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Distribution of LDL subgroups in patients with hyperlipidemia

Ebru TAYLAN*, Emine Pınar TUNCEL
Department of Medical Biochemistry, Faculty of Medicine, Dokuz Eylül University, İzmir, Turkey

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Background/aim: Low-density lipoproteins (LDLs) have been shown to be a major risk factor for coronary artery disease. Multiple distinct subspecies have been identified among LDL particles on the basis of differences in size, density, and chemical composition. Particles with a diameter of <25.5 nm are defined as small dense LDL (sdLDL) and have been shown to be associated with increased risk of coronary disease. Subjects with predominance of sdLDL (pattern B) tend to have higher levels of triglyceride (TG) and lower levels of high-density lipoprotein cholesterol (HDL-C). In this study, we investigated the distribution of LDL subgroups in subjects with different types of hyperlipidemia, such as hypertriglyceridemia (hyperTG), hypercholesterolemia (hyperCHO), and combined hyperlipidemia (HL).

Materials and methods: We used gradient gel electrophoresis and a precipitation method with heparin-magnesium reagent to determine LDL subgroups.

Results: It was found that there was a significant ($P < 0.02$) association between the lipid panel and LDL subgroups. The percentage of sdLDL in all HL groups was higher than in controls, and the ratio of sdLDL was highest in patients with hyperTG.

Conclusion: The predominance of sdLDL is closely related to hyperTG and low HDL-C levels.

Key words: Hyperlipidemia, atherosclerosis, small dense low-density lipoproteins.

1. Introduction

Atherosclerosis is an inflammatory disease characterized by the accumulation of lipids in arteries, which undergo gradual thickening, causing decreased elasticity and reduced blood supply and resulting in constriction of the lumen. It can lead to ischemia, heart failure, stroke, and myocardial infarction (1–4). Low-density lipoproteins (LDLs) are primary plasma lipid carriers in humans that comprise two distinct subfractions: large buoyant LDL (lbLDL) and small dense LDL (sdLDL) particles that differ in size, density, physicochemical composition, metabolic behavior, and atherogenicity (5).

In studies, the most widely used technique is the determination of the peak LDL particle diameter by gradient gel electrophoresis (GGE). Based on the particle diameter, individuals can be subdivided into those with a preponderance of dense particles, referred to as pattern B phenotype, and those with a preponderance of lbLDL particles, referred to as pattern A phenotype (6).

It has been reported that sdLDLs possess several proatherogenic properties including greater arterial wall retention (7), longer half-life in plasma, decreased receptor-mediated uptake, increased proteoglycan binding (8), and susceptibility to oxidation (9,10). LDL size seems to be an important predictor of cardiovascular events and predominance of sdLDL has been accepted as a cardiovascular risk factor by the National Cholesterol Education Program Adult Treatment Panel III (11). However, it is still a matter of debate whether to measure the LDL particle size as a cardiovascular predictor (5). For this reason, we investigated the distribution of LDL subgroups in subjects with different kinds of dyslipidemia [hypertriglyceridemia (hyperTG), hypercholesterolemia (hyperCHO), and combined hyperlipidemia (HL)] without considering the reason for dyslipidemia, and we analyzed its correlation with other lipid parameters. We used a widely used technique, GGE, to determine LDL particle diameter, and heparin-magnesium precipitation to have direct information on the concentration of the atherogenic sdLDL particles. Furthermore, we compared the two methods by correlation analysis to find out if the precipitation method was useful for the quantitation of sdLDL particles.

* Correspondence: ebru.ezer@deu.edu.tr

2. Materials and methods

2.1. Subjects

Subjects were chosen among patients who applied to the Central Laboratory of Dokuz Eylül University Hospital for lipid profile analysis. Laboratory results [high density lipoprotein cholesterol (HDL-C), triglyceride (TG), total cholesterol (TC), and LDL cholesterol (LDL-C)] were overviewed and serum samples of patients with different kinds of dyslipidemia were collected, stored at 4 °C, and analyzed for LDL subgroups within 3 days. All samples were obtained after overnight fasting. Patient demographics were not used because the relationship between the LDL subgroups and lipid parameters, and not the etiology of lipid disorder, was the point of interest.

