

# Atherogenic Lipoprotein Particles in Atherosclerosis

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**Abstract**—The importance of low-density lipoprotein (LDL) cholesterol in the development of atherosclerosis has long been recognized, and LDL cholesterol remains the primary target of therapy for the prevention of coronary heart disease. Nevertheless, increasing research attention over the past decade has been devoted to the heterogeneity of LDL particles and the atherogenicity of lipids and lipoproteins other than LDL. Particularly atherogenic forms of LDL include small, dense LDL particles and oxidized LDL. All lipoproteins that contain apolipoprotein B, such as LDL, very-low-density lipoprotein, and intermediate-density lipoprotein, tend to promote atherosclerosis; however, these particles differ in their apolipoprotein and triglyceride content. High levels of plasma triglycerides increase the risk of acute coronary events. Lipoprotein(a) is now considered an independent risk factor in both men and women. Ultimately, better understanding of the roles of these lipid particles and subfractions in the initiation and progression of atherosclerosis may affect treatment decisions. (*Circulation*. 2004;109[suppl III]:III-2–III-7.)

**Key Words:** apolipoprotein B ■ lipoprotein(a) ■ low-density lipoprotein ■ triglyceride-rich lipoproteins ■ triglycerides

Epidemiological studies have shown a positive relationship between total cholesterol concentrations and mortality from coronary heart disease (CHD). Total cholesterol does not accurately predict the risk of CHD in many patients, however, because it is the sum of all cholesterol carried not only by atherogenic lipoproteins (eg, very-low-density lipoprotein [VLDL], low-density lipoprotein [LDL], intermediate-density lipoprotein [IDL]) but also by antiatherogenic lipoproteins (ie, high-density lipoprotein [HDL]). Therefore, the decision to treat is based on LDL-cholesterol values. Yet, treatment decisions may also need to take into account LDL heterogeneity, which has been recognized for many years. Small, dense LDL particles are more atherogenic than large, buoyant LDL particles, and oxidation of LDL also increases its atherogenicity. In addition, LDL belongs to the group of lipoproteins that contain apolipoprotein (apo) B-100. Some of the particles in this highly heterogeneous group contain other apolipoproteins, such as apo C-II, apo C-III, and apo E. Furthermore, some particles are larger and rich in triglycerides (large VLDL), whereas others are smaller and rich in cholesteryl esters (small VLDL, IDL). It is now known that remnant lipoproteins containing apo C-III are highly atherogenic, as is lipoprotein(a) [Lp(a)], another member of the apo B-100 group. This article reviews recent studies involving LDL subclasses and atherogenic lipoproteins, many of which used novel methods of lipoprotein subfractioning.

## Small, Dense LDL

### Analytical Methods

LDL heterogeneity is characterized according to different physical properties of the LDL particles: lipoprotein size, flotation rate, and density. Features of some common assays used to characterize LDL subfractions (as well as to measure other lipids and lipoproteins) are summarized in the Table. Gradient gel electrophoresis using nondenaturing conditions is commonly used to characterize the distribution of LDL particles by size.<sup>1</sup> LDL subclass phenotype A is characterized by a predominance of large LDL particles, whereas LDL subclass phenotype B is characterized by a predominance of small LDL particles. A majority (85% to 90%) of subjects can be classified as having either LDL subclass phenotype A or B, whereas the remainder have an intermediate phenotype.<sup>2</sup>

High-performance gel-filtration chromatography<sup>3</sup> and nuclear magnetic resonance (NMR) spectroscopy<sup>4</sup> have recently been applied to determinations of LDL particle size. Several different methods of density gradient ultracentrifugation have been used to characterize LDL flotation rate (Svedberg flotation unit) using the analytical ultracentrifuge. Methods for characterizing LDL subclasses based on flotation rates are modifications of a procedure developed by Chung et al<sup>5</sup> using a vertical rotor in a preparative ultracentrifuge (Vertical Auto Profile [VAP]).<sup>6</sup> When subjects are classified as LDL subclass phenotype A or B, there are significant differences in LDL relative flotation (Rf).<sup>7</sup> Several methods based on discontinuous salt gradients are used to determine LDL subclasses based on density.<sup>8</sup> These various

