Regulation of Apolipoprotein B–Containing Lipoproteins by Vitamin C Level and Dietary Fat Saturation in Guinea Pigs

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Effects of suboptimal and adequate vitamin C, with varying dietary fat saturation, on hepatic cholesterol and plasma lipoprotein concentrations and metabolism were studied in guinea pigs fed 15% (wt/wt) fat/0.04% cholesterol diets. Fat mixtures were either 49% saturated (SFA) (24% lauric acid) or 53% polyunsaturated fatty acid (PUFA) linoleic acid with vitamin C at 50 (suboptimal) or 500 (adequate) mg/kg diet. Guinea pigs fed suboptimal vitamin C had 15% lower hepatic active 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity and 25% lower low-density lipoprotein (LDL; apolipoprotein [apo] B/E) receptor number, 20% higher acyl-CoA:cholesterol acyltransferase (ACAT) activity, 28% higher triacylglycerol (TAG) and cholesteryl ester concentrations, and increased very–low-density lipopoprotein (VLDL) apo B secretion rates in comparison to animals fed adequate vitamin C. Intake of suboptimal vitamin C lowered plasma high-density lipoprotein (HDL) cholesterol concentrations by 45% and increased plasma TAG, total and VLDL/LDL cholesterol, and cholesteryl ester transfer protein (CETP) activity by 40%, 50%, and 30%, respectively. The hyperlipidemic effects of suboptimal vitamin C were more pronounced with intake of the SFA diet. These data demonstrate that low vitamin C intake results in a pattern of changes in whole-body cholesterol and lipoprotein metabolism that are related to increased risk of cardiovascular disease (CVD).

HUMAN STUDIES, MOSTLY EPIDEMIOLOGICAL, suggest a significant negative correlation between vitamin C intake and cardiovascular disease (CVD), as well as all-cause mortality.[1] Vitamin C is related to CVD through distinct, yet interactive mechanisms: vitamin C plays a regulatory role in cholesterol and lipoprotein metabolism; it acts as a plasma antioxidant protecting low-density lipoprotein (LDL) against oxidative modification by maintaining vascular integrity and is involved in the synthesis of vasoactive prostacyclins.[2] While acute vitamin C deficiency (scurvy) is not common in modern western societies, studies have documented that some subgroups of the population present marginal chronic states of vitamin C deficiency: institutionalized elderly patients, smokers, and patients with diabetes mellitus have lower plasma ascorbate levels and increased risk for CVD.[1]

One of the most appropriate animal models to evaluate vitamin C effects on plasma lipids and atherosclerosis is the guinea pig, which, like humans, lacks the ability to synthesize vitamin C. Ginter[3] described a model for chronic marginal vitamin C deficiency in the guinea pig in which ascorbate is added to the diet at either 50 (suboptimal) or 500 mg/kg diet (adequate). Intake of the suboptimal level of vitamin C has been shown to result in normal growth rates with no symptoms of scurvy; however, this level of dietary ascorbate results in significant changes in lipid metabolism and development of atherosclerosis.[3] Chronic vitamin C deficiency in the guinea pig has been associated with increased cholesterol and triacylglycerol (TAG) in plasma and other tissues, higher plasma LDL and very–low-density lipoprotein (VLDL), and lower high-density lipoprotein (HDL) cholesterol levels, and alterations in hepatic enzymes regulating cholesterol homeostasis.[4,5] In addition, low vitamin C intake in the guinea pig has been correlated with increased plasma lipoprotein a [Lp(a)] concentrations, a lipoprotein linked with CVD and thrombosis.[6] These observations are consistent with epidemiological findings in humans of an inverse relationship between vitamin C intake and plasma lipoprotein cholesterol levels.[3]

Studies have shown that the elevated plasma cholesterol levels in guinea pigs fed the suboptimal level of vitamin C are associated with decreased transformation of cholesterol to bile acids due to a decrease in hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis.[5,7] Decreased catabolism of cholesterol to bile acids results in accumulation of hepatic cholesterol, which would be predicted to affect expression of hepatic LDL (apolipoprotein [apo] B/E) receptors. The high hepatic cholesterol levels observed in vitamin C–deficient guinea pigs are not related to increases in the rate of cholesterol synthesis.[3,5] Studies have shown decreases in active 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the regulatory enzyme of cholesterol synthesis by marginal intakes of vitamin C[8,9] due to alterations in the regulation of this enzyme by the phosphorylation-dephosphorylation cascade.

Vitamin C also affects TAG metabolism by participating in the synthesis of carnitine.[10] Carnitine has an essential role in the transport of long-chain fatty acids into mitochondria, where β-oxidation takes place.[10] In vitamin C–deficient guinea pigs, the carnitine levels of various tissues decrease; consequently, the transport of fatty acids into mitochondria is blocked and their metabolism shunted toward TAG synthesis, resulting in accumulation of fat in the liver and other tissues.[11]

The objectives of this study were to define effects of suboptimal and adequate vitamin C intakes, in combination with diets containing either saturated fatty acids (SFA) or polyunsaturated fatty acids (PUFA) on (1) hepatic cholesterol and TAG levels relative to changes in liver HMG-CoA reductase and Acyl-CoA:cholesterol acyltransferase (ACAT) activities and LDL (apo B/E) receptor expression; and (2) plasma

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lipid and lipoprotein concentrations relative to changes in hepatic VLDL secretion rates and plasma lecithin: cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) activities.

In addition to having a requirement for vitamin C, the guinea pig was chosen as the model for this study based on its plasma lipoprotein profile (high LDL/HDL ratio); tissue distribution of cholesterol synthesis, with the liver contributing less than 20%; a hepatic cholesterol pool with most of the cholesterol present as free rather than esterified cholesterol; comparable hepatic ACAT; and plasma LCAT and CETP activities and documented responses to changes in type and amount of dietary fat that are similar to humans.[12]

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: cholesterol and TAG enzymatic assay kits from Boehringer-Mannheim (Indianapolis, IN); free cholesterol and phosholipid enzymatic kits from Wako Pure Chemical Industries (Osaka Japan); ascorbic acid from Sigma (St Louis, MO); halothane from Halocarbon Lab (Hackensack, NJ); [^{125}I]Na from Amersham (Arlington Heights, IL); DL-hydroxy-[3-^{14}C]methylglutaryl coenzyme A (1.92 Bq/mol) and DL-[5-^{3}H]mevalonic acid (91.80 Bq/mol) from Dupont NEN (Boston, MA); glucose-6-phosphate, glucose-6-phosphatase dehydrogenase, and NADP from Sigma; *Escherichia coli* alkaline phosphatase suspended in 2.6 mol/L ammonium sulfate from Worthington Biochemicals (Freehold, NJ); [^{14}C]oleoyl-CoA from Amersham, and Quick Seal ultracentrifuge tubes from Beckman Instruments (Palo Alto, CA).

