ORIGINAL ARTICLE

# HDL subfractions analysis: A new laboratory diagnostic assay for patients with cardiovascular diseases and dyslipoproteinemia

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## Abstract

**OBJECTIVE:** The HDL family forms a protective part of plasma lipoproteins. It consists of large HDL, intermediate HDL, and small HDL subclasses. The large HDL and intermediate HDL subclasses are considered anti-atherogenic parts of the HDL family. The atherogenicity of the small HDL subclass is currently the subject of much discussion. In the patient group with the diagnosis of cardiovascular disease (arterial hypertension, coronary heart disease) and in individuals with a non-atherogenic hypercholesterolemia, a type of lipoprotein profile (either a non-atherogenic phenotype A, or an atherogenic phenotype B) was identified, and a concentration of small dense LDL (sdLDL) was analyzed. The aim of this study was to identify the major representative of the HDL subclasses in the individuals with cardiovascular diseases, who had an atherogenic lipoprotein phenotype B, and in the individuals with the diagnosis of non-atherogenic hyper-betalipoproteinemia LDL1,2, who had a non-atherogenic lipoprotein phenotype A.
**METHODS:** Identification of the specific lipoprotein phenotype and a quantitative analysis of small dense LDL was performed by an electrophoresis method on polyacrylamide gel (PAG), using the Lipoprint LDL system.
For a quantitative analysis of HDL subclasses, i.e., large HDL, intermediatete HDL, and small HDL, in subjects with newly diagnosed cardiovascular diseases (arterial hypertension and coronary heart disease), and in subjects with a non-atherogenic hypercholesterolemia (hyper-betalipoproteinemia LDL1,2), we used an innovative electrophoresis method on polyacrylamide gel (PAG), the Lipoprint HDL system. With regard to lipids, total cholesterol and triglycerides in plasma were analyzed by an enzymatic CHOD PAP method. A control group consisted of a group of healthy normolipidemic volunteers without signs of clinically manifested impairment of the cardiovascular system.
**RESULTS:** In the patient group with the diagnosis of arterial hypertension ($p<0.0002$) and coronary heart disease ($p<0.0001$), (both are classified as

cardiovascular diseases), the large HDL subclass was significantly decreased and the small HDL subclass was increased ($p<0.0001$). The concentration of the intermediate HDL subclass did not differ from that of the control group. These results were in accordance with an atherogenic lipoprotein phenotype B in individuals with the diagnosis of cardiovascular diseases, where, using a Lipoprint LDL analysis, a high concentration of atherogenic small dense LDL ($p<0.0001$) was found. Thus, it seems that the small HDL subclass represents an atherogenic part of the HDL family.
Conversely, an increased concentration of total HDL ($p<0.0001$), large HDL ($p<0.005$), and intermediate HDL subclasses ($p<0.0001$) was found in a group of subjects with a non-atherogenic hyper-betalipoproteinemia LDL1,2.The concentration of the small HDL subclass did not differ from that of the control group. In this non-atherogenic lipoprotein profile, only traces of atherogenic small dense LDL were identified.
**CONCLUSIONS:** The advantages of this new method includes:
(i) Identification of ten HDL subfractions with Lipoprint HDL analysis (large HDL1-3, intermediate HDL 4-7, and small HDL 8-10) .
(ii) Discovery of a high concentration of smal HDL in plasma lipoproteins in patients with cardovascular diseases with an atherogenic lipoprotein phenotype B, confirmes that the atherogenic subclass of HDL family is attributable to small HDL.
(iii) Presence of a low concentration of small HDL in non-atherogenic hypercholesterolemia also confirms the atherogenic characteristics of the small HDL subclass per se.
(iv) Presence of small dense LDL is definitive to diagnose an atherogenic lipoprotein profile. It is valid for hyperlipidemia and for normolipidemia as well.

**Abbreviations:**

| | |
|---|---|
| AH | - arterial hypertension |
| CHD | - coronary heart disease |
| H-βLP | - hyper-betalipoproteinemia LDL1,2 |
| HDL | - high density lipoproteins |
| LDL | - low density lipoproteins |
| oxid-LDL | - oxidized low density lipoproteins |
| sd HDL | - small dense low density lipoproteins |
| T-Chol | - total cholesterol |
| T-HDL | - total high density lipoproteins |

## INTRODUCTION

The HDL family represents a highly heterogeneous group of plasma lipoprotein entities, in a density range from d = 1.063–1.21 g/ml (Kostner & Laggner 1989). Generally, HDL are characterized as a protective anti-atherogenic part of the plasma lipoprotein spectrum, contrary to the low density lipoprotein (LDL) family, which is considered an atherogenic lipoprotein population. Using a preparative ultracentrifuge analysis, the HDL family can be divided into different subfractions, such as HDL1, HDL2, HDL3 and VHDL, with different physical-chemical characteristics, different biological roles in the intermediary metabolism, and also different roles in the anti-atherogenic protective system of the human organism (Kostner *et al.* 2007).

A new innovative electrophoretic methods to analyze plasma lipoproteins on polyacrylamide gel (PAG), the Lipoprint HDL system (Morais *et al.* 2003), can differentiate up to ten HDL subfractions, HDL1- HDL10. However, according to the Lipoprint HDL system interpretation, these ten HDL subfractions are divided into three subclasses of HDL family, expressed as:

A. large HDL subclass, involving HDL1-HDL3 subfractions;
B. the intermediate HDL subclass (HDL4-HDL7 subfractions); and
C. the small HDL subclass (HDL8-HDL10 subfractions) (Morais *et al.* 2003; Morais 2005).