A total of 208 serum samples (96 males and 112 females) were collected and categorized into 4 groups: 1) the control group (n = 54), which consisted of subjects with normolipidemia; 2) the hyperTG group (n = 50), with subjects who had TG of ≥150 mg/dL and TC of <200 mg/dL; 3) the hyperCHO group (n = 52), with subjects who had TG of <150 mg/dL and TC of ≥200 mg/dL; and 4) the combined HL group (n = 52), with subjects whose TG and TC values were higher than 150 and 200 mg/dL, respectively.

2.2. Gradient gel electrophoresis

The particle size of LDLs was determined by 2%–16% nondenaturing polyacrylamide gel electrophoresis using commercially available gels (Alamo Gradient Gels, USA). GGE was performed according to the method of Nichols and Krauss (12). Briefly, it was carried out at 4 °C using Tris base (90 mM) and boric acid (80 mM) buffer, pH 8.3, containing 3 mM EDTA and 3 mM sodium acid (all chemicals from Sigma). The gels were prerun in this buffer at 125 V for 20 min prior to loading samples and then 15 µL of serum was applied to each lane after diluting 4:1 with 40% sucrose and 0.01% bromophenol blue. Voltage was adjusted to 15 V for 15 min, then 70 V for 20 min, and finally 125 V for 24 h.

Calibration was performed with three standards with known physical properties. One standard was composed of a protein mixture of thyroglobulin (17 nm), ferritin (12.2 nm), catalase (10.4 nm), lactate dehydrogenase (8.1 nm), and albumin (7 nm) (HMW Marker Kit, Amersham). The second standard solution consisted of carboxylate-modified Latex particles (Duke Scientific, 32 nm, used at 1:50 dilution). Since several problems were encountered in the visualization of Latex particles, a third solution composed of silica particles (34 nm) bound to fluorescein isothiocyanate (FITC) was used.

After the electrophoresis, for visualization of the bands, first the silica was screened fluorometrically at Ex 465/Em 535 nm by using an imaging system (Kodak 2000MM). Then two lanes containing standard solutions were stained without prior fixation in 0.05% Coomassie R-250 in 50% methanol and 10% acetic acid, and destained in 20% methanol and 9% acetic acid (Sigma). For lipid staining Oil Red O was used and sample lanes were exposed to a solution comprising 0.1% stain in 60% ethanol at 55–60° C for 24 h. Then the gels were rehydrated in 5% acetic acid.

To identify LDL subclasses and standard bands in the lanes, a densitometric scan (ImageMaster Labscan, Amersham) was performed and the center of the most prominent band was marked on each lane. Migration distances were determined, and then the LDL particle diameter corresponding to each band was calculated from the slope value of the calibration curve plotted using three standards of known diameter (thyroglobulin, ferritin, and silica particles) (Figure 1). After the densitometric analysis of the gels, LDL subclasses were classified as sdLDL and lbLDL based on LDL particle size (13), as shown in Figure 2. Type A pattern was characterized by predominance of lbLDLs with LDL peak diameter of >25.5 nm; type B pattern was characterized by predominance of sdLDL particles with LDL particle diameter of ≤25.5 nm.

2.3. Determination of sdLDL cholesterol by simple precipitation method

sdLDL cholesterol (sdLDL-C) was measured quantitatively by heparin-magnesium precipitation procedure according to the method of Hirano et al. (14). Accordingly, the precipitation reagent (0.1 mL), containing 150 U/mL heparin-sodium salt and 90 mmol/L $MgCl_2$, was added to each serum sample (0.1 mL), mixed, and incubated for 10 min at 37 °C. The samples were placed in an ice bath and allowed to stand for 15 min, and then the precipitate was collected by centrifuging at 15,000 rpm for 15 min at 4 °C. An aliquot of the supernatant was removed for LDL-C analyses. Measured cholesterol content was stated as sdLDL-C. sdLDL-C% was determined by calculating the ratio of sdLDL-C to LDL-C in percentages.

