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Correlations between novel biomarkers in endothelial function and lipid parameters in patients with familial hypercholesterolemia

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Introduction

Heterozygous familial hypercholesterolemia (HeFH) is the most frequent metabolic monogenic disorder with high risk for premature cardiovascular disease, caused by markedly elevated low-density lipoprotein (LDL) cholesterol levels. Patients with HeFH show wide phenotypic heterogeneity and a variable prevalance of cardiovascular disease. Appaerance of cardiovascular complications is affected by several risk factors, and as a consequence the cardiovascular risk assesment is quite difficult in this specific patient population. Beside genetic abnormalities and comorbidities, other risk factors, for instance inflammatory processes, prothrombotic agents, disorders of carbohydrate metabolism, qualitative abnormaltities and functional modifications of the lipoprotein particules contribute to the cardiovascular risk. Identifying further risk factors that alter the process and progression of the atherosclerosis support finding patients at very high cardiovascular risk and contribute to the better understanding of the development of cardiovascular complications. Generally high-density lipoprotein (HDL) plays a protective role against atherosclerosis. Atheroprective effect of the HDL, beside other structures, is associated with the apoliporotein M (ApoM), that is a structural protein of the HDL. ApoM is associated mainly to the HDL and plays important role in the transport of the bioactive sphingosine 1-phosphate (S1P), that contributes to the maintenance of endothelial homeostasis. Although the effect of ApoM/S1P in the vascular function is considerably complex, mainly mediated by several inflammatory processes. Stromal cell-derived factor-1 (SDF-1) is a chemokine produced by endotelial cells. Previous studies have shown that SDF-1 plays a role in the process of angiogenesis and atherosclerosis, and its concentration is altered in lipid metabolism disorders. To date, ApoM/S1P, serum SDF-1 and their correlations with lipoprotein subfractions, inflammatory parameters and endothelial function in HeFH have not been investigated, although based on the literature, their regulatory role in the process of severe premature atherosclerosis in HeFH patients is feasible. Better understanding effects of these regulatory proteins support evaluating more precise cardiovascular risk assesment and finding novel therapeutic targets.

Review of literature

Familial hypercholesterolemia

Familial hypercholesterolemia is the most frequent monogenic disorder, caused by mutations in the genes encoding proteins of LDL metabolism. Heterozygous FH is relatively common, occurs in European countries one of every 200-250 people. In Hungary the estimated prevalence in the northern region is 1:340. Homozygous FH is far more infrequent, the estimated prevalence was previously 1:1 million, but according to novel data occurs 1 in 170 000-300 000. Clinical manifestations caused by markedly elevated total cholesterol and low-density lipoprotein cholesterol (LDL-C) even from birth promote accelerated premature atherosclerosis, that induce generalised vascular disease, leading to premature coronary artery disease (CAD) and acute myocardial infarction (AMI). Vascular complications occur early, in childhood in severe homozygous FH, and generally between the age of 45-50, in females before the age of 60, in males before the age of 55, in heterozygous forms. Premature and severe, radidly progrediating atherosclerosis cause complications such as ischemic stroke, peripheral artery disease (PAD), and aortic stenosis. Genetic testing of mutations support the diagnose of FH, but in everyday practice it is not obligatory for establishing the diagnose, considering it as a costly, not easily available method. Instead, guidlines recommend using diagnostic criteria tools. The Dutch Lipid Network Criteria (DLNC) is the most commonly used diagnostic criteria in Hungary, as well as in Europe. The first-line therapy of FH is based on the hydroxymethylglutaryl-CoA reductase inhibitor statins. The next step can be proprotein convertase subtilisin/kexin-9 inhibitors such us evolocumab or alirocumab, and small interfering RNS (inclisiran). In severe HeFH and in HoFH medical treatment can be accompanied by selective LDL apheresis.

Heterogeneity of the Familial hypercholestrolemia

The most important risk factor for premature and acclerated atherosclerosis in FH patient is the serum LDL-C. Risk for atherosclerotic vascular disease is 10-20 fold increased in untreated FH patients compared to the general population. Inspite of these data, vascular complications do not occur in all FH patients. Generally used carciovascular risk assesment scales such us Framingham risk score is not eligible in this patient population for evaluating cardiovascular risk. Occurance and severity of vascular complications is quite heterogenous in FH patients, even is patients with the same genetic abnormalities.

Accordingly, several genetic and non-genetic modifing factors may contribute to the development of cardiovascular complications and phenotypic heterogeneity. Beside pathogenic mutations of LDLR, ApoB100 and PCSK9, several genetic variants can contribute to the variable character of the dyslipidaemia. Epigenetic modifications in the genes encoding proteins of the LDL metabolism may play an important role. Other frequent dieseases such as obesity, hypertension, type 2 diabetes mellitus occur often also in FH patients, that affect the apperance of cardiovascular diseases. Furthermore, endocrin disorders affect the lipid metabolism and as a consequence, modify the occurance of complications in FH patients. Beside LDL-C, other lipid parameters, for instance changes in triglyceride, HDL-C levels also have relevant effect. In the past few years, importance of lipoprotein(a) [Lp(a)] have come to focus, its elevated level is an independent risk factor for cardiovascular disease. Several previous studies have found eleveted Lp(a) levels in FH patients compared to non-FH patients. In the past few years, beside the well-known cardiovascular risk factors several novel factros were identified in FH and non-FH patients that play a role in the process of atherosclerosis. Detecting these novel factors, for example LDL and HDL subfractions, inflammatory citokines or serum progranulin, may contribute to the better understanding of the process of atherosclerosis. Evaluation of traditional and novel cardiovascular risk factors support more precise cardiovascular risk assesment, that promote evaluating personalized targeted therapy and identifying patients at very high cardiovascular risk, who benefit the most of the new costly lipid lowering treatments.

