

## Plasma LDL subtype distribution in patients with or without coronary stenosis

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**Aim:** Plasma low density lipoprotein (LDL) had 3 subtypes according to its separation by polyacrylamide gradient gel electrophoresis. Of these, the small, dense (Sd) LDL particles have been accepted as an emerging cardiovascular risk factor. This study was conducted to assess the LDL subtypes in a group of patients undergoing coronary angiography.

**Materials and methods:** The study involved 56 patients—36 of whom had at least 1 vessel stenosed (stenosis group)—and 20 patients who had no stenosis (non-stenosis group). LDL subtypes were determined according to their migration pattern after non-denaturing gradient gel electrophoresis. Total cholesterol, LDL and high density lipoprotein (HDL), and triglyceride levels were also evaluated.

**Results:** Sd LDL positivity was slightly increased in the stenosis group, but the difference was not significant. Mean HDL levels were lower in the stenosis group; other lipid parameters were similar between the groups. Patients with Sd LDL positivity had significantly higher levels of triglyceride ( $P < 0.005$ ).

**Conclusion:** In the current study Sd LDL positivity was relatively higher in coronary stenosis patients; however, it is not statistically significant.

**Key words:** Lipoprotein, Sd LDL, atherosclerosis, coronary stenosis

### Koroner arter stenozu olan ve olmayan hastalarda plazma LDL alt tiplerinin dağılımı

**Amaç:** Plazma düşük densiteli lipoproteini (LDL), poliakrilamid jel elektroforeziyle 3 alt birime ayırılır. Bunlardan küçük, yoğun LDL partikülü yeni ortaya çıkan bir kardiyovasküler risk faktörü olarak kabul görmektedir. Bu çalışmada koroner anjiyografik inceleme yapılan bir grup hastada LDL alt tiplerinin belirlenmesi amaçlanmıştır.

**Yöntem ve gereç:** Çalışmada en az bir damarında tıkanıklık olan 36 ve tıkanıklığı olmayan 20 hasta yer aldı. LDL alt grupları denatüran olmayan gradiyent jel elektroforezindeki migrasyon paternine göre belirlendi. Ayrıca total kolesterol, LDL, yüksek densiteli lipoprotein (HDL) ve trigliserit düzeyleri belirlendi.

**Bulgular:** Ortalama HDL düzeyleri stenozlu grupta daha düşükken, diğer lipit parametreleri gruplar arasında benzerdi. Küçük, yoğun LDL pozitifliği stenoz grubunda biraz daha yüksekken, fark anlamlı değildi. Küçük, yoğun LDL pozitifliği olan hastalarda trigliserit düzeyleri anlamlı olarak yüksektir ( $P < 0,005$ ).

**Sonuç:** Bu çalışmada küçük, yoğun LDL pozitifliği koroner stenozlu hastalarda rölatif olarak yükseğe de istatistiksel anlamlı fark görülmemiştir.

**Anahtar sözcükler:** Lipoprotein, küçük, yoğun LDL, ateroskleroz, koroner stenoz

Received: 11.10.2010 – Accepted: 11.01.2011

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## Introduction

Atherosclerosis plays a major role in the pathology of cardiovascular diseases. Practically all patients with myocardial infarction, as defined by electrocardiography and enzymatic changes, have coronary atherosclerosis. Coronary artery disease (CAD) has been an important cause of morbidity and mortality in Turkey for the last 2 decades (1,2)

Elevated serum cholesterol, especially low density lipoprotein (LDL) cholesterol, is established as a major risk factor—along with sex, age, family history, hypertension, smoking, and diabetes—in the development of CAD (3). In addition to hypercholesterolemia, hypertriglyceridemia is a risk parameter for atherosclerosis (4). Discovering novel diagnostic markers for early detection and follow up of CAD has been the objective of many studies in this area (5).

LDL particles heterogeneous in respect to size, density, and lipid composition have been divided into 3 subtypes: large, more buoyant LDL particles; medium LDL particles; and small, dense (Sd LDL) LDL particles (6). Sd LDL particles have been found to be atherogenic because of their smaller particle size, diminished recognition by the LDL receptor, prolonged residence time in plasma, and lower resistance to oxidative stress compared to large, buoyant LDL (7,8). Several studies have reported a 2- to 3-fold increase in coronary heart disease risk among patients with this Sd LDL subtype (9). A preponderance of Sd LDL particles has been accepted as an emerging cardiovascular risk factor by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III, USA (10).