2.4. Lipid analysis

Serum TG, TC, HDL-C, and LDL-C concentrations were determined on an Abbott Architect C-16000 by commercially available colorimetric kits (Abbott Architect C-16000 dedicated kits).

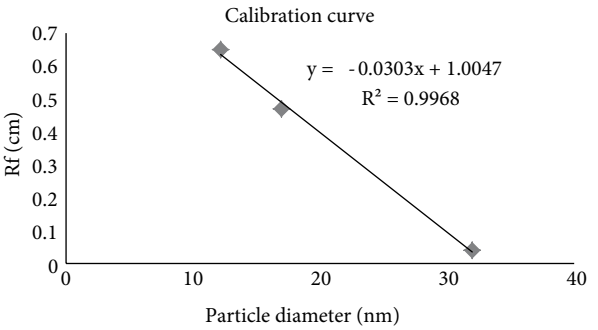

Figure 1. Gradient gel calibration line.

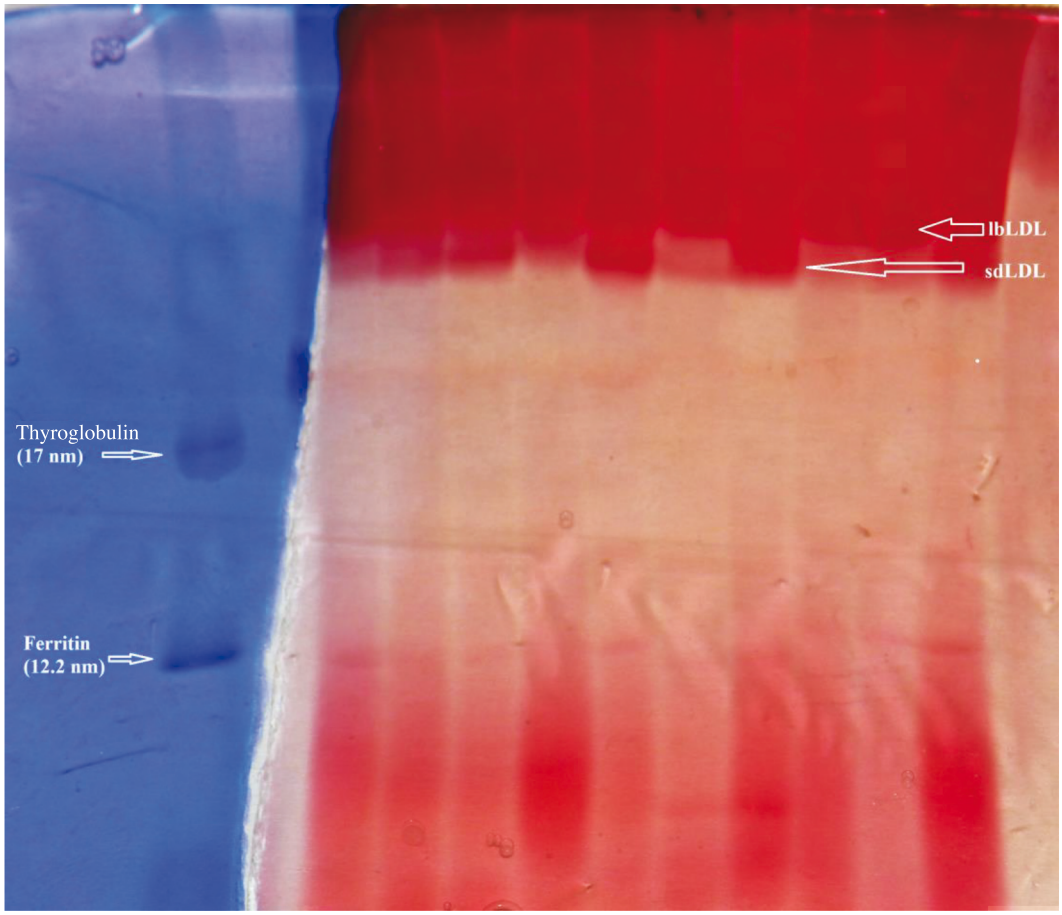

Figure 2. The 2%–16% gel.