Significance of HDL composition and function

HDL is a high density, small (8-10 nm), protein rich lipoprotein particle, with an average density of 1.063-1.21 g/mL. Similar to other lipoprotein particles the surface contains mainly phosphatidylcholine, cholesterol and apolipoproteins, while the hydrophobic core is composed of triglyceride and cholesteryl-ester. HDL particules are extremely heterogenous, contain several micro- and macromolecules, their protein and lipid content is quite variable. Lipid content composed by mainly phospholipid, cholesteryl-ester, free cholesterol and triglyceride, other phospholipids including phosphatidylethanolamine, phosphatidylserine and sphingomyelin can be a component, that may modify the function of these particles. Protein content of HDL is extremely high compared to other lipoprotein particles, and quite various. To date, more than 200 HDL-associated proteins have been identified. Apolipoproteins are structure proteins of HDL particles, and play important role in the lipid transport and

maintaining the stability of the structure. Apolipoprotein A1 (ApoA1) and apolipoprotein A2 give the main proportion of HDL, beside several other apolipoproteins that assume modifying the structrue and function of various HDL particules. Main function of HDL is reverse cholesterol transport, while HDL transport cholesterol from the pheripheral cells, including vascular macrophags back to the liver, where cholesterol is partly discharged with the bile, partly reused. Mainly ApoA1 is responsible for the antioxidant effect of HDL, beside other antioxidant enzymes, for instance human paraoxonase-1 (PON1) and PAFAH, that decrease radical driven lipid peroxidation. Besides, HDL has pleiotropic effects including anti-thrombotic, anti-inflammatoric, anti-apoptotic effects, and improve the endothelial function.

Significance of ApoM

Apolipoprotein M (ApoM) was discovered in 1999. It has a molecular weight of 26 kDa, composed of 188 amino acids, belongs to the lipocalin protein family. In adult its plasma concentrations range between 0.63-1.13 mmol/L. ApoM gene is situated on the 6. chromose on the 21.3 locus, includes 6 exons and is approximately 2.3 kb with a conservative structure. In adults ApoM is expressed in the liver and kidney, although during human embriogenesis it is also expressed in the stomach and in the sceletal muscle cells. ApoM produced by hepatocytes is secreted into the plasma, where it binds to lipoproteins, expecially to HDL particles, but it is detectable on other lipoproteins such as on LDL and chylomicron. Meanwhile renal ApoM is binded to the multiligand receptor megalin of proximal tubular epithelial cells, that is necessary to absorb ApoM secreted in the urine.

Significance of S1P

S1P is a bioactive sphingosine metabolic product, that regulate various cellular functions, such as proliferation, migration and surviving, accordingly modify several inflammatory and vascular processes. S1P is produced of the cell membrane sphingomyelin, forms from ceramide over sphingosine catalised by sphingosine kinase enzyme. Degradation of S1P to E-2-hexadecenal is catalized by sphingosin-1-phosphate lyase enzyme. Plasma concentrations of S1P range between 200-1000 nmol/L. Due to its amphipatic character the transport of S1P requires carrier proteins in the circulation. In 65-80% it is binded to the ApoM of HDL particles, a smaller amount is binded to albumin and other lipoprotein particles. Out of HDL particles only ApoM contained HDL can bind S1P.

However for the binding point of S1P on ApoM, S1P compete with other molecules such as oxidised phospholipids and retinol. Haematopoietic cells, for instance red blood cells store remarkable amount of S1P, while these cells possess no sphingosine-1-phosphate lyase enzyme, presumably these cells recieve S1P directly from the circulating HDL or other lipoproteins coping with their membrane. It can be transported from other lipoprotein particles to HDL by phospholipid transpher protein. Biological efficacy of S1P differ depending on its location on HDL or other proteins, such as albumin. HDL-associated S1P is more effective than the albumin-binded form. Anti-atherogenic function of HDL partially depends on its S1P content. The most important anti-atherogenic functions are promoting endothelial nitric oxide (NO) production by raising endothelial nitric oxide synthetase activity, consequently support NO dependent vasodilatation, impair production of tumor necrosis factor-alpha (TNFα) induced adhesion molecules, enhance endothelial barrier, proliferation and survival of endothelial cells, and promote the process of angiogenesis. Moreover, protect myocardial cells against ischemia reperfusion injury, impair expression of adhesion molecules of vascular smooth muscle cells and increase prostacyclin production. Key role of S1P is confirmed by investigations, in which S1P was removed by enzymatic delipaseing, administering neutralizing antibodies against S1P, or generating arteficial S1P-free HDL markedly impair the HDL function.

Significance of stromal cell-derived factor-1

Stromal cell-derived factor-1 (SDF-1), also known as chemokine C-X-C ligand 12 is a conservative chemokine produced by several type of cell, bone marrow, endothelial and epithelial cells and malignant cells. The most important receptor for SDF-1 is C-X-C Motif Chemokine Receptor 4 (CXCR4), that is a member of the G-protein coupled receptors, structurally composed of seven transmembrane domains. CXCR4 is presented on the surface of several cells including smooth muscle cells, endothelial cells, haematopoietic cells, astrocytes, microglias and neuronal cells. The role of SDF-1 has been investigated intensively in the past few years in physiological and pathological circumstances. Based on the result of these investigations, SDF-1 play a direct role in the process of angiogenesis, connecting endothelial progenitor cells through endothelial CXCR4. Moreover SDF-1 can promote the angiogenesis indirectly, promoting proangiogenic citokine production of endothelial cells, including CXCL1, CXCL8 and VEGF. SDF-1 was detected in the atherosclerotic plaques and as a consequence, may have a probable role in the process of the atherosclerosis. Promoting

expression of intracellular adhesion molecule-1 (ICAM-1) support endothelial progenitor cells connecting to the endothelium and transendothelial migration of monocytes.

