Combined Effects of Lipid Transfers and Lipolysis on Gradient Gel Patterns of Human Plasma LDL

Laurent Lagrost, Philippe Gambert, Christian Lallemant

Abstract The triglyceride content of the plasma very-low-density lipoprotein (VLDL) fraction is the most important factor affecting the size of low-density lipoprotein (LDL) in humans. Because cholesteryl ester transfer protein (CETP) can influence the size distribution of LDL particles in human plasma, the implication of lipid transfers in the formation of small-sized LDL patterns, which have been associated with elevated plasma triglyceride levels, was investigated. The size distribution of LDL particles in 15 plasma samples was determined by electrophoresis of the plasma LDL fraction on 20 to 160 g/L polyacrylamide gradient gels. The apparent diameter of the major LDL subfraction was shown to correlate negatively with triglyceride concentrations \((r = -0.706, P < 0.005)\) and positively with both high-density lipoprotein cholesterol levels \((r = 0.637, P < 0.02)\) and the high-density lipoprotein/VLDL + LDL cholesterol ratio \((r = 0.768, P < 0.001)\). In addition, LDL size correlated negatively with both the ability of plasma LDL to donate choleseryl esters \((r = -0.79, P < 0.001)\) and its ability to acquire triglycerides \((r = -0.72, P < 0.005)\). Whereas these observations indicated that CETP-mediated alterations of the triglyceride/cholesteryl ester ratio of the LDL core would contribute to changes in LDL diameter, they suggested that the formation of small-sized gradient gel LDL patterns would require another biochemical event, such as lipolysis, in addition to neutral lipid transfers. To test this hypothesis, total plasma samples with or without added VLDL (added triglyceride concentration, 2.0 g/L) were preincubated for 24 hours at 37°C. Preincubation mainly induced the replacement of triglycerides in the LDL core, and changes in LDL composition were greater when total plasma was supplemented with VLDL. Subsequently, isolated LDL was incubated in the presence of bovine milk lipoprotein lipase as a source of triglyceride hydrolysis activity. Lipolysis tended to reduce the size of the major LDL subpopulation, and the mean change in LDL diameter was significantly greater when plasma was preincubated with VLDL supplementation than when it was not \((-0.6 \pm 0.3 \text{ versus } -0.2 \pm 0.2 \text{ nm, respectively; } P < 0.01)\). Moreover, sequential effects of lipid transfer and lipolysis activities induced dramatic changes in the general shape of gradient gel LDL patterns. The largest plasma LDL subpopulations tended to disappear, and the formation of new, small LDL particles could be observed. The combined effects of neutral lipid transfers and triglyceride hydrolysis could account for variations of gradient gel LDL profiles in human plasma. In particular, these biochemical processes could favor the formation of the small-sized LDL patterns that are associated with an increased risk for coronary artery disease. (Arterioscler Thromb. 1994;14:1327-1336.)

Key Words • LDL • lipids transfers • lipolysis • polyacrylamide gradient gel electrophoresis
CETP activity. As observed in vitro, the CETP-mediated size redistribution of plasma LDL particles is characterized mainly by a shift of the small particles toward the largest LDL subpopulation. In contrast, the appearance of newly formed LDL subpopulations of smaller size has not been observed, suggesting that CETP may not be the sole factor responsible for alterations in plasma LDL distribution profiles. Thus, formation of small-LDL patterns in human plasma might also relate to other enzyme activities such as lipolysis, which in vitro reduces the size of isolated lipoprotein particles.

In the present study, the implication of neutral lipid transfers in the formation of small LDL particles, which in vivo are associated with high plasma triglyceride levels, was investigated by incubating total human plasma in the presence or absence of increasing concentrations of isolated VLDL particles. In some experiments LDL particles were incubated in the presence of bovine milk lipoprotein lipase (LPL) as a source of triglyceride hydrolysis activity. Analysis of resulting LDL patterns by polyacrylamide gradient gel electrophoresis (PAGGE) suggested that the combined effects of neutral lipid transfers and lipolysis could account for variations in LDL patterns among various plasma samples.

