Low-Density Lipoprotein Subfractions and Cardiovascular Risk in Hypertension

Relationship to Endothelial Dysfunction and Effects of Treatment*


Abstract—Although hypertensive patients are at particular risk of vascular complications, the possible contribution of an atherogenic lipoprotein profile and endothelial dysfunction to this risk is unclear. We investigated this by measuring LDL subfractions and flow-mediated dilation (FMD) (reflecting endothelial dysfunction) in a cohort of high-risk hypertensive patients. We studied 84 hypertensive patients (74 men; mean age, 64 years; SD 8). Chylomicron-free LDL subfractions were analyzed by disc polyacrylamide gel electrophoresis, producing an LDL score, with higher scores being equivalent to a greater proportion of the more atherogenic LDL subfractions. High-resolution ultrasound was used to assess endothelium-dependent brachial artery FMD after reactive hyperemia after vessel occlusion. Baseline levels were compared with 61 age- and gender-matched healthy normotensive control subjects. Mean LDL score was higher and FMD impaired in hypertensive subjects compared with control subjects. These indexes were significantly improved after 6 months of cardiovascular risk factor management. LDL score correlated significantly with the 10-year Framingham coronary heart disease risk score, with a negative correlation with FMD (both P<0.001). Abnormal atherogenesis and endothelial dysfunction are both present in hypertension and appear to be related to each other, potentially leading to vascular complications. The abnormal LDL scores also correlate with the 10-year cardiovascular risk and can be positively influenced by cardiovascular risk management. (Hypertension. 2003;41:528-533.)

Key Words: endothelium ■ flow-mediated dilatation ■ LDL subfractions

Both case-controlled and prospective epidemiological studies have demonstrated that LDL cholesterol is an independent and modifiable factor for cardiovascular disease.1,2 Nevertheless, LDL is not a single entity but a heterogeneous group of particles, varying in size with a predominance of smaller particles that are more atherogenic. The increased atherogenicity is due to reduced affinity for the LDL receptor, resulting in delayed clearance from the liver.3 There is also increased arterial intima permeability for these smaller LDL particles, thus initiating enhanced foam cell formation and hence, atherosclerosis. Because the size of LDL particles is inversely correlated to their atherogenicity, patients with predominantly small LDL particles are considered to have a more “atherogenic profile” than those with larger particles, despite identical total LDL levels.4 Patients with ischemic heart disease have a higher proportion of dense LDL particles than healthy control subjects.5,6 Abnormal LDL subfractions may also be important in hypertensive patients, whose cardiovascular risk is already increased, especially since their lipid profile may also be more atherogenic than those of normotensive patients.7,8 Hyperlipidemia is known to affect endothelial function,9,10 one measure being a reduction in endothelium-dependent dilation, as assessed by noninvasive ultrasound of the brachial artery in response to reactive hyperemia, commonly referred to as flow-mediated dilatation (FMD).11 This method has allowed the evaluation of endothelial dysfunction in relation to various cardiovascular risk factors such as hypercholesterolemia, diabetes mellitus, smoking, and hypertension.11,12 Loss of endothelial integrity in hypertension is not only an early marker for potential complications (such as stroke) but may be contributory to hypertension.

The current communication considers 2 hypotheses. First, that the pathophysiological processes of atherogenesis and endothelial dysfunction are interrelated in hypertension, and second, that likely adverse pathophysiological profiles are ameliorated by treatment. To test the first hypothesis, we measured LDL and its subfractions (as indexes of atherogenesis) and FMD (an index of endothelial dysfunction) and assessed their relationship to cardiovascular and stroke risk (with the use of the Framingham equation13) by correlation. The second hypothesis was tested by intensive cardiovascular risk factor management in a cohort of the high-risk hypertensive patients to reduce the research indexes. A control group of healthy individuals were included to provide a perspective for the measured indexes: No case-control study or hypothesis is presented or tested.

*This investigation is a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT).

