio by weight of said C16:1n-7 content to the combined content of all C20:x and C22:y fatty acids, where $x = 0$ to 5 and $y = 0$ to 6, is at least about 8:1.

19. The method of claim 17 or claim 18, wherein said composition is an edible oil.

15 20. A method of improving the metabolic processing of lipids in a human or an animal comprising formulating a composition for administration to said human or animal comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, or salts or esters of said monounsaturated fatty acids, and mixtures thereof, said lipid species present in said
 20 composition in amounts sufficient to improve the metabolic processing of lipids within said human or animal upon systematic administration; and systematically administering said composition to said human or animal to improve the metabolic processing of lipids within said human or animal.

21. A method of any one of claims 7 to 16, wherein said composition contains
 25 macadamia nut or macadamia oil as a source for said monounsaturated fatty acid.

....
 22. A formulated composition for administration to a human or an animal, said composition comprising at least one lipid species containing at least one short chain monounsaturated fatty acid selected from the group consisting of C16:1n-7, C16:1n-6, C16:1n-5, C16:1n-4, C16:1n-3, C14:1n-5, C14:1n-4, C14:1n-3, and C12:1n-3, or salts
 30 or esters of said monounsaturated fatty acids, and mixtures thereof, which composition is substantially as herein described with reference to Example 1.

23. A method of improving the metabolic processing lipids in a human or an animal requiring said improvement, which process comprises systematically administering to said human or animal a composition of claim 22 in an amount which improves said metabolic
 35 processing of said lipids.

