Low Density Lipoprotein Particle Size Phenotyping in Healthy Persons and Patients with Myocardial Infarction

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Aim. To determine distribution, size, and phenotype of low density lipoprotein (LDL) subclasses and examine the influence of plasma lipid concentrations on lipoprotein particle size in both healthy population and patients with myocardial infarction.

Methods. Nondenaturing gradient (3-31%) gel electrophoresis for lipoprotein separation was used to determine the distribution and size of LDL subclasses in 132 patients with myocardial infarction and 334 healthy control subjects.

Results. Large LDL subclasses (LDL1, LDL2, phenotype A) were dominant in 88.5% of the healthy population, whereas in most patients with myocardial infarction (81%) the dominant subclasses were LDL3 and LDL4 (phenotype B). Only 19% of the patients belonged to the phenotype A (LDL1 and LDL2). Mean LDL subclass size (nm) was significantly smaller in patients with myocardial infarction than in controls (24.38±1.07 nm vs 25.94±0.89 nm; p<0.001). In both groups, LDL size was independent of LDL plasma cholesterol but associated with high triglyceride plasma concentrations.

Conclusion. Coronary artery disease is associated with the predominance of small LDL particles and high plasma triglyceride concentrations. The risk of development of cardiovascular disease can be assessed more accurately by determining lipoprotein subclasses.

Key words: arteriosclerosis; lipoproteins, LDL; electrophoresis, polyacrylamide gel; lipids; apolipoproteins; myocardial infarction

The role of lipoprotein particles in the development of arteriosclerosis and coronary artery diseases has been studied for years. More recently, it has become clear that increased blood concentration of total and low-density lipoprotein (LDL) cholesterol is not a characteristic of individuals who suffer from coronary artery disease and that a large portion of patients treated with plasma lipid lowering medications continue to have clinical events (1). Part of the explanation for this paradox is the fact that routine tests for total and LDL cholesterol do not detect most metabolic disorders contributing to arteriosclerosis, the most common of which is abnormal distribution of LDL and high density lipoprotein (HDL) subclasses (2).

LDLs are present in plasma as a heterogeneous population of particles of various sizes, densities, and compositions (2). The presence of small, dense LDL particles (sLDL) is particularly atherogenic (2,3). However, it is unclear whether these particles are independent risk factors for coronary artery disease or they simply act as markers of other atherogenic lipid abnormalities (4-6). Recent studies revealed an independent association of LDL size with coronary events and LDL subclass distribution as the best predictor of atherogenetic outcome (1,3). The mechanisms that contribute to atherogenicity are reduced clearance of sLDL particles from the bloodstream, their penetration into the artery intima, and retention in the artery wall (7). Due to their longer retention in the circulation, sLDL particles become oxidized, which compromises their binding to the LDL receptors. As a result, they bind to the macrophage scavenger receptor. This provides the basis for the formation of plaques. Reduced connection spots for sLDL particles on LDL receptors speak in favor of this explanation (7-9).

The factors often associated with the decreased LDL size are male gender, increased triglyceride concentrations, and decreased HDL cholesterol concentrations (10). Recent prospective studies have shown that decreased LDL size is associated with greater risk of coronary artery disease (11,12). In the Stanford-City Project study, LDL size was associated with 3-fold greater risk of heart attack, irrespective of age, sex, and triglyceride, HDL, and LDL plasma concentrations (11). The Physician Study showed that LDL particle size was also associated with the risk of myocardial infarction and that it correlated with high concentrations of triglycerides and ApoB-100 and low concentrations of HDL cholesterol and ApoA-1 (12).
The aim of our study was to determine the particle size distribution of LDL subclasses in healthy population and patients with myocardial infarction in FR Macedonia, and to examine the influence of plasma lipid concentrations on lipoprotein size.

