# Oxidized Lipids in the Diet Are a Source of Oxidized Lipid in Chylomicrons of Human Serum

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*Abstract* We examined whether oxidized lipids in the diet determine the levels of oxidized lipid in human postprandial serum chylomicrons. After we fed subjects control corn oil containing low quantities of oxidized lipid, the levels of conjugated dienes in the chylomicron fraction were low (9.67±0.92 nmol/μmol triglyceride), and no thiobarbituric acid–reactive substances (TBARS) could be detected. However, when subjects were fed a highly oxidized oil, the conjugated diene content in chylomicrons was increased 4.7-fold to 46±5.63 nmol/μmol triglyceride, with 0.140±0.03 nmol TBARS/μmol triglyceride. When subjects were fed medium-oxidized oil, the degree of oxidation of the chylomicron lipids was moderately increased (21.86±2.03 nmol conjugated dienes/μmol triglyceride). Additionally, we found that chylomicrons isolated after ingestion of oxidized oil were more susceptible to $CuSO_4$ oxidation than chylomicrons isolated after ingestion of the control oil. The lag time for oxidation decreased from 4.30±0.40 to 3.24±0.51 hours ($P<.05$). These data demonstrate that in humans dietary oxidized lipids are absorbed by the small intestine, incorporated into chylomicrons, and appear in the bloodstream, where they contribute to the total body pool of oxidized lipid. (*Arterioscler Thromb.* 1994;14:1900-1905.)

*Key Words* • lipid peroxides • oxidized dietary fat • chylomicrons • oxidized serum lipoproteins

Several observations suggest that oxidized lipoproteins are important in the development of atherosclerosis.[1-3] Incubation of oxidized low-density lipoprotein (LDL) with macrophages leads to formation of foam cells, whereas incubation with native LDL does not. Oxidized LDL is cytotoxic to endothelial cells and therefore could induce arterial wall injury.[4] Finally, oxidized LDL stimulates the release of chemotactic proteins, cytokines, and growth factors from endothelial and other arterial wall cells, promoting the inflammatory milieu that characterizes atherosclerotic plaque.[5-7]

Lipoprotein oxidation is postulated to occur within the microenvironment of the artery wall[1-3]; however, the source of oxidized lipoproteins in the serum of humans[8-10] and animals[11,12] has not been identified. Diet has been suggested as the source of oxidized lipoproteins in the intestinal lymph drainage of rodents.[13,14] Studies in our laboratory have shown that the levels of oxidized lipids in the serum very-low-density lipoprotein (VLDL) plus LDL fraction of rats correlated with the quantity of oxidized lipids in the diet.[15] Moreover, we observed a direct relation between the levels of oxidized lipid in the diet and the amounts of oxidized lipid in mesenteric lymph chylomicrons,[14,15] suggesting that in rats oxidized lipids in the diet are absorbed by the small intestine, packaged in chylomicrons, and transported to the bloodstream. Furthermore, our studies have shown that these oxidized chylomicrons are metabolized similarly to normal chylomicrons, with a substantial portion of oxidized lipids delivered to the liver, where they could potentially be repackaged and secreted in VLDL. Thus, the quantity of oxidized lipids in the diet of rodents has a major effect on the levels of oxidized lipoproteins in the serum. Naruszewicz et al[10] have presented evidence that in humans the ingestion of heat-treated soy oil results in oxidized lipid containing chylomicrons whose uptake and degradation are increased in macrophages.

The hypotheses of the present study were that the amount of oxidized lipid in the diet correlates with the levels of oxidized lipid in human postprandial serum chylomicrons and that the levels of dietary oxidized lipid alter the susceptibility of postprandial chylomicrons to further oxidation.

## Methods

### Characterization of Study Subjects

All subjects (n=6) were male volunteers, 24 to 45 years of age, with serum triglyceride and cholesterol levels within the normal ranges. The average body weight of the subjects was 84.75±8.97 kg; the average age was 33.00±6.68 years. In the fasted state, the serum cholesterol and triglyceride concentrations were 4.90±0.17 and 0.78±0.17 mmol/L, respectively. The HDL cholesterol concentration was 1.18±0.09 mmol/L.

All were nonsmokers, were physically active, and consumed standard American diets. None took excessive amounts of vitamin supplements or any lipid-lowering or hypertension medications.