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*Circulation* is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000131511.50734.44

**Methods Used to Measure Lipid/Lipoprotein Subfractions**

Name of Test	Technology Used	Lipids/Lipoproteins Measured
Vertical Auto Profile (VAP) (Atherotech)	Vertical ultracentrifugation technique — spins down plasma using centrifuge	LDL density (pattern A or B) by rate of descending particles; IDL and HDL subtypes; VLDL density and Lp(a). TGs are estimates, so LDL not accurate when TGs >400 mg/dL
Segmented Gradient Gel Electrophoresis (sGGE) (Berkeley HeartLab)	Electrophoresis gradient gel — calibrated against the analytical ultracentrifugation (ANUC)	Basic Panel: apo B; LDL (calculated peak particle size and % distribution); 5 subclasses of LDL, 2 subclasses of IDL, and 3 subclasses of VLDL Advanced Panel: above plus 6 subtypes of HDL
Lipoprint LDL System (Quantimetrix Corp)	High-resolution 3% polyacrylamide gel tubes	Three subclasses of IDL; 7 subclasses of LDL (LDL particle size and phenotype)
Nuclear Magnetic Resonance (NMR) (LipoMed Inc)	Measures the spin or electrical resonance of various particles	No., quantity, and size of LDL particles (calculated measurements of 4 LDL subclasses); HDL (large and small); VLDL particle sizes (low, medium and large)

apo indicates apolipoprotein; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); TG, triglyceride; VLDL, very-low-density lipoprotein.

methods of determining LDL subclasses show a high degree of correlation, despite the fact that they measure different physical properties of LDL.

**Clinical Significance**

Several cross-sectional studies have reported differences in LDL particle size, density, and composition between patients with CHD and healthy controls. Prospective, nested case-control studies have since confirmed that the presence of small, dense LDL particles is associated with a more than 3-fold increase in CHD risk.

In the Québec Cardiovascular Study, men with an LDL particle size <25.6 nm had a significant 2.2-fold increase in the 5-year rate of ischemic heart disease (IHD) compared with men having an LDL particle size >25.6 nm.<sup>9</sup> Multivariate and subgroup analyses indicated that small, dense LDL particles predicted the rate of IHD independently of LDL cholesterol, triglycerides, HDL cholesterol, apo B, and the total cholesterol–HDL cholesterol ratio. The magnitude of the increase in IHD risk attributed to lipid risk factors was modulated to a significant extent by variations in LDL particle size. This study suggests that information on LDL diameter may improve the ability to predict IHD risk accurately over traditional lipid variables.<sup>9</sup>

On the other hand, data from the Physicians' Health Study suggest that small, dense LDL may not be an independent risk factor.<sup>10</sup> A nested case-control study with prospectively collected samples examined whether a predominance of small, dense LDL particles and elevated triglyceride levels are independent risk factors for myocardial infarction (MI). Cases had a significantly smaller LDL diameter (mean [SD], 25.6 [0.9] nm) than did controls (mean [SD], 25.9 [8] nm;  $P<0.001$ ). Cases also had higher median triglyceride levels (1.9 versus 1.5 mmol/L [168 versus 132 mg/dL];  $P<0.001$ ).<sup>10</sup> The LDL diameter had a high inverse correlation with triglyceride level ( $r=-0.71$ ), and a high direct correlation with HDL-cholesterol level ( $r=0.60$ ). After simultaneous adjustment for lipids and a variety of coronary risk factors, LDL particle diameter was no longer a statistically significant risk indicator. However, triglyceride levels remained significant. The total-cholesterol level, but not HDL-cholesterol

level, also remained significant. These findings indicate that nonfasting triglyceride levels appear to be a strong and independent predictor of future risk of MI, particularly when total cholesterol is also elevated. In contrast, LDL particle diameter was associated with risk of MI, but not after adjustment for triglyceride level.<sup>10</sup>

