Bratisl Lek Listy 2008; 109 (4)
155 – 159

CLINICAL STUDY

Low density lipoprotein subclass distribution in children with diabetes mellitus

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Abstract: *Background:* The role of small dense low-density lipoprotein (sLDL) subclasses in atherosclerosis has been demonstrated in many studies. Among other metabolic changes, the alteration in LDL lipoprotein subclass distribution and size has been proved in diabetic adults. Because there is not enough literature data presenting LDL subclass distribution in childhood, the aim of this study was to examine LDL subclass profile in diabetic children compared with healthy control.
Material and methods: Plasma LDL subclasses in 30 children with type I diabetes mellitus and 100 healthy children aged 9–18 years were analyzed using non-denaturing polyacrilamide gradient (3–31 %) gel electrophoresis. Conventional plasma lipid and apoprotein parameters which are thought to affect LDL size were determined as well.
Results: Analysis of LDL phenotype has shown that a great percentage of healthy children (89 %) yield bigger LDL1 with LDL2 subclasses being dominant (phenotype A), whereas 11 % of the children belong to phenotype B characterized by the presence of small, atherogenic LDL3 and LDL4 subclasses. In diabetic children despite no significant differences in their plasma lipid profile when compared with healthy control, the frequency of LDL phenotype B was increased (86.7 %), and the mean LDL diameter was smaller ($p<0.0001$). LDL size was inversely correlated with plasma levels of triglycerides, and positively correlated with plasma HDL cholesterol and BMI.
Conclusion: Although plasma levels for lipid and apoprotein were within the normal range, the increased frequency of LDL phenotype B confirms a grater risk of atherosclerosis development in children with diabetes mellitus. LDL size measurement may potentially help to assess cardiovascular risk and adapt the treatment goals thereafter *(Tab. 3, Ref. 38).* Full Text (Free, PDF) www.bmj.sk.
Key words: LDL subclasses, LDL size, gradient gel, electrophoresis, diabetes mellitus, children.

Small, dense low-density-lipoprotein (LDL) subclasses are strongly associated with increased risk of cardiovascular diseases and diabetes mellitus and a reduction in LDL mean particle size has been reported in patients with coronary and non-coronary forms of atherosclerosis (1–5). For example, a preponderance of small LDL subclasses has been associated with diabetic nephropathy, (6) whereas both small LDL (7) and small HDL (8) have been associated with atherosclerosis. Modifications of lipoproteins by glycation and oxidation (9) and variations in size (i.e., diameter) distributions of lipoprotein particles within the major lipoprotein classes are not reflected in conventional profiles (10, 11). In spite of normal levels of LDL cholesterol in diabetic patients with good glucose control, they have an increased prevalence of smaller, denser LDL particles (12, 13, 14), which are more easily oxidized and therefore their binding affinity for LDL receptor is compromised. Because of this, they are present in plasma for a longer time and taken up by macrophages in extra vascular spaces, which is an important step in the development of arterial fatty streaks (15). Reduced connection spots for small LDL particles (sLDL) on LDL receptors speak in favor of this explanation (16). In accordance with these findings, it has been reported that individuals with predominance of small LDL particles (phenotype B) are at high risk of CAD development (17–21).

Although clinical manifestation of atherosclerosis, such as coronary artery disease (CAD) and stroke occur in middle or later age, studies have shown that atherosclerotic development begins in childhood (21–27). There are only several studies addressing the LDL subclass distribution in children (26–29), and only one of them deals with children with diabetes mellitus type 1 (30).

We have recently showed the LDL subclass distribution in healthy children (26). The present study is aimed at assessing whether children with type 1 diabetes mellitus have a different LDL subclass distribution that is associated with grater risk of atherosclerosis. In addition, for the first time we report the association between conventional plasma lipid and apoprotein levels and LDL size.

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Source of support: This work was supported by The Ministry of Science of R.Macedonia, research grant no. 08-2901/1; 2001

Material and methods

Subjects and blood samples collection

Thirty children with type 1 diabetes mellitus (16 boys and 14 girls) aged 9 – 17 years and 100 healthy children (51 boys and 49 girls) aged 9 – 18 years were included in this study. The children with diabetes were recruited from the Endocrinology Department at Pediatric Clinic, University Clinical Center (Skopje, Macedonia). The inclusion criteria were: age in range of 9 to 18 years and diabetes duration >12 months, nonsmoker, and no chronic disease other than type 1 diabetes. None of the children were taking medications other than daily insulin dose (1.02±0.28 IU/kg). None of the patients had evidence of microvascular complications (retinopathy, neuropathy, microalbuminuria). The children with metabolic acidosis or other chronic diseases and receiving medication other than insulin therapy affecting the lipid metabolism were excluded from the study. The healthy control children were systematically recruited for the study during their regular checkups at the Institute of Physiology and Anthropology, Medical Faculty (Skopje, Macedonia). None of the children had clinical signs or symptoms of any disease or were receiving medication, and all were nonsmokers.

