Clinical Significance of Lipoprotein Size and Risk for Coronary Atherosclerosis

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Correlation between coronary heart disease and lipoprotein size and composition is well documented. Within the low-density lipoprotein (LDL) family the small LDL particles are associated with increased risk of coronary heart disease. These particles also have increased apolipoprotein (apo) B content. The appearance of these small LDL particles is the manifestation of complex alteration of plasma lipoprotein metabolism. The LDL size is influenced by genetic, endocrine, and environmental factors. Within the high-density lipoprotein (HDL) family the decrease of larger HDL₂ particles is associated with coronary heart disease. HDLs can also be separated according to their apoprotein composition into particles containing lipoprotein (LP)A-I only and particles containing LPa-I and LPa-II. Most studies have shown that the concentration of LPa-I-only particles decreases in coronary heart disease. HDLs are remodeled in the circulation and this remodeling continues in vitro after the blood is taken. Therefore adequate preservation of blood samples is necessary.

Indexing Terms: lipoprotein subfractions/genetic variation/hyperapobetalipoproteinemia/two-dimensional electrophoresis

Atherosclerosis is a multifactorial disease that encompasses both genetic and environmental factors. Numerous epidemiologic studies have shown that lipids/ lipoproteins have a major role in the evolution of atherosclerosis. From these studies we have learned that increased plasma concentrations of low-density lipoproteins (LDLs) are associated with coronary heart disease. Low plasma concentrations of high-density lipoproteins (HDLs), even in the absence of increased LDL concentrations, are also frequently associated with coronary heart disease.

Here we review the possible mechanisms responsible for the atherogenic or anti-atherogenic characteristics of lipoprotein subpopulations. These mechanisms include correlations between coronary heart disease and lipoprotein subpopulations, based not only on their size, density, or both, but also on data obtained by other measurements of lipoprotein subpopulations (1–5).

Low-Density Lipoproteins

LDLs have a hydrated density of 1.019 to 1.063 kg/L and a diameter of 20 to 30 nm. On paper or agarose electrophoresis, they display β mobility (6). The structural integrity of LDL is provided by apolipoprotein B (apoB-100), a 550-kDa glycosylated protein synthesized by the liver, as opposed to apoB-48, a 264-kDa protein synthesized in the intestine. ApoB-48 is present in chylomicrons (7), B-100 in LDL. The major function of LDL is the transport of lipid, mainly cholesterol, from the liver to the periphery. LDLs are removed by the circulation in the liver by the high-affinity LDL receptors. LDLs contain several distinct lipoprotein subpopulations that vary in size, hydrated density, molecular mass, and composition (6, 8).

Using non-denaturing polyacrylamide gel electrophoresis, investigators have identified several LDL subpopulations (6, 8). Two major LDL subclass patterns are usually compared in terms of their association with coronary heart disease. The larger, more buoyant LDL has a peak diameter >25.5 nm; the smaller LDL has a peak diameter <25.5 nm. Austin et al. refer to these as phenotype A and phenotype B, respectively (9), which account for 65% and 25% of the human population. The remaining 10% is intermediate size. In women, the smaller LDL is less prevalent than in men. It increases with age in both sexes (10–14).

There is a general agreement that the smaller LDL (phenotype B) is associated with an increased risk of coronary heart disease (6, 8, 11, 15). Phenotype B is usually accompanied with increased plasma triglyceride, decreased HDL, and decreased apoA-I concentrations and with insulin resistance. A threefold relative risk of myocardial infarction of subjects with a phenotype B pattern has been reported in both men and women (11). Sniderman et al. (16) identified subjects with small, dense LDL particles enriched in apoB and depleted in cholesteryl esters. The presence of these LDL particles is strongly associated with premature coronary heart disease (16, 17), a condition referred to as hyperapobetalipoproteinemia. Most likely, phenotype B and hyperapobetalipoproteinemia refer to similar lipoprotein subpopulations.

The conditions that influence LDL subclass patterns are as follows: Populations consuming a low-fat, high-carbohydrate diet have a greater prevalence of the smaller LDL particles (18). This prevalence is also increased in obesity, especially in abdominal obesity (19). Hyper tension, diabetes, and smoking are associated with the presence of the smaller LDL particles. Observations in several studies were complicated by the use of
Mechanisms possibly contributing to the atherogenicity of the smaller LDL are: decreased clearance of smaller LDL in vivo; increased oxidizability; decreased binding to LDL receptors; and decreased neutral carbohydrate content, increasing the binding to proteoglycans. The smaller particles are cleared from the circulation in vivo more slowly than the larger, lighter LDL both in normal persons and those with hyperapobetalipoproteinemia (20). Thus, the smaller LDL has longer residence time in the circulation, where it could be subject to oxidation longer; it is also more easily oxidized in vitro (21–23). When the LDL is oxidized, it is not removed well by the LDL receptor, but rather by way of the scavenger pathway, which may provide the basis for the formation of plaques. Consistent with these explanations is the finding of decreased binding of smaller LDL to the high-affinity LDL receptor (24).