Animals

Male Hartley guinea pigs (Sasco Sprague Dawley, Omaha, NE) weighing between 600 and 700 g were randomly assigned (at least eight per group) to one of four experimental diets for 6 weeks to achieve a metabolic steady-state before analysis. Animals were housed in groups in a light-controlled room with constant temperature and humidity. Animals were fed ad libitum and adapted to the experimental diets over a period of 6 days. At the end of the 6-week experimental period, nonfasting guinea pigs were anesthetized with halothane vapors, exsanguinated by cardiac puncture, and livers were excised for analysis. The study protocol was performed in accordance with Public Health service and US Department of Agriculture guidelines and experimental protocols were approved by the University of Arizona Institutional Animal Care and Use Committee.

Diets

Diets were prepared and pelleted by Research Diets (New Brunswick, NJ). All diets contained identical ingredients, except for the fat source and level of vitamin C (Table 1), and were formulated to meet National Research Council–specific nutritional requirements of the guinea pig. Diets consisted of 15% (wt/wt) fat (35% calories) and cholesterol added at 0.04% (wt/wt), which provides the physiological equivalent of a human intake of 300 mg/d. Protein was 60% casein:40% soy protein to mimic the average American diet. Fat mixtures were either 49% SFA (24% 12:0):20% monosaturated fatty acids (MUFA): 26% PUFA or 24% SFA:24% MUFA:53% PUFA (18:2) (Table 2). The fat mixtures were analyzed by gas chromatography to verify the fatty acid profile of the diets. Vitamin C was added to the diets at either 500 mg/kg diet (adequate) or 50 mg/kg diet (suboptimal), which, based on daily food intake,[13] would be the equivalent of 1.5 or 15 mg/d. Intake of vitamin C at the level of 50 mg/kg diet resulted in normal growth rates in guinea pigs with no symptoms of scurvy. The two levels of vitamin C used in this study are referred to throughout this report as +AA (adequate) and −AA (suboptimal).

Table 1. Composition of Test Diets

Component	Weight (g/100 g)	Energy (% kcal)
Protein (casein:soy)*	22.4	23.0
Fat (12:0 or 18:2)	15.1	35.0
Carbohydrate (sucrose/starch)†	39.6	42.0
Fiber (cellulose/guar gum)‡	13.6	
Mineral mix§	8.2	
Vitamin mix§	1.1	
Added cholesterol	0.04	

*Ratio of casein:soy = 1.5/1.
†Ratio of sucrose:starch = 1.43/1.
‡Ratio of cellulose:guar gum = 4.00/1.
§Mineral and vitamin mixes were formulated to meet NRC-specified requirements for the guinea pig. A detailed composition has been published elsewhere.[34]

Hepatic Vitamin C Concentrations

Liver vitamin C concentrations were determined by the α,α′dipyrydyl method of Zonnoni et al.[14] Liver samples (0.5 g) were homogenized in a Potter-Elvehjem homogenizer with 20 vol of iced-cold 5% trichloroacetic acid (TCA) and following centrifugation the solution was used for analysis.[14]

Hepatic Lipid Assays

Liver samples were extracted overnight with chloroform:methanol (2:1)[15] and extracts were used to determine total cholesterol, free cholesterol, and TAG by enzymatic procedures using microtiter plates and a plate reader.[16] Esterified cholesterol levels were calculated as the difference between free and total cholesterol values.

Plasma Lipid Assays

Plasma samples were obtained from guinea pigs under halothane vapor by cardiac puncture with EDTA (1.5 mg/mL) as anticoagulant with a mixture of aprotonin (0.5 mg/mL), phenylmethylsulfonyl fluoride (PMSF; 0.1 mg/mL, and sodium azide (0.1 mg/mL) to minimize changes in lipoprotein composition during isolation. Plasma cholesterol and TAG concentrations were determined using commercial kits from Boehringer-Mannheim. Plasma HDL cholesterol was determined following $MgCl_2$-dextran sulfate precipitation of apo E and B lipoproteins,[17] and VLDL/LDL cholesterol was calculated as the difference between total and HDL cholesterol.

Hepatic HMG-CoA Reductase Activity

Guinea pigs were killed and livers were excised and a portion was used for microsomal isolation. Tissue was pressed through a tissue grinder into 1:3 (wt:vol) homogenization buffer (50 mmol/L KH_2PO_4,

Table 2. Fatty Acid Composition of Fat Mixes (g/100 g)

	Diet	
Fatty Acid	Polyunsaturated	Saturated
Lauric (12:0)	0	23.8
Myristic (14:0)	0.5	7.8
Palmitic (16:0)	16.2	9.2
Stearic (18:0)	7.1	8.6
Oleic (18:1)	23.5	19.9
Linoleic (18:2)	52.7	26.4
Other	0	4.3

NOTE. Values are the average of 2 determinations.

0.1 mol/L sucrose, 50 mmol/L KCl, 30 mmol/L EDTA, 50 mmol/L NaCl, and 2 mmol/L dithiothreitol, pH 7.2), and homogenized using a Potter-Elvenjhem homogenizer. Microsomal fractions were obtained after two 15-minute centrifugations (Ti-50 rotor; Beckman Instruments) at 10,000 × *g* and a 1-hour centrifugation at 100,000 × *g* at 4°C. The microsomal pellets were resuspended in homogenization buffer and washed (1 hour, 100,000 × *g*). Microsomal HMG-CoA reductase activity was measured by incubation of 200 mg microsomal protein with 7.5 nmol (0.33 GBq/nmol) [3-^{14}C]HMG-CoA, 4.5 mmol glucose 6-phosphate, 3.6 mmol EDTA, 0.45 mmol NADP, and 0.3 IU glucose 6-phosphate dehydrogenase to a final volume of 0.20 mL for 15 minutes at 37°C and using [^{3}H]mevalonic acid as an internal standard (0.024 GBq per assay). HCl was added to stop the reaction and samples were further incubated for 30 minutes. After incubation, microsomal protein was precipitated by microfuging for 1 minute. An aliquot of the supernatant was applied on thin-layer chromatography (TLC) silica gel plates (Alltech, Deerfield, IL) and developed in acetone:benzene (1:1, by vol). The area containing the mevalonate was removed and mixed with 5 mL aquasol and counted for radioactivity in a scintillation counter. Recoveries of the internal standard were between 55% and 75%. Active reductase values were determined by collecting microsomes in the presence of 50 mmol/L NaF to inhibit endogenous phosphatases and preserve the ratio of phosphorylated (inactive) to dephosphorylated (active) reductase values.[18] Total microsomal reductase activity was determined in the presence of *E coli* alkaline phosphatase (34 U/mg protein) as described by Brown et al.[19]