### Experimental Diets

As test meals, one control and two oxidized corn oils were used. The control oil was conventional corn oil that contained vitamin E (0.14 mg/g oil) and low amounts of oxidized lipid (6.5 to 10 nmol conjugated dienes/mg oil). Oxidized oils were α-tocopherol (vitamin E)–depleted corn oil purchased from

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ICN Biochemicals and contained either medium (30 to 50 nmol conjugated dienes/mg oil) or high (80 to 120 nmol of conjugated dienes/mg oil) levels of oxidized lipids, depending on storage and air exposure time before we added vitamin E. Usually, freshly purchased $\alpha$-tocopherol–depleted oil was used as the medium-oxidized lipid-containing oil, and highly oxidized lipid-containing oil was obtained by air exposure for 6 to 8 weeks. When we examined dietary oils for thiobarbituric acid–reactive substances (TBARS),[12] no significant amount was detected in oils with low-oxidized lipid content. Oil with the medium- and highly oxidized lipid content contained 0.041 and 0.103 nmol TBARS/mg oil, respectively. Direct measurement of peroxides using a color reaction with methylene blue derivative[15] indicated that highly oxidized oil contained 66.0 nmol peroxide/mg oil. We could not detect peroxides in other oils with this method.

Oxidation of dietary oils was further confirmed by demonstrating a reduction of linoleic acid content. Analysis of triglyceride composition showed that the control oil contained 62.45% linoleic acid (18:2) and that oxidized oil containing high levels of peroxides (called highly oxidized oil) contained 57.13% linoleic acid. The oil with medium peroxide concentration (called medium-oxidized oil) contained 59.86% linoleic acid. Thus, as expected, the relative concentration of polyunsaturated linoleic acid decreased in oxidized oils. It should be noted that highly oxidized oil is a relative term compared with the control oil. In absolute terms, the highly oxidized oil used in our experiments is only very mildly oxidized because the oil was not heated and oxidation was achieved by air exposure only. Only approximately 5% of the fatty acids had been degraded in the oxidized oil. To make all ingested oils comparable to the control oil in vitamin E concentration, vitamin E was added to oxidized oils to the same concentration as in the control oil.

## Determination of Oxidation

Conjugated dienes were measured with second-derivative UV spectroscopy in a Perkin Elmer 555 Spectrophotometer as described by Corongiu et al.[16] For conjugated diene determination, lipids from the chylomicron fraction were extracted by the method of Dole.[17] Heptane extracts were scanned for absorbance from 260 to 220 nm, and second-derivative spectra were obtained. Absorbance range selected was ±0.05. Minima at 233 and 242 nm were identified and quantitated in millimeters by measuring the peak from minimum to the adjacent maximum at the higher wavelength on the printout. This method has been shown to be specific for lipid hydroperoxides. Oxidized linoleic acid was used to generate a standard curve,[18] and the extinction coefficient of 32 300 $L \cdot mol^{-1} \cdot cm^{-1}$ was obtained and used to calculate the results. The results were expressed as nanomoles of conjugated dienes per micromole of triglyceride in the chylomicron fraction. The triglyceride concentration in the heptane extracts was measured as described by Fletcher.[19] All measurements were performed on the same day of lipoprotein isolation. Measurement of conjugated dienes for estimating lipid oxidation was chosen not only because of the specificity but also because of its convenience and sensitivity. As little as 1 nmol of conjugated dienes could be measured. This method allows measurement of peroxide levels in our samples not detectable by direct measurements of lipid peroxides.[15] TBARS were measured by the standard procedure as described by Morel and Chisolm.[12] Additionally, oxidation was confirmed by measuring the linoleic acid degradation in gas-liquid chromatography (GLC).[20] Lipid peroxides in the oil with high-oxidized lipid content were determined with a color reaction using methylene blue derivative[15]; however, the method was not sufficiently sensitive to detect peroxides in low- or medium-oxidized lipid-containing oils or any of the chylomicron preparations.

## Study Protocol

Informed consent was obtained from the study subjects, and the study was approved by the Human Studies Committee of the University of California, San Francisco. Lipid peroxidation in the postprandial serum chylomicron fraction was determined by measuring conjugated dienes 4 hours after administration of the test meal. All six subjects were administered all three oils (the control corn oil, medium-oxidized corn oil, and highly oxidized corn oil). The time interval between administration of each test oil was at least 14 days, and the order of test meal administration was randomly assigned.

