Comparison of Various Electrophoretic Characteristics of LDL Particles and Their Relationship to the Risk of Ischemic Heart Disease

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Background—Several cross-sectional studies and 3 prospective, nested, case-control studies have indicated that individuals with small, dense low density lipoprotein (LDL) particles are at increased risk for ischemic heart disease (IHD). However, whether LDL particle size is an independent risk factor for future IHD events remains controversial. The objective of the present study was to further analyze the cardiovascular risk associated with various electrophoretic characteristics of LDL particles in men.

Methods and Results—LDL particles were characterized by polyacrylamide gradient gel electrophoresis (PAGGE) in a cohort of 2034 men of the Quebec Cardiovascular Study. All men were initially free of IHD and were followed up for a period of 5 years, during which 108 first IHD events were recorded. Among all LDL characteristics investigated by PAGGE, including LDL peak particle size, the cholesterol concentration in LDL particles with a diameter smaller than 255 Å showed the strongest association with the risk of IHD (relative risk = 4.6 in men in the third vs first tertile of the distribution, \( P < 0.001 \)). Multivariate logistic and survival models indicated that the relationship between LDL cholesterol levels in particles with a diameter <255 Å and IHD risk was independent of all nonlipid risk factors and of LDL cholesterol, high density lipoprotein cholesterol, triglyceride, and lipoprotein(a) levels.

Conclusions—Results from this large, population-based, prospective study suggest that further characterization of LDL particles by PAGGE, in addition to the traditional lipid profile, may improve our ability to predict IHD events in men. (Circulation. 2001;104:2295-2299.)

Key Words: lipids ■ epidemiology ■ lipoproteins ■ ischemia ■ heart diseases ■ nutrition

Although increased plasma LDL cholesterol concentrations are considered 1 of the most important risk factors for ischemic heart disease (IHD), many individuals who develop atherosclerosis and IHD have LDL cholesterol levels in the normal range. LDLs are known to exhibit heterogeneity in size, density, and physicochemical properties. Several cross-sectional case-control studies have indicated that the presence of small, dense LDL particles may be associated with an increased risk of IHD, although this is not a unanimous finding.

Three prospective, nested, case-control studies have recently provided further evidence supporting the inverse relationship between LDL particle size determined by polyacrylamide gradient gel electrophoresis (PAGGE) and the risk of an IHD event. Data indicated, however, that the relationship between variations in LDL particle size and incident IHD was not independent of concomitant changes in other lipid risk factors, such as the total-to-HDL cholesterol ratio or nonfasting plasma triglyceride levels. Similar observations were recently reported by Austin et al in older Japanese-Americans involved in the Honolulu Heart Program. These results suggested that measuring LDL particle size by PAGGE may not improve our ability to predict IHD events beyond that achieved with more traditional risk factors. It must be stressed that these previous studies characterized LDL size on PAGGE by using the particle diameter of the most abundant LDL subclass within an individual, the so-called LDL peak particle size. It is not known whether a more comprehensive characterization of LDL particles by PAGGE would lead to a more accurate assessment of IHD risk.

The objective of the present study was to investigate the relationship between several new characteristics of LDL particles obtained by PAGGE and the risk of developing IHD events in a cohort of 2034 IHD-free men of the Quebec Cardiovascular Study who were followed up for 5 years.

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Methods

Study Population and Follow-Up

The Quebec Cardiovascular Study cohort is an ongoing prospective study that has been described in detail previously.10,11 Data collected in 1985 were used as the baseline characteristics for the present prospective analyses, which were conducted in a sample of 2034 men (46 to 76 years) without IHD in 1985. The diagnosis of diabetes was made for men who self-reported the disease. Only 1% of men were using hypolipidemic medication in 1985, whereas 8% of men were using β-blockers or diuretics as antihypertensive medication on a regular basis at the 1985 evaluation. In 1990 to 1991, participants included in the study were contacted by mail. They provided information on smoking habits, medication use, history of cardiovascular diseases, and type 2 diabetes. For those who reported such diseases and those who had died, hospital charts were reviewed. Mortality and morbidity data were obtained in 99% and 96%, respectively, of the participants.

Definition of IHD Events

The diagnosis of a first IHD event, which included typical effort angina, coronary insufficiency, nonfatal myocardial infarction, and coronary death,10,11 have also been described in detail previously.

