

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

Determination of dysfunctional HDL and other cardiovascular risk factors in systemic lupus erythematosus

by Krisztina Gaál, MD

Supervisor: György Paragh, MD, PhD, DSc



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Supervisor: György Paragh, MD, PhD, DSc

Doctoral School of Health Sciences, University of Debrecen
(Programme of Metabolic and endocrine diseases)

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

Members of the Examination Committee: Márta Hőgye, MD, PhD
Sándor Szántó, MD, PhD

The Examination takes place at the Conference Room in Department of Preventive Medicine, Faculty of Medicine, University of Debrecen at 11:00 AM, November 28, 2017

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

Reviewers: Edit Bodolay, MD, PhD, DSc
Anikó Somogyi, MD, PhD, DSc

Members of the Defense Committee: Márta Hőgye, MD, PhD
Sándor Szántó, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1:00 PM, November 28, 2017

INTRODUCTION

Over the years cardiovascular diseases have been the leading cause of mortality in Hungary. They also took the infamous second place in the aggregated mortality data of European Union. The diseases falling most frequently in this group, such as heart attack and stroke, evolve on the ground of *atherosclerosis*. According to the newest theoretical approach a low grade inflammation existing permanently in the arterial wall is accountable for the development of atherosclerosis and over time results in more and more serious structural and functional aberrations. The process mainly begins under the effect of altered endogen substances triggering the response of the innate and acquired immune system that leads to the overturn of vascular homeostasis. Oxidized low density lipoprotein (oxLDL), accumulating different cytotoxic lipid oxidation products such as lipid hydroperoxides, -aldehydes and -carbonyls – has a prominent role among the modified endogenous substances that induce the immune response.

Lipoxygenase produced by endothelial and macrophage cells converts polyunsaturated fatty acids into lipid peroxides while myeloperoxidase produced by activated mononuclear cells produce tyrosyl radicals, hypochlorous acid, chloramine and nitrite. Both pathways play a role in the generation of oxidative agents, though the oxidation of lipid content of LDL can be induced by reactive free radicals (ROS) and catalytically active metal ions, too. Iron is present in the circulation in large quantities as a component of heme in erythrocytes. It is proven that heme can be easily released from methemoglobin intravascularly during erythrocyte lysis, and due to its hydrophobic character it can enter the LDL particle, where it induces heme oxygenase-1 (HO-1) and ferritin production. HO-1 releases iron, while ferritin can bind the free iron which otherwise would be a potent oxidant. Elevated levels of catalytically active iron and ferritin have been demonstrated in atherosclerotic plaques and their presence has been associated with cardiovascular disease.

Oxidized LDL exerts its effect on endothelial cells through lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and scavenger receptor CD36 enhancing the expression of LOX-1 and vascular adhesion molecules, and the release of monocyte chemoattractant protein-1 (MCP-1). Furthermore it decreases nitric oxide bioavailability via inhibition of endothelial nitric oxide synthase expression and elevation of ROS production in addition its role of provoking endothelial apoptosis. Chemoattractant and cell adhesion molecules induce the migration of circulating monocytes and T-lymphocytes into the intima layer of the arterial wall, where monocytes differentiate into macrophages under the influence of cytokines produced by the present lymphocytes. Macrophages and also vascular smooth muscle cells migrating here take up oxLDL through their scavenger receptors, which then induces several atherogen processes, among them production of inflammatory mediators by cells. The appearance of fatty streaks in the arterial wall is one of the early macroscopic morphological abnormalities of atherosclerosis. These are composed of foamy cells piled up in the intima layer and are macrophages full to overflowing with oxLDL.

Our body defend itself against LDL oxidation by the help of another lipoprotein, the *high-density lipoprotein (HDL)*. HDL is a lipoprotein with the smallest size and the largest density due to its high protein content (35-65%). It consists of an external single-layer hydrophilic sheath containing phospholipids, non-esterified cholesterols and proteins and of a hydrophobic core containing mainly cholesterol-esters and smaller amount of triglycerides. According to our recent knowledge HDL carries 80 proteins at least. Beside the structural proteins, it contains enzymes with antioxidant activity, participants in reverse cholesterol transport (RCT) and certain acute phase proteins as well. Its main structural protein, apolipoprotein A-I (ApoA-I) synthesized mainly in the liver and the bowels can be found on almost every HDL subpopulation. It is the base molecule of HDL synthesis and RCT and has antioxidant, antiinflammatory and anti-atherogenic effects. Among antioxidant enzymes of HDL the human paraoxonase 1 (PON1) is accountable for

the inhibition of LDL oxidation partly due to its quantity and partly its activity. The mechanisms of this are the transfer of oxidized lipids such as lipid hydroperoxides (LOOH) and lysophosphatidylcholine from oxLDL to HDL and the inactivation of LOOHs by hydrolysis. PON1 also hydrolyses other substrates including the also atherogenic homocysteine thiolactone and paraoxone of the organic phosphate metabolites. Both the macromolecular medium provided by intact HDL and the presence of ApoA-I are necessary for the enzyme function. Its concentration and activity varying with diverse substrates shows great individual variation. Its activity is mainly determined by the Q192R and L55M polymorphisms, but because of the significant variability of the genotype phenotype determination is more important in everyday practice.

