

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**INVESTIGATION OF ENDOGENOUS AND EXOGENOUS  
FACTORS AFFECTING THE QUALITY OF HDL**

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Supervisor: Mariann Harangi, MD, PhD



UNIVERSITY OF DEBRECEN  
DOCTORAL SCHOOL OF HEALTH SCIENCES

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Members of the Examination Committee: Mária Tünde Magyar, MD, PhD  
László Márk, MD, PhD

The Examination takes place at the 2<sup>nd</sup> floor Conference Room in the Bldg. of the Faculty of Public Health, University of Debrecen  
11 AM, 31 March, 2017.

Head of the **Defense Committee:** Róza Ádány, MD, PhD, DSc

Reviewers: Judit Barta, MD, PhD  
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen  
1 PM, 31 March, 2017.

## INTRODUCTION

High density lipoprotein (HDL) performs a variety of tasks, including its prominent role in the defense against atherosclerosis through, primarily, its antioxidant effect, as well as by transporting the cholesterol content of peripheral cells to the liver (reverse cholesterol transport, RCT). A growing number of epidemiological data and clinical studies support the idea that HDL may be the next target for the reduction of residual cardiovascular risk in high risk patients receiving statin therapy.

For over three decades now, HDL-cholesterol (HDL-C) quantitative assays have been the primary approach when it comes to assessing HDL. However, a number of studies from the recent years have proven that, in addition to circulatory HDL-C levels, the qualitative assessment of HDL is even more important since HDL-C levels alone do not provide meaningful information about HDL particle composition and function. It has also been proven that in treating cardiovascular disease, it is rather the improvement of circulatory HDL function than the elevation of HDL-C levels that is of key importance.

Due to HDL's physico-chemical and functional heterogeneity, researchers are facing substantial challenges in developing laboratory and clinical methods that are more efficient, and more accurate in estimating cardiovascular risk, than HDL-C quantitative assays. In spite of the great volume of research, no reliable, widely accepted and applied method has been found so far for this purpose.

HDL is a small, dense, protein rich lipoprotein with an average size of 8 to 10 nm and average specific gravity of 1.063 to 1.21 g/mL. HDL particles are complex and heterogeneous macromolecules; their protein content (proteome) and lipid composition (lipidome) are both diverse, resulting in inhomogeneity in terms of size, density, structure, and function, thus they can be classified into sub-fractions, which are in a continuous process of transforming into one another. A substantial

portion of proteome constituent proteins are those required for lipid transport or lipoprotein structural unity (apolipoproteins), but there are also a number of other proteins active in HDL's anti-inflammatory, anti-atherogenic and pleiotropic effect. In terms of structure and function, apolipoprotein AI (apoAI) is the most important protein of HDL. Key functions of apoAI include stabilizing the structure of the HDL particle and the activation of lecithin–cholesterol acyltransferase (LCAT). In addition to these, most of HDL's anti-atherogenic and anti-inflammatory potential, – i.e. neutralization of lipid hydroperoxides, ATP-binding cassette protein A1 (ABCA1) mediated cholesterol efflux, as well as interaction with the ATP-binding cassette protein G1 (ABCG1) transporter and scavenger receptor class B type I (SR-BI) – is also linked to apoAI.

Through its antioxidant effect, HDL can protect low density lipoprotein (LDL) from oxidative damage caused by free radicals through the inhibition of the formation of oxidized lipids with pro-inflammatory effects – mainly lipid hydroperoxides but also, to a partial extent, short-chain oxidized phospholipids. HDL receives lipid hydroperoxides from the oxidized LDL, with HDL's protein and lipid components themselves undergoing modification in the process. HDL, while transporting lipid hydroperoxides to SR-BI receptors of the liver, reduces and thus inactivates them as well. A prominent role in this is played by apoAI's methionine side chains. Nonetheless, the antioxidant effect of HDL is contributed to by other apolipoproteins as well as enzymes related to HDL. Several studies have proven that the distribution of apolipoprotein variants and antioxidant enzymes differs across HDL sub-fractions.

One of the most important enzymes involved in the antioxidant effect HDL is paraoxonase 1 (PON1). Its effects include arylesterase, phosphodiesterase (paraoxonase) and lactonase activities. The arylesterase activity of PON1 strongly correlates with the quantity of the circulatory PON1 protein and is an indicator of the antioxidant effect of the latter, while paraoxonase activity is significantly

influenced by inter-individual differences caused by genetic polymorphism. It has long been known that, with the assistance of PON1, HDL can inhibit LDL oxidation by hydrolyzing oxidized phospholipids; furthermore, it is hypothesized that PON1 can increase the free radical reduction capability of monocytes/macrophages, enhances the removal of cholesterol coming from macrophages, and can enhance HDL's capability to stop LDL oxidation by macrophages. In addition to these, PON1 may modify the activity of myeloperoxidase (MPO) which, in turn, is an inhibitor of PON1. A major discovery of the last few years was that HDL, MPO, and PON1 form a three-element unit, in which it is not only that MPO inhibits PON1's activity, but PON1 also partially reduces MPO's activity. Prospective studies show convincing evidence in relation to the role played by PON1 in cardiovascular diseases. It has been proven beyond doubt that reduced PON1 activity is a risk factor of vascular diseases.