Subjects and Methods

We examined LDL size distribution in 132 patients (96 men and 36 women) aged 25 to 63 years (47±10.4), with a history of myocardial infarction. The condition had been diagnosed upon their admission to the Clinic for Cardiovascular Disease, Skopje University School of Medicine, on the basis of the following criteria: chest pain of more than 20 min in duration, characteristic electrocardiogram changes, and increased concentrations of cardiac enzymes. The patients were systematically recruited for the study in the period between October 1999 and March 2001, during their regular checkups at the Clinic. Since myocardial infarction can change blood lipid concentration in the first two weeks after the event, the patients were assessed for the inclusion in the study a month after the hospitalization. Individuals with diabetes mellitus, renal diseases, neoplastic disorders, and those treated with lipid-lowering drugs were excluded (Fig. 1).

Electrophoretic Separation of LDL Subclasses

Although Pharmacia GE-4 Gel Electrophoresis Apparatus has been the most commonly used instrument for lipoprotein size phenotyping (4,14), the sources of Pharmacia’s specialized electrophoresis chambers have recently become uncertain and Pharmacia Apparatus was not available to us. Therefore, we used Bio-Rad MiniProtein II Apparatus (15) and applied an alternative method with nondenaturing polyacrilamide gradient (3-31%) gel electrophoresis for LDL subclasses separation. During the setup of the method, we developed a new casting protocol in our laboratory and made glass cassettes fitting the Bio-Rad electrophoretic chamber (15). Two peristaltic pumps (Masterflex L/S, Cole-Parmer Instrument Company, Vernon Hills, IL, USA) were used to cast the gradient gels. The pumps were controlled by Compaq 386 Computer with Cole Parmer software to convey the gradient characteristics of 3-31% gradient in 13 linear segments separately. The total flow coming from both pumps in each segment was 8 mL/min, and the procedure lasted for 22 min. Gels were kept in a running buffer refrigerated for approximately 2 months.

Plasma samples and human standard were prestained with Sudan Black B for 18 h for analysis of cholesterol-stained lipoproteins, as described elsewhere (16). Twelve samples were loaded to each gel. Human plasma standard, high molecular weight protein standard (Pharmacia HMW protein standard), and carboxylated polystyrene microspheres (beads, 38 nm diameter) were loaded to calibrate particle size. Pharmacia HMW protein standard was run on the central line. Beads were prestained with Sudan Black B 6 h before loading. They were also loaded in the central line 2 h after the beginning of electrophoresis to avoid mixing with HMW protein standard. In the first and last position, human plasma standard with 4 LDL and 3 HDL subclasses was loaded. After separation, HMW protein standard was stained with Coomassie brilliant blue G-250. Gels were then soaked in buffer and could be stored in sealed plastic bags for several years with no loss of stain. Gels were scanned with a Pharmacia Biotech laser scanner at wavelength of 632 nm, using Image Master Software. Peak particle sizes were calculated from standard curve obtained from the absorbance profiles of HMW Calibration Kit, beads, and human standard by the log of the known diameters and their migration distances (RI) from the starting point.

Statistical Analysis

Two-sample unpaired Student t-test was used to compare the age, plasma lipid concentrations, and LDL particle sizes of patients and healthy controls. The differences between the groups in distribution of LDL subclasses and sex were compared by chi-square test. Pearson correlation and multiple regression analysis were performed to investigate association between LDL size and other plasma lipid parameters. The value of p<0.05 was considered significant. Statistica for Windows software (version 5.0 A, Stasoft Inc. 1984-95; Tulsa, OK 74104, USA) was used for all statistical analyses.

Results

Plasma Lipids and Apoproteins

Patients with myocardial infarction had significantly higher mean total cholesterol, LDL cholesterol, triglyceride, and ApoB concentrations than control subjects (p<0.001 for all). HDL cholesterol and ApoA-1 concentrations were significantly lower in patient group than in control group (Table 1).