HDL particles are not identical. They can be divided into subpopulations of different size, density, electrical charge, lipid and protein composition and functions, and transform into one another during the maturation of HDL. Their classification is also heterogeneous, depending on the method used for their separation. By the most commonly used density-gradient ultracentrifugation or gradient gel electrophoresis, in order of their size and density HDL fractions 2b>2a>3a>3b>3c can be separated. PON1 and the other antioxidant enzymes are not distributed equally on HDL subpopulations but primarily they can be found on dense HDL3 and within that on HDL3c particles.

It has long been established from population based studies that HDL cholesterol level is inversely related to the risk of coronary artery disease. However, cardiovascular risk of some families with very low HDL-C concentrations does not differ from that of general population since their HDL protects more effectively against oxidation of phospholipids. In contrast, in familial hyperalphalipoproteinemia increased HDL-C levels compensate for the reduced antioxidant activity of HDL subclasses, while in acute or chronic inflammatory diseases normal HDL-C levels often imply reduced antiatherogenic property, in other words *dysfunctional HDL*. In a study that examined patients with coronary artery

disease they found that despite lipid concentrations were in the normal range most HDLs did not protect LDL from oxidation, thus were proinflammatory (piHDL).

Free radicals, enzymes and lipid oxidation products secreted during inflammation oxidize not only the lipid content of LDL, but if their amount exceeds the threshold that can be counterbalanced by healthy HDL it can be oxidatively modified as well. By the oxidation of HDL lipids lipid hydroperoxides are formed, the cholesterol ester content of oxHDL becomes lower due to the reduced lecithin:cholesterol acyltransferase activity similarly to its phosphatidylcholine content, while the amount of lysophosphatidylcholines increases. All these processes reduce the fluidity of the lipid envelope and lead to the deterioration of cholesterol efflux capacity. The amount and activity of HDL's constitutional proteins in dysfunctional HDL is decreased. This is caused partly by their reduced synthesis, as in the course of inflammation the production of acute phase proteins becomes predominant, and partly by the suffered oxidative structural modification that leads to the impairment of their functions. Differing oxidant effects alter different parts of ApoA-I and ApoA-II and form cross-links between the molecules. In acute phase HDL, there can be found larger quantities of several proteins that are not present in native HDL or just occur in very small amounts. These proteins include serum amyloid A (SAA), ceruloplasmin, ApoC-III, hemoglobin and its binding proteins, haptoglobin and hemopexin which contribute to the reduction of HDL's antiatherogenic function partly due to their own proinflammatory effect and partly by the displacement of anti-inflammatory proteins from HDL.

In vitro experiments demonstrated that HDL and its lipid content are readily oxidized while immunohistochemical studies demonstrated the presence of oxidized HDL, ApoA-I and ApoA-II in the atherosclerotic arterial wall. The variable deficit of the antiatherogenic functions exerted by oxHDL has also been confirmed in animal models and in human patients. The presence of inflammatory milieu is of primary importance for dysfunctional HDL formation. Its presence has been demonstrated in chronic inflammatory diseases such as coronary artery disease, acute coronary

syndrome, type 2 diabetes, metabolic syndrome, chronic renal failure, rheumatoid arthritis, systemic sclerosis, Crohn's disease, obstructive sleep apnea and acute stroke. Changes in the concentration and function of HDL in distinct diseases do not necessarily affect the various HDL fractions proportionally. Obese women had significantly less large and more intermediate HDLs than their normal weight counterparts while there was no difference among groups in their small HDL levels. In patients with metabolic syndrome, anti-apoptotic activity of HDL3c on endothelial cells is reduced by 35% compared to that of healthy subjects due to the increased triglyceride and reduced cholesterol ester content of the particles. These observations emphasize the importance of functional testing in addition to quantification of HDL.

Among the many anti-atherogenic properties of HDL it is the antioxidant effect that is able to prevent LDL oxidation induced by inflammatory mediators, can neutralize already oxidized lipids and protect its own structural elements from oxidative modifications, thus inhibiting the initial stage of atherosclerosis. The methods used to investigate the antioxidant properties of HDL so far are diverse, as are the results obtained by them. The common feature of these tests is that they oxidize LDL by using a prooxidant, compare the results obtained during LDL oxidation in the presence and absence of HDL and the antioxidant effect of HDL is characterized by their difference. However, the type of method used (cellular or cell-free, end-point or kinetic), the actual LDL: HDL ratio, the method of HDL separation and the type and concentration of the used prooxidant can excessively influence the results obtained. Among the prooxidants used in previous studies, there are some which are not present in the subendothelial space (eg. AAPH-2,2'-azo-bis- (2-amidinopropane) dihydrochloride) while others are detectable though their role in atherosclerosis is not yet sufficiently clarified (eg. Cu^{2+}). Referring to the same types of study groups, differences in the tests simultaneously led to congruous and extremely divergent data. In the absence of standardization, it happened that the same test used by individual research teams delivered significantly different

measurement results and conclusions for their users. Although *Balla J et al* demonstrated in detail the role of free iron released from heme as an oxidant in the process of atherosclerosis, yet in experiments on HDL antioxidant activity the heme has not yet been used. Because of the above shortcomings, we felt necessary the development and standardization of a new method for HDL antioxidant capacity measurement, which is more representative of the in vivo oxidation conditions.