Proton NMR spectroscopy was used to simultaneously quantify levels of subclasses of VLDL, LDL, and HDL in men to examine whether NMR-derived lipoprotein subclass levels improve the prediction of coronary artery disease (CAD).<sup>11</sup> It was found that a global measure of CAD severity was positively associated with levels of large VLDL and small HDL particles and inversely associated with intermediate-sized HDL particles. Men with relatively high (higher than the median) levels of either small HDL or large VLDL particles were 3 to 4 times more likely to have extensive CAD than were men with lower levels; men with high levels of both large VLDL and small HDL were 15 times more likely to have extensive CAD. In contrast, adjustment for levels of triglycerides or HDL cholesterol greatly reduced the relation of small LDL particles to CAD. These findings suggest that large VLDL and small HDL particles may play important roles in the development of occlusive disease.<sup>11</sup>

Relationships between incident cardiovascular disease and lipoprotein subclass measurements by NMR spectroscopy were evaluated in the Cardiovascular Health Study (CHS)<sup>4</sup> in a nested case-cohort analysis. By univariate analysis, the median levels for healthy participants versus participants with incident MI and angina pectoris were 0 versus 7 mg/dL for small LDL, 1501 versus 1680 nmol/L for the number of LDL particles, and 21.6 versus 21.3 nm for LDL size; these values were significantly different between healthy participants and those with incident MI and angina for women but not for men. The level of less dense LDL, which represents most of the LDL cholesterol in women, was not related to incident MI and angina. For men and women, levels of total and VLDL triglycerides were higher in the case group than in the healthy group. In multivariate models for women that included triglycerides and HDL cholesterol, the number of LDL particles (but not LDL size) remained significantly related to MI and angina. This study showed that the size of LDL

particles and the greater number of LDL particles are related to incident CHD among older women.<sup>4</sup>

A prospective, nested case-control study among healthy middle-aged women was conducted to assess LDL particle size and concentration (NMR) as risk factors for future MI, stroke, or death from CHD.<sup>12</sup> Median baseline levels of LDL particle concentration (NMR) were higher and LDL particle size (NMR) was lower (21.5 versus 21.8 nm;  $P=0.046$ ) among women who subsequently had cardiovascular events than among those who did not. Of these factors, LDL particle concentration (NMR) was the stronger predictor.<sup>12</sup>

It has been shown recently that LDL particle size, but not susceptibility of LDL to oxidation in vitro, is independently associated with carotid intima-media thickness in asymptomatic familial combined hyperlipidemia family members.<sup>13</sup>

#### ***Insulin Resistance, Diabetes, and Small, Dense LDL***

The small, dense LDL phenotype rarely occurs as an isolated disorder. It is most frequently accompanied by hypertriglyceridemia, reduced HDL-cholesterol levels, abdominal obesity, and insulin resistance (all of which are components of the metabolic syndrome) and by a series of other metabolic alterations predictive of an impaired endothelial function and increased susceptibility to thrombosis.

Results from the Québec Cardiovascular Study have indicated that persons displaying elevated plasma concentrations of insulin and apo B together with small, dense LDL particles showed a remarkable increase in CHD risk.<sup>14</sup>

NMR spectroscopy was used to define the effects of insulin resistance on lipoprotein subclasses in insulin-sensitive, insulin-resistant, and untreated subjects with type 2 diabetes mellitus.<sup>15</sup> In the group as a whole, insulin resistance had profound effects on lipoprotein size and subclass particle concentrations for VLDL, LDL, and HDL. When compared with insulin-sensitive subjects, the insulin-resistant and diabetes subgroups exhibited a 2- to 3-fold increase in concentrations of large VLDL particles (no change in medium or small VLDLs), which resulted in an increase in serum triglycerides. They also showed a decrease in LDL size (as a result of a marked increase in small LDL particles and reduced large LDL) plus an increase in overall LDL particle concentration. Taken together, these latter changes led to no difference (insulin sensitive versus insulin resistant) or a minimal difference (insulin sensitive versus diabetes) in LDL-cholesterol levels. In type 2 diabetes, these alterations could be attributed primarily to the underlying insulin resistance. These changes in the NMR lipoprotein subclass profile predictably increased the risk of cardiovascular disease but were not fully apparent in the conventional lipid panel.<sup>15</sup>