Based on this new interpretation, the large HDL class is considered the most protective for the arteries, and truly the ´good´ HDL cholesterol, in the HDL lipoprotein spectrum (Asztalos *et al*. 2004; Muniz & Morais 2005; Morais 2005). The intermediate HDL class also represents a protective part of the HDL spectrum, but small HDL, however, may likely make up an atherogenic part of the HDL family (Despres 2007).

The new lipoprotein electrophoresis method on PAG, the Lipoprint HDL system, can be a potent diagnostic complement to LDL subfraction analysis, especially in those cases where an atherogenic lipoprotein phenotype, with a high concentration of small dense LDL, has already been identified.

To date, clinical information has been lacking about the predictive value of HDL subclass analysis for the determination of the degree of cardiovascular risk in those individuals with cardiovascular diseases and an impaired lipoprotein metabolism.

The aim of this pilot study, therefore, was to identify a major representative HDL subclass in the HDL family, that is associated with cardiovascular diseases (arterial hypertension and coronary heart disease). In the first step of the study, all individuals were tested, using the Lipoprint LDL system to determine the lipoprotein phenotype. This identified the individuals with a diagnosis of cardiovascular diseases combined with an atherogenic lipoprotein phenotype B, also the individuals with a non atherogenic lipoprotein profile but with hyper-betalipoproteinemia LDL1,2 and also the volunteers of the control group. In the second step, the concentration of HDL subclasses in all tested groups was determined.

## PATIENTS AND METHODS

A group of newly diagnosed arterial hypertension patients revealed 72 hypertensive individuals who were not receiving pharmaco-therapy. The group consisted

**Tab. 1.** Lipids and lipoprotein concentrations of HDL subgroups and small (dense) LDL. (See also Figs. 1–8).

| | T-Chol | TG | T-HDL | large HDL | intermediate HDL | small HDL | small LDL |
|---|---|---|---|---|---|---|---|
| | mmo/l ± SD | | | | | | |
| Control (n=103) | 4.16±0.59 | 1.04±0.35 | 1.31±0.29 | 0.59±0.27 | 0.56±0.10 | 0.15±0.08 | 0.036± 0.035 |
| AH (n=72) | 5.07±0.90 | 2.41± 1.10 | 1.24±0.30 | 0.44±0.24 | 0.56±0.11 | 0.24±0.07 | 0.38±0.35 |
| Control vs. AH | $p<0.0001$ | $p<0.0001$ | n.s. | $p<0.0002$ | n.s. | $p<0.0001$ | $p<0.0001$ |
| CHD (n=96) | 5.24± 1.19 | 2.28±1.07 | 1.18±0.29 | 0.41±0.20 | 0.56±0.11 | 0.22±0.07 | 0.42±0.35 |
| Contr. vs. CHD | $p<0.0001$ | $p<0.0001$ | $p<0.001$ | $p<0.0001$ | n.s. | $p<0.0001$ | $p<0.0001$ |
| H-pLP (n=110) | 6.71±0.90 | 1.29±0.49 | 1.51±0.34 | 0.70±0.46 | 0.65±0.42 | 0.15±0.12 | 0.010±0.010 |
| Contr. vs. H-PLP | $p<0.0001$ | $p<0.0001$ | $p<0.0001$ | $p<0.005$ | $p<0.0001$ | n.s. | $p<0.0001$ |

T-Chol: total cholesterol, TG: triglycerides, T-HDL: total HDL

of 36 men (average age, 47 ± 10 years) and 36 women (average age, 49 ± 12 years).

A newly diagnosed coronary heart disease was demonstrated in 96 individuals. This group of patients consisted of 60 males (average age, 56 ±12 years) and 36 females (average age, 59 ± 11.6 years).

The third group of examined individuals were individuals with untreated hypercholesterolemia, who created a hyper-betalipoproteinemia LDL1,2 group of 110 individuals. This group involved 26 men (average age, 50 ± 9.3 years) and 84 women (average age, 56 ± 12.2 years).

The control group consisted of 103 normolipidemic probands, all nonsmokers, who had no clinically apparent impairment, or laboratory signs of cardiovascular disease, and were recruited from the pool of outpatients from the department of internal medicine. The average age of the control group was 47.5 ± 8.5 years, with 48 males and 55 females.

All subjects gave written, informed consent, and the study was approved by the local ethics committee.

**Tab. 2.** Atherogenicity of small dense LDL (Bernais & Krauss 2002, Packard 2003).

| Small dense LDL are highly atherogenic for: |
|---|
| * low recognition by LDL-receptors (configuration alterations Apo B ) → |
| * enhanced aptitude for oxidation and acetylation → |
| * Oxid-LDL → release of pro-inflammatory cytokines<br>→ muscle cell apoptosis |
| * Oxid-LDL → release of metalloproteinase<br>→ collagen degradation |
| * Oxid-LDL → enhanced aptitude for trapping by macrophages (scavenger-receptors)<br>→ stimulation of foam cell formation |
| * easier penetration into the subendothelial space and formation of cholesterol deposits |

A blood sample from a cubital vein was collected in the morning after a 12 hour fasting period. EDTA-$K_2$ plasma was obtained and a concentration of total cholesterol and triglycerides in plasma was analyzed using the enzymatic CHOD PAP method (Roche Diagnostics, Germany).