Functional impairment of HDL caused by inflammation is most likely to be present in pathological states with pronounced persistent systemic inflammation. This is the case in systemic autoimmune diseases, among which the most common is *systemic lupus erythematosus (SLE)*. In SLE, cardiovascular morbidity and mortality significantly - 6-9 times - exceeds that of general population. However, examined by age groups the hazard may be much higher; for the 35 to 44-year-old women the presence of the disease means 52-fold higher risk of myocardial infarction compared with age matched population. The process of atherosclerosis is accelerated in patients and that is indicated by their increased carotid intima-media thickness, presence of carotid plaques, endothelial dysfunction, and impaired flow-mediated dilation of the brachial artery. Although traditional cardiovascular risk factors are more frequent among SLE patients, they alone do not provide sufficient explanation for accelerated atherosclerosis, thus the role of disease-specific risk factors associated with inflammation also emerged in its development.

Inflammation is a key factor both in SLE and atherosclerosis. Autoimmune disorders, similarly to atherosclerosis, are characterized by a shift of Th1/Th2 lymphocyte equilibrium in Th1 direction accompanied by macrophage activation and by the release of inflammatory cytokines. In case of systemic inflammation, inflammatory mediators in the circulatory system may also provoke inflammation in the arterial wall, which may contribute to the development of accelerated atherosclerosis in patients, in accordance with the "low grade inflammation" theory of atherosclerosis. Among acute phase proteins, increased C-reactive protein (CRP), and SAA, furthermore higher erythrocyte sedimentation rate (ESR) values were

reported in SLE patients and also elevated MCP-1 and interleukin-6 (IL-6) concentrations, which correlated with the rate of coronary calcification. In SLE, SAA is a more sensitive marker of inflammation than CRP. Linked to HDL, it causes structural alteration with a concomitant functional deficiency, manifesting itself in reduced inhibition of MCP-1 production of vascular smooth muscle cells. Antioxidant protection by HDL is also decreased. In several studies, in addition to the total antioxidant capacity of serum the activity of PON1, main antioxidant enzyme of HDL, had also been found to be lower in SLE patients than in their healthy counterparts.

First, *McMahon M et al* studied the antioxidant properties of HDL in patients with **SLE and** determined the incidence of **piHDL**. According to their results HDL was pro-inflammatory in 45-48% of SLE patients and its presence was associated with the increased prevalence of coronary artery disease and subclinical atherosclerosis and with specific inflammatory markers. In the background of the changed antiatherogenic effect of HDL, alterations in proportions of its subpopulations and increased involvement of distinct fractions were also suggested. To date, however, only a few studies have investigated the distribution of HDL subclasses in SLE and the results obtained are controversial. *Hua X et al* found decreased amount of small HDL (sHDL) in SLE patients than in healthy subjects. On the other hand, in a group of 69 SLE patients sHDL particles had a positive correlation with carotid intima-media thickness and with complement levels. The above illustrates that although several studies have dealt with the background of accelerated atherosclerosis in SLE, the role of inflammation, of change in HDL quality, i.e. dysfunctional HDL and of possible subpopulation changes, has not yet been clarified in the process.

OBJECTIVES

1. Development of a method for the determination of HDL antioxidant capacity, which in contrast to previously published methods approximates better the in vivo HDL-LDL interaction.
2. Standardization of our method – HDL antioxidant capacity measurement during the hemin-induced LDL-oxidation – according to current laboratory guidelines.
3. Testing HDL antioxidant capacity in a group of healthy volunteers and also testing their HDL fractions. Comparison of the obtained data with previous results gained by other methods.
4. Determination of lipoprotein levels, HDL antioxidant capacity, HDL subpopulations, concentrations and activities of specific HDL associated proteins (ApoA-I, PON1, SAA) in the groups of SLE patients and their healthy controls in order to characterize corresponding HDL quality.
5. Characterization of systemic inflammation in patients with SLE via assessment of inflammatory markers (CRP, ESR, SAA, IL-6, MCP-1) and disease activity index (SLEDAI).
6. To analyse the relations of inflammatory markers and qualitative and quantitative HDL characteristics with special focus on HDL antioxidant capacity.

PATIENTS AND METHODS

Participants of methodological studies and of HDL antioxidant capacity studies of healthy subjects and their HDL subfractions

53 young healthy volunteers (19-44 years) - free of renal impairment, liver and cardiovascular disease or dislipidemia - took part in our study.