Methods

Subjects
Fifteen subjects (11 women and 4 men; age range, 24 through 50 years; plasma cholesterol concentrations, 151 to 274 mg/dL; and plasma triglyceride concentrations, 47 to 265 mg/dL) were selected for the study. With the exception of four women who took oral contraceptives, the subjects were not taking medications.

Blood Samples
Venous blood from all subjects was collected after a 12-hour overnight fast. Blood samples were drawn into EDTA-containing glass tubes, and plasma was promptly separated by a glass tubes, and plasma was promptly separated by a centrifugal analyzer (Roche). Protein concentrations were measured by using bicinchoninic acid reagent (Pierce) and albumin, HDL and triglyceride concentrations were measured by using Boehringer reagents. Plasma HDL-C concentrations were measured after selective precipitation of apolipoprotein (apo) B-containing lipoproteins with Boehringer phosphotungstic acid/MgCl₂ reagent as recommended by the manufacturer. Plasma VLDL+LDL cholesterol concentrations were obtained by difference.

Lipoprotein Preparation
Lipoprotein fractions were isolated from total plasma by sequential ultracentrifugation. Details of the isolation procedure and definition of various lipoprotein fractions should be given in the Methods section. The apparent diameters of the separated LDL subfractions were determined by comparison with ferritin (diameter, 12.20 nm), thyroglobulin (diameter, 17.00 nm), thyroglobulin (diameter, 17.00 nm), and carboxylated latex beads (diameter, 38.00 nm; Duke Scientific) that were subjected to electrophoresis with the samples. The reproducibility of the gel analysis of LDL size was evaluated by analyzing the same LDL preparation eight times on the same gel and by running eight different gels independently. Standard deviations of mean LDL diameter (26.50 nm) within the same gel and between runs were 0.06 and 0.19 nm, respectively. Throughout the study different experimental conditions applied to the same plasma sample were analyzed on the same gel.

Enrichment of LDL With Triglycerides
Human LDL was progressively enriched with triglycerides by supplementing total plasma with various amounts of ultracentrifugally isolated VLDL. The final triglyceride concentration of added VLDL ranged from 0 to 2.0 g/L. Subsequently, mixtures were incubated for 24 hours at 37°C to allow the substitution of triglycerides for cholesteryl esters in LDL particles through the CETP-mediated neutral lipid transfer reaction (see "Results"). To block lecithin:cholesterol acyltransferase activity, all plasma samples contained 1.5 mmol/L iodoacetate.

Electrophoretic Separation of LDL Subfractions
LDL was separated by electrophoresis on nondenaturing gradient gels ranging from 20 to 160 g/L polyacrylamide (PAA 2202 Ultroscan laser densitometer (LKB) attached to a 2202 integrator (LKB)) that directly indicated the position of the predominant LDL peak.
TABLE 1. Characteristics of Subjects

<table>
<thead>
<tr>
<th>Subject</th>
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<th>Gender</th>
<th>Plasma Triglycerides, mg/dl</th>
<th>Cholesterol, mg/dl</th>
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<td></td>
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<tr>
<td>SD</td>
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<td>18</td>
<td>33</td>
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</table>

HDL indicates high-density lipoprotein; VLDL, very-low-density lipoprotein; and LDL, low-density lipoprotein.

*The apparent diameter of the major LDL subtraction is presented.

Correlation of Plasma LDL Size With Neutral Lipid Transfers

To determine whether plasma LDL size could relate to its ability to exchange cholesteryl esters for triglycerides, the apparent diameter of the major LDL subtraction in native plasma samples was compared with changes in the LDL cholesteryl ester/protein ratio and the LDL triglyceride/protein ratio induced by the incubation of total plasma for 24 hours at 37°C.

LDL size correlated positively with changes in the LDL cholesteryl ester/protein ratio (r=.79, P<.001 using regression analysis; ρ=.60, P<.03 using Spearman rank correlation analysis) and negatively with changes in the LDL triglyceride/protein ratio (r=-.72, P<.005 using regression analysis; ρ=-.44, P<NS using Spearman rank correlation analysis) (Fig 1). These observations suggested that an elevated capacity of plasma LDL to acquire triglycerides and to donate cholesteryl esters would be associated with a preponderance of small-LDL subpopulations in human plasma.