Lipoprint LDL analysis using the Lipoprint LDL system (Quantimetrix Corp., CA, USA) (Hoefner *et al.* 2001; Oravec 2006) was performed in all groups. In the arterial hypertension and coronary heart disease groups, an atherogenic lipoprotein phenotype was confirmed (Austin *et al.* 1990, Van *et al.* 2007), in the non-atherogenic hyper-betalipoproteinemia LDL1,2 a non-atherogenic lipoprotein profile was confirmed (Oravec *et al.* 2011)

The quantitative analysis of HDL subfractions, which included large HDL, intermediate HDL and small HDL, was performed by the Lipoprint HDL system (Quantimetrix Corp., CA, USA) (Morais *et al.* 2003; Muniz & Morais 2005).

Statistical evaluation of obtained values was performed by an unpaired students´ t-test. The level of significance was accepted at $p<0.05$.

## RESULTS

Table 1 and Figures 1-8 show the lipid and lipoprotein parameters obtained in 72 examined subjects with arterial hypertension (AH), 96 patients with coronary heart disease (CHD), and 110 hypercholesterolemic subjects with hyper-betalipoproteinemia LDL1,2 (H-βLP). In the AH group, and in the subjects with CHD, lipid parameters were significantly higher ($p<0.0001$), compared to the control group.

In the groups with cardiovascular diseases where large HDL concentrations were lower, both reached statistical significance: in the group with AH at $p<0.0002$, and in the CHD group at $p<0.0001$. The concentrations of intermediate HDL were not changed, compared to the controls, but the concentrations of small HDL were significantly increased ($p<0.0001$) in both diagnostic