Determining LDL and HDL subfractions

LDL and HDL particles are a heterogenous group, as far as their size, charge and composition concerned, and can be devided to subfractions. Determination of lipoprotein subfractions is not a part of the everyday clinical practise, meanwhile better understanding of the subfractions assume clarifing more precisely the function of LDL and HDL particles. Several laboratory methods are available separating lipoprotein subfractions, although the gold standard method is the analytical ultracentrifugation. During ultracentrifugation LDL means 1.019-1.063 g/ml density, while HDL means 1.063-1.021 g/mL liporptein density. Several other methods are available in the commercial trade, including density-gradient ultracentrifugation, gradient gel electrophoresis, 2D gel electrophoresis and nuclear magnetic resonance spectroscopy and high resolution ion mobility technique. Apperance of the Lipoprint method (Quantimetrix. Corp. Redondo Beach, CA, USA) was a remarkable impovement, that is a commercial, standardized, widely available non gradient gel electrophoresis.

Based on their size Lipoprint separate LDL to 7 subfractions, while HDL is separated to 10 subfractions. In case of LDL two groups: large (LDL1-2) and small (LDL3-7) LDL is separated. In case of HDL 3 groups: large (HDL1-3), intermediate (HDL4-7) and small (HDL8-10) HDL subfractions can be separated. Separating HDL subfractions is similar, VLDL, IDL and LDL particles do not separate, remain on the take-off place, while the most mobile part of the albumin is detected on the bottom of the tube. Between these 2 bands 10 HDL subfractions separate.

Separating subfractions with various methods result in dissimilar quality of subfractions, that cause difficulties in comparison of the results of different studies and understanding relevance of lipid subfractions.

Aims of the study

In our research, we aimed in untreated HeFH patients and in a matched control population:

- determining serum S1P and ApoM levels,
- examination of the correlation of S1P and S1P/ApoM ratio with the serum lipid parameters,
- within this, examination of the ratio of HDL and LDL subfractions,
- furthermore, examination of the correlation of S1P/ApoM ratio with inflammatory and endothelial function parameters.

In addition, we aimed in the same study population:

- determining serum SDF-1 levels,
- examination of the correlation of SDF-1 with lipid parameters,
- within this, examination of the ratio of HDL and LDL subfractions,
- as well as inflammatory and endothelial function parameters.

Materials and methods

Study population

Eighty-one subjects (55 females and 26 males) with HeFH were enrolled in our study at the Lipid Outpatient Clinic of the Department of Internal Medicine, University of Debrecen. All HeFH patients were heterozygous and fulfilled the Dutch Lipid Clinic Network diagnostic criteria for FH or had a confirmed LDL receptor gene mutation. The patients were referred to our Lipid Outpatient Clinic by general practitioners, neurologists, cardiologists to verify the diagnosis of HeFH and start the optimal therapy. The patients arrived after 12 hours fasting and the blood samples were collected from 08:00-10:00 AM. All HeFH patients were newly diagnosed without ongoing lipid-lowering drug treatment. Thirty-two gender- and aged-matched healthy individuals were enrolled as controls in the study from our General Outpatient Clinic Department of Internal Medicine, University of Debrecen. In controls, main inclusion criteria were normal cholesterol, glucose and liver enzyme levels; normal body mass index, moreover, the subjects were free of medications and had no chronic or acute diseases in the past 3 months. Physical examination and electrocardiogram of controls did not show any abnormalities. Exclusion criteria were type 1 or 2 diabetes, alcoholism, malignancy, known liver, autoimmune and endocrine diseases, pregnancy, lactation. All participants provided written informed consent before enrollment. The study was carried out in accordance with the Declaration of Helsinki and was approved by the local and regional ethical committees (DE RKEB/IKEB 4775-2017, date of approval: 3rd April 2020 and ETT TUKEB 34952-1/2017/EKU, date of approval: 30th June 2017).

Blood sampling

Peripheral venous blood samples were drawn into Vacutainer tubes and sera were centrifuged at 3500 RPM for 15 minutes. Routine laboratory parameters including LDL-C, HDL-C, Lp(a), triglyceride, total cholesterol, fasting glucose and hsCRP were analyzed using a Cobas c600 autoanalyzer (Roche Ltd., Mannheim, Germany) in the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen. Tests were performed according to the recommendation of the manufacturer. The sera were kept frozen below -70°C in 300 µl aliquots for subsequent laboratory assays.

Measurement of S1P

Serum S1P was measured by ELISA (Echelon Biosciences, Salt Lake City, USA), according to the recommendation of the manufacturer, and expressed as µg/mL.

Measurement of ApoM

Serum ApoM levels were determined with an ELISA kit (BioVendor – Laboratorni medicina a.s., Brno, Czech Republic) and expressed as μg/mL. Intra-assay coefficients of variation ranged from 4.9-5.22% and inter-assay coefficients of variation from 5.7-5.8%.

Measurement of SDF-1

Serum SDF-1 was determined using a commercially available duoset enzyme linked immunoassay (ELISA, Cat. No. DY350-05, R&D Systems Europe Ltd., Abington, UK) according to the instructions of the manufacturer. The values were expressed as pg/mL. Intra-assay coefficients of variation ranged between 3.4-3.9 % and inter-assay coefficients of variation were 8.2-13.4 %. The assay range was 31.2-2,000 pg/mL. In this assay undiluted serum samples were used.