The atherogenicity of oxidized LDL is supported by the findings that it increases the concentration of cholesteryl esters in macrophages by direct deposition of lipoprotein-derived cholesteryl esters and by the stimulation of cholesterol esterification (25, 26). The deposited lipoproteins are more resistant to lysosomal proteolysis and induce cytokine production, events that may lead to formation of foam cells (26). A significant decrease of neutral carbohydrate content is present in the smaller LDL subfractions (27). The lower carbohydrate content of apoB also could influence its interaction with the high-affinity LDL receptors. Proteoglycans isolated from human aorta have greater avidity to LDL than do lower neutral carbohydrates (28). Genetic influence on LDL patterns has also been shown, suggesting a moderate heredity of phenotype B (9, 13, 29–31).

The consensus is that the phenotype B subpopulation of LDL is not an independent risk factor but rather is an indication of a broader metabolic defect. Its presence is often associated with increased plasma triglyceride concentrations, decreased HDL cholesterol and apoA-I concentrations, and increased insulin resistance (9, 11, 12, 14, 30). Small, dense LDL also has a lower cholesteryl ester content and a greater apoB content than the larger particles. A simplified metabolic scheme for small, dense LDL has been proposed (8, 15). Fig. 1 shows the effects of an increased plasma concentration of very-low-density lipoprotein (VLDL) triglyceride; the increased net transfer of cholesteryl esters by the cholesteryl ester transfer protein (CETP) results in a decreased HDL concentration (32). In the same mechanism, the triglyceride will replace the transferred cholesteryl esters, and the LDL will be enriched in triglyceride. This triglyceride-rich LDL is subjected to the action of lipoprotein lipase, resulting in a lipoprotein lipase.
be obtained on nondenaturing gel electrophoresis and column chromatography (41).

The formation and interconversion of HDL2 and HDL3 are modulated by the actions of hepatic and lipoprotein lipases as well as CETP. The actual participation and relative activities of these components result in the final distribution of HDL subpopulations.

The majority of the epidemiologic studies on the correlation of coronary heart disease and HDL subfractions have been retrospective studies. In most studies, HDL2 and HDL3 concentrations were compared in survivors of myocardial infarctions (5), or in patients with chest pains and angiographically established atherosclerosis, with matched control subjects (42). These data showed that both HDL2 and HDL3 concentrations were reduced in coronary heart disease, the reduction of HDL2 being proportionally greater than for HDL3. Similar observations were reported by Gofman et al. in a prospective study in 1966 (43); they found that subjects who developed coronary heart disease had a decrease of HDL2 and HDL3, but the association of coronary heart disease was greater with the decrease of HDL2. A recent prospective study (44) and other studies confirmed the decrease of HDL2 and HDL3, but the decrease in HDL3 was a stronger predictor of coronary heart disease than HDL2.

A major contributor for the variance in the reported findings are the differences in the techniques of the isolation of HDL subfractions (ultracentrifugation or polyanion precipitation). Most studies did not consider the role of sample preservation, which influences the results. Careful attention should be placed on the collection and storage of blood. Samples should include, in addition to bactericidal compounds, inhibitors of protease and LCAT.

Currently, it is difficult to conclude unequivocally whether the decrease of HDL2 or HDL3 is a better predictor of coronary heart disease. Total HDL cholesterol is as good, if not better, a predictor of coronary heart disease as HDL2 or HDL3 (44); certainly, it is less controversial and easier to measure.

Twenty years ago, Alaupovic established the concept of lipoprotein families and demonstrated that several entities of lipoprotein families exist with a specific combination of apolipoproteins (45). He postulated that the individual lipoprotein families have unique physiologic functions as well as pathologic significance. Cheung and Albers separated HDL lipoproteins by immunoaffinity chromatography containing only apoA-I, calling them lipoprotein (Lp)A-I-only particles (46). They also identified lipoprotein particles containing both apoA-I and apoA-II, referring to them as LpA-I with LpA-II particles. The LpA-I-only particle is larger (9.98-nm diameter vs 7.65 nm for the LpA-I with LpA-II) (47). LpA-I-only is believed to be mainly associated with HDL3, whereas the LpA-I with LpA-II particles predominate in HDL2 (48). This arrangement may contribute to the findings that a decrease of HDL2 correlates better with coronary heart disease than does HDL3. In control plasma, 30% to 40% of total apoA-I is present in LpA-I-alone particles, and the LpA-I with LpA-II represents 60% to 70% of the total apoA-I (49).