Hepatic ACAT Activity

Hepatic ACAT activity was determined by the method of Smith et al.[20] The assay was conducted by preincubating isolated hepatic microsomes (0.7 to 10 mg protein per assay) with albumin and ACAT buffer (50 mmol KH_2PO_4/L, 1 mol sucrose/L, 50 mmol KCl/L, 30 mmol EDTA/L, and 50 mmol NAF/L). After 5 minutes at 37°C, 20 mL (500 mmol/L) oleoyl-[1-^{14}C]-coenzyme A (0.15 GBq/pmol) was added and the reaction proceeded for 15 minutes at 37°C. The reaction was stopped by adding chloroform:methanol (2:1). The recovery standard [^{3}H]cholesteryl oleate (0.045 GBq/assay) was added. Tubes were mixed and left to stand overnight. The aqueous phase was removed and, after evaporation of the organic phase to dryness, samples were resuspended in chloroform containing unlabeled cholesteryl oleate. Samples were applied to 20- X 20-cm glass silica gel TLC plates, which were developed in hexane:diethyl ether. Cholesteryl esters were visualized with iodine vapors, scraped from the TLC plates, and counted in a scintillation counter after the addition of 5 mL liquifluor.

In Vitro LDL-Binding Assays

Pooled samples of LDL from each of the four dietary groups were radiodinated by the iodine monochloride method of Goldstein et al[21] to give a specific activity between 250 and 400 cpm/ng LDL protein. Isolated hepatic membranes (200 mg protein) were incubated with the radiolabeled LDL over a concentration range of 10 to 80 μg/mL in the presence of absence of 1 mg/mL of unlabeled human LDL, for 2 hours at 4°C. After incubation, the membranes were pelleted by ultracentrifugation, and the pellets counted in a gamma counter. Incubation without excess human LDL represented total binding, and receptor-mediated binding was calculated as the difference between total and nonspecific binding. The affinity of hepatic membrane apo B/E receptors for LDL (K_d) and maximal receptor binding (B_{max}) were calculated from Woolf plots[22] by plotting bound (x axis) versus free/bound (y axis) radiolabeled LDL. The slope = $1/B_{max}$ and the intercept = K_d.

TAG and Apo B Secretion Rates

VLDL TAG and apo B secretion rates were determined by blocking VLDL catabolism with Tyloxapol (Sigma, St Louis, MO), a detergent that coats VLDL particles and interferes with the action of lipoprotein lipase (LPL) in vivo.[23] TAG secretion rates were calculated by regression analysis as mg TAG secreted/kg body weight per h. Apo B secretion rates were calculated by multiplying VLDL TAG secretion rates times the apo B concentration (%) divided by VLDL TAG (%).[23] Plasma from Tyloxapol-injected animals was used for isolation of nascent VLDL. VLDL was collected at a density of 1.006 g/mL and further purified by washing with a buffer solution at 1.006 g/mL. The composition of nascent VLDL was analyzed by measuring protein, TAG, total cholesterol, and phospholipid as previously described.[24] Apo B in VLDL was determined by selective precipitation with isopropanol, and the number of component molecules of nascent VLDL was calculated assuming one apo B per VLDL particle.[23]

Plasma LCAT and CETP Measurements

CETP activity was determined in freshly isolated plasma samples by analysis of the change in HDL cholesteryl ester mass when cholesteryl ester synthesis by LCAT was inhibited with 1.5 mmol/L dithiodinitrobenzoic acid (DTNB).[25] Samples were incubated at 37°C for 0 and 6 hours, and CETP activity was determined as the change in cholesteryl ester levels in HDL samples incubated at 0 and 6 hours. Plasma LCAT activity was determined in fresh plasma by analysis of the change in total plasma free cholesterol in samples incubated at 37°C for 0 and 6 hours.[25]

Statistical Analysis

Significant differences caused by fat type or vitamin C level in plasma and interactive effects on hepatic lipids, activities of HMG-CoA reductase and ACAT, plasma apo B, and hepatic apo B/E receptors were analyzed by two-way ANOVA. Differences in apo B secretion rates between vitamin C adequate and suboptimal groups were determined by Mann-Whitney nonparametric *t* test. Data are presented as the mean ± SD unless otherwise stated, and differences were considered significant at $P < .05$.

RESULTS

Growth Rates and Hepatic Vitamin C Levels

There were no significant differences in body weight gains of animals from the four dietary groups in this study (Table 3). Suboptimal vitamin C resulted in fourfold to fivefold reductions of hepatic ascorbate concentrations in comparison with adequate vitamin C intake (Table 3), demonstrating the efficacy of the dietary treatment to produce differences in hepatic vitamin

Table 3. Body Weight Gains and Liver Ascorbate Concentrations in Guinea Pigs Fed Diets Rich in 12:0 or 18:2 Fatty Acids With Either Adequate (+AA) or Suboptimal (−AA) Vitamin C

Diet (n)	Weight gain (g/d)	Liver Ascorbate (mg/100 g)
12:0/+AA (16)	6 ± 3	26 ± 6
12:0/−AA (16)	6 ± 2	5 ± 2
18:2/+AA (6)	7 ± 4	24 ± 6
18:2/−AA (6)	6 ± 2	6 ± 2
Statistics*		
Fat type	NS	NS
AA level†	NS	$P < .001$
Interactions	NS	NS

NOTE. Values are means ± SD for the indicated (n) number of guinea pigs. Different superscripts in the same column indicate differences ($P < .05$) among the dietary groups.