Oxidized LDL measurement

Serum concentrations of oxidized LDL (oxLDL) were detected by commercial sandwich ELISA (Mercodia AB, Uppsala, Sweden) based on a direct sandwich technique where two monoclonal antibodies were directed against separate antigenic determinants of the oxidized apolipoprotein B molecule. Values were presented as U/L and sera were used for a final dilution of 1/6561 according to the recommendation of the manufacturer. Intra- and inter-assay coefficients of variation were 5.5-7.3% and 4-6.2% respectively. Sensitivity of oxLDL measurements was <1 mU/L.

Measurement of sICAM-1, sVCAM-1 and sCD40L

Serum sICAM-1, sVCAM-1 and sCD40L were determined with sandwich ELISAs (R&D Systems Europe Ltd., Abington, England). ELISA procedures were carried out according to the recommendations of the manufacturer. Intra-assay coefficients of variations were 3.7-5.2% (sICAM-1), 2.3-3.6% (sVCAM-1) and 4.5-5.4% (sCD40L), while the inter-assay coefficients of variation ranged between 4.4-6.7% (sICAM-1), 5.5-7.8% (sVCAM-1) and 6.0-6.4% (sCD40L). All concentration values were expressed as ng/mL.

Myeloperoxidase measurement

Serum MPO concentration was measured by a sandwich ELISA technique (R&D Systems Europe Ltd., Abington, UK) according to the instructions of the manufacturer. Intra- and inter-assay coefficients of variation were 6.5–9.4% and values are expressed as ng/mL.

Determination of PON1 Enzyme Activities

Serum PON1 paraoxonase and salt stimulated paraoxonase activity were measured in Greiner microtiter plates using a kinetic, semiautomated method. Hydrolysis of paraoxon (O,O-diethyl-O-p-nitrophenyl phosphate, Sigma-Aldrich, Budapest, Hungary) as a substrate was followed at 405 nm at room temperature. Serum PON1 arylesterase activity was measured using phenylacetate as a substrate (Sigma Aldrich, Budapest, Hungary), and the hydrolysis of the substrate was monitored at 270 nm at room temperature, as previously described.

Determinations of LDL and HDL subfractions

Circulating LDL and HDL lipoprotein subfractions were detected using a commercially available polyacrylamide gel tube electrophoresis (Lipoprint System, Quantimetrix Corporation, Redondo Beach, CA, USA) as previously described. Briefly, 25 µl sera were taken into polyacrylamide gel tubes with 200 and 300 µl loading gel containing Sudan Black, respectively. After 30 minutes of photopolymerization, the gel tubes were electrophorized in an electrophoresis chamber with 3 mA/each tube. Each electrophoretic cycle was loaded with a high purity lipoprotein quality, control provided by Quantimetrix (Liposure Serum Lipoprotein Control, Quantimetrix Corp., Redondo Beach, CA, USA). After a half hour but no longer than two hours rest, the lipoprotein bands were scanned with an ArtixScan M1 digital scanner (Microtek International Inc., CA, USA) and analyzed with the Lipoware Software developed by the manufacturer (Quantimetrix Corp., Redondo Beach, CA, USA).

Up to seven LDL subfractions were assessed between the VLDL and HDL peaks during the LDL subfraction test (Cat.No. 48-7002). The proportion of large LDL (large LDL %) was defined as the summed percentages of LDL1 and LDL2, whereas the proportion of small LDL (small-dense LDL %) was defined as the sum of LDL3-LDL7. Cholesterol concentrations of LDL subfractions were determined by multiplying the relative area under the curve (AUC) of subfractions by total cholesterol concentration. Calculated total LDL-C was the sum of cholesterol in midbands C through A (which are mainly comprised with IDL) plus LDL subfractions (LDL1-LDL7). Calculated LDL-C correlated with the directly measured LDL-C (Lipoprint LDL: 130.8±30.14 mg/dL vs. β-Quant LDL: 130.0±30.42 mg/dL, r^2=0.887) as described previously.

In case of HDL subfraction analysis, ten HDL subfractions were detected (Cat. No. 48-9002). Large (HDL1-3), intermediate (HDL4-7) and small (HDL8-10) HDL subfractions were determined between VLDL/IDL/LDL and albumin bands. Cholesterol content of HDL subfractions was also calculated by the Lipoware Software according to the relative AUC of subfraction bands.

Statistical analyses

Statistical analyses were performed using the Statistica 13.5.0.17 (TIBCO Software Inc., Tulsa, OK, USA) and graphs were prepared using the GraphPad Prism 6.01 (GraphPad Prism Software Inc., San Diego, CA, USA). We also calculated the statistical power with SPH Analytics online calculator (SPH Analytics LTD., Alpharetta, GA, USA) to validate the difference of circulating ApoM, S1P and SDF-1 levels in HeFH (group 1) and control subjects (group 2). The statistical power was above 0.8 (0.98). The difference between genders in the two studied groups was calculated with Chi^2 test. Normality of continuous variables were tested using Kolmogorov–Smirnov test. In case of normal distribution, the comparison between groups was analyzed with Student's unpaired t-test and with the Mann-Whitney U-test in case of variables with non-normal distribution, respectively. Data were presented as means±standard deviation or medians (upper and lower quartiles). The relationship between normally distributed variables was performed with Pearson tests. Backward multiple regression analysis was performed to define which variables are the best predictors of S1P and SDF-1 levels. $P \leq 0.05$ probability values were considered statistically significant.

Results

Lipid parameters, inflammatory and oxidative parameters in HeFH patients and in controls

Compared to controls, HeFH patients had significantly higher total cholesterol, LDL-C, triglyceride, apoB100 and Lp(a) levels; while circulating HDL-C and ApoA1 did not differ significantly. Significantly higher PON1 arylesterase activity was found in HeFH patients compared to controls, but there were no differences in PON1 paraoxonase and salt-stimulated paraoxonase activities between the two study groups. Circulating oxLDL, myeloperoxidase, sICAM-1 and TNFα was significantly higher in HeFH patients than in nonFH subjects. However, there were no significant differences in sVCAM-1, sCD40L and hsCRP between patients and controls.