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 11.0 for Windows. Variables were normally distributed. Variables between groups were compared using one-way ANOVA. Pearson's single linear regression analysis was used to assess the correlation between two parameters. Data are presented as mean ± standard deviation (SD). $P \leq 0.05$ was considered significant.

3. Results

Plasma lipid profiles of the studied individuals are shown in the Table. Mean predominant sdLDL diameters obtained by GGE results are shown in the diameter column, and quantitative sdLDL-C results obtained by simple precipitation method are presented in the sdLDL-C column. lbLDL-C values were calculated by subtracting sdLDL-C from the total LDL-C value. The control group's sdLDL-C level was the lowest among all groups, but the largest particle diameter was in the hyperCHO group (Table). The smallest LDL particle diameter was in the hyperTG group and only this group's mean LDL diameter was the LDL size that was considered as sdLDL (<25.5 nm). TG, TC, HDL-C, and LDL-C levels were not analyzed statistically, since groups were formed according to these lipid parameter values. The ANOVA test indicated that the rate of sdLDL in all HL groups was higher than in the control group, and the highest rate of sdLDL was in the hyperTG group.

Linear regression analysis was performed to determine the possible relationship between LDL particle size and lipid parameters. A significant negative correlation between LDL particle diameter and plasma TG ($P = 0.01$) (Figure 3) and significant positive correlations between the particle diameter and TC ($P = 0.05$) and HDL-C ($P = 0.01$) were found. Both sdLDL-C and sdLDL-C% positively correlated with LDL-C ($P = 0.05$), TG ($P = 0.01$), and TC ($P = 0.01$) and negatively correlated with HDL-C ($P = 0.01$).

When subjects were classified according to their patterns (pattern A or B) without group separation, only TG levels were statistically different between the two patterns (Figure 4).

Table. Distribution of lipid panel between the groups.

Groups	n	TG (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	sdLDL-C (mg/dL)	Diameter (nm)
Group 1 Control	54	92.70 ± 28.58	163.11 ± 29.62	44.98 ± 11.09	88.96 ± 25.80	33.81 ± 12.39[1]	25.95 ± 0.79[3]
Group 2 HyperTG	50	216.84 ± 79.79	170.30 ± 19.79	35.72 ± 7.75	90.22 ± 19.97	39.42 ± 9.02[2]	25.16 ± 0.72[2]
Group 3 HyperCHO	52	113.52 ± 29.26	233.06 ± 31.76	52.69 ± 11.50	136.31 ± 32.14	54.50 ± 13.75[4]	26.07 ± 0.82[4]
Group 4 Combined HL	52	222.60 ± 86.89	247.81 ± 34.43	43.27 ± 8.52	145.00 ± 31.20	63.06 ± 14.94	25.74 ± 0.79

Values are mean ± standard deviation. [1]lower than all groups $P \leq 0.05$, [2]lower than group 3 and 4, [3]lower than group 2, [4]lower than group 4.
hyperTG: Hypertriglyceridemia, hyperCHO: hypercholesterolemia, combined HL: combined hyperlipidemia.