Selection of patients with SLE and their controls

51 patients with diagnosis of SLE (mean age 31.82 ± 6.40 years; 44 women and 7 men; mean disease duration 6.59 ± 5.26 years) and 49 healthy controls (mean age 31.8 ± 6.81 years; 41 women and 8 men) were enrolled in our investigations. Patients were attended at the Division of Clinical Immunology, Department of Internal Medicine, University of Debrecen. All patients fulfilled at least four of the revised American College of Rheumatology (ACR) classification criteria for SLE. Controls were recruited from volunteering health care professionals and local habitants. Subjects with renal failure ($GFR < 60$ ml/min/1.73 m²), diabetes mellitus or statin use were excluded due to their impact on the anti-inflammatory function of HDL. Disease activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score, its mean value in our patient population was 4.0 [2.0 – 6.0]. Patients and controls were matched in age, gender and smoking habits, since those affect lipid profile and HDL antioxidant enzyme activities.

The studies were approved by the Ethical Committee of University of Debrecen and ETT-TUKÉB, respectively and all participants gave written informed consent.

Sample collection and routine laboratory tests

Venous blood samples of study participants were collected after a 12-h fast. Routine laboratory parameters, including fasting blood glucose level, triglyceride (TG), total cholesterol (TC), LDL-C, HDL-C, ApoA-I, ApoB, high-sensitivity C-

reactive protein (hsCRP), ESR were determined from fresh sera by standard laboratory methods in the Department of Laboratory Medicine, University of Debrecen. Autoantibodies specific for SLE and immune complex concentrations were tested with commercially available enzyme-linked immunosorbent assay (ELISA) kits in the Regional Immunologic Laboratory.

Determination of inflammatory markers

In our SLE study, we tested IL-6, SAA, MCP-1 and oxLDL levels with commercially available ELISA assays.

Determination of HDL antioxidant capacity

LDL and HDL samples used in the test were isolated with density gradient ultracentrifugation. In every case 4 mL HDL was collected, but for the purpose of antioxidant capacity examination of HDL subfractions with different densities the HDLs were collected in 8 consecutive 0.5 mL fractions and was numbered in the order of collection (numbers increasing with density from 1 to 8). LDLs from different volunteers were pooled, and the same LDL compound was used in every test.

The inhibitory effect of HDL on LDL oxidation was assessed by measuring $\Delta T_{V_{max}}$ of hemin-catalyzed LDL oxidation in the absence and in the presence of HDL. $\Delta T_{V_{max}}$ represents the time necessary to reach the maximum reaction velocity in the propagation phase. The reaction mixture contained HEPES-buffer (6 mmol/L; pH 7.4; Sigma), hemin solution (5 $\mu\text{mol/L}$; Sigma), LDL-cholesterol (22 mg/dL), with or without HDL-cholesterol (2.2 mg/dL). A 1:10 HDL:LDL ratio was established as optimal for the examination of HDL fractions with highly different antioxidant activity. The mixture was incubated for 15 minutes at 37 °C, and the reaction was started with the addition of hydrogen peroxide (75 $\mu\text{mol/L}$; Sigma), which accelerates the reaction by splitting the hem ring and releasing redox-active iron. The given concentrations indicate final concentrations in a total volume of 200

μL . LDL oxidation was followed by measuring hemin degradation at 405 nm spectrophotometrically with a multimode microplate reader (Beckman Coulter DTX 880 Multimode Detector) in every 3 min for 5 hours. In the initial phase of the reaction the LDL barely oxidized, hence the amount of hemin is just slowly decreased, then in the propagation phase the intense oxidation resulted in considerable hemin degradation, accompanied by significant fall in absorbance. Finally, in the terminal phase there were no substantial changes in absorbance, because all the hemin was consumed. The time necessary to reach the maximum reaction velocity ($\Delta T_{V_{\max}}$) of hemin degradation was calculated by the Multimode Analysis Software. The HDL antioxidant capacity was expressed as the percentage ratio of $\Delta T_{V_{\max}}$ in the presence and absence of HDL. Using HDL samples of identical cholesterol concentrations, the inhibitory effect of HDL on LDL oxidation was termed specific HDL antioxidant capacity. The total HDL antioxidant capacity were calculated by multiplying specific HDL antioxidant capacity values with the HDL concentrations of study participants. We tested HDLs of 41, and HDL fractions of another 8 healthy volunteers, while samples of 51 SLE patients and 49 controls were examined in the second study.