Serum S1P and ApoM levels in HeFH patients and in controls

ApoM and S1P levels were significantly higher in HeFH patients compared to controls. There were no significant differences in ApoM and S1P concentrations between HeFH patients with (VC) or without vascular complications (nonVC), including previous acute myocardial infarction, stroke, carotid artery disease or peripheral arterial disease (ApoM: VC 3.79±0.62 vs. nonVC 3.73±0.53 μg/mL, p=0.7; and S1P: VC 7.63±1.08 vs. nonVC 7.75±2.06 ng/mL, p=0.8, respectively). We could not find significant differences in S1P/ApoM ratio between FH patients and controls (2120±700 vs. 2290±740; p=0.22).

Amount and proportion of LDL and HDL subfractions in HeFH patients and in controls

The amount of VLDL and IDL subfractions, and the concentration of IDL subfraction were significantly higher in HeFH patients compared to the controls. The concentrations of large- and small-density LDL subfractions were both significantly higher in HeFH patients compared to the controls, while the amount of mean LDL size was significantly lower in HeFH patients compared to the controls. Lower large and intermediate HDL subfractions, while higher concentration of small HDL was found in HeFH patients compared to tthe controls.

Correlation of serum S1P and HDL subfractions in HeFH patients and in controls

Significant negative correlation was found between large HDL subfractions and S1P levels in the whole study population and in HeFH patients. No correlation was observed between

intermediate HDL and S1P levels. There were significant positive correlations between small HDL subfractions and S1P concentrations in the whole study population and in HeFH patients.

Correlations of serum S1P and sVCAM-1, PON1, sCD40L and MMP-9 levels in HeFH patients and in controls

We found significant negative correlation between sVCAM-1 and S1P in the HeFH population.

Significant positive correlation was found among PON1 arylesterase activity, s CD40L, MMP-9 and S1P concentrations. S1P/ApoM ratio showed significant negative correlation with sVCAM-1 ($r=-0.197$; $p=0.037$), while we found positive correlation between S1P/ApoM and sCD40L ($r=0.28$; $p=0.00027$).

Best predictors of S1P

Multiple regression analysis using a backward stepwise method showed that the best predictor of S1P was PON1 arylesterase activity ($\beta =0.281$; $p<0.01$) and the pecentage of large HDL subfractions ($\beta =0.35$; $p<0.001$). The model contained logTg, percentage of large HDL, percentage of small HDL, sVCAM-1, ApoM, PON1 arylesterase activity, MMP-9 and log sCD40L.

SDF-1 levels in HeFH patients and in controls

We found significantly lower SDF-1 levels in HeFH patients compared to controls (71.3 ± 39.7 vs. 150.6 ± 55.4 pg/mL, $p<0.001$). Furthermore, females had tendetiously higher serum SDF-1 compared to males. No significant difference was detected in serum SDF-1 between females and males in HeFH patients (78.2 ± 39.9 vs. 59.1 ± 36.9 pg/ml, $p=0.07$), and in controls (166.5 ± 51.3 vs. 129.8 ±66.6 pg/mL, $p=0.14$).

Correlations between SDF-1 and lipid parameters, lipoprotein subfractions, oxidative and inflammatory parameters in HeFH patients and in controls

We found significant negative correlations between TC, triglycerides, LDL-C and ApoB100 in the whole study population. No significant correlation was found between HDL-C, ApoA1 and SDF-1. We detected negative correlations between the amount of VLDL, IDL and large LDL subfractions, while positive correlation was found between mean LDL size and SDF-1 in the whole study population. Significant positive correlation was found between SDF-1 and

large and intermediate HDL subfractions. SDF-1 correlated negatively with oxLDL ($r=-0.51$; $p=0.01$) and the logarithm of serum MPO activity (lgMPO) ($r=-0.33$; $p<0.001$). Although, we found no significant correlations between other oxidative markers including either hsCRP, TNFα, sICAM-1, sVCAM-1, sCD40L or PON1 arylesterase and paraoxonase activity.

Best predictors of SDF-1

Muliple regression analysis using backward stepwise method showed that the best predictors of serum SDF-1 are VLDL and oxLDL in both statistical models. The first model contained TC, logarithm of serum triglyceride, LDL-C, ApoB100, concentration of VLDL, IDL, large LDL, mean LDL size, large HDL, intermediate HDL, small HDL, oxLDL and lgMPO. Meanwhile, in the second model, the variables were selected on the basis of the biological traits of the items and contained the TC, logarithm of serum TG, ApoB100, concentration of VLDL, mean LDL size, large HDL, small HDL, oxLDL and lgMPO. Best predictors of SDF-1 turned out to be VLDL and oxLDL in both statistical models. Correlations in HeFH patients and in controls were calculated separately. We found similar tendencies in HeFH patients and in controls. We found significant positive correlations between mean LDL size, large HDLand SDF-1 in HeFH patients, while significant negative correlations were found between SDF-1 and triglycerides and VLDL in controls.

Summary of novel findings

During the examination of untreated HeFH patients and in matched control individuals:

- Significantly higher serum ApoM and S1P levels were found in HeFH patients compared to the controls.
- Significant negative correlation was observed between large HDL subfractions and SDF-1 in the whole study population and in HeFH patients.
- Significant positive correlation was found between small HDL subfraction and S1P in the whole study population and in HeFH patients.
- Significant negative correlation was detected between sVCAM-1 and S1P in HeFH patients. S1P correlated negatively with PON1 arylesterase activity and sCD40L.
- S1P/ApoM ratio showed significant negative correlation with sVCAM-1, while we found positive correlation between S1P/ApoM and sCD40L.
- Multiple regression analysis showed that the best predictor of S1P was PON 1 arylesterase activity and the pecentage of large HDL subfractions.