LDL Subclass Distribution and Size Phenotyping

The relative migration distances of lipoprotein subclasses in eight human plasma samples with indication of the lipoprotein regions, protein standard, and human standard are presented in Figure 2.

Large LDL subclasses (LDL1 and LDL2, phenotype A) were predominant in 88.5% of healthy controls, whereas small LDL subclasses (LDL3 and LDL4, phenotype B) were predominant in only 11.5% of the

Figure 1. The selection of subjects for the study.

For comparative analysis, 334 healthy blood donors (195 men and 139 women) aged 25 to 63 years (47±10.4) were selected as a control group. All participants were free of acute illness and recruited from the Republic Institute of Transfusion in Skopje in the same study period. Histories of treated hypertension, diabetes mellitus, coronary artery disease, renal disease or lipid-lowering medications were exclusion criteria for controls. The groups did not significantly differ in age (p=0.081), but they differed in sex (Fig. 1, chi-square = 8.3, p = 0.004). All participants included in the study signed informed consent forms.

Plasma Samples

Blood samples were obtained by venipuncture after 12-h overnight fast and anticoagulated with K3 EDTA. Plasma samples were separated at 3,000 rpm and kept at -80°C until analysis.

Plasma Lipid and Apoprotein Measurements

Plasma total cholesterol and triglyceride concentrations were determined by enzymatic methods (Randox Laboratories, Crumlin, UK). Determination of plasma HDL cholesterol concentrations with dectran sulfate-magnesium precipitation was followed by enzymatic determination of cholesterol. Friedewald formula was used to calculate LDL cholesterol concentrations (13). ApoA-1 and ApoB concentrations were determined by immunonephelometry (DADE Behring, Marburg, Germany).
population. Large LDL subclasses (phenotype A) were predominant in 79% and small LDL subclasses in 21% of healthy men included in the study. Greater proportion of larger particles was found in 93% of healthy women, whereas small LDL subclasses were predominant only in 7% of them (Table 2). There was a significant difference in the distribution of LDL subclasses between men and women in the control group (chi-square = 24.36, p < 0.001).

In most patients with myocardial infarction (81%), LDL3 and LDL4 (phenotype B) were predominant subclasses due to the presence of smaller, atherogenic subclasses (sLDL). Only 19% of the patients belonged to the phenotype A (LDL1 and LDL2). A significant difference in the distribution of dominant LDL subclasses was found between the patient and control group (p < 0.001). Frequency distribution of predominant LDL subclasses was different between men and women with myocardial infarction. Phenotype A was more prevalent in women (30% vs 15% in men), whereas phenotype B was more prevalent in men (85% vs 70% in women) (Table 2). There was a significant difference in the distribution of LDL subclasses between sexes, with predominance of smaller sLDL particles in men (chi-square = 14.4, p < 0.01).

The analysis of LDL subclass size as one of the significant risk factors for the development of arteriosclerosis showed a significant difference in LDL size distribution between patients and controls (Fig. 3).

Mean LDL size of dominant LDL subclass was significantly smaller in patients with myocardial infarction than in control group (24.38±1.07 vs 25.94±0.89; p < 0.001). Men with myocardial infarction had significantly smaller LDL diameter than men in the control group. Similarly, there was a significant difference in mean LDL size between women with myocardial infarction and women in the control group (Table 3).
There was a significant difference in the mean LDL size between men and women in both groups, with smaller LDL size in men (Table 3).

Pearson correlation analysis revealed a correlation between dominant LDL subclass size and plasma lipid and apoprotein concentrations in each sample (Table 4). In the control group, lower LDL size diameters correlated with higher concentrations of triglycerides and lower concentrations of HDL cholesterol. Similarly, in patients with myocardial infarction, lower LDL size diameters correlated with higher concentrations of triglycerides and lower concentrations of HDL cholesterol. There was no correlation between LDL size and ApoA-1 concentrations, but negative correlation was found between LDL size and ApoB concentration. No correlation between LDL size and plasma LDL cholesterol was found in either group. However, after multiple regression analysis, the only remaining explanatory variables associated with LDL size were triglycerides in both the control (r=-0.34, p<0.001) and patient group (r=-0.30, p<0.01) (Fig. 4) and HDL cholesterol in control group (r=-0.27; p<0.01). In both groups, significantly negative correlation was found with plasma triglyceride concentration, which was an independent predictor of LDL size.