These lipoprotein subclasses can be isolated in substantial quantities by immunoaffinity chromatography and their biologic and metabolic characteristics can be investigated. In tissue-culture studies, LpA-I-only particles promote cholesterol efflux from peripheral cells, whereas LpA-I with LpA-II lipoproteins do not (50). However, these observations are controversial, and some investigators have found no difference in cholesterol efflux between the two particles (51, 52). The different experimental conditions used in these studies make the comparisons difficult.

These lipoprotein families can be measured relatively easily by enzyme-linked differential antibody immuno sorbent assay (53) or by differential electroimmunoassay (54). The relative ease as to how these assays can be performed greatly facilitated the clinical studies on the role of LpA-I-only and LpA-I with LpA-II particles.

Study of the in vivo metabolism of the LpA-I-only and particles of LpA-I with LpA-II found that the residence time of LpA-I-only particles are substantially shorter (4.4 days) than the residence time of LpA-I with LpA-II (52.2 days) (55).

In a study comparing controls with normolipidemic patients with angiographically proven coronary heart disease, the concentrations of LpA-I-only particles were lower and the concentrations of LpA-I with LpA-II were similar to those in the controls (56). Similar studies were performed on patients with higher triglyceride concentrations than those of controls (57), contrary to the previous findings, both LpA-I-only and LpA-I with LpA-II particles were found to be decreased in a similar degree in coronary heart disease patients.

Women also have slightly higher LpA-I-only and lower concentrations of LpA-I with LpA-II particles than men (49). A comparison of populations from separate geographic areas with different mortality rates of myocardial infarctions showed that populations with the highest mortality rate for myocardial infarction had the lowest LpA-I-only concentrations (58).

Diets and drugs also influence the concentrations of these particles (Table 1). Diets with high ratios of polyunsaturated to saturated fatty acids lead to a decrease of LpA-I-only particles, whereas concentration relationship of LpA-I with LpA-II did not change (59). Nicotinic acid increases LpA-I-only concentrations (48), whereas 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors increase both LpA-I-only and LpA-I.

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<th>Table 1. Influence of diet and drugs on HDL composition.</th>
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<td>Factor</td>
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<td>Increased polyunsaturated/saturated fat</td>
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<td>Nicotinic acid</td>
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<td>Ethanol</td>
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with LpA-II particles (49). Fenofibrate, as well as alcohol consumption, decreases LpA-I-only particles and increases LpA-I with LpA-II (49, 60).

In a study of octogenarians Bard and Fruchart (49) measured the concentration of these two particles. Compared with younger subjects, the octogenarians showed a significant increase in LpA-I-only concentration, along with a decrease in LpA-I with LpA-II.

These data make it clear that different approaches are needed for the separation of lipoprotein subpopulations for HDL. A separation of HDL subpopulations where the functional characteristics of the subpopulations are emphasized should be considered.

Characterization of HDL Subpopulations According to Charge and Size

Using two-dimensional electrophoresis, Fielding and colleagues showed that whole plasma can be separated into several apoA-I-containing subpopulations that differ in charge and size (61, 62). When plasma was incubated for 1 or 2 min with fibroblasts labeled with tritiated cholesterol and subjected to two-dimensional electrophoresis, the labeled cholesterol was transferred to a pre-β1-migrating, small-sized, apoA-I-containing particle. Subsequently, the labeled cholesterol was transferred from the pre-β1 particle to other, larger, pre-β-migrating particles and, finally, to α-migrating HDL. During this process, the labeled free cholesterol originating from the peripheral cells was esterified preferentially over the unlabeled free cholesterol in plasma. Fielding postulated that the initial stage in the reverse cholesterol transport is the transfer of free cholesterol from peripheral cells to pre-β1 particles, followed by several steps of transfer and esterification to the α-migrating HDL.

From Fielding's data, the two-dimensional electrophoresis system seemed to be an excellent choice for separating HDL subpopulations according to functional characteristics (61, 62). This system would allow us to identify separate particles with known physiologic functions. Additionally, this method provided fine resolution of the different HDL subpopulations.