  Furthermore:
- We found significantly lower SDF-1 levels in HeFH patients compared to controls.
- Significant negative correlations were found between TC, triglycerides, LDL-C and ApoB100 in the whole study population.
- Significant negative correlations were observed between the amount of VLDL, IDL and large LDL subfractions, while significant positive correlation was found between mean LDL size and SDF-1 in the whole study population.
- SDF-1 correlated negatively with oxLDL and the logarithm of serum MPO activity, although, we found no significant correlations between other oxidative markers including either hsCRP, TNFα, sICAM-1, sVCAM-1, sCD40L or PON1 arylesterase and paraoxonase activity.
- Multiple regression analysis showed that the best predictors of serum SDF-1 are VLDL and oxLDL in both statistical models.

Discussion

Based on epidemiological reviews, low serum HDL-C is a risk factor for cardiovascular diseases, although clinical trials aiming at raising HDL-C have not been successful proving the reduction of cardiovascular risk. Thus, the HDL function, rather than HDL-C concentration is responsible for cardioprotective effects of HDL. As a consequence, improving the function of HDL turned to be therapeutic target since HDL has several atheroprotective functions.

Clarifying regulatory pathways and identifying biomarkers responsible for these anti-atherogenic effects is an essential step forward. HDL-associated S1P has been shown to causally contribute to many HDL functions, such as the maintenance of endothelial homeostasis, arterial vasodilatation and cardioprotection. Most of the previous studies examined HDL enrolling healthy individuals, as a consequence the contribution of disease associated alterations of HDL-S1P to numerous aspects of HDL dysfunction remained unclear. To date, this is the first clinical study evaluating S1P levels, HDL subfraction distribution, HDL function, and inflammatory markers in HeFH. In contrast to previous data on smaller HeFH groups, we found higher S1P and ApoM levels in our HeFH population. However, opposite to this previous study, S1P and ApoM concentrations were similar in HeFH patients with and without vascular complications. To date, ApoM and S1P concentrations have not been determined in larger, unrelated FH patient population. Previous prospective studies have shown that numerous molecular proinflammatory biomarkers from foam cell formation to plaque rupture may be applied to predict future cardiovascular events. While the elevation of serum inflammatory cytokines and adhesion molecules such as sVCAM-1 and sICAM-1 can be detected in the early phase of atherogenesis, increased levels of oxLDL, MMPs and sCD40L can be seen in the late phase indicating plaque destabilization and imminent rupture. In our HeFH patients S1P concentration correlated negatively with sVCAM-1, but there was a positive correlation between sCD40L and MMP-9 concentrations. It has been reported that S1P, transported by HDL-associated ApoM may act on S1P1 and 3 receptors inducing anti-atherogenic and vasculoprotective effects, while S1P carried by HDL-ApoM can also bind to S1PR2 leading to macrophage retention in the atherosclerotic plaques and the promotion of atherosclerosis. Therefore, in our HeFH patients the effect of higher ApoM and S1P concentrations might not necessarily be beneficial. Keul et al. proved that HDL-bound S1P exerts a potent anti-inflammatory effect on smooth muscle cells by inhibiting the induction of TNFα-stimulated inflammatory genes, including MMP-9.

However, we found positive correlation between S1P and MMP-9 concentrations, which may indicate the responsive expression of S1P in mature atherosclerotic plaques. The function of HDL-associated enzymes is often impaired in HeFH patients. A key role in the antioxidant properties of HDL is exerted by the enzyme PON1 associated to HDL surface. PON1 hydrolyses oxidized lipids and protects LDL and biological membranes from lipid peroxidation, resulting in decreased endogenous oxidative stress and the prevention of atherogenesis. Previously, decreased PON1 arylesterase activity has been reported in FH patients. In contrast, MPO is a pro-oxidant enzyme produced mainly by neutrophils and monocytes and generates reactive intermediates. In this present study, we unexpectedly found higher PON1 arylesterase activity in HeFH patients compared to controls, although we enrolled untreated patients to exclude the previously reported effect of statins on PON1 activities. We could not find significant differences between PON1 paraoxonase and salt stimulated paraoxonase activities of HeFH patients and controls, while higher oxLDL and increased MPO activity demonstrated increased oxidative stress in our HeFH population, which was similar to some previous observations. Of note, PON1 arylesterase activity was found to be a predictive factor of S1P based on the result of multiple regression analysis, indicating relationship between S1P and HDL-associated antioxidative processes. The lower percentage and concentration of large and intermediate HDL subfractions, in contrast to higher percentage and concentration of small HDL subfractions in HeFH patients compared to control subjects have been described previously. Recently, higher concentrations of large HDL particles have been found in CHD-free elderly HeFH patients, potentially indicating that these particles have other functions than smaller HDL particles, and that separation of HDL subfractions might provide better risk profiles in HeFH than the currently used HDL-C concentration. Some limitations of the study must be mentioned. Direct association of S1P with HDL particles was not measured. HDL-S1P could be determined by liquid-chromatograpy-mass spectrometry, but this time consuming and costly method is not available in the everyday clinical practice, therefore, it could not be used as a biomarker. Use of imaging modalities to identify and quantify the burden of atherosclerosis in the aorta, carotid arteries, coronary arteries, and peripheral vasculature would improve the value of the study. However, the results underline the potential importance of studying HDL function and the feasible regulatory role of S1P in HeFH.