Effect of Lipid Transfers Between VLDL and LDL on the Size and Composition of LDL Particles

To investigate the implication of lipid transfers between triglyceride-rich lipoproteins and LDL in determining plasma LDL pattern, total human plasma containing a lecithin:cholesterol acyltransferase inhibitor (iodoacetate 1.5 mmol/L) was incubated for 24 hours at 37°C in the absence or presence of various concentrations of VLDL (added triglyceride concentrations ranged from 0.5 to 2.0 g/L). At the end of the incubation, plasma LDL patterns were determined by gradient gel electrophoresis of the 1.019<rf< 1.063 g/mL plasma fraction.

Incubation of total plasma tended to induce the transformation of polydisperse patterns into monodisperse profiles<sup>30</sup> (Fig 2). This phenomenon was characterized by a decrease in the relative abundance of discrete, minor LDL subpopulations. When plasma was supplemented with increasing concentrations of VLDL before incubation for 24 hours at 37°C, the general shape of the LDL
distribution profile was not affected further (Fig 2). However, with the highest VLDL concentration studied (added triglyceride concentration, 2.0 g/L) a slight but significant increase in the mean size of the major LDL peak was observed compared with the peaks observed in both 4°C control plasma and incubated plasma without VLDL supplementation (Table 3).

Changes in LDL size were accompanied by alterations in composition of the particles. Incubation of total plasma induced a significant decrease of the unesterified cholesterol/protein and cholesteryl ester/protein ratios in LDL particles with a concomitant increase in the triglyceride/protein ratio. These changes in LDL composition were greater when total plasma was supplemented with VLDL (added triglyceride concentration, 2.0 g/L) (Table 3). No significant differences in the mean size of the major LDL subpopulation between the Control and Transfer groups were observed, suggesting that changes in lipid composition of LDL particles as induced by plasma incubation without VLDL supplementation were not high enough to induce detectable variations in LDL diameter (Table 3).

Significant relationships between changes in size and the lipid/protein ratio in LDL particles were observed. Indeed, changes in LDL size correlated positively with changes in the triglyceride/protein ratio but negatively with changes in the cholesteryl ester/protein ratio (r = 0.57, $P<0.001$ and $r = -0.55, P=0.0015$, respectively). By contrast, no significant correlations were observed with changes in either the unesterified cholesterol/protein, phospholipid/protein, or total lipid/protein ratios (Fig 3).

Whereas the results described above indicate that the replacement of cholesteryl esters by triglycerides in the LDL core may be an important process in determining the size and distribution of plasma LDL, they alone cannot account for the negative correlation between the size of the major LDL subfraction and plasma triglyceride levels that has been observed in population studies.

### Concentration-Dependent Effect of LPL on LDL Patterns

To investigate the implication of triglyceride hydrolysis in changing plasma LDL patterns, the concentration-dependent effect of bovine milk LPL on the size distribution of either native or triglyceride-enriched LDL particles was studied. To this end, LDL particles were isolated from...
plasma samples that were (Transfer+VLDL samples) or were not (Control samples) preincubated in the presence of VLDL (added triglyceride concentration, 2.0 g/L). Isolated LDL was then incubated for 1 hour at 37°C in the presence of increasing concentrations of bovine milk LPL as a source of triglyceride hydrolysis activity. LPL did not induce marked changes in the size distribution of native LDL that was isolated from normolipidemic plasma (Fig 4). In contrast, when LDL was isolated from plasma preincubated in the presence of VLDL, LPL induced a concentration-dependent shift of the gradient gel distribution profile of triglyceride-enriched LDL toward subpopulations of a smaller size (Fig 4). Thus, the LPL-mediated reduction of LDL size would require as a first step the substitution of triglycerides for cholesteryl esters through neutral lipid transfer reactions.

Combined Effects of Neutral Lipid Transfers and Lipolysis on LDL Patterns

To further investigate the possible interaction of neutral lipid transfers and lipolysis in the transformation of plasma LDL patterns, total plasma with or without VLDL (added triglyceride concentration, 2.0 g/L) was preincubated for 24 hours at 37°C. Subsequently, isolated LDL was incubated for 2 hours at 37°C in the presence of bovine milk LPL (final concentration, 6.2 μg/mL) as a source of triglyceride hydrolysis activity. Finally, the mean apparent diameter of the major LDL subpopulation was determined by PAGE of the LDL fraction.