A recent study examined whether NMR lipoprotein spectroscopy improves the prediction of CAD in patients with type 1 diabetes, independently of conventional lipid and other risk factors. In a prospective, nested case-control study of subjects with childhood-onset type 1 diabetes (Pittsburgh Epidemiology of Diabetes Complications Study),<sup>16</sup> univariate analyses showed that both lipid mass and particle concentrations (NMR spectroscopy) of all VLDL subclasses, small LDL, medium LDL, and medium HDL were increased in CAD cases compared with controls, whereas large HDL was

decreased. Mean LDL and HDL particle sizes were less in CAD cases.<sup>16</sup>

The Diabetes Atherosclerosis Intervention Study (DAIS)<sup>17</sup> showed that lipid-modifying treatment decreased the angiographic progression of coronary atherosclerosis in subjects with type 2 diabetes. This effect was related in part to the correction of lipoprotein abnormalities. Compared with placebo, fenofibrate treatment significantly increased LDL particle size and HDL cholesterol and decreased plasma total cholesterol, LDL cholesterol, and triglyceride concentrations. The final LDL particle size was inversely correlated with the increase in percentage diameter stenosis.<sup>17</sup>

#### **Oxidized LDL**

It has been suggested for 20 years that oxidative stress, and particularly LDL oxidation, could induce atherosclerosis<sup>18</sup> and that markers of LDL oxidation in plasma (circulating oxidized LDL, autoantibodies against oxidized LDL) could be used to assess the development of atherosclerosis in patients. Circulating oxidized LDL is additive to the global risk score based on age, sex, total and HDL cholesterol, diabetes mellitus, hypertension, and smoking as a useful marker for identifying persons at risk for CAD.<sup>19,20</sup> In one study, circulating oxidized LDL was associated with both subclinical atherosclerosis (clinically silent ultrasound-assessed atherosclerotic changes in the carotid and femoral arteries) and inflammatory variables (C-reactive protein and the inflammatory cytokines interleukin-6 and tumor necrosis factor- $\alpha$ ), supporting the concept that oxidatively modified LDL may play a major role in atherosclerosis development, although no causality could be shown.<sup>21</sup> It has been proposed that, because of the antigenic properties of oxidized LDL, the anti-oxidized LDL antibody titer could represent a useful index of in vivo LDL oxidation. Autoantibodies against oxidized LDL have been reported to be associated with atherosclerosis. However, the data are not consistent: some studies have reported a positive relationship between autoantibodies against oxidized LDL and CHD,<sup>22,23</sup> whereas another did not.<sup>24</sup> There is a strong cross-reactivity between autoantibodies against oxidized LDL and anticardiolipin antibodies, which have been positively associated with CHD.<sup>23</sup>

Despite the plausible role of oxidative stress in atherogenesis, results of controlled, prospective trials examining the effects of antioxidant supplements on clinical end points have been disappointing.<sup>25</sup> Recently, Steinberg and Witztum, who were early proponents of the role of oxidized LDL in the development of atherosclerosis,<sup>26</sup> have argued for further research, suggesting that the failure of these clinical trials does not disprove the role of lipoprotein oxidation in atherogenesis and citing a number of limitations. For example, the clinical trials conducted to date may not have used the right antioxidants at the right dosages. In addition, the trials may not have been started early enough in the disease process or continued long enough to demonstrate a favorable effect.<sup>27</sup> This subject will undoubtedly remain controversial.

#### **Apolipoprotein B**

As first suggested by Lee and Alaupovic,<sup>28</sup> lipoprotein particles can be differentiated on the basis of their constituent