HDL function is also exposed to negative interference by a number of molecules. A prominent one of these is MPO which, by virtue of its bactericide effect, constitutes an important component of congenital immunity. Several studies also investigate MPO's role played in the pathomechanism of atherosclerosis: by oxidizing LDL, MPO facilitates LDL uptake by macrophages, thus it contributes to the formation of foam cells. MPO also oxidatively modifies apoAI, resulting in damage to HDL binding to ABCA1, which in turn leads to a disruption of ABCA1-dependent reverse cholesterol transport, and inhibits HDL's anti-inflammatory and anti-apoptotic abilities. In addition, MPO directly damages the function of HDL-bound PON1, as well as ABCG1-dependent cholesterol transport. Consequently, elevated MPO levels have been implicated in intensified atherosclerosis; there are even a number of studies evaluating MPO as a cardiovascular marker.

It has recently been proven that another enzyme, matrix metalloproteinase 9 (MMP-9), is also capable of forming a complex with HDL, especially HDL2, and

thus potentially contributes to the formation of dysfunctional HDL. Members of the zinc-dependent endopeptidase family, MMP enzymes play a key role in the breakdown of extracellular matrix, thereby contributing to the generation and destabilization of vulnerable atherosclerotic plaques, and thus the onset of cardiovascular events. Tissue inhibitors of metalloproteinase (TIMP) regulate MMP activity by binding to the enzyme molecules. Several studies prove that patients with cardiovascular disease have elevated TIMP-1 levels; it has also been proven that TIMP-1 is an independent predictor of cardiovascular mortality. It is hypothesized that such elevated TIMP-1 levels may reflect a compensatory response to increased MMP activity.

Another highly important function of HDL is the delivery of excess cellular cholesterol from peripheral tissues to the liver via the process of reverse cholesterol transport; such cholesterol then either gets secreted into the bile and passes with feces or ends up in the adrenal glands, testes or ovaries for the purpose of steroid hormone synthesis. Through this process, HDL is able to counterbalance the processes leading to atherosclerotic plaque formation and development. HDL sub-fractions vary in the extent to which they can facilitate cholesterol efflux.

During cholesterol efflux, which is the first step of RCT, free cholesterol is released from cells. This is produced by passive and active processes acting in a parallel fashion. Transport protein mediated active processes play a substantially greater role in reverse cholesterol transport. Three major transporters have so far been identified: ABCA1, ABCG1, and SR-BI. The most important function is served by ABCA1, which transports, in addition to non-esterified cholesterol, phospholipids – mainly, phosphatidylcholine – to lipid-free or lipid-poor apoAI, thus contributing to the formation of pre-beta-HDL as well. While ABCA1 transports cholesterol primarily to lipid-poor apoAI, ABCG1 and SR-BI do so mainly to mature HDL. During reverse cholesterol transport, HDL itself also undergoes structural and functional changes. As a first step, lipid-poor apoAI joins

together with plasma membrane derived phospholipids and non-esterified cholesterol to form pre-beta-HDL. Upon cholesterol uptake, small pre-beta1-HDL molecules turn into larger, but still discoidal, pre-beta2-HDL particles; these in turn evolve, by cholesterol esterification and further apoAI uptake, into spherical alpha3-HDL, then alpha2-HDL. A number of enzymes work to ensure the smooth functioning of reverse cholesterol transport (cholesteryl ester transfer protein (CETP), LCAT, phospholipid transfer protein (PLTP), hepatic lipase (HL), endothelial lipase (EL)). At the end of the process residual lipid-free or lipid-poor apoAI particles then either contribute again to HDL formation, or get filtered through renal glomeruli, which takes place via endocytosis mediated by cubilin and megalin receptors found in the proximal tubules.

The key role of apoAI in reverse cholesterol transport is highlighted by the fact that cells found in the intima of atherosclerotic arteries secrete a variety of proteases that may modify HDL, partly by the proteolysis of apoAI found in pre-beta-HDL, leading to the inability of apoAI to bind to ABCA1, which may result in a reduction of apoAI's potential to facilitate ABCA1 mediated cholesterol efflux and HDL biogenesis. Some studies even show that the proteolysis of apoAI may damage its potential to facilitate ABCG1 mediated cholesterol efflux. It is also widely known that one of the targets of nitration and chlorination catalyzed by MPO is apoAI; it is hypothesized that oxidative damage to apoAI thus limits apoAI release from HDL in a lipid-free form, which is detrimental to its recycling – another mechanism contributing to the reduction of ABCA1 mediated cholesterol reflux.

Beyond these, HDL is known to affect cell survival, proliferation and migration, and to have an antithrombotic effect; HDL may contribute to endothelial repair processes and can directly stimulate the nitric oxide (NO) production, as well as playing an important role in the body's immunological potential.

The composition and function of HDL may also be endogenously affected by genetic factors, and exogenously by the presence and activity of enzymes/molecules binding to it to form a functional unit (e.g. PON1, MPO); selected diseases and medicinal therapy can likewise be influential.