According to a large body of clinical evidence, markedly elevated LDL-C and accumulation of these particles in the arterial wall has been considered to promote cardiovascular risk in

patients with HeFH. Previously, several predictive score systems were suggested for FH patients, such as the FH-Risk-Score and the SAFEHEART registry. These score systems were based on classical risk factors including TC and LDL-C. While these risk stratifications could be integrated into the clinical practice of HeFH, future investigation is needed to determine if other elements of risk stratification could improve HeFH outcomes. Therefore, in addition to LDL-C, evidence for other potential cardiovascular risk factors in patients with FH is also emerging. Since several former observations in HeFH populations failed to find associations between LDL-C and atherosclerotic cardiovascular events, indicate that other lipoprotein fractions and inflammatory cytokines might be strong driving factors of cardiovascular risk in the HeFH population. Therefore, the potential role of novel factors including recently identified and characterized chemokines should be investigated in this special patient population. SDF-1, also known as CXCL12, is involved in inflammatory responses and neuromodulation in the brain by acting on its receptors CXCR4 and CXCR7. After cerebral ischemic stroke SDF-1α and CXCR4 are upregulated in the ischemic penumbra. Meanwhile, remote ischemic postconditioning increases SDF-1 in the peripheral blood, the increase in the production of the protein is a possible part of ischemic adaptation. Previous studies have reported that the homing or recruitment of circulating endothelial progenitor cells (EPCs) to injury or ischemic sites by SDF-1 is an important process for executing their angiogenic and repair functions. SDF-1 may be involved in regulating the mobilization, proliferation and adhesion capacity of EPCs through binding to CXCR4 and CXCR7. Of note, data indicate that effects of SDF-1 on atherosclerosis rely on production of SDF-1 in arterial endothelial cells, identifying endothelial cell-derived SDF-1 as a crucial driver of atherosclerosis and an important contributor to circulating SDF-1. As this contribution only amounted to 25% of total plasma SDF-1, other cellular sources, e.g. adipocytes or hepatocytes likely produce a substantial component of unknown functional relevance. In our HeFH population serum SDF-1 amounted to approximately half of the control population's SDF-1. Therefore, the cause of decreased circulating SDF-1 in our HeFH patients can be an impaired production by endothelial cells accompanied by other mechanisms affecting SDF-1's production. Although serum SDF-1 was moderately higher in females compared to males, the difference was not significant. Previous data on the correlation between serum SDF-1 and lipid parameters are scant and contradictory. In a small previous study serum SDF-1 was significantly higher in male patients with borderline dyslipidemia compared to control subjects. However, SDF-1 in male patients with clinically significant dyslipidemia was lower than in borderline dyslipidemia. Furthermore, there was a non-significant negative correlation between SDF-1

and TC/HDL-C ratio. SDF-1 correlated positively with HDL-C only in female patients. In another study, increased quartiles of SDF-1 were associated with higher LDL-C and triglycerides, while HDL-C decreased across SDF-1 quartiles in Framingham Heart Study participants. Interestingly, mean serum SDF-1 was 1894 pg/mL (range 742 pg/mL to 17.633 pg/mL), approximately five to ten times higher than usually detected in other human studies. In contrast, we found a strong negative correlation between SDF-1 and ApoB100 containing lipoproteins, and SDF-1 was predicted by VLDL. Recently, it has been shown that the hazard risk for having cardiovascular outcomes is greater for VLDL than LDL, since VLDL carries more cholesterol per particle than smaller LDL and VLDL remnants are trapped more easily in the intima of the arterial wall, where they cause low-grade inflammation by direct and indirect mechanisms and may suppress the expression of SDF-1 as well. Although an elevated amount of triglyceride-rich particles is not characteristic for FH, the disorder is often associated with other conditions including overweight, which may lead to the elevation of triglyceride-rich particles such as VLDL and IDL, leading to elevated serum triglyceride. Formerly, data suggested that inflammatory cytokines (TNFα and IL-6) may have a suppressive effect on SDF-1 in male patients with hyperlipidemia. Although we could not find correlations between inflammatory markers and SDF-1, higher hsCRP indicating enhanced systemic inflammation may diminish SDF-1's production in HeFH. To date, associations between lipoprotein subfractions and SDF-1 have not been studied. Significantly higher small dense LDL concentrations, lower mean LDL sizes, as well as lower large and intermediate HDL subfractions in contrast to higher small HDL was previously reported in HeFH compared to control subjects. Based on our result SDF-1 correlates positively with mean LDL size and large and intermediate HDL subfractions. However, the significance of these findings warrants further investigation. OxLDL enhances coronary atherosclerosis by promoting cellularity, macrophage activation, and differentiation into foam cells, stimulating smooth muscle cell proliferation, and decreasing endothelial nitric oxide (NO) production. MPO is one of the leading agents inducing oxidative stress, which is the basis for oxLDL-generation. OxLDL, or endogenous antibodies against oxLDL have been found to relate to CVD, functioning as biomarkers. However, its prognostic role in FH is not well established. The degree of LDL oxidation was not associated with the history of cardiovascular disease in adult male patients with HeFH, and there was no significant correlation between the plasma concentration of LDL-C and LDL's degree of oxidation. Moreover, no association was found between carotid intima-media thickness and oxidation parameters or circulating antibodies and oxLDL in a larger cohort of HeFH patients. In another previous study, in which antibody