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Methods

Participants

Eighty-four “high-risk” hypertensive patients (74 men, 10 women; mean age 64 years, SD 8.4) were recruited as part of the Anglo Scandinavian Cardiac Outcome Trial (ASCOT) at our dedicated research clinic at City Hospital, Birmingham, UK. The methods for the ASCOT study have previously been described. In brief, the inclusion criteria for ASCOT were patients between the age of 40 and 80 years with either newly diagnosed untreated hypertensive patients with systolic blood pressure (BP) >160 mm Hg and/or diastolic BP >100 mm Hg or treated hypertensive patients with systolic BP >140 and/or diastolic BP >90 mm Hg. The blood pressure was measured after 10 minutes of rest in a quiet room. Three consecutive blood pressure readings were taken, and the average of the last 2 readings was used. We excluded patients with secondary hypertension or malignant hypertension, ongoing angina, as well as those with renal or hepatic impairment, infection or inflammatory disorders, and concurrent therapy with warfarin. The study was approved by the West Birmingham Ethics Committee, and inpatients and healthy control subjects gave their informed consent for our study.

The hypertensive patients were assessed for their future cardiovascular risk requiring ≥3 risk factors to be included into ASCOT. Risk factors included (1) left ventricular hypertrophy according to Cornell voltage duration product (>2440) or Sokolow Lyon criteria (>38 mV); (2) other ECG abnormalities (left ventricular strain pattern, abnormal Q waves, left bundle branch block, ST-T changes compatible with ischemic heart disease); (3) history of diabetes mellitus according to World Health Organization criteria; (4) past medical history of cerebrovascular event, including transient ischemic attack; (5) male gender; (6) age ≥55 years; (7) microalbuminuria/proteinuria; (8) smoking; (9) plasma total cholesterol >5.5 mmol/L; (10) family history of cardiovascular disease in a first-degree relative before the age of 55 (men) or 60 years (women); and (11) peripheral vascular disease according to Edinburgh claudication questionnaire. The 10-year coronary heart disease (CHD) and stroke (cerebrovascular disease, CVA) risks were calculated by means of the Framingham equation.

All patients were given lifestyle advice regarding diet, smoking, alcohol, and exercise in both verbal and written forms. Smoking cessation was strongly encouraged. Patients with 3 or more cardiovascular risk factors and untreated hypertension with systolic BP >160 mm Hg and/or diastolic BP >100 mm Hg or treated hypertension with systolic BP >140 and/or diastolic BP >90 mm Hg, eligible to enter the treatment phase of the trial, were allocated on a randomized, single-blind basis to antihypertensive therapy (with either amlodipine with/without perindopril or atenolol with/without bendroflumethiazide); previous antihypertensive medication was withdrawn. If total cholesterol was <6.5 mmol/L (250 mg/dL), patients were further randomly assigned into the lipid arm in a double-blind manner to receive 10 mg atorvastatin once daily or placebo. Patients with total cholesterol >6.5 mmol/L (250 mg/dL) were referred back to their general practitioner for treatment of their hypercholesterolemia. The antihypertensive treatment was adjusted accordingly by us to aim for target blood pressure for nondiabetic patients of <140 mm Hg systolic and <90 mm Hg diastolic. In patients with diabetes mellitus, the goal was <130 mm Hg systolic and <80 mm Hg diastolic.

The “healthy control” group consisted of 61 healthy normotensive control subjects (48 men, 13 women; mean age 61 years, SD 8.7) recruited from healthy hospital staff, relatives of the patients, and those attending the hospital of routine cataract surgery. The subjects were healthy and free of medications and without clinical evidence of vascular, metabolic, neoplastic, or inflammatory disease by careful history, examination, and routine laboratory tests.

Laboratory Methods

On the day of the ultrasound procedure, blood was drawn after an 8-hour fasting period with minimal trauma from the antecubital vein. Samples were put on ice for 5 minutes and then centrifuged at 3000 rpm for 20 minutes. The plasma and serum were stored at −70°C until batch analyses. In the laboratory, serum was analyzed by standard techniques for total cholesterol, triglycerides, and HDL cholesterol. LDL cholesterol was calculated by use of the Friedewald formula.