Discussion

This study involving patients with myocardial infarction and matched healthy controls showed that LDL size was associated inversely and prospectively with the incidence of myocardial infarction. Our results were in agreement with other case-control studies reporting an increased prevalence of small LDL particles in patients with coronary artery disease (4-6,10,11) and confirmed the association of small LDL particles and coronary artery disease.

Analysis of LDL subclass distribution showed that healthy Macedonians had higher prevalence of large LDL subclasses (phenotype A, 88.5%) than patients with myocardial infarction, who showed a predominance of small LDL subclasses (phenotype B, 81%). The frequency of small LDL particles was significantly higher in the patient group (p<0.001). In agreement with previous reports (3,11,18), our data showed a difference in the subclass distribution between sexes in both study groups, with larger subclasses found in women.

Mean LDL particle size was 24.38±1.17 nm in patients with myocardial infarction and it was significantly smaller than in the control subjects (25.94±0.89 nm, p<0.001). In our study, men had generally smaller LDL particles than women, and individuals with documented myocardial infarction generally had smaller LDL particles than the individuals without myocardial infarction. Our results are close to those obtained by Austin et al (4) and Gardner et al (11), indicating that LDL size is associated inversely with the incidence of cardiovascular disease. The gender difference in LDL size distribution might explain some of the well-known differences between men and women in risk of premature coronary artery disease, with the risk of cardiovascular disease being higher in men.

In accordance with previous findings (5,19), patients with myocardial infarction had significantly increased triglyceride concentrations. A significantly inverse correlation between LDL particle size and triglyceride concentration was also observed. Current theories suggest that very low-density lipoproteins

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**Table 3.** Mean LDL sizes in patients with myocardial infarction and control subjects (mean ± SD) (nm)

<table>
<thead>
<tr>
<th>Sex</th>
<th>n patients</th>
<th>n controls</th>
<th>LDL size (nm, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>96</td>
<td>195</td>
<td>24.2±1.1</td>
</tr>
<tr>
<td>Women</td>
<td>36</td>
<td>139</td>
<td>24.9±0.8</td>
</tr>
<tr>
<td>Men vs women</td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>132</td>
<td>334</td>
<td>24.4±1.1</td>
</tr>
</tbody>
</table>

*aUnpaired Student-t test.

**Table 4.** Correlation between the dominant LDL subclass size and respective variables in patients with myocardial infarction (n=132) and control subjects (n=334)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients r</th>
<th>Controls r</th>
<th>p*</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>-0.13</td>
<td>0.150</td>
<td>-0.06</td>
<td>0.230</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.33</td>
<td>&lt;0.001</td>
<td>-0.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.27</td>
<td>0.038</td>
<td>0.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>-0.06</td>
<td>0.490</td>
<td>-0.01</td>
<td>0.800</td>
</tr>
<tr>
<td>ApoB</td>
<td>-0.29</td>
<td>0.046</td>
<td>-0.19</td>
<td>0.230</td>
</tr>
<tr>
<td>ApoA-1</td>
<td>0.08</td>
<td>0.500</td>
<td>0.30</td>
<td>0.042</td>
</tr>
</tbody>
</table>

*aMultiple regression analysis.