More than 40 genes that affect HDL cholesterol levels have been identified to this day. Located in the brush border of jejunal epithelial cells, the Niemann–Pick C1-like 1 (NPC1L1) protein came to the forefront of attention when it had turned out that it is this protein to which ezetimibe binds to inhibit cholesterol absorption. Upon sequencing the *NPC1L1* gene, it became known that in carriers of certain *NPC1L1* variants, cholesterol absorption rates are significantly lower than in non-carriers of those variants. Cholesterol enters the epithelial cells of the small intestine through the NPC1L1 protein, during which process NPC1L1 determines the quantity of cholesterol ending up in circulation. Cholesterol is esterified in the intestinal epithelial cells and, via the ABCA1 transporter located at the basolateral margin of the cell, enters the lymphatic system, which in turn transports it to the liver. In a situation of low cellular cholesterol, NPC1L1 gets expressed in the membrane; and when the cells become saturated with cholesterol, the receptor becomes internalized into endosomes. To this date, in excess of 140 single nucleotide polymorphisms (SNP) and five insertions/deletions have been identified within the *NPC1L1* gene. Several of these SNPs affect therapeutic responses to statin (hydroxy-methyl-glutaryl-coenzyme A reductase inhibitor) or statin-ezetimibe combined treatment. It has recently been proven that c.-133A>G has a significant effect on *NPC1L1*'s promoter activity; the mutation significantly modified the lipid lowering effect of statin therapy during pravastatin treatment. Up until now, a substantial proportion of studies have researched the effect of ezetimibe in combination with statins only; the effects of individual SNPs on the response to ezetimibe monotherapy have not yet been studied.

In several pathologies (such as coronary heart disease, type 2 diabetes, metabolic syndrome, systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease, antiphospholipid syndrome, psoriasis, chronic renal disease, and selected environmental pollutions), the presence of a so called dysfunctional HDL has been established, a variety with notable pro-oxidative and pro-inflammatory characteristics. In dysfunctional HDL, a reduced level of atheroprotective, and an elevated level of proatherogenic molecules are present; this is accompanied by a higher oxidized fatty acid and malonaldehyde content, with – partly after chlorination or nitration caused by MPO – oxidative modification of apoAI, and detectable changes to PON1 activity. Dysfunctional HDL is incapable of preventing LDL oxidation; reverse cholesterol transport is compromised, adhesion of white blood cells to activated endothelial cells is reduced, endothelial nitrogen monoxide (NO) synthesis induction is cut, endothelial repair functions are damaged.

Based on the reverse association between HDL-C and cardiovascular events, boosting HDL-C levels might appear to be an attractive therapeutic target, which explains the great number of medicines being developed and studied. Still, a number of clinical trials in coronary disease patients failed to achieve substantial cardiovascular risk reduction in spite of an elevation in HDL-C levels. A substantial proportion of patients on statin therapy develop some level of intolerance to the medication used. Although the frequency of side effects caused by statins is no greater than that with other drugs, the importance of the issue has grown due to the high number of patients on statin treatment. In the era preceding the wide availability of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, cardiovascular risk reduction in statin intolerant patients remains some of a challenge. The cardiovascular risk reduction effect of ezetimibe is moderate in comparison to that of statins; however, in statin intolerant patients, ezetimibe is one of the most frequently recommended therapies amongst currently available

medications, thus ezetimibe monotherapy is in widespread use in such patients. Ezetimibe is a highly tolerable medicine; on average, it delivers 15 to 20% in LDL-cholesterol (LDL-C) reduction and about 3% in HDL-C elevation. Ezetimibe decreases the amount of cholesterol en route to the liver; the number of LDL receptors increases, which leads to reduced LDL-C levels through intensifying the catabolism of LDL. It has also been proven that ezetimibe also enhances several constituent processes of reverse cholesterol transport.

There is a growing body of evidence indicating that the measurement of a single HDL component (such as cholesterol content) is insufficient to estimate the cardioprotective function of HDL. HDL-C level alone may be indicative of the number of particles in question, but it fails to inform about the subgroup with the greatest involvement in the protection against cardiovascular disease. Therefore, isolated measurement of HDL-C levels (i.e., cholesterol content carried by HDL) does not appear to be the most reliable predictor of HDL function. A number of experimental and clinical data also suggest the possibility that the composition of the HDL particle may change in selected diseases or upon certain therapies, with such changes not necessarily being reflected by HDL-C levels. Based on all these, it is evident that future studies will need to investigate the relationship between HDL structure and function, leading to more accurate ways to estimate cardiovascular risks and therapeutic effects; these findings may also even offer new treatment options.

## OBJECTIVES

1. Our aim was to examine the effect of 3, 6 and 12 months of of 10 mg daily ezetimibe monotherapy on lipid, hsCRP and creatinine-kinase levels in statin-intolerant, hyperlipidemic patients.
2. Our additional objective was to study the genotype distribution of SNP c.-133A>G at the *NPC1L1* gene.
3. Furthermore, we aimed to evaluate the efficacy of SNP c.-133A>G at the *NPC1L1* gene on lipid levels in the same study population
4. In the second part of our studies we measured and compared the levels of PON1, MPO and other two biomarkers involved in the atherosclerotic process (MMP-9 and TIMP-1) in overweight hyperlipidemic, lipid-lowering therapy-naive patients with and without vascular complications.
5. Our additional objective was the investigation of association of MPO - PON1, MPO – MMP-9 and MPO – TIMP1 in the same study population.