*Differences due to dietary fat type, vitamin C level and interaction as determined by 2-way ANOVA. NS, not significant ($P > .05$).

†+AA > −AA.

Table 4. Hepatic TAG, Free Cholesterol, and Esterified Cholesterol Concentrations in Guinea Pigs Fed Diets Richin in 12:0 or 18:2 Fatty Acids With Either Adequate (+AA) or Suboptimal (−AA) Vitamin C

	Hepatic Lipids (mg/g)		
Diet (n)	TAG	FC	CE
12:0/+AA (6)	7.5 ± 1.0	0.7 ± 0.2	0.2 ± 0.1
12:0/−AA (6)	9.5 ± 1.6	0.6 ± 0.1	0.3 ± 0.1
18:2/+AA (6)	5.3 ± 1.2	1.1 ± 0.3	0.2 ± 0.1
18:2/−AA (6)	6.9 ± 1.1	0.9 ± 0.2	0.4 ± 0.1
Statistics			
Fat type*	$P < .001$	$P < .001$	NS
AA level†	$P < .005$	NS	$P < .005$
Interactions	NS	NS	NS

NOTE. Values are means ± SD for the indicated (n) number of guinea pigs. Differences due to dietary fat, vitamin C level, and interaction as determined by 2-way ANOVA; NS, not significant ($P > .05$).

Abbreviations: FC, free cholesterol; CE, cholesteryl ester.

*12:0 > 18:2 for hepatic TAG and 18:2 > 12:0 for FC.

†−AA > +AA for CE.

C levels. There was no fat effect on hepatic vitamin C concentrations as indicated by two-way ANOVA (Table 3).

Hepatic Lipids and Enzymes

Hepatic TAG concentrations were affected by both fat type and level of vitamin C (Table 4). Intake of 12:0/−AA and 18:2/−AA diets resulted in 26% and 30% higher concentrations of TAG in the liver than intake of 12:0/+AA and 18:2/+AA diets. Hepatic free cholesterol was affected by fat type only; guinea pigs fed 18:2 fat had higher hepatic concentrations of free cholesterol than animals fed the 12:0 diet (Table 4). In contrast, hepatic cholesteryl ester concentrations were affected only by vitamin C, since intake of 12:0/−AA and 18:2/−AA resulted in 50% and 100% higher concentrations of cholesteryl ester than 12:0/+AA and 18:2/+AA diets, respectively (Table 4).

Hepatic total HMG-CoA reductase activity was not affected by fat type or vitamin C level (Table 5). In contrast, active reductase activity was affected by vitamin C intake, with lower

Table 5. Hepatic Enzyme Activities of Guinea Pigs Fed Diets Rich in 12:0 or 18:2 Fatty Acids With Either Adequate (+AA) or Suboptimal (−AA) Vitamin C

	Hepatic Enzymes (pmol/min · mg)		
	HMG-CoA Reductase		
Diet (n)	Total	Active	ACAT
12:0/+AA (3)	18.6 ± 3.1	5.3 ± 0.4	21.4 ± 3.0
12:0/−AA (4)	21.9 ± 5.8	4.8 ± 0.9	24.7 ± 2.1
18:2/+AA (3)	20.3 ± 4.1	5.3 ± 0.6	25.4 ± 3.1
18:2/−AA (3)	18.1 ± 3.4	4.3 ± 0.5	32.3 ± 2.1
Statistics			
Fat type	NS	NS	NS
AA level*	NS	$P < .05$	$P < .01$
Interactions	NS	NS	NS

NOTE. Values are means ± SD for the indicated (n) number of guinea pigs. Differences due to dietary fat, vitamin C level, and interaction as determined by 2-way ANOVA; NS, not significant ($P > .05$).

*+AA > −AA for active HMG-CoA reductase and −AA > +AA for ACAT activity.

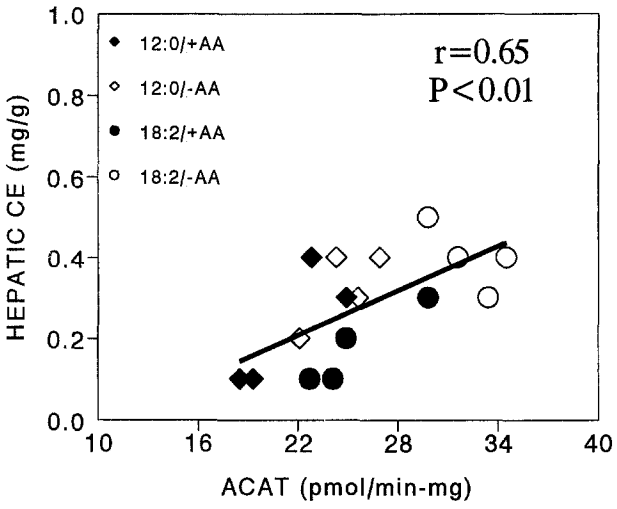

Fig 1. Relationship between hepatic ACAT activities and cholesteryl ester (CE) concentrations in guinea pigs fed diets rich in 12:0 or 18:2 fatty acids with either adequate (+AA) or suboptimal (−AA) vitamin C. Each point represents an individual guinea pig fed 1 of the 4 dietary treatments. The correlation equation is y = 0.02 × −0.19.

activities (17%) in the groups fed suboptimal vitamin C (Table 5). Guinea pigs fed the 18:2/−AA diet had the highest ACAT activity, and the lowest activity was found in the 12:0/+AA group (Table 5). Hepatic ACAT activity and cholesteryl ester concentrations in the liver were positively correlated ($r = .652$, $P < .01$) (Fig 1), consistent with the role of ACAT in regulating the concentration of cholesteryl ester in the liver.