After a 12-hour fast (time 0), blood was drawn from each subject for measurement of baseline serum triglycerides and cholesterol. Subjects then were given corn oil (1 g/kg body weight), which was accompanied by 100 g carbohydrate (three to four slices of white bread). The subjects were not permitted to consume any food for the duration of the test period. Water was allowed ad libitum. The subjects tolerated the test meal well; no one had diarrhea or other symptoms of malabsorption. At 4 hours, 50-mL blood samples were obtained and conjugated dienes were measured in the chylomicron fraction ($S_f$>1000) to determine the oxidized lipid levels. The results were expressed as nanomoles of conjugated dienes per micromole of triglyceride in the chylomicron fraction. For determining the postprandial clearance of conjugated dienes in serum, four subjects were administered the control oil and highly oxidized oil, and blood samples were obtained every 2 hours over an 8-hour period for oxidized lipid measurements. The results were expressed as conjugated dienes (in nanomoles) in the chylomicron fraction per micromole of triglyceride.

## Chylomicron Isolation

Chylomicron fraction ($S_f$>1000) in the postprandial serum was isolated as described previously for lymph.[21] Briefly, serum samples were overlaid with saline and centrifuged in a swinging bucket rotor (Beckman, SW 40) for 60 minutes at 25 000 rpm at 8°C. The floating chylomicron fraction was removed, layered under saline, recentrifuged, and analyzed immediately for triglycerides, fatty acid composition, and conjugated diene content. When examined by gel electrophoresis,[14] the chylomicron fraction contained a small amount of apolipoprotein B-100, indicating some contamination by large VLDL particles (data not shown).

## Oxidation of Chylomicrons by $CuSO_4$

The susceptibility of chylomicrons to metal oxidation was determined by monitoring the production of conjugated dienes as described by Esterbauer et al.[22] Freshly isolated chylomicrons (0.5 mg triglyceride/mL incubation mixture) were incubated with $CuSO_4$ (final concentration, 0.01 mmol/L) and 0.15 mol/L NaCl containing 0.02 mol/L phosphate buffer, pH 7.2, at 37°C for 8 hours in a total volume of 10 mL. Before incubation, the buffer was purged with oxygen for 30 minutes. Conjugated dienes were measured every 60 minutes in a 1-mL aliquot as described above. Dienes present before addition of copper were subtracted, and the baseline was adjusted to zero. Oxidation rate was expressed as lag time (during which absorption increased minimally) and was determined from an intercept of lines drawn through the linear portions of the lag and propagation phase. During the propagation phase, absorption increased rapidly, indicating the formation of lipid peroxides.[22] The lines representing the propagation phase for each subject were generated by a computer (CA CRICKET GRAPH III) using 5-, 6-, 7-, and 8-hour time points (linear portion of the graph). The data were presented as an average lag time for each diet calculated from the results of four subjects.

## Analytical Methods

Total cholesterol, HDL cholesterol, and triglycerides in serum were determined by enzymatic assays as described.[21]

**Percent Fatty Acid Concentration in Serum Chylomicron Fractions After Ingestion of Oil Containing Low, Medium, or High Quantities of Oxidized Lipid**

| Fatty Acid | Low | Medium | High |
|---|---|---|---|
| 16:0 | 14.66±0.64 | 13.74±0.27 | 17.79±1.79 |
| 18:0 | 2.70±0.45 | 3.13±0.14 | 6.05±0.95 |
| 18:1 | 26.38±0.48 | 28.82±0.44 | 33.31±4.69 |
| 18:2 | 56.27±0.35* | 54.31±0.03 | 41.45±1.45 |

Results are mean±SEM and are derived from three preparations.

*$P<.005$ and $P<.001$ when chylomicrons isolated after ingestion of low-peroxide oil are compared with chylomicrons isolated after ingestion of medium- and high-peroxide oil, respectively.

Vitamin E concentration in oils was measured by high-performance liquid chromatography.[23] Fatty acid composition of oils and chylomicrons was determined by GLC (Hewlett Packard 5890).[20] Fatty acids were transmethylated with boron trifluoride methanol and were analyzed by GLC equipped with a hydrogen flame ionization detector and an SP-2330 fused silica capillary column (Supelco). The retention time and area of each peak were determined by an HP-3390 integrator. A mixture of fatty acids supplied by Matreya, Inc, was used as standard.