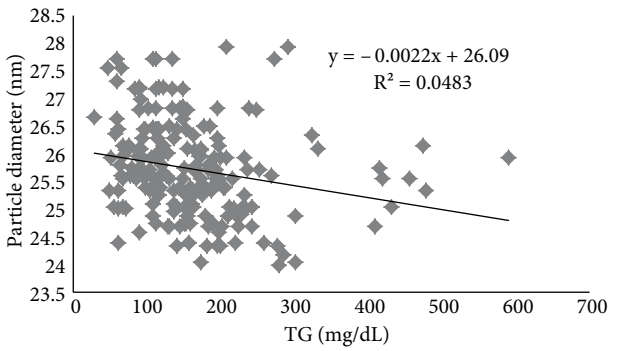

Figure 3. Plots of Pearson's single linear regression analysis between LDL particle size and plasma TG levels in 208 subjects.

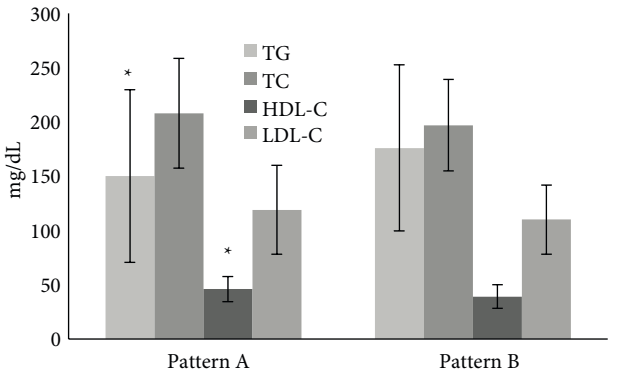

Figure 4. Comparison of the lipid parameters between two patterns (A and B).

Lastly, we categorized samples according to sex and found that only TC and HDL-C were significantly different between the two groups (P = 0.011 and P = 0.001, respectively).

4. Discussion

The present study shows that the more atherogenic particle, sdLDL, is found predominantly in hyperlipidemia, in accordance with previous reports (15–17). The increased rate of sdLDL is associated with high levels of TG and low levels of HDL-C in plasma. In this methodology, silica was used as a standard for GGE for the first time. However, our results showed that although the heparin-magnesium method was very useful in evaluating sdLDL-C, it was not reliable enough.

Plasma LDL particles play an important role in the formation of atherosclerotic lesions. Previous studies have shown that LDLs comprise a heterogeneous group of particles that differ in their biological and physicochemical properties (6,18,19). Experimental data have suggested that sdLDL has a greater atherogenic potential when compared to lbLDL subgroups (5,19–21); for this reason, it may be appropriate to determine LDL subfractions to evaluate cardiovascular risk (22). In the present study, besides LDL-C, LDL subgroups were determined by GGE according to their particle sizes and their cholesterol content, and were also measured quantitatively with a simple precipitation method in different HL groups. The results were compared with the lipid profile of patients to show the distribution of LDL subgroups.

Many studies have been conducted to find out whether it is possible to predict coronary heart disease according to the distribution of LDL subgroups. Concordantly, a Quebec cardiovascular study showed that increased cholesterol levels in the sdLDL subfraction were largely associated with an increased risk of ischemic heart disease in men (23).

In the present study, when the groups were examined for sdLDL dominance, the highest frequency was in the hyperTG group. The mean LDL particle diameter of this group was the smallest, and only this group had a particle diameter that corresponded to sdLDL. There was also a negative correlation between TG and LDL particle diameter, and TG levels had a positive correlation with sdLDL-C and sdLDL-C%. The latest studies searching for the best marker for reducing the risk of atherosclerotic cardiovascular diseases remarked on the importance of LDL particle size (24). All these results strengthen the conclusion that increased TG concentration causes high levels of atherogenic sdLDL in plasma. Although hyperTG by itself as an atherogenic risk factor is still controversial, its consequences, such as TG rich remnants, and its association with apoC-III and a higher number of sdLDL particles are considered atherogenic (25,26). Our results also demonstrated significant negative correlation between TG and HDL-C. Similar results were obtained by Griffin et al. and Rizzo et al. (27–29). Austin et al. (30) found that pattern B was associated with a 2-fold increment in plasma TG, higher plasma apoB, and intermediate density lipoproteins levels as well as reduced HDL-C and apoA-I concentration. Campos et al. (31) reported a highly significant correlation between LDL size and plasma TG levels. Plasma LDL subtype distribution has also been studied in Turkish people with coronary stenosis, and Er Öztaş et al. (32) found that patients with sdLDL positivity had significantly higher levels of TG.