With native LDL (Control samples), the sole effect (if any) of LPL was characterized by a slight decrease in the mean apparent diameter of the major LDL subpopulation. Indeed, in eight plasma samples studied, the mean variation in lipolyzed compared with nonlipolyzed samples (Δ[−LPL]−[+LPL]) was a 0.2-nm reduction in the mean apparent diameter of LDL (Table 4). When LDL was obtained from preincubated plasma samples without VLDL supplementation (Transfer samples), LPL decreased the LDL diameter in all samples studied; in those instances, the mean reduction of LDL size was nonsignificantly greater compared with control samples (0.45 versus 0.20 nm, respectively) (Table 4). When plasma samples were preincubated in the presence of VLDL (Transfer+VLDL samples), LPL induced a significantly greater reduction in LDL size compared with Control homologues (−0.60 versus −0.20 nm, respectively; P<.01) (Table 4).
Table 4. Effect of Lipolysis and Lipid Transfers on the Size of the Major LDL Subpopulation

<table>
<thead>
<tr>
<th>Subject</th>
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<th>Transfer</th>
<th>Transfer+VLDL</th>
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<tr>
<td>Mean±SD</td>
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<td>25.8</td>
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<td>23.5</td>
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<td>15</td>
<td>25.6</td>
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</table>

Whereas the combined effects of lipid transfers and lipolysis induced consistent changes among the various plasma samples studied, some variations could be observed according to the starting plasma LDL pattern. Fig 5 represents two series of LDL profiles obtained either from a normolipidemic plasma that initially showed a typical LDL pattern A (subject 11) or from a moderately hypertriglyceridemic plasma that initially showed a typical LDL pattern B (subject 14). Whereas in vitro lipolysis of LDL isolated from nonincubated plasma (Control+LPL profiles) reduced only slightly the mean apparent diameter of the major but not the minor LDL subpopulation of pattern A, it more dramatically reduced the apparent diameter of each of the three LDL subpopulations of pattern B. However, in both cases, the general shape of LDL distribution profiles remained virtually the same.

Incubation of total plasma from subject 11 (Transfer profile) induced only minor alterations in LDL pattern A; the smallest LDL subpopulation (24.4 nm diameter) tended to shift toward the largest one (Fig 5). More pronounced transformations were observed in LDL pattern B, since incubation of total plasma from subject 14 (Transfer profile) induced the redistribu-
of the somewhat different effects of plasma incubation on the LDL phospholipid/protein ratio is unclear and might relate to interindividual variations in the exchangeable phospholipid pool, a parameter that we did not investigate. Incubation of LDL in the presence of LPL was characterized mainly by a significant reduction of the triglyceride/protein ratio, whereas the unesterified cholesterol and cholesteryl ester contents remained unchanged (Table 5). LPL also slightly reduced the phospholipid content of LDL particles compared with nonlipolyzed homologues (Table 5). The ability of LPL to hydrolyze LDL triglycerides appeared to be dependent on the triglyceride content of LDL particles. Indeed, compared with nonlipolyzed homologues, the mean reduction of the LDL triglyceride/protein ratio was 0.06, 0.27, and 0.48 in LDL isolated from Control, Transfer, and Transfer+VLDL plasma samples, respectively.

### Discussion

Results from the present study revealed that in human plasma, both lipid transfer and triglyceride hydrolysis may constitute two key events in the transformation of gradient gel LDL patterns. Whereas lipid transfer activity or lipolysis alone promoted only minor changes in the mean size of the major plasma LDL subpopulation, their combined effects induced the formation of small LDL particles. The resulting LDL patterns presented the main features of LDL pattern B, which is associated with an increased risk for coronary artery disease.12

In earlier in vitro studies, Deckelbaum and coworkers27 proposed a general model for the size reduction of isolated LDL particles involving triglyceride hydrolysis in addition to neutral lipid transfers. Moreover, recent data indicate that the presence of small LDL particles in human plasma could be related to the efficiency of the lipolytic system.34-37 In vitro changes in the size of LDL particles as induced during the incubation of isolated LDL isolated from preincubated plasmas, both lipid transfer and triglyceride hydrolysis may constitute two key events in the transformation of gradient gel LDL patterns.