A number of methods have been developed to characterize LDL heterogeneity. Density gradient ultracentrifugation of plasma or isolated LDL has been commonly used to separate the LDL particles according to density (11). An advantage of the ultracentrifugation technique is the possibility for compositional studies of LDL subtypes. Non-denaturing polyacrylamide gradient gel electrophoresis, on the other hand, separates LDL according to particle size, is comparably easy to perform, and has been extensively used in clinical studies.

This study aimed to detect LDL subtypes in the plasmas of patients undergoing coronary angiography by non-denaturing gradient gel electrophoresis and to search for an association between Sd LDL positivity and coronary stenosis.

## Materials and methods

### Subjects

The study was approved by the institutional review board and the patients who attended cardiology clinic consented to a full diagnostic workup. Enrolled in the study were 56 consecutive patients who had undergone coronary angiography at the cardiology laboratory of Ibn-i Sina Hospital, Ankara, Turkey, during the same month. Patients older than 75 years and those with severe renal, hepatic, infectious or malignant disease or any other clinical instability after angiography were excluded from the study. Blood samples were drawn after an overnight fast. After routine tests had been performed, plasmas were stored at  $-80^{\circ}\text{C}$  for LDL subtype analysis.

### Lipid analysis

Total cholesterol (TC), triglyceride (TG), and high density lipoprotein cholesterol (HDL) levels were determined on a Technicon Dax-96 autoanalyzer using enzymatic reagents (Biotrol Diagnostics) and anti-human  $\beta$  lipoprotein precipitation (Sigma Diagnostics) (12,13). LDL cholesterol levels were estimated for all subjects by using Friedewald's formula (14). When TG levels were higher than 400 g/dL, LDL levels were determined by enzymatic assay (Centronic).

### Gradient gel electrophoresis

LDL was separated by gradient gel electrophoresis with a linear non-denaturing polyacrylamide gradient of 3% to 7.5%. Gradient gels were cast using a manual gradient maker (170-9042 Model 475 Gradient Delivery System, Bio-Rad, Richmond, CA, USA). Just before pouring, freshly prepared 10% (w/v) ammonium persulfate (Merck) was added to the acrylamide (Sigma) solutions to attain a polymerization time of 90 min. Poured into each injector of the gradient maker were: 20 mL of 3% acrylamide solution (acrylamide, 29.25 g/L; bisacrylamide, 0.75 g/L; Tris, 0.375 mol/L, pH 8.35;

Temed, 0.6 mL/mL; ammonium persulfate, 1 g/L) and 20 mL of 7.5% acrylamide solution (acrylamide, 73.125 g/L; bisacrylamide, 1.875 g/L; Tris, 0.375 mol/L, pH 8.35; Temed, 0.4 mL/mL; and ammonium persulfate, 0.5 g/L). The acrylamide gradient was formed by allowing the gradient mixture to fill the gel casting cassette (Bio Rad Protean Xi Cells; 1.0 mm spacers, 15-well combs) from the bottom by hydrostatic pressure for 15 to 20 min.

The vertical slab gels were run in the Bio Rad Protean Xi Cells apparatus. A total volume of 20  $\mu$ L of plasma sample mixed in a 1:1 volume ratio with a sample buffer containing 20% sucrose and 0.25% bromophenol blue was loaded onto the gels. Electrophoresis was performed by using the running buffer [Tris (180 mmol/L), boric acid (160 mmol/L), and Na<sub>2</sub>-EDTA (6 mmol/L pH 8.35)], with cooling from a thermostatic circulator set at 10 °C for 24 h at 125 V for a total of 3400 volt-hours, as previously described (15). Control samples were from 2 well-characterized subjects, 1 with large LDL and 1 with Sd LDL, as previously described (16). The gels were stained for lipid with Oil Red O (Allied Chemical) dissolved in 60% ethanol for 24 h at 55-60 °C and destained in a 5% solution of acetic acid. Gel images were analyzed with ImageJ software (NIH, USA). LDL subtype of a patient is determined according to the relative, or predominant, distribution of lipoprotein particles.