## **PATIENTS AND METHODS**

### ***Enrollment of statin intolerant patients***

One hundred and one (69 females, 32 males), Fredrickson type IIa and IIb hyperlipidemic patients with previously diagnosed statin induced adverse effects were enrolled. Patients were recruited from our lipidology outpatient clinic at Institute of Medicine, University of Debrecen. The mean age of studied patients was  $61.23 \pm 9.87$  years, BMI of our patients was  $28.18 \pm 4.29$  kg/m<sup>2</sup>. In 42 cases statin-induced myopathy (myalgia with or without creatine-kinase (CK) elevation), in 28 cases statin-induced liver enzyme elevation (hepatopathy), in 15 cases statin-induced severe gastrointestinal symptoms were proved. Sixteen patients had more than one statin-induced symptom: two patients had myopathy, hepatopathy and gastrointestinal symptoms, seven had myopathy and hepatopathy, five had myopathy and gastrointestinal symptoms, and two patients had hepatopathy and gastrointestinal symptoms. Patients with alcoholism, drug dependence, malignancy, pregnancy or lactation, as well as patients on anticoagulant or lipid-lowering therapy were excluded. After 6 weeks on the National Cholesterol Education Program step 1 diet, patients received 10 mg/day ezetimibe (Ezetrol) for 12 months. Informed consent was obtained from all patients after explaining the nature and the purpose of the study. The Ethics Committee of the University of Debrecen, the National Institute of Pharmacy approved the study. The study is registered by the European Clinical Trials Database (EudraCT number 2009–017732–40).

### ***Enrollment of overweight, therapy naive patients***

In the second part of our study 167 (89 females and 78 males) overweight adult Caucasian patients with Fredrickson type IIa and IIb hyperlipidemia were

enrolled. The mean age of studied patients was 50.0 (41-61) years, BMI of our patients was  $28.49 \pm 5.56 \text{ kg/m}^2$ . Participants were recruited from our lipidology outpatient clinic at Institute of Medicine, University of Debrecen. Overweight was defined according to the subjects' body mass index ( $\text{BMI} \geq 25 \text{ kg/m}^2$ ).

Physical examination and carotid ultrasound were performed regularly. Other imaging techniques (Doppler ultrasound, echocardiography and computer tomography) were performed in case of complaints or abnormal physical and electrocardiography (ECG) examinations. We determined the presence of hypertension, type 2 diabetes mellitus and smoking habits in all patients. Hypertension was defined as the recurrent use of antihypertensive drugs or systolic blood pressure  $\geq 140 \text{ mmHg}$ , diastolic BP  $\geq 90 \text{ mmHg}$ . The diagnosis of type 2 diabetes mellitus was made by recurrent use of antidiabetic drugs or insulin or a fasting blood glucose level  $\geq 7 \text{ mmol/l}$ . Smoking was defined as previous (in the last 10 years and longer-than-six-months-lasting) and current smoking habits. Study subjects were divided into two gender-matched subgroups as patients with pre-existing vascular complications (VC) and patients without vascular complications (NVC). (Patients with vascular complications: mean age:  $60.49 \pm 10.05$ ; 22 females and 19 males; BMI:  $29.34 \pm 3.94 \text{ kg/m}^2$ . Patients without vascular complications: mean age:  $46.93 \pm 12.40$ ; 67 females and 59 males; BMI:  $28.18 \pm 6.00 \text{ kg/m}^2$ ). Vascular complications were defined as known ischemic heart disease (myocardial infarction or coronary sclerosis), ischemic cerebrovascular disease (ischemic stroke, transient ischemic attack, carotid artery stenosis/occlusion) and peripheral arterial disease. Vascular complications were established by the history data of patients or the results of imaging techniques. Patients were divided into the "patients with vascular complications" group if they had at least one complication. At the time of the enrollment, patients were free of acute complaints. Exclusion criteria included (at least 6 weeks) previous and ongoing lipid lowering therapy, autoimmune disease, chronic inflammatory states,

active liver or endocrine disease including type 1 diabetes mellitus, malignancy and end-stage kidney failure. The study was carried out according to the Declaration of Helsinki and informed consent was obtained from all patients after approval of the local ethics committee.

### ***Sample collection and laboratory measurement of statin intolerant patients***

At baseline and after 3, 6 and 12 months of treatment with ezetimibe, after 12 h of fasting, a 10-ml venous blood sample was taken between 07.30 and 08.00 a.m. Serum cholesterol, triglyceride, HDL-C, LDL-C, apoAI, apolipoprotein B (apoB), high-sensitivity C reactive protein (hsCRP) levels and creatinine-kinase (CK) activity were determined from fresh sera with standard laboratory measurements in the Institute of Laboratory Medicine, University of Debrecen.

### ***NPC1L1 genotype analysis***

Genomic DNA was isolated from EDTA (ethylenediaminetetraacetic acid) or citrate-anticoagulated blood in the the Institute of Laboratory Medicine, University of Debrecen. The presence of the c.-133A>G polymorphism (rs17655652) was tested by enzyme digestion of PCR amplified products.

### ***Statistical analyses of the results in statin intolerant patients***

Statistica forWindows 6 and IBM Statistical Package for the Social Sciences (SPSS) Statistics Version 19 computer softwares were used for statistical analysis. Normality of distribution was tested by the Kolmogorov-Smirnov test. In case of normal distribution the differences between parameters were analyzed with one-way analysis of variance (ANOVA), followed by post hoc comparisons using the Newman-Keuls test. In cases of non-normal distributions the differences were compared with Kruskal-Wallis and Mann-Whitney U tests. A value of  $P < 0.05$

was considered to be statistically significant. The genotype-dependence of changes was analyzed by Welch's robust test. The data of persons with various genotypes and alleles were compared using the chi-square and Fisher's exact test.