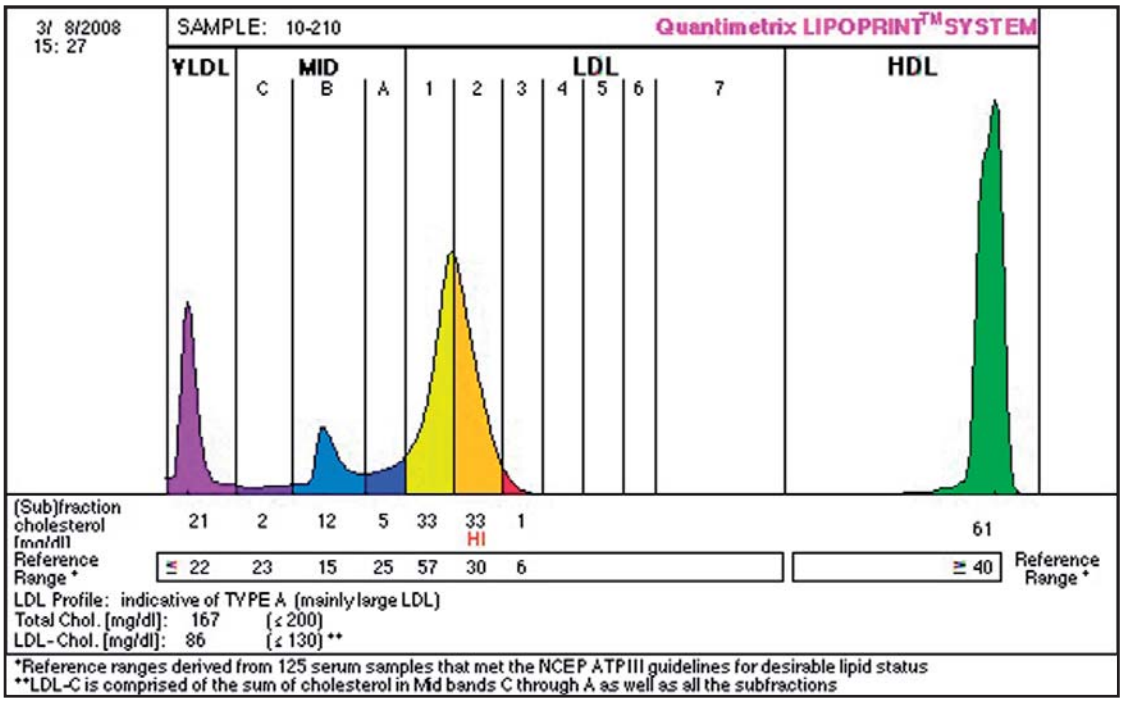


**Fig. 1.** Control, LDL subfractions.

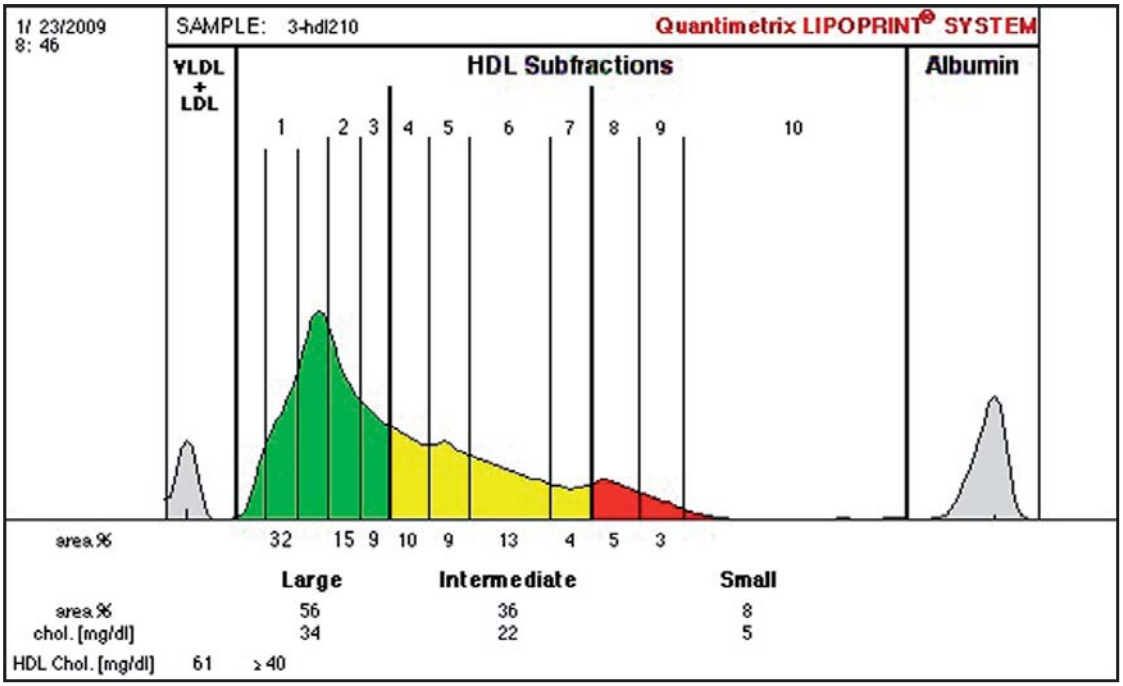


**Fig. 2.** Control, HDL subclasses.

groups, together with an increased concentration of small dense LDL ($p<0.0001$) compared to the controls.

In the group with a non-atherogenic H-βLP, the total cholesterol and triglyceride concentrations were significantly increased (both at $p<0.0001$), together, with the increased large HDL ($p<0.005$) and intermediate HDL ($p<0.0001$) subclasses. The concentration of the small HDL subclass did not differ from that of the control group and there were only traces of small dense LDL in H-βLP ($p<0.0001$) compared to the controls.

## DISCUSSION

The scientific idea of atherogenic and non-atherogenic lipoproteins is not a new idea in the pathophysiology of the onset and development of degenerative atherosclerotic processes in arterial walls, where plasma lipids play an important role. However, lipoprotein research has recently been focused more intensively on the phenomenon of atherogenic/non-atherogenic lipoproteins, and atherogenic and non-atherogenic lipoprotein pro-