apolipoproteins. Apo B-100 is the major apolipoprotein component of the atherogenic lipoproteins (VLDL, LDL, IDL). Although epidemiological data relating apo B concentrations to CHD are limited, some case-control studies in patients with CHD have found plasma apo B levels to be more discriminating than other plasma lipids and lipoproteins.<sup>29</sup> Nevertheless, the extent to which plasma apo B levels were better predictors of risk varied.<sup>30</sup> Some of these discrepancies may have resulted from difficulties in standardizing assays for measuring plasma apo B levels; however, the World Health Organization–International Federation of Clinical Chemistry (IFCC) has now developed standardized methods for measurement of apo B and apo A-I,<sup>31</sup> and population results have subsequently been reported.<sup>32</sup> The prospective Québec Cardiovascular Study<sup>33</sup> and other investigations have demonstrated the importance of apo B in estimating coronary risk. Recently, the large prospective Apolipoprotein-related Mortality Risk (AMORIS) study<sup>34</sup> showed that the age-adjusted values of apo B and the apo B/apo A-I ratio were strongly and positively related to increased risk of fatal MI in men and women. In multivariate analyses, apo B was a stronger predictor of risk than LDL-cholesterol in both men and women. This study suggests that apo B and the apo B/apo A-I ratio should be regarded as highly predictive in evaluating cardiac risk. Apo B might be of greatest value in the diagnosis and treatment of persons with normal or low concentrations of LDL cholesterol.<sup>34</sup> For example, patients with type 2 diabetes mellitus frequently have hypertriglyceridemia together with hyper-apo B, an atherogenic lipid profile that is often unappreciated because of concomitant low or normal levels of LDL cholesterol.<sup>35</sup>

### Triglyceride-Rich Lipoproteins

A meta-analysis of 17 prospective population-based studies found hypertriglyceridemia to be an independent risk factor for cardiovascular disease.<sup>36</sup> Nevertheless, it is possible that, rather than being actual atherogenic agents in themselves, elevated triglycerides merely serve as a marker for increases in triglyceride-rich remnant lipoproteins and that it is these latter particles that are involved in the development of atherosclerosis.<sup>37,38</sup>

Triglyceride-rich lipoproteins comprise a great variety of nascent and metabolically modified lipoprotein particles differing in size, density, and lipid and apolipoprotein composition. Studies have shown an inverse relationship between the size of lipoproteins and their ability to cross the endothelial barrier to enter the arterial intima. Chylomicrons and large VLDLs (Svedberg flotation unit [ $S_f$ ] 60 to 400) are probably not capable of entering the arterial wall. On the other hand, small VLDLs ( $S_f$  20 to 60) and IDLs ( $S_f$  12 to 20) can enter the arterial intima. Therefore, certain triglyceride-rich lipoproteins are atherogenic, whereas others are not. A large body of evidence suggests that small VLDLs and IDLs are independently associated with atherosclerosis.

A study<sup>39</sup> in 174 patients with type 2 diabetes mellitus who were not receiving lipid-lowering therapy concluded that the severity of CHD (as examined by angiography) was related to the number of circulating fasting triglyceride-rich lipoprotein particles. This relationship was stronger in women than in

men and was independent of HDL and LDL. Remnant-like particles (RLPs) can be isolated from the plasma or serum by an antibody-based assay. In the Framingham Offspring Study,<sup>40</sup> mean RLP cholesterol and RLP triglycerides were highly significantly ( $P < 0.0001$ ) increased in diabetic women and significantly ( $P < 0.001$ ) increased in diabetic men compared with respective controls.

Apo C-III in VLDL is associated with denser, smaller VLDL subclasses and is believed to be particularly atherogenic. Remnants associated with apo C-III are more related to the development of atherosclerosis than are triglycerides per se. Fasting remnant lipoproteins could reflect postprandial remnant lipoproteins and predict future clinical coronary events independently of other risk factors.<sup>41</sup> The concentration of apo C-III in VLDL and IDL particles has been significantly correlated with development of coronary stenosis.<sup>42</sup>

Results of the Monitored Atherosclerosis Regression Study (MARS)<sup>43</sup> provide evidence for a strong positive relationship between the progression of CAD and concentrations of triglyceride-rich lipoprotein remnants. Lipoproteins in the  $S_f$  12 to 60 range (IDLs and small VLDLs) were independently correlated with development of coronary atherosclerosis. In MARS, triglyceride-rich lipoproteins such as small VLDLs and particles rich in apo C-III were related to the progression of mild or moderate ( $< 50\%$  diameter stenosis) coronary artery lesions rather than severe ( $\geq 50\%$  diameter stenosis) lesions.<sup>43</sup> Lesions of  $< 50\%$  diameter stenosis appear to highly predict clinical coronary events.