### ***Sample collection and laboratory measurement of overweight, therapy naive patients***

Venous blood samples were taken between 08:00 - 10:00 a.m. after an overnight fast and sera were prepared immediately. Routine laboratory analyses (hsCRP, triglyceride, total cholesterol, LDL-C, HDL-C, ApoAI, ApoB, lipoprotein (a) (Lp(a)), hemoglobin A1C (HbA1C), uric acid and ultrasensitive thyroid-stimulating hormone (sTSH) levels) were determined with standard laboratory measurements in the Institute of Laboratory Medicine, University of Debrecen. The sera for MPO, MMP-9, TIMP-1 and PON1 paraoxonase and arylesterase measurements were kept at -70°C until analysis, samples were used within 2 months. Serum concentrations of MPO, MMP-9 and TIMP-1 were measured by sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Europe Ltd., Abington, England) according to the recommendations of the manufacturer.

### ***Paraoxonase-1 measurements***

PON1 paraoxonase and arylestarse activity was analyzed by spectrophotometrically. Paraoxonase activity was assayed on a microtiter plate, utilizing paraoxon (O,O-diethyl-O-p-nitrophenyl-phosphate, Sigma) as a substrat and the generation of 4-nitrophenol was measured on a microtiter plate at 405 nm. PON1 paraoxonase activity was expressed in U/l, where 1 unit equals 1  $\mu$ mol of substrate hydrolyzed per minute. Arylesterase activity was analyzed by the

hydrolysis of phenylacetate (Sigma) at 270 nm. Arylesterase activity was expressed as units per liter of serum. 1 unit of arylesterase activity is defined as 1  $\mu$ mol phenylacetate hydrolyzed per minute.

PON1 (Q192R) phenotype was calculated by the dual substrate method. The hydrolysis of paraoxon is faster by the R allele than by the Q allele. In contrast, both R and Q alleles had similar arylesterase activity. The ratio of the hydrolysis of paraoxon in the presence of 1 mol/l NaCl (salt-stimulated paraoxonase) to the hydrolysis of phenylacetate was used to assign individuals to one of the three possible PON1 phenotypes: low activity, intermediate activity and high activity.

### ***Statistical analyses of the results in overweight, therapy naive patients***

Statistical analysis was performed by SAS<sup>TM</sup> for Windows<sup>TM</sup> 8.2 computer software (SAS Institute Inc., Cary, NC, USA). Normality of distribution was tested with Kolmogorov-Smirnov test. Comparisons between groups with normal distribution were performed by a Student t-test whereas comparisons between non-normally distributed parameters were performed with Mann-Whitney U-test. Correlations between the investigated parameters were assessed by Pearson's correlation analysis; the above mentioned non-normally distributed parameters were transformed logarithmically to correct their skewed distributions. Multiple regression analysis (backward-stepwise method) was performed to determine variables that best predicted MPO levels in the whole patient group as well as in the VC and NVC subgroups. We used two sided p-values;  $p < 0.05$  was accepted as the level of significance.

## RESULTS

### *The effect of c.-133A>G polymorphism in Niemann–Pick C1-Like 1 gene on the efficiency of ezetimibe monotherapy*

After 3 months of ezetimibe treatment the total cholesterol ( $p<0.001$ ), LDL-C ( $p<0.001$ ) and hsCRP ( $p<0.01$ ) levels significantly decreased, while the other studied parameters (triglyceride, HDL-C, ApoB, ApoAI and CK levels) did not change significantly in the whole study population. Six months of treatment significantly decreased the total cholesterol ( $p<0.001$ ), LDL-C ( $p<0.001$ ), hsCRP ( $p<0.01$ ), triglyceride ( $p<0.01$ ), and ApoB ( $p<0.01$ ) levels compared to the initial results. After 12 months of treatment we found significantly decreased total cholesterol ( $p<0.001$ ), LDL-C ( $p<0.001$ ), triglyceride ( $p<0.05$ ) and ApoB levels ( $p<0.01$ ). The HDL-C and apoAI levels did not change significantly after 12 months of ezetimibe monotherapy in the whole study population.

In the studied population the *NPC1L1* c.-133A>G genotype distribution was as follows: 57.42 % were AA, 34.65% were AG and 7.92% were GG genotype. Because of the low ratio of GG patients we divided the study population into two groups: AA (G non-carriers) and AG+GG (G carriers). There were no significant differences in initial lipid levels between AA and AG+GG patients. We could not find significant differences in age, body mass index (BMI) and waist circumference between the two groups.

Plasma levels of ApoA1 did not change significantly after 3, 6 and 12 months of ezetimibe treatment (1.96; 3.39 and 2.74%, respectively) in AA patients. However, significant elevation in ApoA1 levels has been found after treatment in AG+GG patients (9.15; 8.54 and 13.58%, respectively). The effect of *NPC1L1* - 133A >G on the efficacy of ezetimibe treatment on ApoA1 level was significant ( $p < 0.05$ ). HDL-C levels remained unchanged in both groups, and there was no significant difference between the two groups.

Comparing the two groups we could not find any significant difference in the efficacy of treatment with ezetimibe on other plasma lipid parameters after 3, 6 or 12 months. After 3 and 12 months of ezetimibe treatment LDL-C and total cholesterol levels significantly decreased both in the AA and in the AG+GG groups ( $p<0.05$ ). The ApoB level decreased significantly only in the AA group after 6 months and 12 months therapy, but there was no significant difference between the two groups.