When all cases were classified as either pattern A or pattern B, TG and HDL-C levels were significantly different between the two groups, but there were no differences in their TC and LDL-C levels. In the pattern B group, TG was higher and HDL-C levels were lower than in the pattern A group. These results indicated a typical atherogenic dyslipidemia phenotype and confirmed the relationship between sdLDL and TG concentration once again. Similarly, Maruyama et al. reported similar TC and LDL-C values between LDL subgroups, but they suggested a combined parameter, the TG/HDL-C ratio, as a more beneficial parameter to assess the presence of sdLDL (33). They determined that TC and LDL-C values were not significantly different between LDL subgroups. Another study showed that in renal transplant recipients, despite LDL-C values that were similar with those of the controls, sdLDL-C was higher, and this patient group had impaired distribution of LDL particles (13).

One of the methods preferred for analysis of LDL and HDL subgroups is GGE. It is a reliable method and presents good accuracy with low sample consumption. In this method, mostly Latex beads are used as a reference marker, but we encountered some problems in the application and visualization of this material. They either did not enter the gel or failed to focus on the gel. Instead, we used FITC-labeled silica particles, which have a similar particle diameter to the Latex beads. Except for the difficulty of working in the dark from the beginning to the end of the experiment (since the material is florescent), silica particles were stable and easy to load and visualize. Skoglund-Andersson et al. reported the same problem with the Latex particles, and they suggested isolated the Lp(a) fraction as a reference protein, even though it had difficulties with stability and standardization (34). We did not find any other application of silica particles in the application of GGE in our literature review, so this is probably the first time silica particles have been used for analysis for this purpose.

GGE is considered convenient to use to determine LDL subgroups, and previous studies have shown that the results of electrophoretic separation were very similar between isolated LDL and serum samples (35). Although it is regarded as a 'gold standard' for identifying sdLDL particles (36), it does not provide direct information concerning the concentration of the atherogenic sdLDL particles. Therefore, we used another method, heparin-magnesium precipitation, to determine the concentration of the atherogenic sdLDL particles. This simple precipitation method has a short assay time, a large number of serum samples can be separated in the same run, and the required equipment is found very easily. Hirano et al. studied their method in patients with different lipid phenotypes and confirmed the accuracy of their method by comparing it with GGE and suggested it for use in routine analysis (14,22,37,38). We compared Hirano's method with GGE by correlation analysis to verify the heparin-magnesium method, but we did not get similar results with the GGE and precipitation methods. In particular, an increased level of sdLDL, found especially in the hyperTG group by GGE, could not be shown by Hirano's method. Our mean sdLDL-C levels (control: 33.8 mg/dL, hyperTG: 39.42 mg/dL, combined HL: 63.1 mg/dL) were close to those of another study (control: 30 mg/dL, hyperTG: 41 mg/dL, combined HL: 62 mg/dL) (39) that used the same method, and that study also stated that sdLDL-C levels in hyperTG and combined HL samples were higher in the Hirano et al. study than in their results. During the application of the method we also noticed that, after precipitation of the samples, in some samples the supernatant was not clear enough. Hirano et al. mentioned in a review (40) the need to modify the protocol by using a filter in hyperTG serum to overcome this turbidity. Therefore, although the precipitation method yields quantitative results and is easy to perform, GGE should be preferred in order to obtain more reliable results in studies related to LDL subclasses.

Although studies have shown that genetic and environmental changes affected the distribution of LDL subgroups (19), we could not find any differences between women and men.

A potential limitation of this study was the deficiency of patient demographics. This meant we could not establish a connection with clinical aspects of LDL phenotype.

In conclusion, a high predominance of sdLDL is closely related to hyperTG and low HDL-C levels.

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