Optimization and standardization of HDL antioxidant capacity measurement

In order to optimize the assay we assessed the impact of various hemin (3–9 $\mu\text{mol/L}$) and hydrogen peroxide (50-125 $\mu\text{mol/L}$) concentration on $\Delta T_{V_{\max}}$ and on HDL antioxidant capacity. The precision of our method was determined according to the EP5-A2 guideline of Clinical Laboratory Standards Institute (CLSI, Wayne, PA). Two HDL samples were tested in duplicates for twenty days. The linearity of our results was studied on three fractions of an HDL sample using seven different concentrations of each fraction. For comparison of the methods studies we used the method of measuring conjugated diene formation, which is the most frequently adopted one in the literature to follow LDL oxidation hence we considered it as “gold standard”. In the course of hemin-induced LDL oxidation 30 samples were tested

with simultaneous detection of hemin degradation and conjugated diene formation at 405 nm and at 234 nm, respectively. $\Delta T_{V_{max}}$ of both reaction was calculated by the Multimode Analysis Software. HDL antioxidant capacity by both method was expressed as the percentage ratio of $\Delta T_{V_{max}}$ in the presence and absence of HDL.

HDL subfraction analysis of SLE patients and controls

HDL subfractions were separated from human serum on the basis of their size by an electrophoretic method on polyacrylamide gel with preloaded tubes (Lipoprint System, Quantimetrix Corp., CA, USA). After the electrophoresis, distributions of HDL subfractions of samples dyed with Sudan Black were analyzed with Lipoware software (Quantimetrix Corp.). The 10 differentiated HDL subfractions were grouped into three major classes: large (from HDL1 to HDL3), intermediate (from HDL4 to HDL7) and small (HDL8 to HDL10) HDL subfractions. Cholesterol concentrations of the HDL particle subsets were calculated multiplying the total cholesterol concentration of the samples by the relative area under the curve (AUC) of the subfraction bands.

Determination of human paraoxonase-1 (PON1) enzyme activities and phenotype distribution

PON1 paraoxonase activity was analyzed by a kinetic, spectrophotometric method monitoring its hydrolysing ability of paraoxon (O,O-diethyl-O-p-nitrophenyl-phosphate, Sigma) as a substrate, while PON1 arylesterase activity was assayed with a phenylacetate substrate. Enzyme activity was expressed as U/L, and 1 unit activity was equal to 1 μmol substrate hydrolysed per minute. The dual substrate method was used to determine the Q192R phenotypic distribution of PON1. Subjects were categorised by the ratio of salt-stimulated paraoxonase (paraoxonase activity in the presence of 1 mol sodium chloride) to the hydrolysis of phenyl acetate into three potential group of phenotypes: ratio <3.0 for AA – low

activity, ratio between 3.0 and 7 for AB – intermediate activity, and ratio >7.0 for BB – high activity phenotype.

Statistical analysis

The dichotomous variables were analyzed by chi-squared test or Fisher's exact test. The normality of continuous variables was assessed by the Kolmogorov-Smirnov test. Normally distributed data were expressed as mean (\pm SD), while data not normally distributed were expressed as median (interquartile range [IQR]). Continuous variables were compared by Student's t-test or Mann-Whitney U test, as appropriate. Associations between continuous variables were assessed by Pearson's or Spearman's correlation coefficient, depending on the normality of data. The level of significance was set at $P < 0.05$. Linearity of HDL antioxidant capacity measurement was analyzed by linear regression analysis, the effect of hemin and hydrogen peroxide concentration on HDL antioxidant capacity was calculated with multiple regression analysis. Additionally, multiple regression analysis (backward-stepwise method) was performed to determine variables best predicted the total HDL antioxidant activity. Comparison of methods was performed with Deming and Passing-Bablok regression by MedCalc Software – Version 12.2.1. (MedCalc Software). With the previous exception, analyses were performed by Statistica – Version 8 computer software (StatSoft Inc.).

RESULTS

Optimization and standardization of HDL antioxidant capacity measurement

During optimization of our method both increasing hemin and hydrogen peroxide concentration in the reaction mixture equally shortened $\Delta T_{v_{max}}$ of the reaction, but only hemin reduced the HDL antioxidant capacity values ($r=-0.71$; $p<0.0001$), hydrogen peroxide did not affected them ($p=0.55$). Assessing the precision of our test the within-run CVs were 1.87% and 1.72%, respectively, while the within-laboratory CVs were 4.09% and 4.93 %. Investigating linearity, we found strong positive correlation between HDL concentration and antioxidant capacity values ($r\geq 0.98$, $p<0.0001$). The results gained by our method were compared to those obtained by measuring conjugated diene formation and no significant difference was found between them ($p=0.73$).

HDL fractions and antioxidant capacity of healthy subjects

All HDL fractions of healthy volunteers prolonged the time period until reaching maximum reaction velocity during LDL oxidation with 6.12%-94.12% ($p<0.5$). The antioxidant capacity of consecutive HDL fractions gradually increased, in parallel with their growing density (1. fraction: $115.26\pm 6.86\%$ → 8. fraction: $174.05\pm 13.38\%$). HDL antioxidant capacity of healthy volunteers were between 120% and 140.23%, their mean value was $127.67\pm 4.88\%$.