The tolerability of ezetimibe was excellent. No patient had to discontinue the drug due to side effects. CK level did not significantly change after 12 months of ezetimibe monotherapy as well.

***The association between the paraoxonase-1 activity, myeloperoxidase level and the levels of other vascular biomarkers (MMP-9 and TIMP-1) in overweight, therapy naive patients***

Compared to the patients without vascular complications (NVC subgroup), patients with vascular complications (VC subgroup) were significantly older (NVC:  $46.93\pm 12.40$  years vs. VC:  $60.49\pm 10.05$ ,  $p<0.001$ ), while there were no significant differences in BMI or waist circumference between the two subgroups. Measuring the laboratory parameters, we did not find significant differences in fasting glucose or HbA1C levels between the subgroups. The VC subgroup had a more atherogenic lipid profile with significantly higher total cholesterol ( $p<0.01$ ), LDL-C and TG levels ( $p<0.05$ ); however, HDL-C, apoA1, apoB and Lp(a) levels did not differ significantly between the VC and NVC subgroups. Also, there was no significant difference in the hsCRP concentrations.

PON1 Q192R phenotype distribution and allelic frequencies were also evaluated. The PON1 phenotype distribution was as follows: in the whole patient group 80 % ( $n=134$ ) were AA, 20 % ( $n=33$ ) were AB phenotype, and there were no patients with BB phenotype. The phenotype distribution (AA-AB) was 83%

(n=104), 17% (n=22) in NVC patients and 73% (n=30), 27% (n=11) in VC subgroup, respectively. The allelic frequencies followed the Hardy-Weinberg equilibrium and no significant differences were found between the subgroups.

There was no significant difference in MMP-9 levels between the two investigated subgroups; however, MPO and TIMP-1 levels were significantly higher (MPO: 728 (367.25-1177.90) mg/ml vs. 315.9 (176.05-687.40) mg/ml;  $p < 0.001$ ; and TIMP-1: 172.7 (157.7-197.7) ng/ml vs. 152.6 (129.3-172.3) ng/ml;  $p < 0.0001$ ) and the MMP-9/TIMP-1 ratio (3.02 (1.95-4.22) vs. 3.50 (2.34-5.20),  $p < 0.0001$ ) was significantly lower in VC patients when compared to NVC subjects. We did not find significant differences in PON1 activities between the two subgroups, in contrast, the MPO/PON1 ratio was significantly higher in VC patients than in NVC patients ( $p < 0,05$ ).

MPO level showed a significant negative univariate correlation with PON1 arylesterase activity in the whole patient group ( $r = 0.42$ ,  $p < 0.0001$ ), as well as in both subgroups (VC:  $r = 0.44$ ,  $p = 0.01$ ; NVC:  $r = 0.39$ ,  $p < 0.0001$ ). Investigating the relationship between MPO levels and PON1 paraoxonase activity, we did not find any significant correlations in the patient groups. MPO concentration showed a significant positive univariate correlation with MMP-9 level ( $r = 0.37$ ,  $p < 0.0001$ ) in the whole patient group as well as in the NVC subgroup ( $r = 0.42$ ,  $p < 0.0001$ ). Although not reaching the level of significance, perhaps due to the low number of individuals in this subgroup, MPO levels tended to correlate positively with MMP-9 levels in VC patients ( $r = 0.29$ ,  $p = 0.07$ ). MPO and TIMP-1 levels showed a significant positive correlation in the whole patient group ( $r = 0.42$ ,  $p < 0.0001$ ) as well as in the subgroups (NVC:  $r = 0.41$ ,  $p < 0.0001$ ; VC:  $r = 0.33$ ,  $p < 0.05$ ). Also, PON1 arylesterase activity displayed a significant negative correlation with TIMP-1 levels both in the whole patient group ( $r = -0.24$ ,  $p < 0.01$ ) and in the NVC subgroup ( $r = -0.26$ ,  $p < 0.01$ ).

We performed multiple regression analysis with MPO levels as the dependent variable. PON1 arylesterase activity, MMP-9 and TIMP-1 levels were proven to be independent predictors of MPO levels both in the whole patient group betegcsoportban (PON1 arylesterase activity:  $\beta=-0.350$ ; MMP-9:  $\beta=0.315$ ; TIMP-1:  $\beta=0.292$ ;  $p<0.0001$ ) and NVC subgroup (PON1 arylesterase activity:  $\beta=-0.330$ ; MMP-9:  $\beta=0.351$ ; TIMP-1:  $\beta=0.262$ ;  $p<0.0001$ ). In contrast, only PON1 arylesterase activity was proven to be an independent predictor of MPO levels in the VC subgroup ( $\beta=-0.570$ ,  $p<0.05$ ).

## DISCUSSION

In the first part of our examinations the effect of c.-133A>G polymorphism in Niemann–Pick C1-Like 1 gene on lipid levels especially on HDL-C and apoA1 levels was studied in statin intolerant, hyperlipidemic patients with ezetimibe monotherapy.

In former studies different HDL-C raising effect of ezetimibe has been shown. Some researchers found that ezetimibe could increase the HDL-C level significantly, while in a study of healthy men there were no difference in HDL levels between the placebo group and the ezetimibe monotherapy group. Nevertheless, the effect of *NPC1L1* polymorphisms on the HDL-C response to ezetimibe monotherapy has not been studied yet.