### Combined Effects of Neutral Lipid Transfers and Lipolysis on LDL Composition

Changes in LDL size were accompanied by changes in the composition of LDL particles (Table 5). Indeed, incubation of total plasma for 24 hours at 37°C with or without VLDL supplementation induced a significant decrease in the LDL cholesteryl ester/protein ratio but a significant increase in the triglyceride/protein ratio. A slight reduction in unesterified cholesterol and phospholipid contents of LDL particles could also be observed (Table 5). Since the data presented in Tables 3 and 5 were obtained under similar experimental conditions, the cause of the somewhat different effects of plasma incubation on the LDL phospholipid/protein ratio is unclear and might relate to interindividual variations in the exchangeable phospholipid pool, a parameter that we did not investigate. Incubation of LDL in the presence of LPL was characterized mainly by a significant reduction of the triglyceride/protein ratio, whereas the unesterified cholesterol and cholesteryl ester contents remained unchanged (Table 5). LPL also slightly reduced the phospholipid content of LDL particles compared with nonlipolyzed homologues (Table 5). The ability of LPL to hydrolyze LDL triglycerides appeared to be dependent on the triglyceride content of LDL particles. Indeed, compared with nonlipolyzed homologues, the mean reduction of the LDL triglyceride/protein ratio was 0.06, 0.27, and 0.48 in LDL isolated from Control, Transfer, and Transfer+VLDL plasma samples, respectively.

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lipoprotein fractions with a crude source of lipid transfer activity and purified LPL have been investigated by using electron microscopy and rate zonal ultracentrifugation. 25 Since these two techniques do not allow the investigation of the specific aspects of plasma LDL heterogeneity that can be observed by using PAGGE, 26 in the present study we investigated the combined effects of lipid transfers and lipolysis on the gradient gel electrophoretic patterns of the plasma LDL fraction.

The mean apparent diameter of the major LDL subfraction in human plasma in the population studied, as determined by laser densitometric scanning of polyacrylamide gradient gels, correlated negatively with triglyceride levels but positively with HDL-C levels. These observations agree with previous data that show that the abundance of small LDL particles correlates positively with plasma triglyceride, total cholesterol, VLDL cholesterol, and apoB concentrations but negatively with plasma HDL-C and apoA-I levels. 31 In addition, the preponderance of small LDL particles in human plasma appears to be significantly associated with an increased risk for coronary artery disease. 12-14 However, adjustment for triglyceride concentration reduces the risk ratio of the small-LDL pattern (pattern B) to a nonsignificant level. 12 In fact, the triglyceride content of LDL particles is a predictable function of plasma triglyceride concentration, 39 and the amount of circulating triglycerides is the single most important factor affecting LDL particle size. 24 Whereas a strong, negative correlation between plasma triglyceride levels and LDL size clearly exists, 14, 24 the biochemical mechanism by which triglycerides can affect the gradient gel pattern of the plasma LDL fraction is still not completely understood. Interestingly, in the present study the diameter of the major plasma LDL subfraction correlated positively with the plasma HDL/VLDL + LDL cholesterol ratio, which correlates negatively with plasma CETP activity. 31 These observations suggest, therefore, that CETP-mediated neutral lipid transfers could play a determinant role not only in determining the polydispersity or monodispersity of plasma LDL pattern 26 but also in modifying the mean size of the major LDL subclass in human plasma. This hypothesis was sustained by another finding of the present report, which revealed that the size of the major plasma LDL subfraction correlates negatively with the ability of LDL particles to acquire triglyceride and to donate cholesteryl esters during the incubation of total plasma. In human plasma, VLDL concentrations can influence the CETP-mediated lipid transfer process; Mann and coworkers 40 report a threefold increase in lipid transfers between plasma LDL + HDL and VLDL fractions in hypertriglyceridemic subjects compared with normolipidemic control subjects. In hypertriglyceridemia, increased neutral lipid exchanges can result in the replacement of cholesteryl ester by triglyceride as the predominant neutral lipid component in the LDL core. 41 When circulating triglyceride levels are lowered, the composition of hypertriglyceridemic LDL can be normalized as the result of a reduction of the rate of transfer of neutral lipids between the VLDL and HL + HDL fractions. 13, 25