Traditional cardiovascular (CV) risk factors and inflammatory markers of SLE patients and healthy controls

There was no difference between groups in BMI, blood pressure, TC, LDL-C and Lp(a) values. Patients had significantly higher blood glucose ($p<0.001$), triglycerides ($p=0.005$) and ApoB ($p<0.05$), while lower HDL-C (1.26 ± 0.47 mmol/l vs. 1.70 ± 0.45 ; $p<0.001$) and ApoA-I (1.39 ± 0.35 g/l vs. 1.66 ± 0.41 ; $p<0.001$) concentrations than controls.

The levels of hsCRP ($p=0.002$), ESR ($p<0.001$), SAA ($p<0.001$), IL-6 ($p=0.002$) and MCP-1 ($p<0.001$) were equally significantly elevated in patients with SLE, but their oxLDL concentrations did not differ from controls⁷.

HDL antioxidant capacity, PON1 activity and phenotype distribution, and HDL subfraction distribution in patients with SLE and healthy controls

Examining antioxidant protection by HDL, SLE patients had highly and significantly decreased total HDL antioxidant capacity ($165.82\pm 58.28\%$ vs. 217.71 ± 54.36 ; $p<0.001$), and to a lesser extent increased specific HDL antioxidant capacity also reaching statistical significance ($132.42\pm 8.56\%$ vs. 127.67 ± 4.88 ; $p=0.002$) compared with their healthy counterparts.

SLE patients displayed significantly decreased PON1 arylesterase activity (125.65 ± 26.87 U/L vs. 148.35 ± 39.34 ; $p=0.001$), while their paraoxonase activity was similar to control's. To assess whether altered PON1 activity was due to the decreased HDL level found in SLE group, we standardized the enzyme activity for the HDL concentration (PON1/HDL ratio). We found that the standardized enzyme activities were significantly higher in the SLE patients compared to controls ($p<0.05$).

The PON1 Q192R AA phenotype, indicating the lowest PON1 enzyme activity was present in 94 % of the SLE patients and 87.23 % of controls. AB phenotype with intermediate enzyme activity was found in 6 % of the patients vs. 12.77 % of the controls. BB phenotype, the indicator of the highest PON1 enzyme activity, was not present in either of the study groups. Allele B of PON1 associated with higher enzyme activity was twice as frequent in healthy subjects as in SLE patients (6.38 % vs. 3 %).

Compared to the controls, HDL subfraction analysis showed uniformly lower amount of each HDL fraction in SLE patients ($p\leq 0.01$). However, there was no significant difference in the distribution of the HDL subfractions between the two groups, small HDL/large HDL ratio tended to be significantly decreased in patients.

Correlations between markers of inflammation and oxidative stress in patients with SLE and healthy controls

Both HDL-C and total HDL antioxidant capacity correlated positively with PON1 arylesterase ($r=0.38$ and $r=0.43$; $p<0.01$) and paraoxonase activity in SLE patients, but not in controls. In the patient group, specific HDL antioxidant capacity showed positive correlation with small HDL/large HDL ratio ($r=0.38$, $p<0.01$), while PON1 arylesterase activity associated positively with concentrations of all HDL subpopulations, and it was most pronounced in the case of small HDLs ($r=0.40$, $p<0.01$). In the SLE patients, PON1 paraoxonase activity correlated positively with the proportion of large HDL % ($r=0.35$, $p=0.01$) while negatively with the proportion of intermediate HDL ($r=-0.38$, $p<0.01$).

Not only HDL-C but total HDL antioxidant capacity showed significant negative correlations with the inflammatory markers, including ESR ($r=-0.42$; $p<0.01$), hsCRP ($r=-0.35$; $p<0.01$), IL-6 ($r=-0.50$; $p<0.0001$), MCP-1 ($r=-0.41$; $p<0.0001$), and SAA ($r=-0.28$; $p<0.01$). Though total HDL antioxidant capacity showed a significant inverse relationship with immune complex level ($r=-0.47$, $p<0.001$), we could not find significant correlations between disease activity index and ApoA-I, HDL-C or total HDL antioxidant capacity.

Disease activity was in a direct relationship with oxLDL concentration ($p=0.01$). In the patients oxLDL levels correlated positively with SAA ($p<0.05$) and fibrinogen concentrations, ESR, hsCRP and ApoB levels. Furthermore, oxLDL level was related with decreased large HDL ratio ($p=0.06$) and with increased intermediate HDL ratio ($p<0.01$), respectively.

To test whether the associations detected in the univariate analyses were independent of other laboratory parameters, we carried out a multiple regression analysis with total HDL antioxidant activity as the dependent variable. The model included IL-6, hsCRP, MCP-1, SAA, ESR and PON1 arylesterase activity. Total HDL antioxidant activity turned out to be best predicted by the PON1 arylesterase activity ($p<0.05$, $\beta=0.348$) and ESR ($p<0.01$, $\beta=0.61$).