Our challenge was to standardize the system and make it reproducible (63). We chose to characterize HDL subpopulations according to (a) their mobility relative to albumin, and (b) their size in relation to that of internal standards run simultaneously. The individual steps used in the standardization of two-dimensional electrophoresis were, first, agarose electrophoresis that did not contain albumin in a vertical cassette, with constant cooling to prevent synergies; this was then followed by non-denaturing polyacrylamide gel electrophoresis in a 2% to 35% concave gradient run to completion. Internal protein standards with known sizes were also run simultaneously. Electrophoresis on gradient gel was followed by electrophoretic transfer of proteins to blotting media. Finally, we immunolocalized apolipoproteins with labeled anti-apoA-I antibody and quantified the distribution of apoA-I in the phosphoimager (63).

Figure 2 shows a two-dimensional electrophoresis in our system (63). The upper part of the figure includes a duplicate agarose strip, which was immunolocalized with apoA-I. Three subpopulations can be differentiated on agarose as pre-β, α, and pre-α particles. The duplicate of this agarose gel was placed on the top of the nondenaturing polyacrylamide gel and electrophoresed. The localization of albumin (marked with a star in the middle of the picture) enabled us to calculate R1 values relative to albumin for each subpopulation. The protein standards run simultaneously in the same gel provide a reference for size determination. The schematic representation of the individual subpopulation in Fig. 2 identifies several pre-β, α, and pre-α particles. For each subpopulation, an R1 value relative to albumin, size, and quantitation of apoA-I can be obtained.

First, we evaluated the influence of ultracentrifugation on the distribution of apoA-I. Two-dimensional electrophoretic patterns of whole plasma and lipoproteins isolated at a density of 1.21 kg/L and the d > 1.21 fraction were compared. During ultracentrifugation, most of the pre-β particles were lost from HDL; a compression of sizes and an increase in the pre-α mobilities were observed in the d < 1.21 lipoproteins. The pre-β particles were recovered in the d > 1.21 fraction. When the d < 1.21 and d > 1.21 fractions were combined, the original distribution was not reproduced (63).

These data and many others in the literature suggest that ultracentrifugation alters HDL lipoproteins (64, 65). If we accept that the pre-β particles have an important role in reverse cholesterol transport, then correlation of coronary heart disease with ultracentrifugally isolated subfractions will not provide adequate information on potentially important subpopulations. In vitro incubations of plasma followed by two-dimensional electrophoresis demonstrated that major changes occur in the distribution of HDL subpopulations during incubation. One of the most labile particles is the pre-β
fraction. In 2 h of 37°C incubation of plasma, pre-β particles disappear and reappear in the next several hours. When LCAT inhibitor is added to the incubation, instead of the disappearing, the pre-β particles increase several-fold. These data suggest that both LCAT and CETP are participating in the metabolism of pre-β (66, 67). These data support the concept that HDL is a meta-
stable lipoprotein; its subpopulations are remodeled continuously. Moreover, the physiologic and pathophys-
ologic roles of HDL may be greatly influenced by the dis-
tribution of their subpopulations.

Adequate preservation of samples can be achieved by adding cocktails containing bactericidal agents, pro-
 tease inhibitors, and LCAT inhibitors and immediate freezing in liquid nitrogen.

This system can also be used to localize other apoli-
does not necessarily mean these apolipoproteins are on the same particles; however, absence of co-localization with apoA-II is an indication that it is a particle that contains apoA-I only. Interestingly, this is the case for pre-β1: The apoA-II did not co-localize with apoA-I, sug-
gest that pre-β1 is an LpA-I-only particle (unpub-
lished observation).

In conclusion, the correlation between coronary heart disease and small, dense LDL (phenotype B) or hypera-
betalipoproteinemia is well documented and gener-
ally accepted; it is influenced by genetic, environmental, and endocrine factors. The small size of LDL is not an independent atherogenic risk factor; instead, it is closely tied to triglyceride metabolism and is a manifestation of a complex alteration of plasma lipoprotein metabolism. The findings on the correlation of HDL subpopulations and coronary heart disease are clearly less consistent. Tentatively and with reservation, we think the available data suggest that LpA-I-only particles may repre-
sent the anti-atherogenic particle within HDL. One ex-
planation for the differences in the results obtained for HDL subpopulations is related to the physiology of HDL: HDLs undergo continuous remodeling in vivo, which continues in vitro after blood samples are drawn and will influence the distribution of subpopulations. Thus, appropriate preservation of samples is necessary to obtain quality information. Currently, two-dimen-
sional electrophoresis is only a research tool and cannot be used for large-scale epidemiologic studies. However, well-controlled studies using this system should contrib-
ute to a better understanding of HDL subpopulations and their possible connection to coronary heart disease.

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