titers were compared to oxLDL in patients with homozygous and heterozygous FH demonstrated no significant relationship between the degree of atherosclerosis and the antibody titer. However, the presence of scavenger receptor lectin-like oxLDL receptor-1 (LOX1)'s rs11053646 genotype enhanced the release of the soluble receptor resulting in increased plaque instability and predicted coronary artery disease in adult patients with HeFH. These incongruent results may demonstrate the fact that the interpretation of oxLDL can be challenging in clinical research. Indeed, oxLDL is a general term that covers heterogeneous oxidative changes to both LDL's lipid moieties and ApoB. Of note, non-oxidizing modification of LDL including desialylation can also have an atherogenic effect, and thus should also be taken in consideration. In line with the previous literature serum oxLDL and MPO were significantly higher in our HeFH patients compared to controls. Interestingly, we found negative correlation between oxLDL and both MPO and SDF-1 in our study population, which is a novel finding. Moreover, oxLDL was the strongest predictor of circulating SDF-1 suggesting a possible regulatory role of oxidative stress in SDF-1's production. It has been reported that atorvastatin increased SDF-1α's expression in vivo under ischemia-reperfusion injury. Moreover, SDF-1α's upregulation by atorvastatin in rats with acute myocardial infarction via NO-production conferred anti-inflammatory and anti-apoptotic effects. It has been reported that circulating microRNA miR-548j-5p contributes to the pathological process associated with angiogenesis by promoting migration and tube formation in EPCs, which are associated with the expression of eNOS and SDF-1. Therefore, upregulation of miR-548j-5p improve neovascularization, which implies that SDF-1 may be a potential therapeutic target of the treatment of peripheral artery disease (PAD). Furthermore, the NO-donor MPC-1011 stimulates angiogenesis and arteriogenesis and improves hindlimb ischemia via a cGMP-dependent pathway involving VEGF and SDF-1α. Based on these data, there are some well-established and novel strategies to increase circulating SDF-1 in HeFH, although its potential benefit in cardiovascular prevention needs further investigation.

Some limitations of the study must be mentioned. The direct mechanisms of common regulatory pathways in SDF-1 and lipoprotein metabolism were not investigated. Furthermore, the Dutch Lipid Clinic Network diagnostic (DLCN) criteria system is one of the most widely used diagnostic algorithms for FH, which incorporate LDL-C, clinical signs and family history of premature atherosclerotic cardiovascular disease including coronary artery disease and PAD to generate a score that leads to a classification of either "definite," "probable" or "possible" FH. Moreover, detection of a pathogenic DNA-mutation in any of the FH-related genes leads to a diagnosis of "definite FH." However, some important

limitations of the DLCN criteria must be noted. Clinical manifestations are infrequent, and family history is sometimes unavailable or unreliable. Moreover, DNA-testing is often not readily available and, in some cases, not concordant with the FH-phenotype. Additionally, in young FH-patients the DLCN criteria might underestimate the probability of FH. However, the results underline the importance of studying the potential role of SDF-1 in the process of atherogenesis in HeFH.

Summary

High-density liprotein (HDL)-associated apolipoprotein M/sphingosine 1-phosphate (ApoM/S1P) complex act as a bridge between HDL and endothelial cells in cardiovascular disease, maintaining a healthy endothelial barrier. Stromal cell-derived factor-1 (SDF-1) is a chemokine that play diversified roles in the process of atherosclerosis. Although, its assoctiation with hyperlipidaemia is contradictory.

To date, serum SDF-1, S1P, ApoM and its correlations with lipid and inflammatory parameters and lipid subfractions in HeFH subjects have not been investigated. Eighty-one untreated HeFH patients and thirty-two healthy control subjects were enrolled in this study. Serum SDF-1, S1P, ApoM, oxLDL, MPO, MMP-9, sCD40L, sICAM-1, sVCAM-1 and TNFα contentrations were determined by ELISA. Lipoprotein subfractions were detected by Lipoprint. PON1 activities were measured spectrophotometrically. For the diagnose of FH we used the Dutch Lipid Clinic Network criteria.

Significantly higher S1P and ApoM levels were detected in HeFH patients compared to the controls. There was a negative correlation between S1P and large HDL, and a positive correlation was observed between S1P and small HDL subfractions in HeFH patients and in the whole study population. Significant positive correlations were found between S1P and sCD40L and MMP-9 levels and PON1 arylesterase activity, while significant negative correlation was detected between sVCAM-1 and S1P in HeFH patients.

We found significantly lower SDF-1 concentrations in HeFH patients compared to the controls. We detected significant negative correlations between serum total cholesterol, triglycerides, LDL-C, ApoB100 and SDF-1. Negative correlations were observed between serum SDF-1 and VLDL and IDL, as well as large LDL and large and intermediate HDL subfractions, while positive correlation was found between mean LDL-size, small HDL and SDF-1. There was a negative correlation between SDF-1 and oxLDL and MPO.

A backward stepwise multiple regression analysis showed that the best predictors of serum S1P were large HDL subfraction and arylesterase activity, while the best predictors of serum SDF-1 were VLDL and oxLDL. Higher S1P and ApoM levels and their correlations with HDL subfractions and inflammatory markers in HeFH patients support their potential regulatory role in the endothelial protection. The strong correlation between SDF-1 and lipid fractions and subfractions highlights the potential common pathways of SDF-1 and lipoprotein metabolism, that assume the role of SDF-1 in atherogenesis.

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List of publications related to the dissertation

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1. **Juhász, L.**, Lőrincz, H., Szentpéteri, A., Tóth, N., Varga, É., Paragh, G., Harangi, M.: Decreased Serum Stromal Cell-Derived Factor-1 in Patients with Familial Hypercholesterolemia and Its Strong Correlation with Lipoprotein Subfractions.
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List of other publications

3. Németh, Á., Daróczy, B., **Juhász, L.**, Fülöp, P., Harangi, M., Paragh, G.: Assessment of associations between serum lipoprotein (a) levels and atherosclerotic vascular diseases in Hungarian patients with familial hypercholesterolemia using data mining and machine learning.
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