DISCUSSION

For the examination of HDL antioxidant capacity, we needed a method suitable for testing a large number of HDL samples. We developed a cell-free assay and chose a kinetic method to follow the process of LDL oxidation step by step. Hemin has been chosen as a prooxidant since its role has already been proven in the course of LDL oxidation, along with the role of the catalytically active iron released from hem during erythrocyte lysis. Hydrogen peroxide was used to split the hem ring and to release the iron. It is also synthesized by the NADPH-dependent oxidase of phagocytic cells and thus found in the inflammatory milieu of arterial walls. During the reaction the amount of hem was monitored, because its absorbance can be followed in the visible light spectrum at 405 nm. Investigated HDLs were isolated by density gradient ultracentrifugation in order to ensure sample purity.

It was found during the method optimization, that increasing the hydrogen peroxide concentration, while reducing the time required to reach maximum reaction velocity, did not have a significant effect on HDL antioxidant capacity. Thus, its concentration can be modified as needed in the reaction mixture without affecting the final results. This allows us to study HDL fractions with strong antioxidant properties. In contrast, increasing hem concentration not only reduced $\Delta T_{V_{max}}$, but also significantly reduced the values of HDL antioxidant capacity, so its amount can not be modified in the reaction mixture.

During the standardization of our method, as required by the guideline, we proved its reproducibility, its linearity and comparability with the conjugated diene test considered as reference method. As far as we know our test is the first standardized method to determine the antioxidant capacity of HDL, which on account of the use of physiological prooxidant heme approximates better the *in vivo* LDL-HDL interaction.

Investigating the antioxidant capacity of HDL fractions of healthy volunteers by our method, the subfractions showed increasing values in parallel with

their density. Our findings are in accordance with the results of *Kontush et al*, who detected the prolongation of propagation phase by the different HDL subpopulations elevating with their density in the order of HDL2b<HDL2a<HDL3a<HDL3b<HDL3c during LDL oxidation. The studied subpopulations lengthened the time necessary for LDL oxidation by 15% -99% while in our examination by 6% -94%.

HDL antioxidant capacity of healthy volunteers, measured on average 127.67% also compares to the literature data. In a healthy control group HDL extended the propagation phase by 20% during LDL oxidation, while in two investigations by fluorescent assay the HDL decreased the resulting fluorescent signal by 30%.

In our second study, we focused on traditional and disease-specific cardiovascular risk factors that are involved in the development of accelerated atherosclerosis in SLE. There was no difference between patient and control groups in BMI and blood pressure, so obesity and hypertension have not yet emerged among cardiovascular risk factors in our young SLE patients. In the background of the patients' higher blood glucose levels side effect of steroid therapy was presumed. The results of previous studies suggest that metabolic syndrome and its components, including obesity, hypertension and dyslipidemia, are more frequent in patients with SLE than healthy subjects. However, the studied populations were older (38-50 years) with longer disease duration and higher cumulative dose of steroids, and simultaneously with their more frequent adverse effects (obesity, insulin resistance, hypertension). Calculated by SLEDAI score, disease activity of our patients was relatively high. Accordingly, their lipoprotein profile with increased TG and reduced HDL-C levels showed a lupus pattern, mainly characteristic of active disease, while their total cholesterol, LDL-C and Lp (a) levels did not differ from those of controls. The higher triglyceride levels observed in SLE are attributable to the increased VLDL and chylomicron concentrations as a consequence of decreased chylomicron lipolysis and the slower removal of chylomicron remnant particles caused by the diminished triglyceride splitting activity of lipoprotein lipase as it had been

previously demonstrated.

The low level of HDL-C is an independent cardiovascular risk factor. In SLE, the decline in HDL-C concentration is primarily attributed to inflammation and that is confirmed by the fact that not only the symptoms of the disease improved but also the HDL-C level increased by the effective anti-inflammatory therapy, virtually it become normalized. All the examined inflammatory markers (ESR, CRP, IL-6, SAA, MCP-1, IC) of our patients were significantly elevated and showed strong significant negative correlation with the HDL-C concentration. The amount of ApoA-I was also lower in subjects with SLE, suggesting that low HDL-C levels are partly due to diminished ApoA-I concentration caused by inflammation. For this reason the ABCA1-dependent cholesterol efflux from peripheral cells drops and the amount of cholesterol delivered by HDL also decreases. If the esterification of free cholesterol by lecithin-choline acyltransferase in the presence of its activator ApoA-I is disturbed, that can be a further source of decreased HDL-C level and results in smaller HDL size alongside with less effective RCT.