To study the implication of lipid transfers in modulating the size distribution of plasma LDL particles, total human plasma was incubated for 24 hours at 37°C. In a parallel series of experiments, plasma was supplemented with VLDL particles, which served both as a donor for triglycerides to LDL and as an acceptor for cholesteryl esters that leave LDL. We observed 26, 28 that incubation of total human plasma induced alterations of both the distribution profile and composition of plasma LDL particles. However, lipid transfer reactions, which promoted enrichment of the LDL core with triglycerides, did not promote the formation of the small-LDL subpopulations that have been observed in hypertriglyceridemic states. 42-43 On the contrary, replacement of cholesteryl esters by triglycerides in LDL particles tended to promote the enlargement of LDL particles, which probably reflected the greater volume of triglyceride molecules compared with cholesteryl ester molecules. 42 Therefore, these data indicated that the association of small LDL particles with elevated plasma triglyceride concentrations would involve more than lipid transfer reactions and suggested that an additional event was required to allow the shift of large LDL toward particles of smaller size.

In postheparin plasma, two distinct triglyceride lipases have been identified: LPL, which originates from extracellular tissues, and hepatic lipase (HL), which originates from the liver. 43 Whereas both lipases are able to interact with various plasma lipoprotein fractions, 44 HL has an increasing activity with lipoproteins of decreasing size, 45, 46 and VLDL appears to be a better substrate than LDL for LPL. 44 However, despite differences in the substrate specificity of LPL and HL, in vitro studies reveal that the two enzymes induce very similar alterations in LDL composition that are characterized mainly by a substantial reduction in the core triglyceride content. 45 In the present study, we chose to hydrolyze triglycerides in LDL particles by using a commercially available preparation of purified bovine milk LPL that has structural and functional similarities to human LPL. 46, 47 When plasma LDL was incubated with LPL, the hydrolysis of core triglycerides was accompanied by a concomitant reduction of the size of the major LDL subfraction. This observation was consistent with previous data that demonstrate that the removal of triglycerides from the large, triglyceride-rich LDL subfractions as mediated by either LPL 25 or HL 46 induces their conversion toward LDL subpopulations of smaller size. In the present study the LPL-mediated reduction of LDL size may be accompanied by a small decrease in the LDL phospholipid/protein ratio, which supports the observation by Barter and coworkers 48 that the molar phospholipid/apoB ratio is an important parameter in determining the size of LDL particles. The sequential effects of lipid transfer and lipolysis activities promoted dramatic changes not only in the mean size of plasma LDL but also in the general shape of their gradient gel distribution profiles. In particular, when the effects of lipid transfers and lipolysis were combined, the conversion of LDL pattern A into LDL pattern B could be observed. Therefore, we postulate that in vivo CETP and triglyceride lipases could have synergistic effects in favoring the formation of the potentially atherogenic LDL pattern B. Plasma neutral lipid transfers, which are dependent on plasma VLDL concentrations rather than on CETP mass, 40 might constitute a limiting step in the transformation of the plasma LDL pattern, since in our experiments LPL did not induce marked changes in the distri-
bution of normolipidemic LDL so long as the particles were not enriched with triglycerides. In addition, triglyceride lipases could affect CETP activity indirectly, since nonesterified fatty acids, one of the end products of triglyceride hydrolysis, enhance the CETP-mediated neutral lipid transfer process.50-52

In conclusion, the results of the present study indicated that the combined effects of neutral lipid transfers and triglyceride hydrolysis could account for variations of gradient gel LDL patterns in human plasma. Alterations were primarily characterized by a shift of the large LDL particles toward subpopulations of smaller size. Lipid transfers and lipolysis could have synergistic effects in favoring the conversion of LDL pattern A into the proatherogenic LDL pattern B.

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32. Newhizens HH, Brinton PJ. Synergistic effects of lipids transfer and hepatic lipase in the formation of very small high-density lipoproteins during incubation of human plasma. Biochim Biophys Acta. 1990;1044:57-64.


Abstract.


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