The height and weight of each child was used to calculate their body mass index (BMI; kg/m^2). The groups were matched as to age, sex and BMI. The study was performed according to the Helsinki declaration and approved by the Ethics Committee of the Macedonian Medical Chamber. The parents of all children signed informed consent forms prior to examination.

Venous blood for the analysis was obtained after a 12-hour overnight fast and collected into K3EDTA containing tubes. After centrifugation at 3000 rpm for 10 minutes, plasma samples were stored at -80 °C until analysis. One portion of each sample was used within 48 hours (held on +4 °C) for analyses which require determination in fresh plasma (plasma lipid concentrations).

Lipid and apoprotein measurements

All lipid measurements were performed in fresh plasma samples, within 48 hours, kept at +4 °C. Apoportein measurements were performed in plasma samples stored at -80 °C, 2 – 3 months after collection. Plasma total cholesterol, triglyceride and glucose concentrations were examined using enzymatic methods (Randox, Crumlin, UK). Determination of plasma HDL cholesterol concentrations with dextran sulfate-magnesium precipitation was followed by enzymatic determination of cholesterol. The Friedewald formula was used to calculate LDL cholesterol concentrations (31). ApoA-1, ApoB and Lp(a) concentrations were measured by the immunonephelometry method (DADE Behring, Marburg, Germany). HbA1c was measured with high-performance liquid chromatography (Variant Analyzer, BioRad, CA).

Non-denaturing polyacrylamide gradient gel electrophoresis

Non-denaturing polyacrylamide 3 – 31 % gradient gel electrophoresis (PAGE) was performed to separate LDL subclasses and estimate their size (32). Since sources of specialized Pharmacia GE-2/4 electrophoresis chambers and commercial gels have become uncertain, we used an alternative, Mini-Protean II Electrophoresis Apparatus (BioRad 165-2941, Hercules, CA, USA). Therefore, a new casting protocol was developed and glass cassettes fitting the BioRad electrophoresis chamber were made in our laboratory. The gradient gel characteristics and all details of the method have been presented in our previous publication (33). This new gel format allowed LDL and HDL subclasses separation on the same gel and thus duplication of work was avoided.

Plasma samples and human standard were prestained for 18 hours with Sudan Black B for analyses of cholesterol-stained lipoproteins (34). Ten samples were loaded to each gel. Human plasma standard, high molecular weight protein standard (HMW; 17-0445-02, Pharmacia Biotech, Uppsala, Sweden) and carboxilated polystyrene microspheres (beads; Duke Scientific, Palo Alto, CA) were loaded to calibrate for particle size. Beads were prestained with Sudan Black B, six hours before loading, and were loaded in the same line as HMW standard, 2 hours after beginning of electrophoresis to avoid mixing. HMW protein standard was stained after separation with Coomassie brilliant blue G-250. The gels were sealed in plastic bags and could be stored for several years with no loss of stain.

Lipoprotein profiles were analyzed using a laser densitometer at 632 nm with Image Master Software (version 1,0; 1993; Pharmacia). LDL peak particle sizes were calculated from the calibration curve based on the inverse relationship between the log of the known sizes of the standards on the y-axis and their migration distances from the start of the gel (Rf) on the x-axis. LDL peak particle sizes in the plasma sample absorbance profiles were calculated using Gels Scan software (56-1131-38, Pharmacia). LDL subclasses were classified as phenotype A (diameter >25.5 nm) and phenotype B (diameter <25.5 nm).

Statistical analysis

The data are presented as mean standard deviation (SD). Comparison of mean LDL particle sizes, age, plasma lipid and apoprotein concentrations between groups was performed with the two-sample unpaired Student's t-test. The differences in LDL subclass distribution between two groups were evaluated with chí^2-test. Correlation between LDL particle size and plasma lipoprotein and apoprotein concentrations was assessed by calculating Pearson's correlation coefficients (r) and by simple linear regression. The value of $p<0.05$ was considered significant difference for all analyses in the study. All statistical analyses were done using STATWIN software (version 5,0 A, Statsoft Inc. 1984–95; Tulsa, OK 74104, USA).