In our study we could not find a significant difference in the response to ezetimibe on HDL-C between the patients carrying *NPC1L1* -133 AA and in AG+GG patients. Contrarily, in AA patients plasma levels of ApoA1 did not decrease significantly after 3, 6 and 12 months of ezetimibe treatment. While, a significant elevation in ApoA1 levels has been found after treatment in AG+GG patients. Based on these results we reported firstly that *NPC1L1* c.-133A>G SNP influenced the ApoA1 response to ezetimibe monotherapy, therefore, might alter the effect of ezetimibe on the structure and function of the HDL particles.

In our study we could confirm that ezetimibe monotherapy improved the lipid parameters in statin intolerant hyperlipidaemic patients. Ezetimibe monotherapy significantly reduced the total cholesterol (-11.70 %), LDL-C (-8.52 %) and the triglyceride levels (-3.70 %), while the HDL-C levels (1.45 %) did not increase significantly. A previous meta-analysis found that ezetimibe monotherapy decreased LDL-C level by -18.58 %, and reduces total cholesterol and triglyceride concentration by -13.46 % and -8.06% compared to placebo, respectively.

HDL-cholesterol level was also found to significantly increase by 3%. In our study the reduced cholesterol altering effect of ezetimibe can be – at least partially

- explained by our study population. Other studies have investigated the effects of ezetimibe in patients with diverse lipoprotein abnormalities (e.g. type 2 diabetes and mixed hyperlipidemia), while we enrolled a specific statin-intolerant hyperlipidemic population. In addition, in our patients the distribution of Apolipoprotein E allele could be different from the distribution of former study populations.

In former trials ezetimibe monotherapy decreased hsCRP level compared with placebo, but the differences had generally not been found to be statistically significant as well. We found significantly reduced hsCRP level after 3 and 6 months of ezetimibe monotherapy, but we could not find significant difference after 12 months in all patients. The studied *NPC1L1* polymorphism did not alter the effect of ezetimibe on CRP level significantly.

There are only few data about the genotype distribution of *NPC1L1* c.-133A>G SNP. In a previous study genotype distribution of the c.-133A>G *NPC1L1* polymorphism was similar to our patients. In this study among the autosomal dominant hypercholesterolemic group (n=271), 48% of patients were AA allele carriers, 46 % and 6% were AG and GG allele carriers, respectively. While in the control group (n=272) the genotype distribution was as follows: 58% were AA, 35% were AG and 7% were GG genotype.

The maximum cholesterol-lowering effect of ezetimibe evolved the first 2 weeks, and then it remained the same. Therefore most trials have followed the ezetimibe efficacy only for 12 weeks. Though in short-term (12 weeks) trials the safety profile of ezetimibe monotherapy seems to be like placebo's, there has been limited information about the long-term safety of ezetimibe. In our study a one-year ezetimibe treatment was examined, and it was well tolerated. No patient had to discontinue the drug due to side effects. We did not experience severe side effects. CK level did not significantly change after 12 months of ezetimibe monotherapy as well.

In the second part of our examinations the relationship between two enzymes associated with HDL (PON1 activities and MPO concentration) and the level of two biomarkers involved in the atherosclerotic process (MMP-9 and TIMP-1) was investigated in statin-naïve overweight hyperlipidemic patients. The tight relationship between MPO – MMP-9 – TIMP-1 levels, MPO level – PON1 activity, as well as the chronic inflammation that is characteristic for atherosclerosis, obesity and cardiovascular diseases have been well known for several years, although their associations have not been completely clarified yet. This is the first study in which PON1 activity, MPO, MMP-9 and TIMP-1 levels were examined simultaneously

In this examination individuals with vascular complications had significantly higher MPO levels compared to those without any complications, suggesting increased oxidative stress and a more atherogenic milieu in these subjects.

We demonstrated firstly the significant positive associations between MPO and MMP-9 levels and between MPO and TIMP-1 levels in statin-naïve overweight hyperlipidemic patients. These correlations were observed in subgroups with and without vascular complications as well. Although patients without vascular complications were free of manifest atherosclerotic signs, one might conclude that early-stage asymptomatic atherosclerosis might already be present in them due the obesity-related low-grade chronic inflammation; that atherogenetic process, however, is not diagnosable in the premature stage in these younger individuals.

In our statin-naïve, hyperlipidemic patients MPO concentrations showed significant negative correlations with PON1 arylesterase activities in the whole patient group as well as in both subgroups. Our results correspond with the data of researches in patients with type 2 diabetes mellitus or coronary artery disease. Based on multiple regression analysis, PON1 arylesterase activity was proven to be an independent predictor of MPO concentration in the whole patient population as

well as in both subgroups. Our in vivo results correspond with the in vitro data of Huang et al. These data indicate that MPO and PON1 mutually inhibit each other's effect.

In our patients the MPO/PON1 ratio was significantly higher in the VC subgroup compared to the NVC group, consequently the examination of this parameter may be related to the severity of atherosclerosis. This finding supports the results of a research published in 2014. Moreover, this study also raised the possibility that the MPO/PON1 ratio could be a potential indicator for dysfunctional HDL therefore could be a beneficial marker for secondary prevention of cardiovascular disease.

We did not find any significant correlation between PON1 paraoxonase activities and MPO levels, which may have been due to the high inter-individual variability of paraoxonase activity. We also did not detect any significant difference in PON1 activities (arylesterase or paraoxonase) comparing the two subgroups.