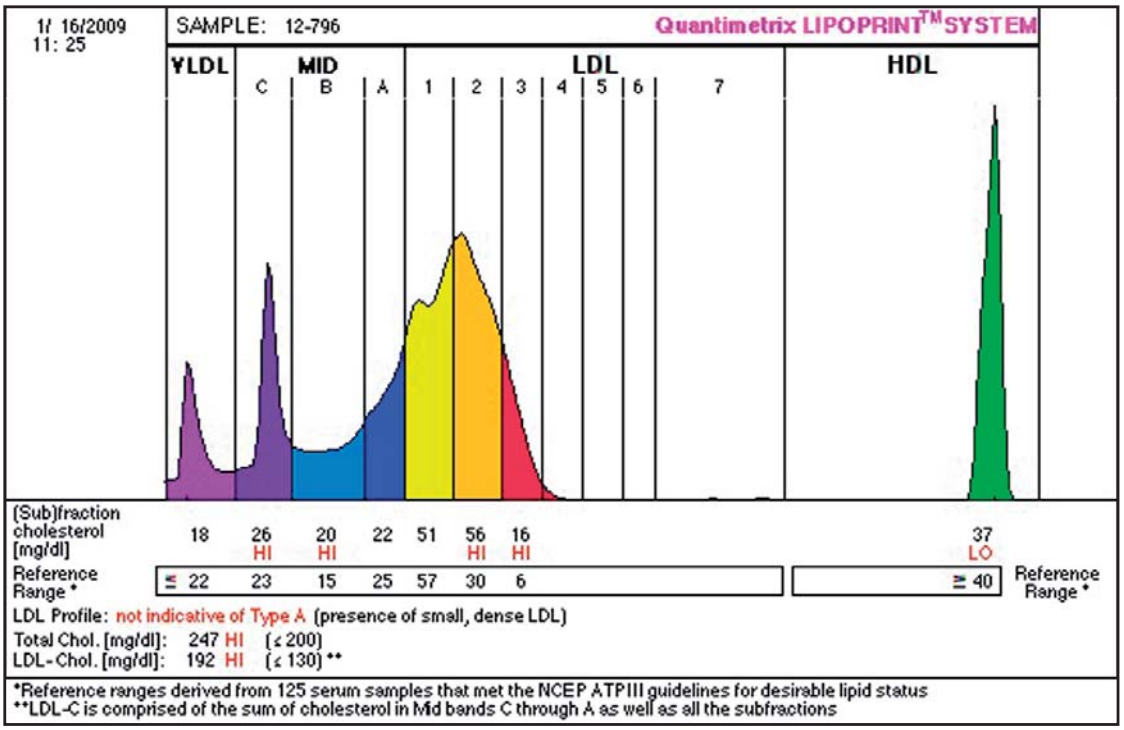


**Fig. 3.** Arterial hypertension, LDL subfractions.

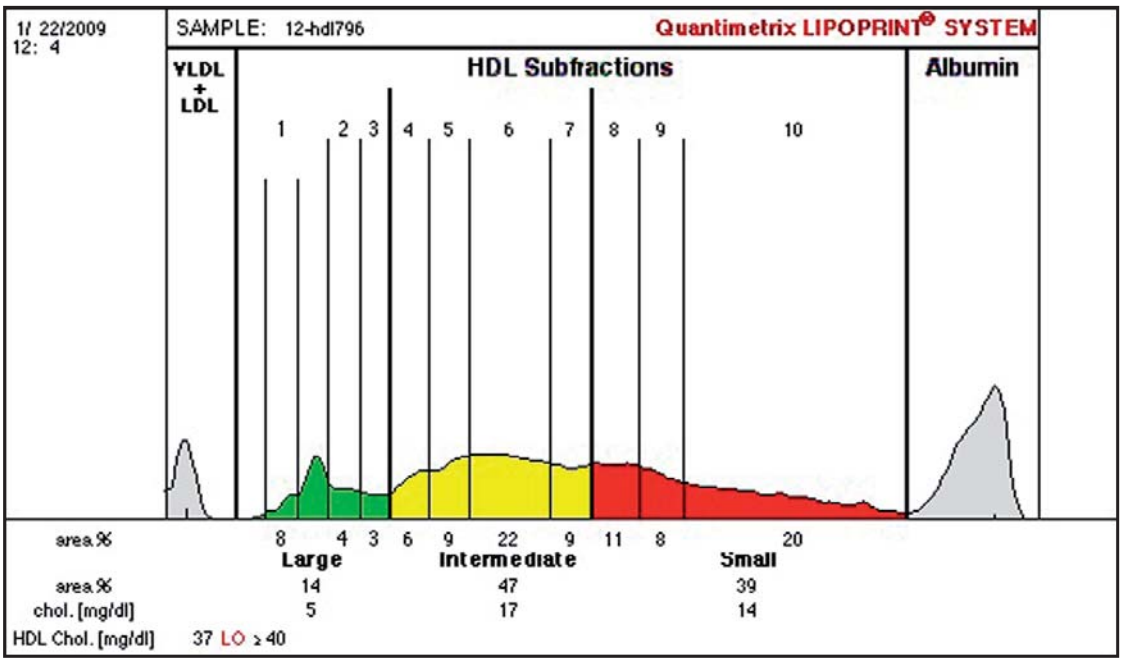


**Fig. 4.** Arterial hypertension, HDL subclasses.

files (Austin *et al.* 1990; Van *et al.* 2007). There are two objective facts that encouraged cardiovascular and lipoprotein researchers to search for other risk factors in plasma, that could cause an acute coronary or cardiovascular event.

The first fact was the discovery of much more sensitive analytical procedures for the identification of new subclasses in lipoprotein families (i.e., gradient gel electrophoretic separation of LDL and HDL; proton nuclear magnetic resonance spectroscopy; linear gel electrophoresis) (Alabakovska *et al.* 2002; Hoefner *et al.* 2001; Otvos *et al.* 1992; Rainwater *et al.*1997).

The second fact was the necessity to re-evaluate a hypercholesterolemia as a traditional atherogenic risk factor in the onset of degenerative diseases of the cardiovascular system. Castelli in 1988, and subsequently, published evidence, that more than 75 percent of the patients with an acute coronary syndrome or a myocardial infarction had normal plasma concentrations of total cholesterol, LDL cholesterol, or HDL cholesterol