Results

Mean levels of age, BMI, glucose, HbA1c, plasma lipid and apoprotein measurements are summarized in Table 1. There were no statistical gender differences in mean values of determined parameters in both examined groups. The groups were matched by age, sex and BMI. Diabetic children had lower HDL choles-

Tab. 1. Age, BMI, Plasma lipid, glucose, HbA1c and apoprotein levels in study groups (x±SD).

Parameter	Children with diabets n=30	Control group n=100	p
Age (years)	12.92±2.68	13.61±2.28	0.196
BMI (kg/m2)	19.54±3.12	20.47±3.43	0.23
Total cholesterol (mmol/L)	3.88±0.49	3.95±0.66	0.463
Triglyceride (mmol/L)	0.94±1.05	0.76±0.34	0.115
LDL cholesterol (mmol/L)	2.3±0.45	2.21±0.57	0.493
HDL cholesterol (mmol/L)	1.21±0.23	1.35±0.28	< 0.05
Glucose (mmol/L)	8.8±2.08	4.58±0.56	< 0.0001
Lp(a) (mg/dL)	15.18±11.28	11.95±5.98	< 0.05
Apo A-1 (mg/dL)	130.44±16.37	134.71±16.25	0.250
Apo B (mg/dL)	97.76±17.38	95.04±16.81	0.475
HbA1c	8.59±1.6	4.38±0.5	<0.0001

Tab. 2. Distribution of dominant LDL subclasses in diabetic and control children (%).

Dominant LDL subclass	Phenotype	Diabetic children (%)	Phenotype (%)	Control children (%)	Phenotype (%)
LDL 1		0		63	
LDL 2	A	11.5	11.5	27	89
LDL 3		65.5		11	
LDL 4	B	23	88.5	0	11

Tab. 3. Correlation between LDL size and other determined parameters in diabetic and healthy children.

Parameter	Diabetic children r	Control children r
Age (years)	0.253	0.063
BMI	-0.199	-0.131
Total cholesterol	-0.154	0.11
Triglyceride	-0.379*	0.05
HDL cholesterol	0.375*	-0.006
LDL cholesterol	-0.266	0.11
Glukose	0.284	-0.006
Lp(a)	-0.19	0.24
ApoB	0.02	0.13
ApoA-1	0.29	0.18
HbA1c	0.256	0.09

* $p<0.05$

terol, higher glucose, HbA1c and Lp(a) concentrations compared with control children. There were no statistical differences in plasma total cholesterol, triglyceride, LDL cholesterol, apoprotein A-1 and Apo B between the groups. Except for glucose and HbA1c, the values for all measured parameters in both groups were within the normal range for age of the children population (27, 29, 35) (Tab. 1).

LDL subclass distribution and size

The results obtained from all 130 children showed that in 5 % of the children only one LDL subclass was present (homogeneous population of LDL). Most subjects had polydispersion of LDL subclasses. In 27 %, a second subclass, in 48 % three subclasses and in 20% four subclasses were detected, but none had more than four. However, only one of the subclasses present in each sample was dominant. The separation on composite gradient gels was presented in our previous publication (24).

As shown in Table 2, the prevalence of small LDL particles (phenotype B) was in 86.7 % of diabetic children when compared to the control group (11 %). There was a significant difference in LDL subclass distribution between the groups ($chí^2=50.45$; $p<0.0001$). The smallest, LDL4 subclass was not found to be dominant in control children whereas in diabetic ones it was noted in 23 % (Tab. 2).

For each subject the LDL size of the dominant subclass was determined. Mean LDL particle size in diabetic children (24.64±0.59) was significantly smaller than in the control children (26.37±0.68 nm; $p<0.0001$).

Correlation of LDL size with other determined parameters

Pearson correlation coefficients between LDL size and selected variables are shown in Table 3, separately for children with diabetes and control subjects. In diabetic children, LDL size was inversely correlated with plasma levels of triglycerides, and positively correlated with plasma HDL cholesterol. Overall, LDL size was not correlated with age, plasma concentrations of total cholesterol, LDL cholesterol, glucose, HbA1c and apoproteins in diabetic children.

In control subjects, no correlation between LDL size and other parameters was noted. The only association observed was a slightly positive association with plasma concentrations of Lp(a).

However, Lp(a) cannot potentially contribute to direct regulation of LDL size (Tab. 3).