**Figure 4.** Correlation between the diameter of dominant LDL size and plasma triglyceride concentrations in control subjects and patients with myocardial infarction.
(VLDL1), which are present in excess in hypertriglyceridemia, take part in neutral lipid exchange with LDL through cholesteryl ester transport protein. Exchange of triglycerides from VLDL core with cholesterol esters from LDL core creates a triglyceride-rich LDL particle, which forms small, atherogenic LDL particle after being hydrolyzed by hepatic lipase (7,9,10). These sLDL particles are denser since they still contain the same amount of protein (ApoB) as other LDL particles, but a smaller amount of lipids. Thus, the ApoB concentration is a measure of the number of LDL particles, whereas LDL cholesterol concentration reflects the number of particles and their cholesterol content (21). The correlation between LDL size and plasma triglyceride concentrations confirms the importance of plasma triglyceride concentration as a factor determining small LDL subclasses formation.

It has been known for over 40 years that low plasma HDL cholesterol concentrations are associated with increased risk of coronary disease. Our findings are in agreement with previous reports on reduced plasma HDL cholesterol and ApoA-1 concentrations in patients with myocardial infarction (10,12). In addition to this, we found a positive correlation between plasma HDL concentrations and LDL size in both study groups. However, multiple regression analysis did not show statistically significant association between LDL particle size and HDL cholesterol concentrations in subjects with myocardial infarction, probably because such patients have lower HDL2 cholesterol fraction (10). In general, subjects with a predominance of large LDL particles have been characterized as having the highest HDL cholesterol concentrations and the lowest triglyceride concentrations, compared with individuals with smaller LDL particles (10,12).

Previous reports showed evidence of association between smaller LDL particles and higher apoB concentrations in cardiovascular diseases (5,21). ApoB is secreted as VLDL by the liver and remains associated with the particle until its clearance from the circulation as intermediate-density lipoprotein (IDL) and LDL. Since there is only one ApoB per particle and most of the fasting ApoB is found in the LDL fraction, its concentration could serve a crude marker of LDL particle number. In our study, ApoB concentrations were increased in patients with myocardial infarction in comparison with controls. The correlation between LDL size and ApoB concentration was inversely in patients with myocardial infarction, but not significantly, whereas in control subjects no association was found. This finding supports the theory that ApoB could rather be a marker of LDL particle number than their size.

The association between plasma LDL cholesterol concentration and LDL particle size is more complex. Whereas some studies indicated that higher LDL cholesterol concentration correlates with smaller LDL size (10,19), other studies reported no such association (5,11,20). In our study, there was no association between plasma LDL concentration and LDL size. The previous study of the family data by complex segregation analysis demonstrated that LDL phenotype is a heritable trait determined by a single major dominant gene (the alp locus) on chromosome 19 (4). In our study, the presence of small LDL particles was also common in patients and control subjects with normotriglyceridemia and in patients with normal LDL cholesterol concentrations.

The previous case-control reports comparing the predictive value of small LDL subclasses with that of lipid and apolipoprotein concentrations concluded that LDL phenotype was not independent of the concomitant variations in plasma triglycerides, HDL cholesterol or LDL cholesterol concentrations (10,12,20). On the other hand, association between LDL particle size and risk of coronary artery disease was considered even after normalization of plasma triglyceride concentrations (2,5,11). Lamarche et al (21) found that coronary artery disease risk associated with smaller LDL particles appeared to be independent of age, body mass index, alcohol consumption, smoking, high blood pressure, and concomitant variations in ApoB concentrations. However, increased ApoB concentrations combined with the greater prevalence of small LDL particles resulted in the 6-fold greater risk of cardiovascular disease (21).

In conclusion, our results suggest that plasma lipid and apolipoprotein plasma variations may not be the only factors influencing LDL particle size and prevalence of small LDL particles. Genetic predisposition, specific diet interactions, and other environmental factors probably contribute to the development of these phenomena. Small LDL particles may be considered as an independent risk factor for development of arteriosclerosis. The knowledge of LDL subclass distribution can be used to individualize patient treatment programs aimed at achieving optimal cardiovascular outcome.

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**References**


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