The LDL subfraction profile was analyzed by disc polyacrylamide gel electrophoresis (DPAGE), with the use of the Lipoprint LDL system (Quantimetrix Corporation). In addition to 200 μL of loading gel, 2.4 g/dL acrylamide, 0.2 g/dL N,N,N,N-tetramethylethylene bis-acrylamide, 3.6 mg/dL Sudan Black B, 25 μL serum was added to each gel tube. After thoroughly mixing, the gels were placed in front of a fluorescent light to polymerize for 30 minutes. Electrophoresis was carried out with a constant current of 3 mA per gel tube for ~65 minutes, being stopped until the HDL fraction was 1 cm from the bottom of the tube. The electrophoresis buffer contained 66.1% Tris (hydroxymethyl) aminomethane and 33.9% boric acid (pH 8.2 to 8.6). The gel tubes were allowed to settle for at least 30 minutes before being scanned at a wavelength of 610 nm (LAB laser densitometer, ImageMater Software, Pharmacia). Their migration distance from the top of the gel each identified the LDL subfractions, which have a specific electrophoretic mobility relative to the HDL fraction.

Naito et al originally illustrated DPAGE of lipoproteins in 1973, which has since been used to describe abnormalities of LDL subfraction profile in a variety of diseases. The LDL score was then calculated by using both the areas under the curve and peak heights of all LDL bands present on the gel. The relative percentage of the area under the curve was multiplied by its band number. The sum of the area under the curve of all the bands present was calculated to produce the final score. Higher scores are equivalent to a higher proportion of small, dense particles. The interassay and intra-assay coefficients of variation were 4.1% and 2.7%, respectively. All laboratory work was performed in a blinded fashion with respect to the identity of the samples.

Flow-Mediated Dilation

High-resolution ultrasound was used to assess changes in brachial artery diameter, as previously described. Measurements were taken in the morning after fasting patients had rested in a supine position for 20 minutes in a quiet room by a single observer. Good-quality images were obtained by a single operator using a 10-MHz vascular ultrasound probe (GE Vingmed Ultrasound, System Five, Slough). The brachial artery was scanned in longitudinal section ~5 cm above the elbow. The transducer remained in a fixed position relative to the patient’s arm throughout the procedure. Vessel diameter was assessed at end diastole, taken from anterior to posterior endothelium. Reactive hyperemia was measured 30 to 90 seconds after release of the cuff (FMD is a measure of endothelium-dependent vasodilation) or 3.5 minutes after sublingual glycerol trinitrate (400 μg GTN spray) (GTN-mediated dilation is a measure of endothelium-independent vasodilation). FMD and GTN-induced dilation were estimated as percent change in diameter relative to their respective baseline measurements. Interobserver and intraobserver variability of the method on 20 subjects was <10%.

Power Calculations and Statistical Analysis

We first hypothesized a relationship between LDL score and FMD. Believing a Spearman correlation coefficient of ~0.3 to be significant, we need to recruit 84 subjects to be able to test this hypothesis with a power of 0.80 and 2P<0.05. In paired analyses, this sample size provides a power of 0.80, with a probability level of <0.05 to detect a difference due to intensive treatment of 0.27 of a standard deviation. Continuous data were subjected to the Ryan-Joiner test to assess distribution. Age and blood pressure were normally distributed and are expressed as mean and standard deviation. LDL scores, CHD, and CVA risk were distributed nonnormally and are therefore expressed as median with interquartile range (IQR). Statistical analysis was performed using the unpaired t test for parametric data and the Mann-Whitney for nonparametric data, as appropriate. Correlations between risk factors and measured parameters were assessed according to the Spearman method. Data before and after intervention were analyzed by paired t test or the Wilcoxon test. A stepwise multiple regression analysis was...
undertaken with FMD as a dependent variable and clinical factors (eg, age, gender, presence of target organ damage (TOD), diabetes, smoking status, lipids, and so forth) as predictors. All statistical calculations were performed on a microcomputer with the use of a commercially available statistical package (SPSS 10.0 for Windows). A probability value of \( p < 0.05 \) was considered statistically significant.