Discussion

As the process of atherosclerosis starts in childhood, the risk factors have to be assessed and prevention of atherosclerosis should start early in life (22–25). In our previous study of healthy children population we showed that the prevalence of phenotype B (small LDL subclasses) was 11 % (27), and is similar with the prevalence of phenotype B (11.5 %) in healthy adult Macedonians (4).

This is the first study that describes the LDL subclass distribution in diabetic Macedonian children aged 9–17 years. The prevalence of small LDL particles (phenotype B) was 86.7 % and was significantly increased compared with healthy controls ($chí^2$=50.45; p<0.0001). The smallest, LDL4 subclass was not found to be dominant in control children whereas in diabetic ones it was noted in 23 %. Mean LDL particle size in diabetic children (24.64±0.59 nm) was significantly smaller than in the control children (26.37±0.68 nm; p<0.0001).

A number of studies reported an increased prevalence of small LDL subclasses in patients with CAD and diabetes mellitus and confirmed the association of LDL phenotype B with atherosclerosis development (1–14). But, the data on LDL subclass distribution are insufficient in children (26–29). In the only study concerning diabetic children, the size of LDL in subjects with apoE 4/3 phenotype was smaller than in subjects with other apoE phenotypes, but LDL size has been designed as phenotype A (30). Increased prevalence of phenotype B (40–54 %, respectively) was found in obese children with increased insulin resistant markers (36, 37).

In our study, the values for all lipid and apoprotein parameters in both study groups were within range that is normal in children population (27, 29, 35). Despite this, as reported in adult diabetic patients (2, 3, 17), sLDL was found to be related with higher plasma triglyceride levels, lower HDL cholesterol and higher BMI. Metabolically, higher triglyceride concentration promotes TG transfer from VLDL to HDL. The TG-enriched HDL transfers TG to LDL and removes cholesterol from LDL. The cholesterol-depleted LDL than becomes smaller and denser. A reduction in LDL particle size associated with decreased HDL cholesterol levels may result from the alteration in lipoprotein metabolism in diabetic children which becomes more pronounced later in life. In obese children, the reduction in LDL size and association with plasma lipid and apoprotein concentrations suggested that already in adolescence phenotype B might be an important risk factor of coronary heart disease mortality and morbidity in future (36, 37). Although a direct comparison between the present study and the studies of obese children is not possible, higher BMI indicates obesity as a modifying factor of LDL particle size.

No association was observed between LDL size and total cholesterol, LDL cholesterol, ApoB, ApoA-1 and Lp(a). Although it is reasonable to expect a positive association between LDL size and LDL cholesterol and ApoB, no correlation was observed in diabetic children. The results obtained in healthy children indicate that LDL phenotype is primarily genetically determined and is not affected by plasma lipid and apoprotein concentrations in childhood (21, 27). Recently it has become clear that increased blood concentration of total and LDL cholesterol is not always characteristic for individuals with atherosclerotic vascular changes and that a large portion of CAD patients treated with plasma lipid lowering medications continue to have clinical events (1, 4). Lahndenpera et al. (17) reported that serum triglyceride had no effect on LDL size below the level of 1.7 mmol/l. In accordance with this, in our previous study (4), as well as in the study of Rajaman et al (10), in some patients with CAD and healthy adults with low LDL cholesterol and normotriglyceridemia, higher prevalence of sLDL particles was noted. In accordance with our findings, in the study of Jarvisalo et al (38), all children with type 1 diabetes had normal plasma lipid concentrations, but increased carotid artery IMT and in vitro susceptibility of LDL oxidation when compared with healthy children. This indicates that there is a relation between early atherosclerotic vascular lesions and diabetic state. Modifications of LDL, such as oxidation, have been reported to be higher in smaller denser LDL particles, which is an important step in the development of arterial fatty streaks (15). Other factors, such as specific diet interactions, exercise and some other environmental factors probably contribute in determining LDL subclass distribution in adulthood (1).

In conclusion, our results show that individuals with similar lipid values have different LDL subclass distribution. In children with type 1 diabetes there are marked abnormalities in LDL subclass distribution with increased prevalence of phenotype B, which is strongly associated with higher risk of CAD. Diabetic children have plasma lipid and apoprotein levels within the normal range, which suggests that these parameters cannot be a relevant predictor for the increased risk of atherosclerosis and emphasizes the importance of LDL phenotype as an independent risk factor of atherosclerosis. The determination of LDL subclass phenotype is important in early detection of atherosclerotic changes and may have implications in the treatment of children with diabetes.

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Received December 10, 2007.
Accepted February 12, 2008.