In a randomly selected subgroup of 63 MARS subjects, lipoprotein particles were isolated by column immunoaffinity techniques.<sup>44</sup> Subjects who showed progression of coronary artery lesions had significantly higher levels of LP-B<sub>c</sub> (a summed measure of triglyceride-rich lipoproteins) and LP-A-II:B:C:D:E particles of small VLDL and/or IDL-like sizes.<sup>44</sup> In a prospective study (Etude Cas Temoins sur Infarctus du Myocarde [ECTIM]), Lp B:C-III was significantly increased in French and Irish MI survivors compared with controls.<sup>45</sup>

Atherogenic lipoprotein particles were evaluated in a prospective, nested case-control substudy<sup>46</sup> of the Cholesterol and Recurrent Events (CARE) trial, a randomized, placebo-controlled trial of pravastatin in patients with previous MI and average LDL concentrations at baseline (3 to 4.5 mmol/L, mean 3.6 mmol/L [115 to 174 mg/dL, mean 139 mg/dL]). Baseline concentrations of VLDL-apo B (the VLDL particle concentration), VLDL lipids, and apo C-III and apo E in VLDL+LDL and in HDL were compared in patients who experienced either a recurrent MI or coronary death with concentrations in patients who did not have a cardiovascular event during the 5 years of follow-up. Results showed that plasma concentrations of VLDL particles and apo C-III in VLDL and LDL were more specific measures of CHD risk than were plasma triglycerides.<sup>46</sup>

### Lipoprotein(a)

Lp(a) particles contain apo(a) and apo B in a 1:1 molar ratio. Apo(a) contains a kringle domain and a carboxyl-terminal domain with 85% amino acid identity with the plasminogen

protease domain. The molecular mass of apo(a) protein varies from 187 kDa for an apo(a) that contains 12 kringle 4 domains, to 662 kDa for an apo(a) that contains 50 kringle 4 domains.<sup>47</sup> Among numerous retrospective case-control studies, virtually all have shown a strong association between elevated Lp(a) levels and CHD. On the other hand, the results of prospective studies have been somewhat discordant—a total of 9 prospective studies concluded that Lp(a) is an independent risk factor for MI or CHD in men, but 4 studies reached the opposite conclusion. These inconsistencies may relate to a lack of standardization and the failure of some immunoassays to measure all apo(a) isoforms equally.<sup>48</sup> Nevertheless, a meta-analysis of prospective studies indicated that plasma Lp(a) concentration is an independent risk factor for CHD in both men and women.<sup>49</sup> Recently, the Prospective Epidemiological Study of Myocardial Infarction (PRIME), a cohort study that included 9133 men from France and Northern Ireland with no history of CHD or use of hypolipidemic drugs, confirmed Lp(a) as a predictor of CHD risk.<sup>50</sup>

### Conclusions

Elevated LDL-cholesterol levels are associated with a high risk of CHD, and LDL cholesterol continues to be the primary target of therapy for the prevention of CHD. However, the heterogeneity of LDL particles and the increasing recognition of the atherogenicity of other lipoproteins, remnant lipoprotein particles, and certain apolipoproteins, demands attention. Physicians need to be aware of these other atherogenic lipoproteins because they may become the targets of therapeutic intervention in the future. Small, dense LDL particles are highly atherogenic, and high levels of circulating oxidized LDL increase the risk of CHD. Lipoproteins that contain apo B are highly heterogeneous in terms of chemical composition and size. Further fundamental and clinical studies are needed to characterize these lipoproteins and their capacity to induce atherosclerosis. For the time being, apo B might be of greatest value in the diagnosis and treatment of men and women with some common lipid abnormalities but normal or low concentrations of LDL cholesterol. The apo B/apo A-I ratio should also be regarded as highly predictive in evaluating cardiac risk. Elevated plasma triglyceride levels increase the risk of acute coronary events and are an independent risk factor, but the concentrations of remnant particles associated with apo C-III are more related to the development of atherosclerosis than are triglycerides per se. Lipoprotein(a) is now considered an independent risk factor for CHD in both men and women. An important challenge is to develop standardized and simple analytical methods to generalize the measurement of different atherogenic lipoprotein particles in clinical biochemistry and in clinical practice.

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