**Results**

**Subjects**

Hypertensive subjects and control subjects were comparable in age, smoking status, gender, and ethnicity, but, as expected, patients had significantly higher mean blood pressures, serum creatinine, and body mass index, and CHD and CVA risk scores than control subjects (Table 1). The control group demonstrated better endothelium-dependent (FMD) and endothelium-independent dilation.

**Correlations Between LDL Scores and Other Markers and Multivariate Analyses**

The patients’ LDL scores correlated significantly with systolic and diastolic blood pressure, total cholesterol, LDL and triglyceride levels, and with 10-year CHD risk. LDL score correlated inversely with age, HDL, and FMD (Table 2). The correlation between LDL score and CVA risk did not reach significance.
TABLE 3. Effects of Cardiovascular Risk Factor Management on Lipids and Indices of Endothelial Dysfunction in Hypertensive Patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>After 6 Months Treatment</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mm Hg</td>
<td>167 (17)</td>
<td>146 (16)</td>
<td>0.005</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>91 (9)</td>
<td>84 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.1 (0.9)</td>
<td>5.4 (1.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.32 (0.30)</td>
<td>1.27 (0.36)</td>
<td>0.014</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.9 (0.8)</td>
<td>3.4 (1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL score, units</td>
<td>1.6 (1.2–2.0)</td>
<td>1.33 (1.0–1.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FMD, %</td>
<td>4.95 (1.56)</td>
<td>7.46 (2.00)</td>
<td>0.001</td>
</tr>
<tr>
<td>GTN-MD, %</td>
<td>17.02 (6.27)</td>
<td>17.05 (6.15)</td>
<td>0.838</td>
</tr>
</tbody>
</table>

Values are mean (SD) or median (IQR). Data were analyzed by Wilcoxon test or paired t test, as appropriate. GTN-MD indicates GTN-mediated dilatation.

A stepwise multiple regression analysis was performed to identify significant predictors of FMD. Systolic blood pressure ($R^2=0.397$, $P<0.001$), total cholesterol ($R^2=0.434$, $P=0.001$), gender ($R^2=0.460$, $P=0.007$), age ($R^2=0.484$, $P=0.008$), LDL score ($R^2=0.506$, $P=0.009$), and history of diabetes mellitus ($R^2=0.524$, $P=0.017$), were all significant predictors of FMD.

Effects of Cardiovascular Risk Factor Management

As expected, the hypertension and lipid risk factor profile of the patients improved after 6 months of cardiovascular risk factor management (all $P<0.001$), although there was also a small decrease in mean HDL cholesterol levels ($P=0.014$). LDL score ($P<0.001$) and FMD ($P=0.001$) were also significantly improved (Table 3 and Figure).

Discussion

In the present study, we have confirmed previous observations of an increased LDL score as well as greater endothelial dysfunction (as indicated by reduced FMD) in hypertension. Indeed, atherogenesis, as indicated by the LDL score, correlated positively with the Framingham CHD risk score and negatively correlated with endothelial dysfunction (as assessed by FMD). In addition, we demonstrated that LDL subfractions (as an index of atherogenesis) and FMD (reflecting endothelial dysfunction) were improved after 6 months of cardiovascular risk factor management.

The importance of LDL subfractions is increasingly recognized, with an increased proportion of small dense LDL particles being correlated with an increased risk of ischemic heart disease, even after controlling for age and gender. Furthermore, several prospective studies have shown that a predominance of small dense LDL particles predicts an increased risk of subsequent coronary artery disease.

The underlying mechanism(s) responsible for the increased atherogenicity of the small dense LDL particles have remained speculative. Previous case-controlled studies suggested that the association between the CHD risk and LDL particle size was closely linked to other lipoprotein and lipid levels, but 2 studies concluded that this effect might be independent of HDL, LDL, and total cholesterol. These independent effects may be related to a reduced affinity of the dense particle to the hepatic LDL receptors, resulting in a delayed clearance. There is also some evidence of a higher affinity of dense LDL particles to bind to intimal proteoglycans. In addition, more dense LDL subfractions are more susceptible to oxidative modification and may therefore contribute more to the generation of macrophage-derived foam cells, the hallmark of atherosclerotic plaques. The present study suggests that LDL subfractions may also be related to endothelial dysfunction, providing further mechanism(s) by which atherogenesis and the endothelium are linked.