Hepatic LDL Receptors

Hepatic apo B/E LDL receptor number (B_{max}) was lower in animals fed the saturated fat and suboptimal levels of vitamin C (Table 6). Intake of 12:0/−AA and 18:2/−AA resulted in 48% and 30% decreases in LDL receptor B_{max} compared with animals fed the 12:0/+AA and 18:2/+AA diets. Intake of 18:2 fat increased receptor number and low vitamin C downregulated hepatic apo B/E receptors to values found in animals fed

Table 6. Kinetics of LDL Binding to Hepatic Membranes of Guinea Pigs Fed Diets Rich in 12:0 or 18:2 Fatty Acids With Either Adequate (+AA) or Suboptimal (−AA) Vitamin C

	Binding Parameters	
Diet (n)	B_{max} (μg/mg)	K_d (μg/mL)
12:0/+AA (4)	4.9 ± 1.3	51 ± 22
12:0/−AA (5)	3.3 ± 0.8	55 ± 18
18:2/+AA (5)	6.0 ± 0.3	60 ± 19
18:2/−AA (3)	4.6 ± 0.5	65 ± 18
Statistics		
Fat type*	$P < .005$	NS
AA level†	$P < .005$	NS
Interactions	NS	NS

NOTE. Values are means ± SD for the indicated (n) number of guinea pigs. Different superscripts in the same column indicate significant differences ($P < .05$) among dietary groups. Differences due to dietary fat, vitamin C level, and interaction as determined by 2-way ANOVA; NS, not significant ($P > .05$).

*18:2 > 12:0.

†−AA > +AA.

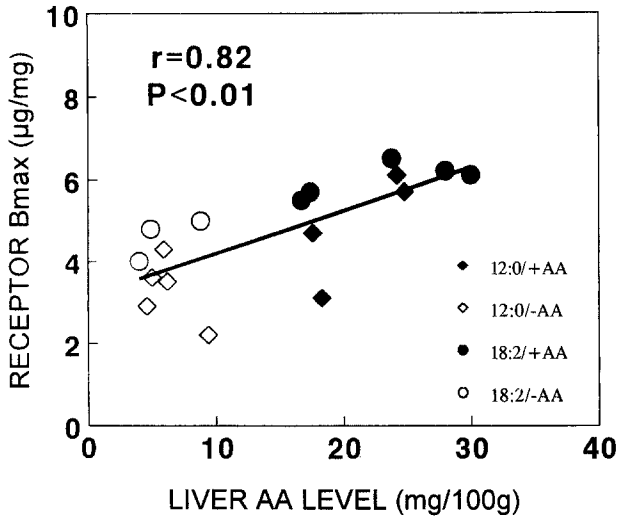

Fig 2. Relation between LDL receptor B_{max} values (mg/mg) and liver vitamin C concentrations (mg/100 g) in guinea pigs fed diets rich in 12:0 or 18:2 fatty acids with either adequate (+AA) or suboptimal (−AA) vitamin C. Each point represents an individual guinea pig fed 1 of the 4 dietary treatments. The correlation equation is y = 0.11 × +3.16.

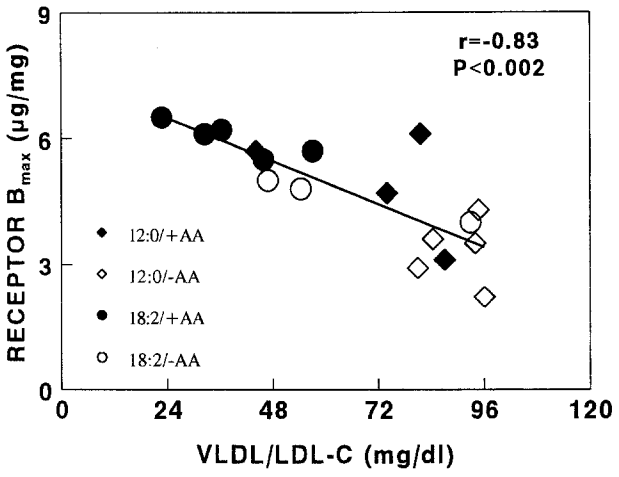

Fig 3. Correlation between LDL receptors B_{max} values (mg/mg) and plasma VLDL/LDL (mg/dL) concentrations in guinea pigs fed diets rich in 12:0 or 18:2 fatty acids with either adequate (+AA) or suboptimal (−AA) vitamin C. Each point represents an individual guinea pig fed 1 of the 4 dietary treatments. The correlation equation is y = −0.04 × +7.52.

12:0 fat. LDL receptor ligand affinity (K_d) was not modified by fat type or vitamin C in the diet (Table 6). In addition, a positive correlation ($r = .82$, $P < .01$) was found between liver ascorbate concentrations and LDL receptor B_{max} values (Fig 2).

Plasma Lipids and Apo B

Feeding 12:0 and suboptimal vitamin C resulted in higher plasma apo B levels than feeding 18:2 and adequate vitamin C, indicating these animals had a higher number of lipoprotein particles in plasma (Table 7). Intake of suboptimal vitamin C resulted in 11% and 18% higher plasma apo B concentrations than adequate vitamin C intake in the 12:0 and 18:0 groups, respectively. Plasma TAG concentrations were increased twofold in vitamin C–deficient animals in comparison to guinea pigs that received adequate vitamin C (Table 7). Fat type had no significant effects on plasma TAG concentrations, although a trend of higher plasma TAG levels with 12:0 was observed ($P = .1$).

Plasma total cholesterol and VLDL/LDL concentrations were increased by feeding 12:0 and suboptimal vitamin C (Table 7). The increment in plasma cholesterol due to low vitamin C intake was more pronounced in the 18:2 dietary group, with increases of 39% and 54% for total and VLDL/LDL cholesterol, respectively. In the 12:0 dietary group, low vitamin C intake resulted in 29% and 38% higher total and VLDL/LDL cholesterol levels in comparison to animals fed adequate vitamin C. Total and VLDL/LDL cholesterol levels were lower in guinea pigs fed 18:2 fat and low vitamin C intake increased plasma VLDL/LDL concentrations to values similar to those found in animals fed 12:0 fat. In addition, plasma VLDL/LDL cholesterol concentrations and LDL receptor B_{max} values exhibited a negative correlation ($r = -.83$, $P < .002$) (Fig 3). Plasma HDL cholesterol concentrations were affected by vitamin C level only, as indicated by the two-way ANOVA analysis (Table 7).