PON1 arylesterase activity, MMP-9 as well as TIMP-1 levels proved to be significant predictors of MPO levels both in the whole patient group and in the NVC subgroup; however, the correlation between MPO and PON1 seems to be the strongest one since only PON1 arylesterase activity was proven to be independent predictor of MPO levels in patients with vascular complications. The correlations found in our overweight, hyperlipidemic individuals suggest cumulative and synergistic atherogenic effects in which elevated levels of MPO contribute to the development of the vascular events by direct enhancement of oxidative stress and by indirect impairment of anti-atherogenic PON1 function. Besides the significant correlations of MPO with MMP-9 and TIMP-1 levels, PON1 arylesterase activity also showed a significant negative correlation with TIMP-1 levels, which suggests that PON1 might also be influenced by inflammatory factors causing enzyme inactivation.

These findings support that MPO-PON1-HDL is a complex system. Therefore, investigating HDL and its related biomarkers (PON1 and MPO) may be a potential marker for the presence of dysfunctional HDL and parallel examination of these parameters – supplemented with other biomarkers involved in the atherosclerotic process - may add further information on the severity of the atherosclerotic process in overweight patients. Parallel investigation of these parameters may be useful for selecting high-risk cardiovascular patients which may allow earlier treatment and may improve treatment or can help to follow-up the effectiveness of therapy.

## SUMMARY

Recently, several researches demonstrated the significant antiatherosclerotic effect of HDL. There is a growing body of evidence indicating that the measurement of a single HDL component (such as cholesterol content) is insufficient to estimate the cardioprotective function of HDL. Based on these results it is obvious that examinations dealing with the association between the structure and function of HDL are needed.

Numerous genes associated with HDL have a so-called endogenous effect on the level and structure of HDL. Furthermore, enzymes/molecules which form a functional complex with HDL can affect exogenously the function of HDL as well.

First, we aimed to examine the effect of SNP c.-133A>G at the *NPC1L1* gene on lipid levels and on the efficacy of 3, 6 and 12 months of 10 mg daily ezetimibe monotherapy in hyperlipidemic patients. Furthermore, we also examined PON1 activities, MPO concentration and the level of two biomarkers involved in the atherosclerotic process, MMP-9 and TIMP-1, in overweight hyperlipidemic, lipid-lowering therapy-naive patients with and without vascular complications.

In patients with ezetimibe monotherapy HDL-C levels remained unchanged in both AA and AG+GG group, and there was no significant difference between the two groups. Interestingly, plasma levels of apoA1 did not change significantly after 3, 6 and 12 months of ezetimibe treatment in AA patients. However, significant elevation in ApoA1 levels has been found after treatment in AG+GG patients. The effect of *NPC1L1* c.-133A>G on the efficacy of ezetimibe treatment on ApoA1 level was significant ( $p < 0.05$ ).

In our overweight hyperlipidemic, lipid-lowering therapy-naive patients there was no significant difference in HDL-C levels between patients with and without vascular complaints. MPO level showed a significant negative univariate correlation with PON1 arylesterase activity in the whole patient group as well as in both subgroups, while MPO concentration showed a significant positive univariate

correlation with MMP-9 and TIMP-1 levels as well. MPO/PON1 ratio was significantly higher in patients with vascular complications than in patients without any complications. We performed multiple regression analysis and PON1 arylesterase activity, MMP-9 and TIMP-1 levels were proven to be independent predictors of MPO levels, moreover, the correlation between MPO and PON1 seems to be the strongest one.

In our studies we did not find significant differences in HDL-C levels. However, *NPC1L1* c.-133A>G SNP influenced the ApoA1 response to ezetimibe monotherapy, therefore, might alter endogenously the effect of ezetimibe on the structure and function of the HDL particles. Moreover, parallel investigation of HDL and its related biomarkers, PON1 and MPO, by modification of HDL function, was a more accurate indicator of the severity of atherosclerosis in overweight patients, and it might be a possible indicator for dysfunctional HDL. Our results confirm that the examination of factors affecting the quality of HDL can assist the estimation of the cardioprotective role of HDL and therefore may be more important than the measurement of HDL-C level alone.



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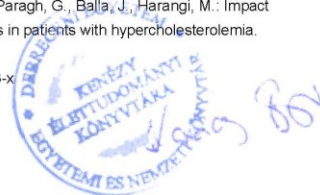
Candidate: Noémi Zsíros  
Neptun ID: DQUOC2  
Doctoral School: Doctoral School of Health Sciences

### List of publications related to the dissertation

1. **Zsíros, N.**, Koncosos, P., Lőrincz, H., Seres, I., Katkó, M., Szentpéteri, A., Varga, V. E., Fülöp, P., Paragh, G., Harangi, M.: Paraoxonase-1 arylesterase activity is an independent predictor of myeloperoxidase levels in overweight patients with or without cardiovascular complications. *Clin. Biochem.* 49 (12), 862-867, 2016.  
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### List of other publications

3. Harangi, M., **Zsíros, N.**, Paragh, G.: Dyslipidaemiák kezelése: új lipidcsökkentő szerek. *Metabolizmus.* 14 (3), 160-163, 2016.
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10. Harangi, M., **Zsíros, N.**, Paragh, G.: A myeloperoxidáz szerepe a szív- és érrendszeri betegségek kialakulásában.  
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*Orv. Hetil.* 154 (3), 83-92, 2013.  
DOI: <http://dx.doi.org/10.1556/OH.2013.29530>

**Total IF of journals (all publications): 14,133**

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