### Statistical analysis

The Mann-Whitney U test was used to compare means of the groups that did not have a normal distribution. The Kruskal-Wallis analysis of variation was used to compare more than 2 groups. Between-group differences in gender and smoking habits; the prevalence of diabetes, hypertension, and hyperlipidemia; family history; and LDL phenotype were analyzed by the chi-square test or Fisher's exact test.

## Results

### Patient characteristics

After angiographic evaluation 36 patients were involved in the stenosis and 20 patients in the non-stenosis group. The male sex, diabetes, and smoking were significantly more prevalent among the stenosis

cases ( $P < 0.05$ ). The distribution of risk factors among 2 groups is summarized in Table 1.

### Plasma lipids

Mean serum TG, TC, and LDL levels were similar between the stenosis and the non-stenosis group, whereas mean HDL levels were significantly lower in the stenosis group than in the non-stenosis group ( $P < 0.05$ ) (Table 1).

Table 1. Characteristics of stenosis and non-stenosis patients.

	Stenosis (n = 36)	Non-stenosis (n = 20)
Age	54.4 $\pm$ 9.4	53.8 $\pm$ 10.7
Sex, male	23 (63.9)*	7 (35)
Hypertension	17 (47.2)	9 (45)
Diabetes mellitus	14 (38.9)*	3 (15)
Hyperlipidemia	26 (72.2)	13 (65)
Smoking	21 (58.3)*	6 (30)
Family history	15 (41.7)	11 (55)
Lipoproteins (mg/dL)		
Total cholesterol	215.4 $\pm$ 53.7	217.3 $\pm$ 41.9
HDL	39.2 $\pm$ 9.3*	46.4 $\pm$ 10.6
LDL	129.2 $\pm$ 45.8	134.4 $\pm$ 37.9
Triglyceride	195.9 $\pm$ 135.8	157.3 $\pm$ 104.5

Values are frequency (%) or mean  $\pm$  SD;

\* $P < 0.05$ .

### LDL subtypes

When the gels were evaluated at the end of 24 h electrophoresis the largest lipid particles remained near the origin, and smaller particles migrated farther in accordance with their particle size. LDL particles were separated over a distance of 25 to 30 mm of the 3% to 7.5% polyacrylamide gel (Figure). The distribution of LDL subtypes in the stenosis and non-stenosis groups is summarized in Table 2. Although Sd LDL positivity was slightly higher in the stenosis group compared to the non-stenosis group, the difference between the groups was not significant (Table 2).

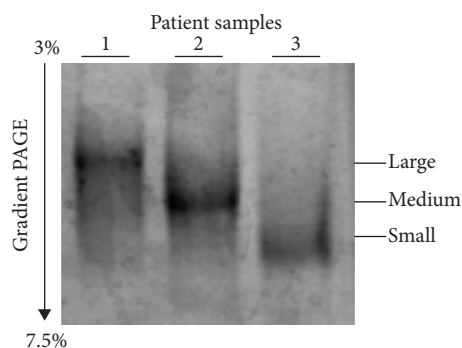


Figure. Representative gel photograph of 3 patient samples with each different LDL subtype: small, dense, medium, and large LDL. Sd LDL particles migrated farthest in the gel with a gradient of 3%-7.5% polyacrylamide.

Table 2. Distribution of LDL subtypes among stenosis and non-stenosis groups.

	Stenosis (n = 36)	Non-stenosis (n = 20)
Sd LDL	11 (30.5)	5 (25)
Medium LDL	6 (16.7)	6 (30)
Large LDL	19 (52.8)	9 (45)

Values are frequency (%).

### Association between LDL subtypes and plasma lipids

Of the 56 patients who underwent coronary angiography 28 had large LDL, 12 had medium LDL, and 16 had Sd-LDL. Mean TG, TC, HDL, and LDL levels for each LDL subtype are shown in Table 3. Mean TG levels were significantly higher in the Sd-LDL group than in the medium and large LDL groups

( $P < 0.005$ ). In 14 patients TG levels were greater than 200 mg/dL, and 10 patients had small LDL.