Table 7. Plasma TAG and Cholesterol Concentrations in Guinea Pigs Fed Diets Rich in 12:0 or 18:2 Fatty Acids With Adequate (+AA) or Suboptimal (−AA) Vitamin C

Diet	TAG (mg/dL)	Apo B (mg/dL)	Cholesterol (mg/dL)		
			Total	VLDL/LDL	HDL
12:0/+AA	106 ± 43 (14)	39 ± 4 (6)	86 ± 20 (14)	75 ± 20 (13)	11 ± 6 (13)
12:0/−AA	217 ± 75 (16)	44 ± 4 (6)	112 ± 37 (16)	105 ± 37 (16)	7 ± 3 (16)
18:2/+AA	83 ± 25 (6)	28 ± 4 (4)	50 ± 12 (6)	41 ± 13 (5)	9 ± 2 (5)
18:2/−AA	178 ± 76 (6)	33 ± 7 (4)	70 ± 16 (6)	64 ± 16 (6)	6 ± 2 (6)
Statistics					
Fat type*	NS	$P < .001$	$P < .005$	$P < .001$	NS
AA level†	$P < .005$	$P < .05$	$P < .01$	$P < .01$	$P < .05$
Interactions	NS	NS	NS	NS	NS

NOTE. Values are means ± SD for the indicated (n) number of guinea pigs. Different superscripts in the same column indicate significant differences ($P < .05$) among dietary groups. Differences due to dietary fat, vitamin C level, and interaction as determined by 2-way ANOVA; NS, not significant ($P > 0.05$).

*12:0 > 18:2 for plasma total, VLDL + LDL, and apo B concentrations.

†−AA > +AA for TAG, apo B, total, and VLDL + LDL; +AA > −AA for plasma HDL cholesterol concentrations.

Table 8. Plasma LCAT and CETP Activities in Guinea Pigs Fed Diets Rich in 12:0 or 18:2 Fatty Acids With Either Adequate (+AA) or Suboptimal (−AA) Vitamin C

Diet	LCAT (nmol/mL · h)	CETP (nmol/ml · h)
12:0/+AA	18 ± 5 (14)	3.5 ± 1.5 (12)
12:0/−AA	16 ± 6 (16)	4.1 ± 1.6 (15)
18:2/+AA	13 ± 3 (6)	1.6 ± 0.2 (6)
18:2/−AA	20 ± 8 (9)	2.8 ± 0.6 (6)
Statistics		
Fat type*	NS	$P < .05$
AA level†	NS	$P < .05$
Interactions	NS	NS

NOTE. Values are means ± SD for the indicated (n) number of guinea pigs. Different superscripts in the same column indicate significant differences ($P < .05$) among dietary groups. Differences due to dietary fat, vitamin C level, and interaction as determined by 2-way ANOVA; NS, not significant ($P > .05$).

*12:0 > 18:2 for CETP activity.

†−AA > +AA for CETP activity.

The reductions in HDL cholesterol levels observed with low vitamin C intake were more pronounced with intake of the 12:0 fat; feeding the 12:0/−AA diet resulted in a significant reduction (65%) of plasma HDL concentrations in comparison to the 12:0/+AA diet (Table 7).

Plasma Enzymes and Transfer Activities

No significant differences were found in plasma LCAT activities among all dietary groups (Table 8). In contrast, both fat type and vitamin C level affected plasma CETP activity (Table 8). Feeding 12:0/−AA and 18:2/−AA increased CETP activity by 22% and 43% in comparison to 12:0/+AA and 18:2/+AA, respectively.

TAG and Apo B Secretion Rates

TAG and Apo B secretion rates were measured only in animals fed the 18:2 diets. VLDL Plasma TAG concentrations increased linearly over the 8-hour period following Triton injection ($r = .99$) in animals fed both levels of vitamin C (Fig 4). TAG secretion rates were not affected by vitamin C level in

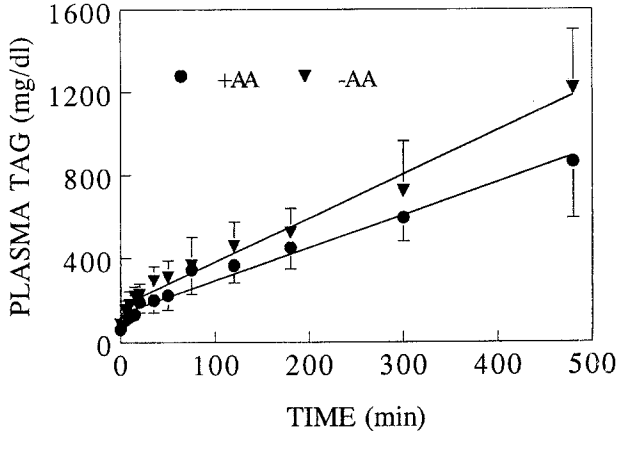

Fig 4. TAG accumulation over time in guinea pigs fed diets rich in 18:2 fatty acids with either adequate (+AA) or suboptimal (−AA) vitamin C (mean ± SD). Each graph represents the mean of ≥5 determinations.

Table 9. TAG and apo B Secretion Rates in Guinea Pigs Fed Diets Rich in 18:2 Fatty Acids With Either Adequate (+AA) or Suboptimal (−AA) Vitamin C

	Secretion Rates (mg/kg · h)	
Diet (n)	TAG	apo B
+AA (6)	42.1 ± 14.0	1.0 ± 0.3[a]
−AA (5)	54.5 ± 13.4	1.9 ± 0.8[b]

NOTE. Values are means ± SD for the (n) number of guinea pigs. Different superscripts in the same column indicate significant differences ($P < .05$) as determined by Mann-Whitney nonparametric t test.

the diet (Table 9), whereas apo B secretion was significantly higher (47%) in animals fed suboptimal vitamin C (Table 9). These data indicate that the vitamin C–deficient animals secreted increased number of VLDL particles into the circulation. Plasma cholesterol concentrations were significantly correlated with VLDL apo B secretion rates, and the best fit was obtained with a log curve ($r = .88$, $P < .01$) (Fig 5). These data suggest that plasma cholesterol levels are in part regulated by the number of particles secreted from the liver into the circulation. Low vitamin C intake resulted in nascent VLDL particles with decreased number of TAG molecules and higher number of total cholesterol molecules ($P < .05$) (Fig 6), indicating that vitamin C–deficient guinea pigs secreted smaller VLDL particles than the control group.