Laboratory Analyses

Twelve-hour fasting blood samples were obtained at baseline evaluation and immediately used for lipid and apolipoprotein measurements as described earlier.11,12 LDL cholesterol levels were estimated by the equation of Friedewald et al.13 Using a modification of the approach described by Tchernof et al.14 LDL particle size was determined on 8×8-cm polyacrylamide gradient gels prepared in batches in our laboratory. Aliquots of 3.5 μL of whole plasma samples were mixed in a 1:1 volume ratio with a sampling buffer containing 20% sucrose and 0.25% bromophenol blue and loaded onto the gels. A 15-minute prerun at 75 V preceded electrophoresis of the plasma samples at 150 V for 3 hours. Gels were stained for 1 hour with Sudan black (0.07%) and stored in a 0.81% acetic acid/4% methanol solution until analysis by the Imagemaster 1-D Prime computer software (Amersham Pharmacia Biotech). LDL size was extrapolated from the relative migration of 4 plasma standards of known diameter.15 The estimated diameter for the major peak in each scan was identified as the LDL peak particle size. An integrated (or mean) LDL diameter was also computed by using a modification of the approach described by Tchennot et al.14 This integrated LDL particle size corresponds to the weighted mean size of all LDL subclasses in 1 individual. It was calculated as a continuous variable and was computed as the sum of the diameter of each LDL subclass multiplied by its relative area. Analysis of pooled plasma standards revealed that measurement of LDL peak and mean particle size was highly reproducible, with an interassay coefficient of variation of <2%. The cumulative number of identifiable LDL subclasses and the number of LDL subclasses with a diameter <255 Å were also computed for each individual. The relative proportion of LDL having a diameter <255 Å was ascertained by computing the relative area of the densitometric scan <255 Å. It has been documented that Sudan black stains mainly nonpolar lipids.15 The absorbance profile with Sudan black staining was also assumed to closely reflect the cholesterol distribution among LDL particles of different sizes.16 Thus, the absolute concentration of cholesterol among particles <255 Å was calculated by multiplying the total plasma LDL cholesterol levels by the relative proportion of LDL with a diameter <255 Å. A similar approach was used to assess the relative and absolute concentrations of cholesterol in particles with a diameter >260 Å.

TABLE 1. Baseline Characteristics of the 1926 Patients Who Did Not Develop IHD During 5-Year Follow-Up and the 108 Incident IHD Cases

<table>
<thead>
<tr>
<th>Variables</th>
<th>IHD-Free Men (n=1926)</th>
<th>IHD Cases (n=108)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56.3±6.9</td>
<td>59.3±7.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.1±3.7</td>
<td>26.8±4.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>130±17</td>
<td>137±18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type 2 diabetes, % (n)</td>
<td>4.4% (85)</td>
<td>14.8% (16)</td>
<td>0.003</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.7±1.0</td>
<td>6.1±1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.04±0.26</td>
<td>0.96±0.24</td>
<td>0.002</td>
</tr>
<tr>
<td>Total cholesterol/HDL</td>
<td>5.8±1.7</td>
<td>6.7±1.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cholesterol (ratio)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides, mmol/L*</td>
<td>1.7±0.7</td>
<td>2.0±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dL</td>
<td>116±30</td>
<td>130±32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lipoprotein(a), mg/dL*</td>
<td>328±347</td>
<td>419±471</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are mean±SD. *Tested by nonparametric ANOVA.

Statistical Analyses

Duration of follow-up was first calculated in person-years by using the follow-up of each participant from the 1985 baseline evaluation until death, onset of IHD, or the 1990 to 1991 last contact. Mean baseline characteristics of incident IHD cases and of men who remained free of IHD during follow-up were compared by Student’s t test for parametric variables and by the Wilcoxon test for nonparametric variables. Differences in frequency data were tested by χ² analysis. Cox proportional-hazards models were used to estimate rates of IHD events. Age, body mass index, systolic blood pressure, type 2 diabetes (presence vs absence), smoking habits (smokers of >20 cigarettes per day vs others), familial history of IHD (presence vs absence), medication use (presence vs absence), LDL and HDL cholesterol levels, and logarithmically transformed triglyceride and lipoprotein(a) levels at baseline were included as potential confounders where indicated. Receiver operating characteristic (ROC) curves were used to examine the additional value of the various LDL size characteristics in discriminating subjects who did vs those who did not suffer a first IHD event during follow-up. The area under the ROC curves was the primary end point for these analyses. Linear predictors of the logistic regression used to generate the ROC data were also used to assess the number of incident IHD cases classified as being in the highest tertile of risk based on a traditional risk factor model with and without the various LDL size characteristics on PAGGE. Statistical analyses were performed with SAS software (SAS Institute).