### Discussion

In the present study, plasma LDL subtypes were determined in patients undergoing coronary angiography to evaluate the positivity of an emerging risk factor for CAD—Sd LDL—in stenosis and non-stenosis cases. While both groups had a similar percentage of Sd LDL in this study, mean TG levels were significantly higher in patients having Sd LDL subtype compared to patients with medium and large LDL.

Considering atherosclerosis risk factors, the stenosis group had 2 times more male patients, more than 2 times the diabetes patients, and almost 2 times more smokers compared to the non-stenosis group. HDL is the only lipid parameter that is different between the groups; stenosis patients had lower mean HDL levels.

Although we expected to find higher Sd LDL positivity in the stenosis group, which also had a higher diabetes incidence, we observed similar LDL positivity between groups that also had similar mean TG levels. While the metabolic origin of the Sd LDL particle is not totally understood, hypertriglyceridemia was proposed as a trigger for its formation (17), and it was shown that LDL size correlated well with TG levels (18). Serum TG concentration was reported to be the most important determinant of the presence of Sd LDL particles in patients with metabolic syndrome (19).

Previously in the Physician’s Health Study Survey it was proposed that Sd LDL increased the risk for

Table 3. Mean values of the lipid parameters in each LDL subtype.

	Large LDL	Medium LDL	Sd LDL
Triglyceride	143.8 ± 65	121.5 ± 41.1	273.6 ± 174*
Total cholesterol	209.3 ± 53	203.1 ± 31.1	243.9 ± 50.6
HDL	42.4 ± 11	41.5 ± 7.7	37 ± 8.3
LDL	134.1 ± 43	121.3 ± 33.8	135.5 ± 51

Values are mean ± SD;  
\* $P < 0.001$ .

coronary artery disease. However, in multivariate analysis it was found that Sd LDL did not have any significant effect in coronary heart disease, but TG levels continued their effect (20). Interestingly, CAD and LDL size were found to be unrelated in a study concerning an older population in Finland (21). Sd LDL positivity was found to be similar between chronic hemodialysis patients and healthy controls, and, additionally, hemodialysis patients with or without coronary artery disease had similar Sd LDL proportion and size (22).

In the current study Sd LDL positivity was higher but statistically insignificant in stenosis patients. The relation between LDL size and incidence of coronary events during follow-ups had been evaluated by prospective studies, which reported Sd LDL positivity before diagnosis of coronary events (20,23). Therefore, the presence of Sd LDL in non-stenosis patients may imply a risk for the development of future stenosis, and these patients should be followed up carefully with risk reduction and drug therapy.

It is surprising that in this study nearly half of the patients in the stenosis group had large LDL. In a previous study LDL size was found to be identical in patients with cardiovascular disease and controls. It was concluded that large LDL size might be an independent predictor of coronary events by different mechanisms than Sd LDL (24). Large LDL had reduced affinity for LDL receptor, which clears LDL from plasma, and has been found capable of depositing more cholesterol into plaque than Sd LDL (25). A report from ~5500 asymptomatic individuals

in the Multi-Ethnic Study of Atherosclerosis implied that higher concentrations of large LDL were significantly associated with carotid intima media thickness, which is a direct and well-validated measure of subclinical atherosclerosis, and concluded that both small and large LDL were “atherogenic” to a similar extent (26)

Although it has been investigated as a marker of atherogenic dyslipidemia and coronary risk factor (10), the ability of Sd LDL to cause heart disease independent of other factors (such as diabetes and hypertriglyceridemia) has not been fully established. Perhaps as a result of this, it has not been recommended as a routine test in daily clinical practice, yet (27). However, studying lipoprotein subtypes is important for advancing research, developing potentially novel therapies, and understanding the pathophysiology of atherothrombotic diseases (28). In the current study Sd LDL positivity was relatively higher in coronary stenosis patients; however, it was not statistically significant. Large prospective cohort studies and intervention studies may help to determine whether Sd LDL should be used as a routine test in the diagnosis and follow up of atherosclerosis and CAD.

### Acknowledgements

This study was carried out during Yeşim Öztaş's residency training in the Department of Biochemistry, Faculty of Medicine, Ankara University. Dr. Mehmet Yapar from Gülhane Military Medical School offered valuable technical contributions to this study.

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