DISCUSSION

Hepatic cholesterol and TAG homeostasis is maintained by a net balance between cholesterol and TAG delivered to the liver from dietary and endogenous sources, as well as rates of biliary cholesterol secretion, catabolism of cholesterol to bile acids, cholesterol efflux in lipoproteins, and rates of TAG synthesis and catabolism. In addition, the liver regulates plasma LDL cholesterol via its function as the site of VLDL synthesis and of LDL catabolism through the apo B/E receptor.[26] The high

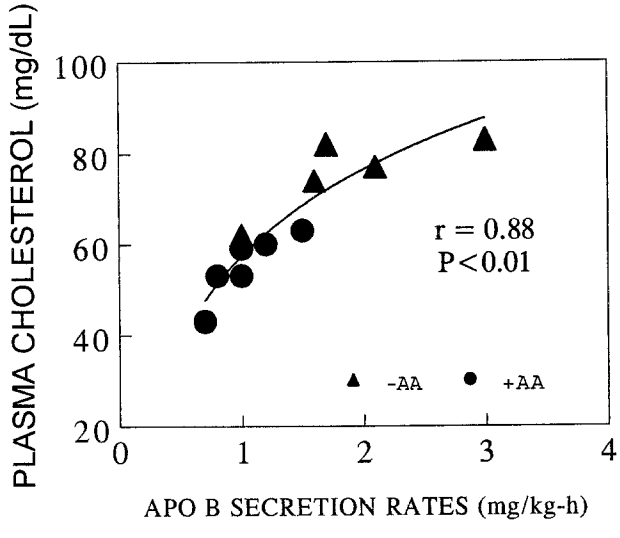

Fig 5. Correlation between apo B secretion rates (mg/kg · h) and plasma cholesterol concentrations (mg/dL) in guinea pigs fed diets rich in 18:2 fatty acids with either adequate (+AA) or suboptimal (−AA) vitamin C. Each point represents an individual guinea pig fed 1 of the 2 levels of vitamin C. The correlation equation is $y = 27.6x^{57.3}$.

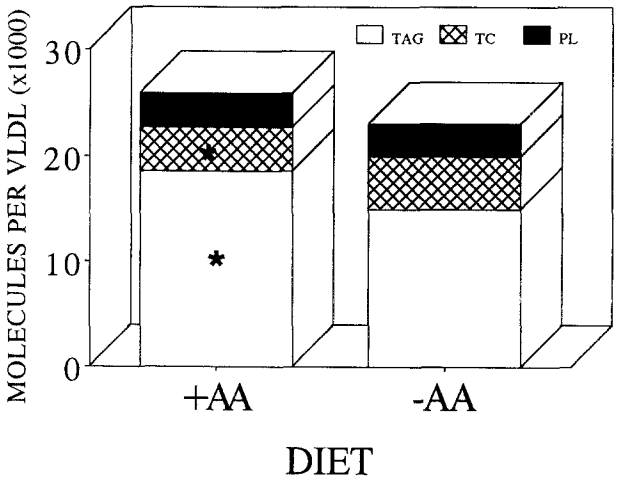

Fig 6. Number of TAG, total cholesterol (TC), and phospholipid (PL) molecules of nascent VLDL isolated from guinea pigs fed diets rich in 18:2 fatty acids with either adequate (+AA) or suboptimal (−AA) vitamin C. The number of TAG molecules was higher ($P < .05$) in the +AA group, while the number of TC molecules in nascent VLDL was higher ($P < .05$) in the −AA group.

hepatic concentrations of TAG and cholesteryl esters observed in vitamin C–deficient guinea pigs indicate major effects of vitamin C status on hepatic TAG and cholesterol metabolism. The accumulation of hepatic cholesterol observed with low vitamin C intake apparently results from reduced catabolism of cholesterol to bile acids associated with lower hepatic cholesterol 7α-hydroxylase activity[3,5,7]; higher concentrations of hepatic TAG in vitamin C–deficient guinea pigs are thought to result from alterations in TAG metabolism due to low carnitine levels.[10] What has not been defined is the contribution of other metabolic parameters in determining hepatic lipid levels and the plasma lipoprotein profile associated with low vitamin C intake. The purpose of the present investigation was to study the effects of adequate and suboptimal intakes of vitamin C, in combination with diets rich in SFA or PUFA, on the regulation of hepatic cholesterol homeostasis and VLDL secretion rates.

Dietary Fat Type, Vitamin C Level, and Hepatic Cholesterol Metabolism

Data indicate that intake of suboptimal vitamin C and saturated 12:0 result in the highest hepatic TAG concentrations, in agreement with our previous report.[5] It appears that both saturated fat and low vitamin C contribute to increased hepatic TAG levels by lowering fatty acid catabolism. Intake of 12:0 could cause accumulation of hepatic TAG, since saturated fatty acids are used predominantly to synthesize TAG, while PUFA preferentially enter the hepatic pathway of β-oxidation.[27] Vitamin C deficiency probably increases hepatic TAG concentrations by decreasing catabolism of fatty acids due to reduction of carnitine synthesis necessary for transport of fatty acids into mitochondria for β-oxidation.[10]

Higher hepatic cholesteryl ester levels were noted in vitamin C–deficient guinea pigs, which suggests that the homeostatic response of the liver to maintain the free cholesterol pool occurs by suppressing cholesterol synthesis and increasing hepatic cholesterol stores, as suggested by the reduction in the active form of HMG-CoA reductase and the increased hepatic ACAT activities in guinea pigs fed suboptimal vitamin C.

Reductions in the active form of HMG-CoA reductase are consistent with previous reports indicating that low vitamin C intake decreases the unphosphorylated form of the enzyme, with no effect on total enzyme activity.[8,9] The absence of effects on total reductase activity suggests that hepatic vitamin C status does not alter the total number of reductase molecules. The mechanism by which vitamin C affects the phosphorylated state of the enzyme is not clear. This regulation could act directly on the reductase molecule, or indirectly by increasing reductase kinase activity, by decreasing reductase phosphatase activity, or a combination of these mechanisms.

Fat type did not affect either total or active reductase, similar to studies in guinea pigs fed saturated lard or PUFA corn oil diets.[5,13,28] In contrast, ACAT activity was upregulated by suboptimal vitamin C intake, probably as a result of increased cholesterol availability for esterification. The hepatic cholesterol available for ACAT would be expanded in vitamin C–deficient guinea pigs because of decreased conversion of cholesterol to bile acids, as suggested by the observed reductions in the activity of hepatic cholesterol 7α-hydroxylase in our previous study.[5] In addition, it is possible that vitamin C also exerts a direct regulatory effect on ACAT through the phosphorylation-dephosphorylation cascade, with the phosphorylated enzyme being active.[4] A significant positive correlation was found between ACAT activity and hepatic cholesteryl ester concentrations, consistent with the regulatory role of ACAT in determining hepatic cholesteryl ester levels.