Results

As shown in Table 1, incident IHD patients had a disturbed risk profile compared with men who remained free of IHD during the 5-year follow-up. The various characteristics of LDL particles on PAGGE among incident IHD cases and IHD-free men are presented in Table 2. LDL peak particle size (P=0.002) as well as the LDL integrated (mean) size (P<0.001) at baseline were significantly smaller in incident IHD cases compared with IHD-free individuals. Approximately 80% of the population had 3 or 4 identifiable LDL subclasses on PAGGE. The mean cumulative number of LDL subclasses was higher among IHD-free individuals compared with incident IHD cases (P=0.01). However, the mean cumulative number of LDL subclasses with a diameter <255 Å was greater among incident IHD cases than among men who remained IHD-free (P=0.005). Approximately half
TABLE 2. Analysis of LDL Size and Distribution by PAGGE

<table>
<thead>
<tr>
<th>LDL Characteristics</th>
<th>IHD-Free Men (n=1926)</th>
<th>IHD Cases (n=108)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL peak particle size, Å</td>
<td>257.1±5.8</td>
<td>255.2±6.4</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL integrated (mean) size, Å</td>
<td>257.2±4.9</td>
<td>254.4±4.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No. of LDL subclasses</td>
<td>3.6±1.0</td>
<td>3.4±0.8</td>
<td>0.01</td>
</tr>
<tr>
<td>No. of LDL subclasses &lt;255 Å</td>
<td>1.5±0.8</td>
<td>1.7±0.8</td>
<td>0.005</td>
</tr>
<tr>
<td>Relative proportion of LDL, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;255 Å</td>
<td>39.4±20.0</td>
<td>50.7±16.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>255–260 Å</td>
<td>23.7±10.0</td>
<td>20.2±5.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;260 Å</td>
<td>36.9±18.1</td>
<td>29.1±14.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L*</td>
<td>3.9±0.9</td>
<td>4.2±1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;255 Å</td>
<td>1.5±0.9</td>
<td>2.1±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>255–260 Å</td>
<td>0.9±0.4</td>
<td>0.8±0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>&gt;260 Å</td>
<td>1.4±0.8</td>
<td>1.2±0.7</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*Total plasma LDL cholesterol levels were obtained with the Friedewald equation as indicated in Methods.

(50.7%) of the LDL particles among incident IHD cases were smaller than 255 Å, whereas 29.1% of the particles had a diameter >260 Å. These proportions were significantly different from those observed among IHD-free individuals, among whom the distribution of LDLs within large (36.9%) and small (39.4%) particle size fractions was relatively homogeneous. The significant difference in baseline LDL cholesterol levels between incident IHD cases and IHD-free men (4.2±1.0 vs 3.9±0.9 mmol/L, P<0.001) was attributable mainly to a 40% elevation in the cholesterol content of small particles (<255 Å) in the former group compared with the latter (2.1±0.8 vs 1.5±0.9 mmol/L, P<0.001).

The LDL peak and integrated particle sizes were correlated (inversely) with plasma triglyceride levels (r=-0.54 and r=-0.51, respectively; P<0.001) but less so with the HDL cholesterol (r=0.39 and r=0.38, respectively; P<0.001) and apolipoprotein B (r=-0.33 and r=-0.31, P<0.001) levels. Although the relative proportion of LDL <255 Å was correlated strongly with LDL peak particle size (r=-0.71) and LDL integrated particle size (r=-0.76), the shared variance did not exceed 50%. The relative proportion of LDL <255 Å was also correlated with HDL cholesterol (r=-0.31, P<0.001), triglyceride (r=0.43, P<0.001), and apolipoprotein B (r=0.25, P<0.001) levels but showed essentially no relationship with LDL cholesterol level (r=0.06, P<0.01). The cholesterol concentration in LDL particles <255 Å was correlated significantly with the total LDL cholesterol level (r=0.43, P<0.001), but the shared variance between these 2 measures of LDL was <25%.