Data are presented as mean±SEM. The mean differences in lipid and oxidation measurements between the control and experimental groups were assessed with Student's $t$ test or paired $t$ test. Significance was expressed as $P<.05$.

## Results

### Fatty Acid Composition of the Chylomicron Fraction

The Table shows the fatty acid composition of chylomicrons obtained 4 hours after ingestion of the various diets. The percentage of linoleic acid in the chylomicron fraction after ingestion of the control oil was 56.27±0.35; after ingestion of highly oxidized oil, it was 41.45±1.45% ($P<.001$). Thus, the linoleic acid content in postprandial chylomicrons decreased with decreasing linoleic acid concentration in the dietary oils.

### Incorporation of Dietary Oxidized Lipids Into Postprandial Chylomicron Fractions

It has been established that after the first meal of the day the peak triglyceride response is usually 3 to 5 hours after the meal.[24,25] When subjects were fed corn oil containing increasing amounts of lipid peroxides, TBARS in postprandial serum chylomicrons isolated at 4 hours could be detected only in chylomicrons isolated after ingestion of an oil that contained high amounts of lipid peroxides. These high-peroxide chylomicrons contained 0.140±0.03 nmol TBARS/$\mu$mol triglyceride. On the other hand, conjugated dienes containing polyunsaturated fatty acid hydroperoxides and hydroxy fatty acids accumulate in much greater amounts (60% to 70% of the degraded polyunsaturated fatty acid) and are the major lipid peroxidation products.[26,27]

When we measured conjugated dienes, we found that subjects fed corn oil with increasing amounts of lipid peroxides contained significantly increasing amounts of conjugated dienes in their serum chylomicron fraction (Fig 1). When subjects were fed control oil containing low quantities of oxidized lipid, the levels of conjugated dienes in the chylomicron fraction were low (9.67±0.92 nmol/$\mu$mol triglyceride). When fed medium-oxidized oil with intermediate quantities of peroxides, the subjects had moderately increased degrees of oxidation of chylomicrons (21.86±2.03 nmol/$\mu$mol triglyceride). However, when fed the highly oxidized oil, the subjects' conjugated diene content in chylomicrons was increased 4.7-fold to 46±5.63 nmol/$\mu$mol triglyceride. Thus, the content of oxidized lipid in the diet determined the levels of oxidized lipid in postprandial chylomicrons. Moreover, approximately 4% to 5% of the triglyceride is oxidized on a molar basis (46±5.63 nmol peroxide/1130 nmol triglyceride), further indicating that the oxidized lipid content in chylomicrons is similar to that of the ingested oil.

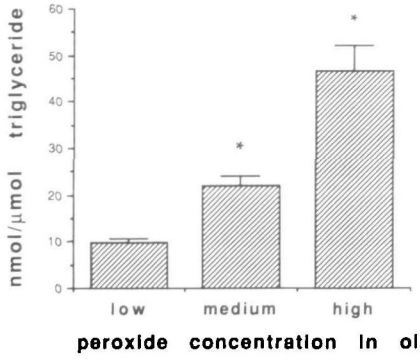


FIG 1. Bar graph shows lipid oxidation in the chylomicron fraction measured as conjugated dienes. Six subjects were administered corn oil containing increasing lipid peroxides. Control, medium-oxidized, and highly oxidized oil contained 6.5 to 10, 30 to 50, and 80 to 120 nmol conjugated dienes/mg oil, respectively. Serum samples were obtained at 4 hours, and the chylomicron fraction was isolated. All data are expressed as nanomoles of conjugated dienes per micromole triglyceride and represent mean±SEM. *$P<.005$ when control group fed control oil was compared with the group fed either medium-oxidized oil or highly oxidized oil.