The finding that hepatic ACAT activity was increased with suboptimal vitamin C intake is of interest, since studies have documented that hepatic ACAT is associated with changes in LDL composition and atherogenicity. Carr et al[29] reported a significant correlation between hepatic ACAT activity and LDL cholesteryl ester content, as well as coronary atherosclerosis in African green monkeys fed various diets.

The present study indicates that both intake of the 12:0 diet and suboptimal vitamin C had an effect in downregulating hepatic apo B/E receptor number. In agreement with the present study, Auslinkas et al[30] reported increases in the number of LDL receptors of cultured aortic smooth muscle cells when supplemented with physiological concentrations of vitamin C. These investigators suggested that vitamin C deficiency might impair the rates of newly synthesized LDL receptor and that the regulation of receptor synthesis by vitamin C may involve activation at either the level of transcription or translation.[30]

The low expression of LDL receptors observed in the 12:0 groups accounts in part for the higher concentrations of plasma total and LDL cholesterol found in these animals and the slower LDL fractional catabolic rate (FCR) values from our previous report.[5] Human studies[31,32] have reported that intake of PUFA versus SFA increases LDL FCR, consistent with the thesis that intake of PUFA increases apo B/E receptor expression. Investigations with experimental animals have demonstrated that intake of PUFA diets compared with SFA intake results in increased expression of hepatic apo B/E receptors.[23,33-36]

In the present study, no changes in VLDL TAG secretion rate were observed in guinea pigs fed the two levels of vitamin C; however, higher secretion rates of VLDL apo B were observed

in animals fed suboptimal vitamin C, as well as higher concentrations of plasma apo B. These data suggest that increased secretion of smaller VLDL particles could contribute in part to the hypertriglyceridemia associated with vitamin C deficiency. Similar findings have been reported in guinea pigs fed diets rich in sucrose compared with those fed starch.[22] Suboptimal intake of vitamin C has also been related to higher conversion rates of VLDL to LDL[5]; thus, the combination of both mechanisms contributes to the higher plasma LDL cholesterol observed in animals fed marginal levels of vitamin C.

Dietary Fat Type, Vitamin C Level, and Plasma Lipoprotein Metabolism

As indicated earlier, the hypertriglyceridemia observed in vitamin C–deficient guinea pigs is probably related in part to an increased secretion of VLDL particles from the liver into the circulation. In addition, decreased removal of TAG from plasma due to changes in lipolytic enzymes may be an important contributing factor. Studies have shown that the higher plasma TAG levels in vitamin C–deficient guinea pigs and baboons are associated with lower tissue LPL activities.[37]

Guinea pigs fed suboptimal vitamin C had higher plasma apo B, TAG, and total and VLDL/LDL cholesterol and lower HDL concentrations compared with animals fed adequate vitamin C. These results are consistent with studies in the guinea pig showing an association between vitamin C deficiency and hypercholesterolemia.[3-5] The results from the present study suggest that the elevated plasma LDL cholesterol levels in vitamin C–deficient guinea pigs are probably related to changes in the synthesis and secretion of VLDL particles and to a decreased receptor mediated clearance of LDL by the liver. Low vitamin C resulted in increased number of small, cholesterol-enriched VLDL particles. There is evidence that small, TAG-poor VLDL particles are directed more efficiently into the LDL pathway.[38] This would suggest that the TAG-poor VLDL particles secreted by vitamin C–deficient animals are converted to LDL, which agrees with our previous report of increased LDL apo B flux in guinea pigs fed suboptimal levels of vitamin C.[5]

In humans, the effect of dietary restriction of vitamin C on lipoprotein metabolism has been examined in just one study,[4] with no consistent effects being observed in plasma cholesterol levels. However, the study lasted for only 1 week and tissue vitamin C levels were not reduced during the test period. Interestingly, vitamin C depletion caused elevation in plasma cholesterol levels in subjects who had the lowest initial vitamin C levels.[4] Due to similarities in cholesterol and lipoprotein metabolism between guinea pigs and humans,[13,32] results of this study are considered relevant to the human situation.

Alterations in the intravascular processing of lipoproteins may contribute to changes in LDL and HDL concentrations associated with low vitamin C intake. Plasma CETP is involved in plasma lipoprotein remodeling in the intravascular compartment and plays essential roles in reverse cholesterol transport.[39,40] Saturated fat and suboptimal intake of vitamin C significantly affected plasma CETP activity. These findings are consistent with the observation that increases in dietary saturated fat are related to increases in CETP activity.[41] The role of CETP in regulating plasma lipoprotein concentrations has been demonstrated in transgenic mice expressing human CETP.[42] The induction of CETP in these animals resulted in lowering (20% to 30%) of HDL cholesterol and marked increases in LDL plus VLDL cholesterol.[43] The present investigation indicates that in guinea pigs fed suboptimal vitamin C, the higher plasma CETP activity could be related to increased transfer of cholesteryl esters from HDL to VLDL, which would explain in part the more elevated levels of VLDL and LDL and the lower concentrations of HDL cholesterol compared with animals fed adequate vitamin C. The mechanisms by which vitamin C deficiency affects CETP activity are not clear. It could be due to higher concentrations of CETP in plasma, which may be influenced by the chemical and physical characteristics of the substrate lipoproteins.[43]

The present study has demonstrated that suboptimal intake of vitamin C results in significant changes in various regulatory parameters of hepatic cholesterol homeostasis and intravascular lipoprotein metabolism associated with alterations in concentration and composition of plasma lipoproteins. In addition, interactions of fat type and vitamin C level appear to have a limited role in the regulation of plasma lipoprotein levels, whereas there are significant independent effects of fat type and vitamin C amount in this regulation.

The cholesterol and lipoprotein changes observed in vitamin C–deficient guinea pigs raise the question whether similar metabolic changes would be found in vitamin C–deficient humans. Since the guinea pig, in addition to requiring vitamin C, has many similarities to humans in cholesterol and lipoprotein metabolism, this animal provides a relevant model to understand the mechanisms by which vitamin C deficiency alters the risk of CVD.

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