Table 3 presents the risk of IHD computed for each tertile of LDL characteristics by using the low-risk tertile as a reference (relative risk [RR]=1.0). This approach allowed a standardized comparison of the IHD risk associated with LDL characteristics that had different scales and distributions. An increased total plasma LDL cholesterol concentration (third vs first tertile) was associated with a 2.7-fold increase in the risk of IHD, which was independent of the individual or combined contribution of other nonlipid and lipid risk factors. The significant association between small LDL peak particle size and the risk of future IHD events (model 1: RR=2.5; 95% confidence interval [CI], 1.5 to 4.0) was no longer significant after adjustment for nonlipid and lipid risk factors (model 3: RR=1.5; 95% CI, 0.9 to 2.7). On the other hand, the relationship between LDL integrated size and IHD risk retained borderline significance even after multivariate adjustment for all nonlipid and lipid risk factors (model 3: RR=1.7; 95% CI, 1.0 to 3.1). Finally, men in the third tertile of relative or absolute cholesterol levels in LDL particles <255 Å had a 4- to 6-fold increase in the risk of IHD compared with men in the first tertile of the distributions. This increase in IHD risk remained highly significant even after adjustment for nonlipid and lipid risk factors. Adjusting for the total-to-HDL cholesterol ratio also did not attenuate the relationship between LDL cholesterol levels in particles <255 Å and incident IHD risk (not shown).

The incremental benefit of adding a measurement of LDL characteristics on PAGGE to the series of traditional risk factors in discriminating incident IHD cases from noncases was also investigated by using ROC curves obtained by logistic regression analysis (Table 4). The area under the ROC curve based on

TABLE 3. Comparison of the Multivariate 5-Year RR of IHD in Men According to Various LDL Characteristics

<table>
<thead>
<tr>
<th>After Adjustment for</th>
<th>LDL Cholesterol</th>
<th>LDL Peak Size</th>
<th>LDL Mean Size</th>
<th>LDL Proportion &lt;255 Å</th>
<th>Cholesterol LDL &lt;255 Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: No adjustment</td>
<td>2.3 (0.002)</td>
<td>2.5 (&lt;0.001)</td>
<td>2.7 (&lt;0.001)</td>
<td>5.5 (&lt;0.001)</td>
<td>6.3 (&lt;0.001)</td>
</tr>
<tr>
<td>2: Nonlipid risk factors†</td>
<td>2.9 (&lt;0.001)</td>
<td>2.1 (0.003)</td>
<td>2.4 (0.001)</td>
<td>5.1 (&lt;0.001)</td>
<td>6.2 (&lt;0.001)</td>
</tr>
<tr>
<td>3: Nonlipid+lipid risk factors‡</td>
<td>2.7 (&lt;0.001)§</td>
<td>1.5 (0.2)</td>
<td>1.7 (0.06)</td>
<td>4.2 (&lt;0.001)</td>
<td>4.6 (&lt;0.001)</td>
</tr>
</tbody>
</table>

P values are presented in parentheses.

*For total LDL cholesterol levels, LDL proportion <255 Å, and LDL cholesterol levels in particles <255 Å, the RR of IHD was obtained by comparing the rate of events in the third vs the first tertile of their respective distribution. For LDL peak and integrated (mean) size, the RR of IHD was obtained by comparing the rate of events in the first vs the third tertile of their respective distribution.

†Nonlipid risk factors included age, body mass index, systolic blood pressure, smoking habits, type 2 diabetes, familial IHD history, and medication use at baseline.

‡Lipid risk factors included LDL cholesterol, HDL cholesterol, and logarithmically-transformed triglyceride and lipoprotein(a) levels.

§This model excluded LDL cholesterol levels as a covariate.
the combination of traditional risk factors was 73.9% (model 1). LDL peak particle size (area under the ROC curve=74.8%) and integrated size (area under the ROC curve=75.0%) added virtually no discriminating power to the model of traditional risk factors. However, when the absolute or relative LDL cholesterol concentration within particle sizes <255 Å was added to model 1, the ability to discriminate incident IHD cases from noncases was significantly increased (area under the ROC curve=76.8% and 77.4%, respectively; P<0.001). Adding information on cholesterol levels in particles <255 Å to the multivariate logistic model (model 5) also identified a greater number of incident IHD cases within the population’s highest tertile of risk compared with the traditional model of risk factors (N=77 vs 72).

The combined impact of concomitant variations in the proportion of LDL particles <255 Å and of LDL cholesterol levels on the risk of future IHD events is shown in the Figure. The increased IHD risk in men with LDL cholesterol levels ≥3.8 mmol/L (median of the cohort) was significant only when >40% of LDL was distributed among particles with a diameter <255 Å (RR=6.5; 95% CI, 3.1 to 13.7). On the other hand, an increased proportion of LDL cholesterol among small LDL particles was associated with a significant 4.0-fold increase in the risk of IHD (95% CI, 1.9 to 8.8), even among men with a total LDL cholesterol <3.8 mmol/L (mean LDL cholesterol in this group was 3.2±0.5 mmol/L). Similar trends were observed when categorizing subjects on the basis of elevated or reduced plasma apolipoprotein B or triglyceride levels (Figure).