Fig 2 shows that there was also a significant increase in conjugated dienes in the circulation over an 8-hour time period after ingestion of the oxidized oil compared with the control oil. The levels of conjugated dienes at 2, 4, and 6 hours were relatively similar, with a decrease seen at 8 hours in the high-peroxide-diet group. Thus, oxidized lipids in the diet result in an increase in oxidized lipids in the circulation for extended postprandial periods, and there is a continuous presence of

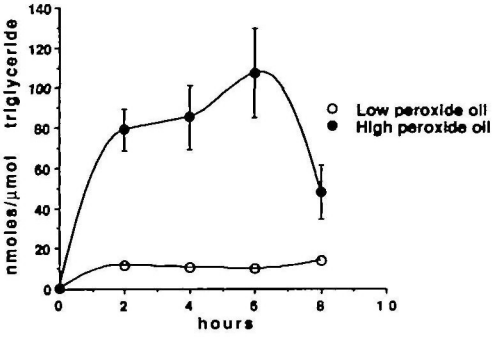


FIG 2. Graph shows profiles of postprandial total conjugated dienes in the chylomicron fraction in four subjects who consumed control (6.5 nmol conjugated dienes/mg oil) and highly oxidized oil (108 nmol conjugated dienes/mg oil). Values are expressed as mean±SEM. $P<.01$ for all time points studied.

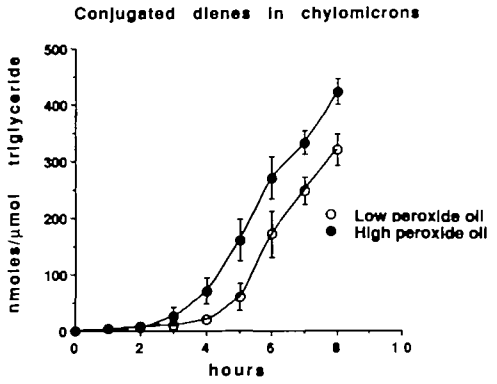


FIG 3. Graph shows the generation of conjugated dienes during copper oxidation of chylomicrons isolated after four subjects were fed oil containing either low- (6.5 nmol conjugated dienes/mg oil) or high- (111 nmol conjugated dienes/mg oil) lipid peroxides. Chylomicrons (0.5 mg triglyceride) were incubated in phosphate-buffered saline, pH 7.2, containing 0.01 mol/L $CuSO_4$ at 37°C. Reaction mixture (1 mL) was removed every 60 minutes and extracted, and the conjugated dienes were measured. The values at time 0 were subtracted from all measurements. Values are expressed as mean±SEM.

possibly atherogenic oxidized lipids in the blood after a meal containing lipid peroxides.

### Oxidation of Chylomicrons by $CuSO_4$

In all four individuals studied, the lag time before onset of oxidation was shorter for chylomicrons isolated after ingestion of an oil containing high levels of lipid peroxides than for chylomicrons isolated after ingestion of the control oil containing low lipid peroxides (3.24±0.51 versus 4.30±0.40 hours) ($P<.05$, paired $t$ test) (Fig 3). There was no significant increase in conjugated dienes when parallel samples were incubated without $CuSO_4$. Thus, oxidized dietary oil results in chylomicrons that not only contain increased lipid peroxides but also are more susceptible to further oxidation. This increased susceptibility to oxidation cannot be explained by a higher linoleic acid content (Table).

## Discussion

The present study was undertaken to determine whether in humans oxidized lipids in the diet are also absorbed and incorporated into postprandial serum lipoproteins. When we fed human subjects a test meal containing low amounts of oxidized lipid (6.5 to 10.0 nmol conjugated dienes/mg oil), we found that the serum chylomicrons contained very low amounts of oxidized lipid. In contrast, when the same subjects were fed a test meal containing large quantities of oxidized lipids (80 to 120 nmol conjugated dienes/mg oil), the levels of oxidized lipids in their serum chylomicrons were increased approximately fivefold (Figs 1 and 2). These results, along with the studies of Naruszewicz et al,[10] indicate that in humans oxidized lipids in the diet are absorbed by the small intestine and are transported in chylomicrons to the circulation, where they can contribute to the total body pool of oxidized lipid.