In the present study, one confounder may be the concomitant use of lipid-lowering therapy as part of the intensive cardiovascular risk factor management. The response of LDL subfractions to lipid-lowering treatment with fibrates and HMG-CoA reductase inhibitors has been controversial. In one study, fenofibrate induced a shift in LDL subtype from small dense LDL to intermediate-dense LDL, whereas atorvastatin reduced all LDL subfractions, with the absolute effects on the more atherogenic small dense particles being comparable. Similarly, pravastatin caused a change in the LDL particles to a more dense form, whereas bezafibrate was more effective in raising HDL cholesterol and altered LDL particle composition to a more favorable form. However, neither of these studies alone would explain the reduction in the LDL score observed in the present study. More than half of our patients were randomly assigned in double-blind fashion to receive 10 mg atorvastatin or placebo, and therefore a significant proportion would have received active treatment. Because the trial is in progress, we are unable to break the blinding code to discover which patients are receiving lipid-lowering treatment. The overall lipid-lowering effect of atorvastatin on the group as a whole would not be sufficient to instigate the decrease in small dense LDL particle observed. Our observations may therefore have an additional if not different underlying explanation. We suggest...
that the combined effect of the intensive cardiovascular risk factor management “care package,” which included blood pressure lowering and diet and lifestyle advice, resulted in the observed (beneficial) changes in lipids as well as indexes of endothelial dysfunction. Although blood pressure lowering, diet, and lifestyle advice have shown to influence lipoproteins, their effects on LDL subfractions have not been investigated in detail.33,34 Interestingly, there was also a small reduction in HDL cholesterol levels after 6 months of cardiovascular risk factor management; although the influence of drug regimes is a possibility, alteration of fat intake (as part of dietary/lifestyle alterations) can also result in a reduction in HDL cholesterol levels.35

As part of our study, we demonstrate a correlation between LDL score and CHD risk, as assessed by the Framingham equation.16 The latter is a validated method for assessing cardiovascular and stroke risk over the next 10 years, based on individual risk factors, and is now widely used for risk stratification to target appropriate patients for lipid-lowering therapy with HMG-CoA reductase inhibitors or initiation of antihypertensive treatment in persons having high-normal blood pressure.36 Broadly similar results have been found in prospective studies when correlating LDL particle size and CHD risk, as assessed by the Framingham equation.15

The LDL score in the present study was negatively correlated with endothelial function, as assessed by FMD. Thus, higher FMD (indicating less endothelial dysfunction) would be correlated with lower (and thus, better) LDL scores; conversely, increased atherogenesis may correlate with greater endothelial dysfunction. Importantly, we have demonstrated in the present study that both FMD and LDL score can be beneficially influenced by cardiovascular risk factor management. The effect of small dense LDL particle on endothelial function has previously been demonstrated, independent of HDL, LDL cholesterol, and triglycerides levels.38,39 In the present study, LDL score remained an independent predictor of FMD on multivariate analysis, with a greater contribution of FMD than total cholesterol. This would suggest that LDL particle size is a more important determinant of endothelial function than other serum HDL, LDL, or total cholesterol levels.

Because this substudy is part of the main ASCOT clinical trial, the investigations and treatment regimes allowed are very much protocol-driven, and the substudy was secondary to the main ASCOT trial. The paired data are also presented as a whole, in fulfillment of our power calculation, after completing a “package of care” of general cardiovascular risk management, which includes education, lifestyle advice, dietary advice, antihypertensive treatment, statin use, and so forth. Further planned analyses from this substudy will occur at the end of 5 years (on completion of the main ASCOT study), when we would hope to present complete data on our research indexes in relation to the full duration of follow-up, study end points, grades of blood pressure reduction, effects of different drug regimes (including statins), and so forth.

Perspectives
Abnormal LDL scores and endothelial dysfunction are both present in hypertension and appear to be related to each other, potentially leading to vascular complications. The abnormal LDL scores also correlate with the 10-year cardiovascular risk and can be positively influenced by cardiovascular risk management.

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