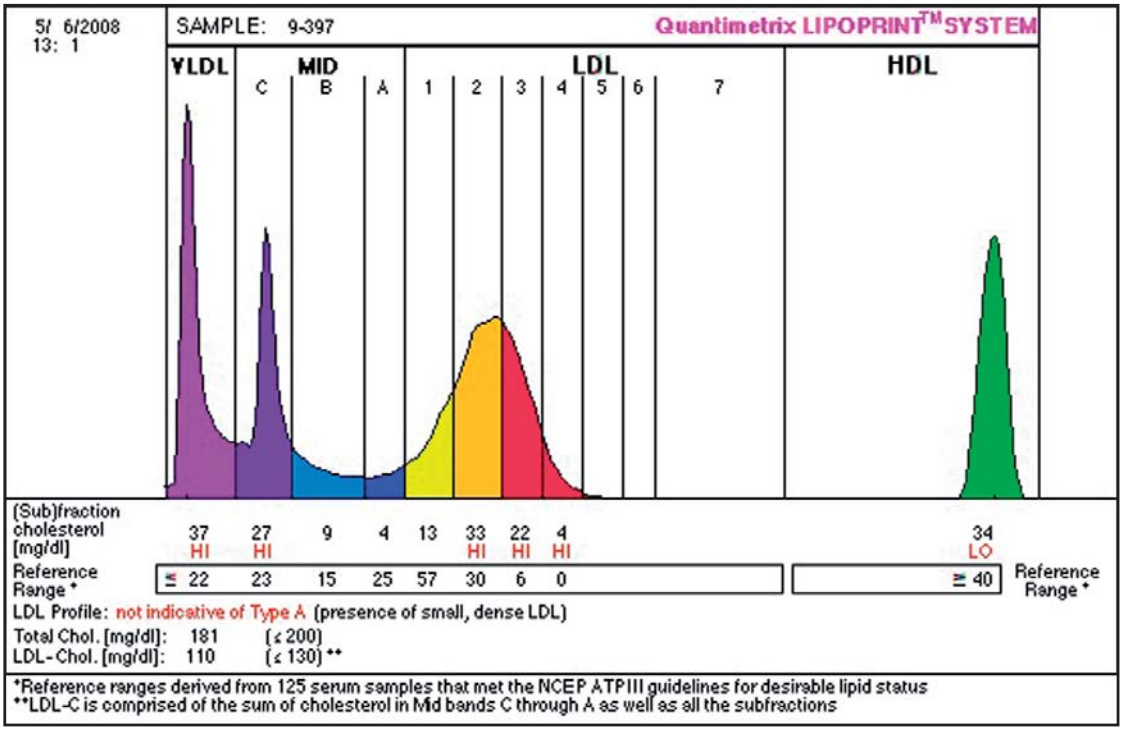


**Fig. 5.** Coronary heart disease, LDL subfractions.

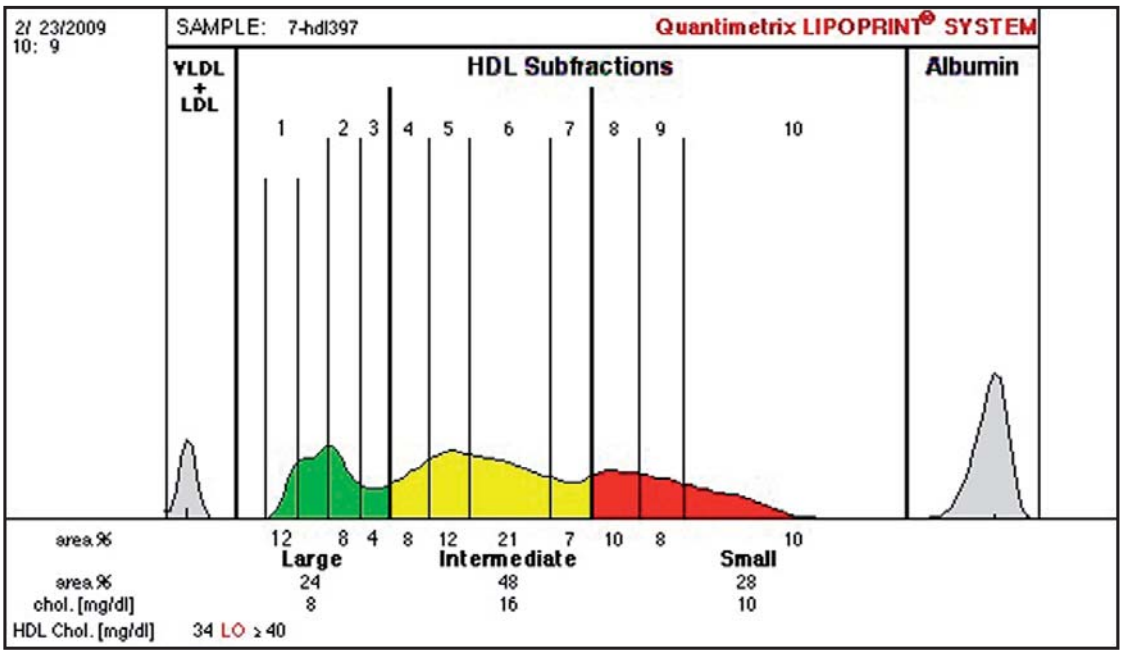


**Fig. 6.** Coronary heart disease, HDL subclasses.

(Castelli 1988; 1992; 1998). Normal values for what was thought to be a traditional risk factor could not then explain the onset of acute myocardial infarction or other cardiovascular event. Therefore, it was necessary to look for other risk factors. A reasonable area to explore was the re-evaluation of atherogenic lipoprotein family subfractions to search for as yet unidentified lipoprotein subfractions in the whole lipoprotein spectrum.

The evidence, that this new theoretical-analytical approach to the lipoprotein subfraction analysis was successful, was the identification of small dense LDL (Table 2, Berneis & Krauss 2002; Packard 2003) and subsequently, the confirmation of their key atherogenic role in the development of cardiovascular diseases because of their predisposition to oxidation on oxidized form, called oxidized LDL (oxid-LDL) (Chait *et al.*1993).