The presence of oxidized lipids in chylomicrons may have adverse consequences. Because of their large size, chylomicrons are not atherogenic, but chylomicron remnants have been implicated repeatedly in the atherogenic process. Zilversmit[28] has proposed that intestinally derived lipoproteins can contribute to atherosclerosis, and experimental animal studies have shown that remnants from triglyceride-rich lipoproteins deposit lipid in the arterial wall.[29] Moreover, in humans, familial dysbetalipoproteinemia, which is characterized by an increase in remnant particles of both hepatic and intestinal origin, is associated with premature atherosclerosis.[30] It was also shown that patients with coronary artery disease have a delayed clearance of postprandial lipoproteins from their serum.[31] *In vitro studies demonstrated that chylomicron* remnants are capable of promoting cholesterol esterification and cholesterol ester storage in macrophages[32] and smooth muscle cells[33] and that in macrophages the intestinally derived remnants were much more potent than hepatic-derived remnants in inducing foam cell formation.[34] Moreover, oxidation of chylomicron remnants has been shown to enhance the delivery of cholesterol to these cells.[10,35,36]

Additionally, we now demonstrate that partially oxidized chylomicrons are more susceptible to further oxidation. This increased susceptibility could be due to decreases in antioxidant content secondary to the presence of oxidized lipids. Alternatively, the presence of oxidized lipid per se may allow accelerated further oxidation, as suggested by several investigators.[37] Of course, it must be recognized that increased susceptibility to copper oxidation may not necessarily correlate with increased potential for oxidation in vivo. The mechanism by which chylomicrons and other lipoproteins are oxidized in vivo is not clear.

A further adverse consequence of the absorption of oxidized lipids is that the levels of oxidized lipids in endogenous lipoproteins may be increased. In our studies in rats, we observed that the levels of oxidized lipid in VLDL+LDL directly correlated with the quantity of oxidized lipid in the diet.[15] Additionally, studies in humans by Reaven et al[38] showed that the fatty acid composition of the diet determines the fatty acid composition of LDL. Whether oxidized lipids in the diet increase the levels of oxidized lipids in the endogenous lipoproteins of humans remains to be determined.

A limited amount of data in the literature suggest that oxidized lipids in the diet may be not only toxic[39] but also atherogenic.[40] Early studies by Kritchevsky et al[41] showed that diets containing polyunsaturated fatty acids heated for 20 minutes at 215°C are more atherogenic in rabbits than diets containing unheated oil. Moreover, similar experiments with olive oil, which contains monounsaturated fatty acids that are not oxidized, did not demonstrate an increase in atherosclerosis with heated oil. These results suggest that oxidized lipids induced by heating of polyunsaturated fatty acids are atherogenic. Unfortunately, these studies were carried out before the potential importance of oxidized lipids was recognized, so the oxidative state of the oils, serum lipoproteins, and lipid in the atherosclerotic lesions were not measured. Some studies also showed that adding fish oils to a cholesterol-rich diet enhances atherosclerosis in rabbits.[42] Since fish oils are highly susceptible to oxidation, oxidized lipids could account for this observation, but again no measurements to determine the state of oxidation were made. Finally, Blankenhorn et al,[43] using dietary recall, reported that development of new coronary artery lesions was increased in patients with a high

consumption of not only saturated but also polyunsaturated fat. It is possible that dietary oxidized lipid contributed to the harmful effects of polyunsaturated fats in this study, but again no data directly addressing the issue of fatty acid oxidation were provided.

The typical American diet contains substantial quantities of polyunsaturated fatty acids that have been subjected to various degrees of processing and heat treatment, particularly deep fat frying, which is known to lead to oxidation. Frankel et al[44] examined the relative percent of oxidation products in frying oils used in fast food and found that the majority of these foods contain oxidized lipids. For example, french-fried potatoes contain up to 8.2% of oxidized material. Alexander[45] showed that oils used in many restaurants are kept at 180°C for extended time periods, which results in the appearance of oxidized lipids. Thompson et al[46] also performed oxidized lipid measurements and showed that oils used for deep-frying in restaurants, institutions, and armed services contained large quantities of oxidized lipids. Finally, Yagi et al[47] measured peroxides in 30 kinds of foods and found that the peroxide content could be as high as 600 nmoles/g food. Thus, there is abundant evidence that oxidized lipids are frequently present in the typical American diet in both commercial and domestic settings. Furthermore, the quantity of oxidized lipids reported in these studies is higher than the levels of oxidized lipids used in our experiments.

In summary, our studies demonstrate that in humans dietary oxidized lipids are absorbed by the small intestine, incorporated into chylomicrons, and appear in the bloodstream, where they contribute to the total body pool of oxidized lipid. Thus, dietary oxidized lipids could potentially contribute to the development of atherosclerosis.

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