**Discussion**

Results from the population-based Quebec Cardiovascular Study confirmed that LDL particle size should be considered an important risk factor for future IHD events. Our data suggest for the first time that a more refined characterization of LDL particle size by PAGGE may provide valuable information on the risk of IHD and could improve significantly our ability to identify high-risk individuals. Specifically, cholesterol levels within small LDL particles (<255 Å), among all features of LDL investigated, appeared to be the best discriminant factor of IHD risk.

Previous case-control studies had suggested that the relationship between LDL phenotype or LDL peak particle size and the risk of IHD was not independent of variations in other lipid risk factors, particularly plasma triglyceride levels and the total-to-HDL cholesterol ratio. Results of the current prospective, population-based study are concordant with these previous observations. Indeed, a reduced LDL peak particle size predicted an increased risk of IHD, independent of nonlipid risk factors but not after controlling for traditional lipid risk factors. These results suggest that assessing LDL peak particle size only by PAGGE may not substantially improve our ability to predict IHD events beyond that achieved with more traditional risk factors.

In the current report, we have extended our analysis of the relationship between LDL size and IHD by characterizing further the heterogeneity of LDL particles on PAGGE. Although the cumulative number of LDL subclasses <255 Å was higher among incident IHD cases than noncases, the number of small LDL subclasses did not predict IHD onset in multivariate analysis (not shown). The integrated (or mean) LDL particle diameter was more strongly associated with incident IHD risk than was the LDL peak size from a statistical point of view. It must be stressed, however, that the magnitude of the difference in risk associated with having small peak (RR=1.5) or integrated (RR=1.7) LDL particle size and the difference in IHD...
risk predictability (area under the ROC curve, 74.8% vs 75.0%) is considered to be only marginal.

The relative and absolute concentrations of LDL cholesterol in particles <255 Å were also investigated as potential risk factors. Men with an increased cholesterol concentration within small LDL particles (<255 Å) had a 4- to 6-fold increase in incident IHD risk compared with men having a low cholesterol concentration within small particles. This increase in risk remained highly significant after adjustment for nonlipid and lipid risk factors. In comparison, the RR associated with high total LDL cholesterol levels was increased by only 2-fold. Finally, the correlations between the absolute concentration of cholesterol in LDL particles <255 Å and LDL peak particle size and total LDL cholesterol levels, though highly significant from a statistical point of view, reflected a shared variance <50%, indicating that these various measures of LDL represent different and fairly distinct characteristics of these particles.

ROC curve analysis provided additional evidence to support the concept that the usual LDL size characteristics such as LDL peak particle size and LDL integrated size may not represent the optimal measure of the atherogeneity of small LDL particles. Indeed, a more comprehensive LDL size characterization that included the estimated relative or absolute cholesterol concentration within LDL particles <255 Å improved significantly our ability to predict IHD events. As indicated in Table 4, adding a measure of cholesterol levels within LDL particles <255 Å to the list of traditional risk factors significantly increased the area under the ROC curve. Measuring cholesterol levels in small LDL particles also contributed to the identification of 5 additional incident IHD cases in the third of the population at highest risk. The preventive efforts required to identify 5 additional cases in one third of the entire population can be considered important. In that context, the cost-benefit of adding a measure of cholesterol levels in small LDL particles as an adjunct to the series of traditional risk factors becomes a critically important but complex issue that will need to be addressed in detail in future studies.

Our results (Figure) also suggest that information on the distribution of cholesterol among LDL of various sizes may contribute to a more adequate characterization of IHD risk among individuals generally considered as being at high risk or at low risk based on the current cholesterol guidelines. Indeed, the risk of incident IHD among men with elevated plasma LDL cholesterol levels was >3 times greater when the relative proportion of LDL with a diameter <255 Å was high (RR = 6.5) as opposed to low (RR = 2.0). Furthermore, an increased proportion of small LDL particles was associated with a 4-fold elevation in the risk of future IHD, even in the presence of relatively normal LDL cholesterol levels (LDL cholesterol <3.8 mmol/L).

In conclusion, this large, prospective, population-based study confirmed that individuals with small LDL particles were at greater risk for IHD. Most important, however, our data suggest that measuring the levels of cholesterol contained within small LDL particles by PAGGE may represent the best approach to characterizing the risk of IHD associated with small, dense LDL and with elevated plasma LDL cholesterol levels. Finally, our results also suggest that further characterization of LDL particles by PAGGE may improve our ability to identify individuals at high risk for future IHD events beyond that achieved by using more traditional risk factors.

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