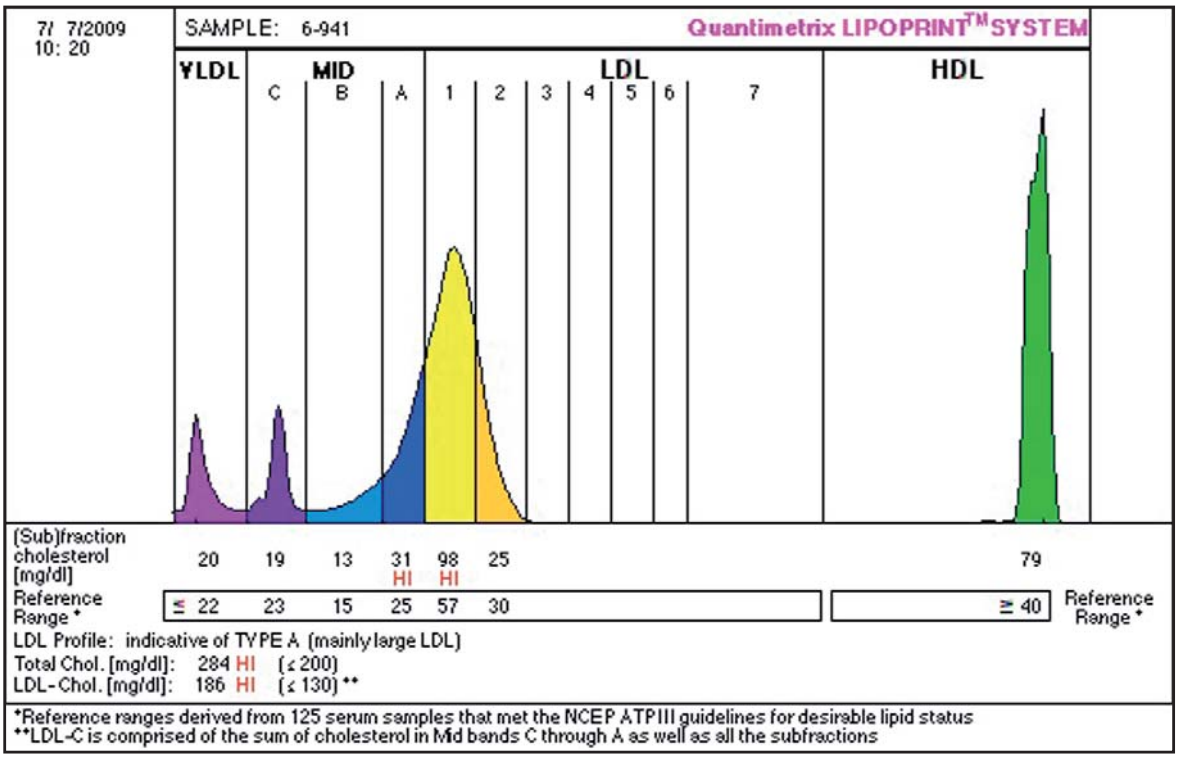


**Fig. 7.** Hyper-betalipoproteinemia LDL1,2, LDL subfractions.

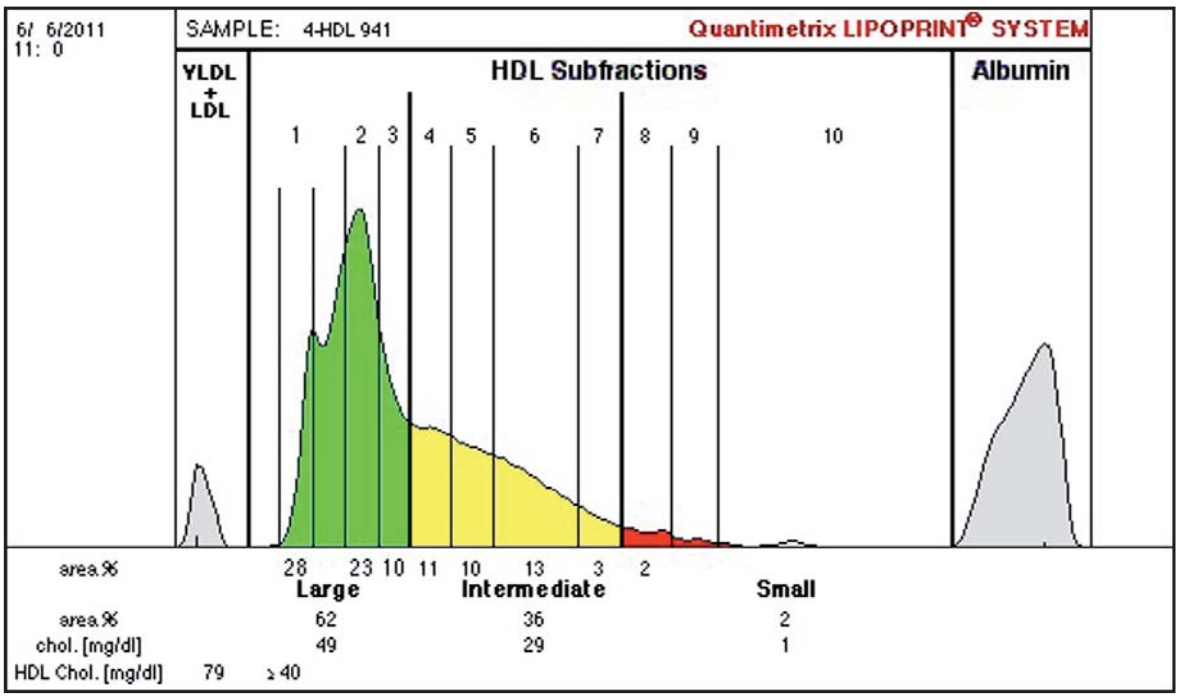


**Fig. 8.** Hyper-betalipoproteinemia LDL1,2, HDL subclasses.

Recently, the anti-atherogenic role of HDL, traditionally considered a non-atherogenic lipoprotein family and a protective part of the lipoprotein spectrum, also became a target for re-evaluation of its role in the atherogenesis. The work from Morais and co-workers, and other authors, reported evidence that subfractions HDL 8-10, called small HDL, might be indicative of an increased CHD risk (Asztalos *et al.* 2004; Morais *et al.* 2003; Morais 2005; Morais & Muniz 2005; Muniz & Morais 2005).

In our clinical study of patients with newly diagnosed arterial hypertension and coronary heart disease, we found a statistically significant increased concentration of small HDL ($p<0.0001$) for both these groups, compared to controls. These findings were in accordance with high concentrations of atherogenic small

dense LDL in lipoprotein profiles that had been identified in arterial hypertension and coronary heart disease subjects.