Dated 2 August, 1994

Lipotech Partners Limited Partnership

Patent Attorneys for the Applicant/Nominated Person

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Figure 1

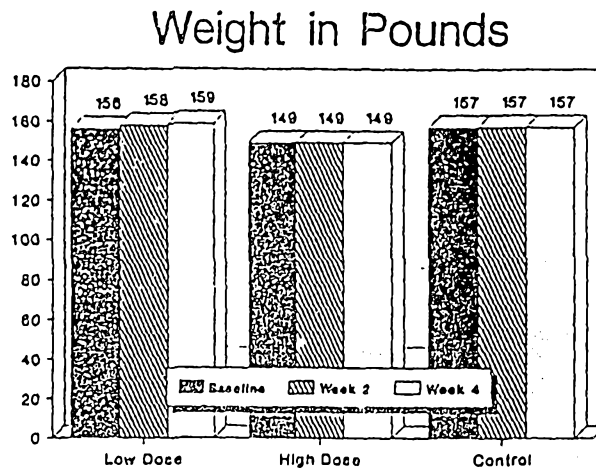


Figure 2

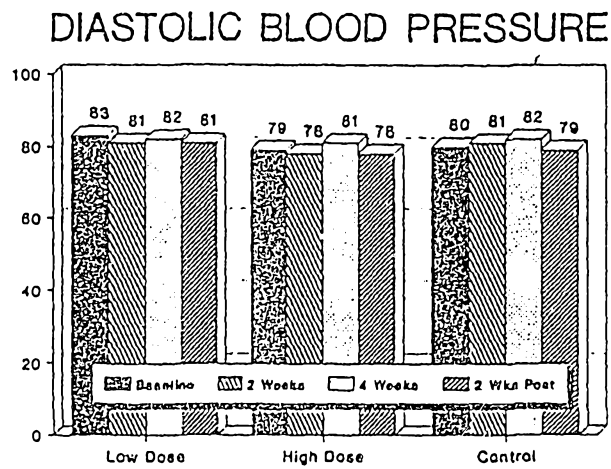


Figure 3

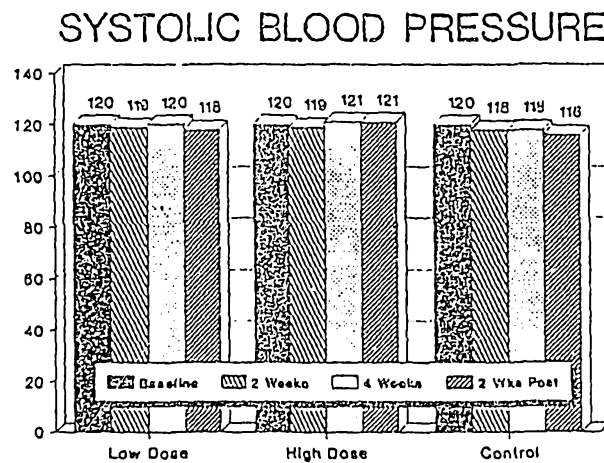


Figure 4

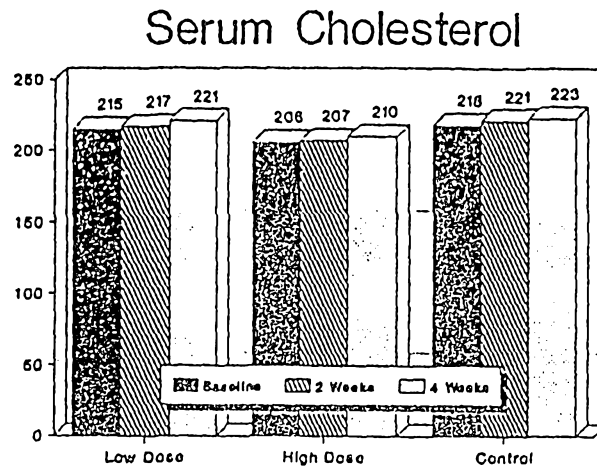


Figure 5

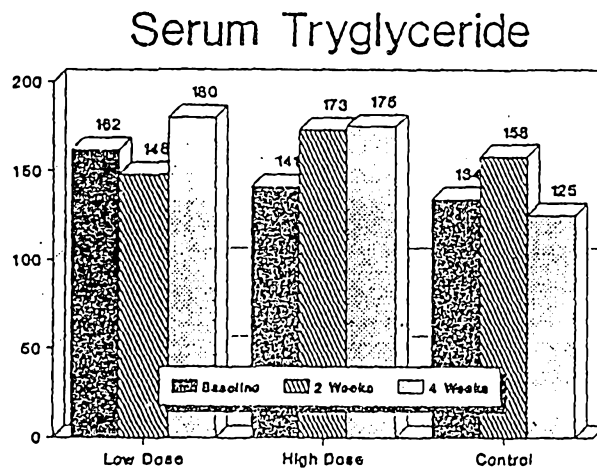


Figure 6

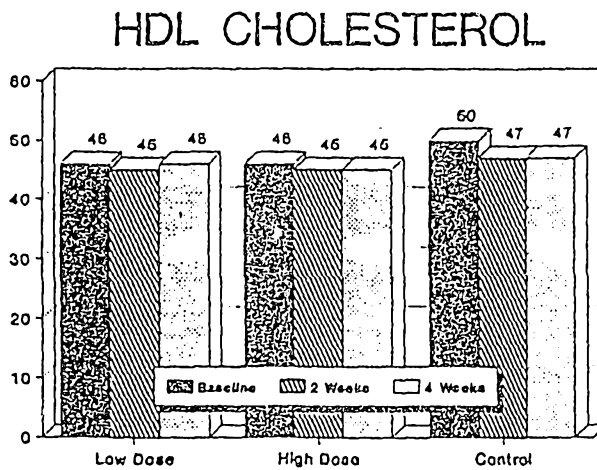


Figure 7

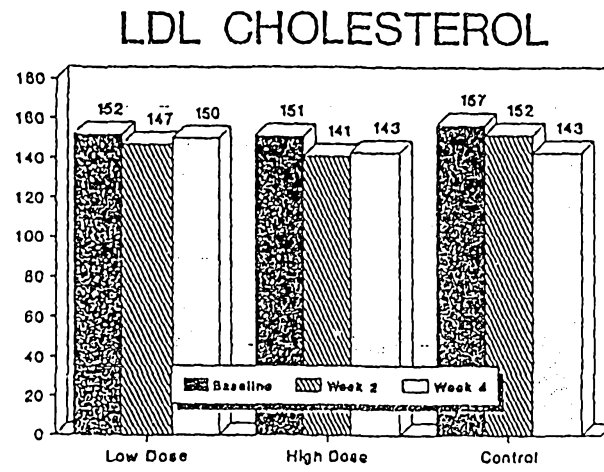
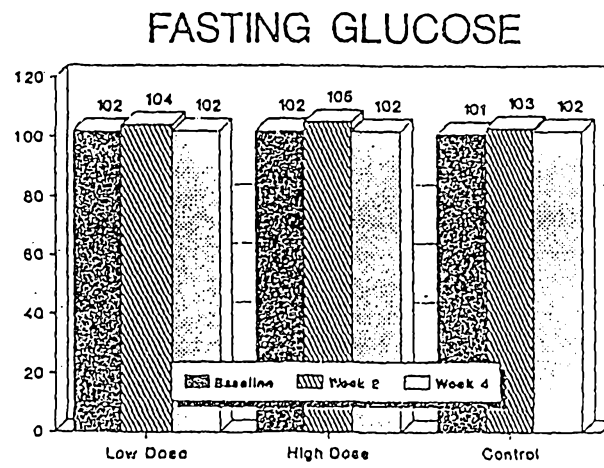
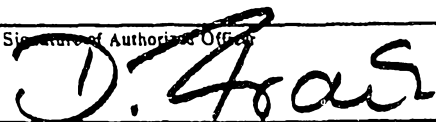


Figure 8



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/04668

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1.5 A 61 K 31/20 A 23 K 1/16 A 23 L 1/03		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1.5	A 61 K A 23 L A 23 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ^o	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemical Abstracts, volume 105, no. 5, 4 August 1986, (Columbus, Ohio, US) see page 397, abstract 49086h, & JP, A, 6176417 (OTSUKA PHARMACEUTICAL CO., Ltd) 18 April 1986 ---	1-20
A	Chemical Abstracts, volume 97, no. 1, 5 July 1982 (Columbus, Ohio, US) Takeuchi Nozomu et al : "Effect of 2-octadecenoic acid, alpha-monounsaturated fatty acid from Corbicula, and its diglycerides on serum and liver lipids of rats", see page 554, abstract 549p, & Oyo Yakuri 1981, 22(6), 819-26 ---	1-20
A	Patent Abstracts of Japan, volume 13, no. 402 (C-633) [3750] 6 September 1989, & JP, A, 1146818 (NIPPON OIL) 8 June 1989, see the whole abstract --- -/-	1-20
^o Special categories of cited documents : ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
17-10-1991		13. 11. 91
International Searching Authority		Signature of Authorizing Officer
EUROPEAN PATENT OFFICE		

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0014910 (GATZEN) 3 September 1980, see abstract; claims ---	1-20
A	EP,A,0271909 (GREEN CROSS CORP.) 22 June 1988, see abstract; column 2, lines 4-48; claims ---	1-20
A	US,A,4472432 (IWAMURA) 18 September 1984, see abstract; claims ---	1-20
A	DE,A,2902160 (HOERRMANN) 24 July 1980, see the whole document, especially page 10, lines 1-2 -----	1-20

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9104668
SA 49755

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/11/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0014910	03-09-80	DE-B- 2905979 AT-T- 3944 US-A- 4272548	08-05-80 15-07-83 09-06-81
EP-A- 0271909	22-06-88	JP-A- 63154618	27-06-88
US-A- 4472432	18-09-84	None	
DE-A- 2902160	24-07-80	None	