Conversely, in a non-atherogenic hyper-betalipoproteinemia LDL1,2, the concentration of small HDL was significantly lower, and was accompanied with a trace amount of small dense LDL.

Our results lead us to conclude that the small HDL subgroup represents a non-anti atherogenic lipoprotein part of the HDL family. An increased concentration of small HDL completes an atherogenic lipoprotein profile for individuals with cardiovascular diseases, and the quantification of small HDL could play an important complementary role in the identification of patients who are at risk for cardiovascular events.

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REFERENCES

1 Alabakovska SB, Todorova BB, Labudovic DD, Tosheska KN (2002) Gradient gel electrophoretic separation of LDL and HDL subclasses on BioRad Mini Protean II and size phenotyping in healthy Macedonians. Clin Chim Acta. **317**(1–2): 119–123.
2 Asztalos BF, Cupples LA, Demissie S, Horvath KV, Cox CE, Batista MC, Schaefer EJ (2004) High density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. Arterioscler Thromb Vasc Biol. **24**(11): 2181–2189.
3 Austin MA, King MC, Vranizan KM, Krauss RM (1990).Atherogenic lipoprotein phenotype. A proposed genetic marker for coronary heart disease risk. Circulation. **82**(2): 495–506.
4 Berneis KK, Krauss RM (2002) Metabolic origins and clinical significance of LDL Heterogeneity. J Lipid Res. **43**(9): 1363–1379.
5 Castelli WP (1988) Cholesterol and lipids in the risk of coronary artery disease – The Framingham Heart Study. Can J Cardiol. **4**(Suppl A): 5A–10A.
6 Castelli WP (1992) Epidemiology of triglycerides; a view from Framingham. Am J Cardiol. **70**(19): H3–H9.
7 Castelli WP (1998) The new pathophysiology of coronary artery disease. Am J Cardiol. **82**(Suppl2): 60–85.
8 Chait A., Brazo RL, Tribble DL, Krauss RM (1993) Susceptibility of small, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. Amer J Med. **94**(4): 350–356.
9 Despres JP (2007) Cardiovascular disease under the influence of excess visceral fat. Crit Pathw Cardiol. **6**(2): 51–59.
10 Hoefner DM, Hodel SD, O´Brien JF, Branum EL, Sun D, Meissner I, McConnell JP (2001) Development of a rapid quantitative method for LDL subfraction with use of the Quantimetrix Lipoprint LDL system. Clin Chem. **47**(2): 266–274.
11 Kostner GM, Laggner P (1989) Chemical and physical properties of lipoproteins. In: Fruchart JC, Shepherd J (eds).Clinical Biochemistry – Human Plasma Lipoproteins. Berlin, New York, Walter de Gruyter, 23–54.
12 Kostner GM, Scharnagl H, Kostner K, März W (2007) Zusammensetzung und Stoffwechsel der Lipoproteine. In: Schwandt P, Parhofer KG (eds) Handbuch der Fettstoffwechselstörungen. Dyslipoproteinämien und Atherosklerose: Diagnostik, Therapie und Prävention, 3.Auflage, Stuttgart, New York, Schattauer, 2–65.
13 Morais J, Neyer G, Muniz N (2003) Measurement and Distribution of HDL subclasses with new Lipoprint® HDL Method (pdf format) Presented at AACC,55th Annual meeting, Philadelphia, PA , July 2003.
14 Morais J (2005) Quantimetrix shows that all HDL subfractions may not protect against heart disease. AACC international congress of Clinical Chemistry, Orlando FL, june 2005.
15 Morais J, Muniz N (2005) Differences in HDL subfraction distribution in normolipidemic versus dyslipidemic individuals (pdf format) Presented at AACC,57th Annula meting, Orlando FL, July 2005.
16 Muniz N, Morais J (2005) Coronary heart disease. High density lipoprotein subclasses associated with heart disease. Medical Letter on the CDL and FDA July 31st 2005.
17 Oravec S (2006) Nová laboratórno-medicínska pomoc v diagnostike dyslipoproteínemií a kardiovaskulárnych ochorení: Identifikácia LDL podskupín ( A new laboratory-medical support in the diagnostics of dyslipoproteinemia and cardiovascular diseases) Med Milit Slov. **8**(1): 28–32.
18 Oravec S, Gruber K, Dostal E, Mikl J (2011) Hyper-betalipoproteinemia LDL 1,2: A newly identified nonatherogenic hypercholesterolemia in a group of hypercholesterolemic subjects. Neuroendocrinol Lett. **32**(3): 322–327.
19 Otvos JD, Jeyarajah EJ, Bennet SW, Krauss RM (1992).Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma protein concentrations and subspecies distribution from a single, rapid measurement. Clin Chem. **38**(9):1632–1638.
20 Packard CJ (2003) Triacylglycerol-rich lipoproteins and the generation of small dense low-density lipoprotein. Biochem Soc Trans. **31**(5): 1066–1069.
21 Rainwater DL, Moore PH jr, Shelledy WR, Dyer TD, Slifer SH (1997) Characterization of a composite gradient gel for the electrophoretic separation of lipoproteins. J Lipid Res. **38**(6): 1261–1266.
22 Van J, Pan J, Charles MA, Krauss R, Wong N, Wu X (2007) Atherogenic lipid phenotype in a general group of subjects. Arch Pathol Lab Med. **131**(11): 1679–1685.