The complex evaluation of HDL quality was performed by determining ApoA-I and SAA concentration, PON1 paraoxonase and arylesterase activity, HDL antioxidant capacity, and HDL subpopulations. In our SLE patients, the level of ApoA-I, major structural protein of HDL, was lower than in the controls, while the level of SAA, characteristic component of acute phase HDL (A-HDL), was much higher. Based on previous studies SAA concentration depends on the pro-inflammatory stimuli and on participants of the cytokine activation cascade, the main inducers of its production being IL-1, TNF- α and IL-6. In our case strong correlation was observed between IL-6 and SAA, so it is likely that IL-6 has a role in triggering SAA production. SAA enhances the activity of secretory phospholipase A2, while A-SAA-HDL is 2-3 times more susceptible to hydrolysis, and thus HDL-C level reduced by up to 30%. Similarly to our SLE population, patients with STEMI had elevated CRP, IL-6 and SAA levels, and their SAA concentration correlated with the decreased antioxidant and cholesterol efflux capacity of their HDL. In

prospective studies of healthy populations, the circulating immune complex (IC) level was directly related to the development of acute myocardial infarction thus proved to be a risk factor for atherosclerosis. We found a strong negative correlation between the IC concentration of SLE patients and the HDL total antioxidant capacity, indicating the role of IC as a disease-specific cardiovascular risk factor.

Examining HDL antioxidant capacity with our new standardized method, all HDL samples protected the LDL from oxidation, piHDL was not detected. Surprisingly, though the total HDL antioxidant capacity of the patients was significantly reduced, their specific HDL antioxidant capacity was found to be slightly but significantly elevated. Our results may indicate the altered RCT of patients, resulting in decreased HDL cholesterol levels, and thus increased protein/cholesterol ratio of their particles. Their higher ApoA-I/HDL-C ratio also supports this theory. Likewise, PON1 arylesterase activity was much lower in the patients' serum, but referring to HDL-C per unit it exceeded the values of controls. The modestly increased enzyme concentration in the reaction mixture was associated with higher antioxidant activity, however, due to the low HDL-C concentration it did not mean greater antioxidant protection for the body. Although there was no difference in the distribution of PON1 phenotypes among our study groups, the allele B with higher activity- similarly to previous results of our research group - was less common among patients than controls. The decreased PON1 paraoxonase activity of patients with SLE was previously detected by our team, but the arylesterase activity and CRP level of the slightly older, overweight patients with inactive disease did not differ from their healthy counterparts, and their lipid profile did not show the lupus pattern, instead they had increased LDL-C levels. Their paraoxonase activity did not correlate with other parameters (SLEDAI, α -PL, α -oxLDL, CRP) other than the occurrence of atherothrombotic events. In our present younger patients with activity symptoms the opposite was found, but in both studies CRP correlated negatively with PON1 arylesterase activity, which supports the deleterious effect of inflammation on HDL antioxidant function. Nevertheless, differences draw our

attention to the fact that the role of risk factors of atherosclerosis varies depending on disease activity during the course of the disease: traditional risk factors are dominating in inactive disease period, while disease-specific factors, connected with inflammation, in the active period.

McMahon et al investigated in several studies the occurrence of piHDL and the antioxidant activity of HDL in groups of SLE patients. The incidence of piHDL in SLE patients was 29%, 45% and 48.2% (plaque+: 86.7%, plaque-: 40.7%), respectively. The value of HDL inflammatory index was 0.68 in healthy subjects and 1.02 in SLE patients. Apart from ESR and oxLDL, piHDL was not associated with values of other inflammatory markers, nor with SLEDAI, ApoA-I and PON1 activity. Compared to the above, in SLE we also found a decrease in HDL antioxidant capacity but it did not reach the level of pro-oxidant activity, however, correlated positively with PON1 activity and negatively with level of inflammatory markers and disease activity. The source of the discrepancy of the results may be the different test method and also the dissimilar test population: *McMahon et al* measured the antioxidant capacity of the ApoB depleted plasma, their patients were older (40-55 years), with lower disease activity and their lipoprotein concentrations were similar to controls'. In our study, we first demonstrated a complex inverse relationship between inflammatory markers and HDL antioxidant capacity or PON1 activity, respectively, in a group of young SLE patients with activity symptoms.

The correlation of oxLDL concentration with the presence and severity of coronary artery disease which is independent of traditional risk factors is already known. We did not find significant difference between the oxLDL levels of our study groups, which suggests that the HDLs of our patients still provide some degree of protection against LDL oxidation. Still, the relationship of inflammatory markers (SAA, ESR, CRP) and SLEDAI with the amount of oxLDL shows that the patients with active disease are exposed to a higher oxidative load.

In our SLE group we found a proportional statistically significant decrease in the amount of all HDL subpopulations which correlated with the lower total HDL

antioxidant capacity and arylesterase activity. The arylesterase activity was most closely related to small HDL ratio, which shows well the primary localization of PON1 within the HDL subfractions. The association of specific HDL antioxidant capacity with the small HDL/large HDL ratio also directs to this, emphasizing the role of small HDL fractions in the protection against LDL oxidation. We demonstrated for the first time a direct relationship between oxLDL and the ratios of decreased large and elevated intermedier HDL and simultaneously an inverse relationship between PON1 activity and the previously mentioned HDL subpopulations in SLE patients. The correlation of oxLDL with the above subpopulations refers to less effective RCT of patients in which may have a role the declining antioxidant protection due to decreased paraoxonase activity caused by inflammation. Although we did not detect piHDL in our investigations, total HDL antioxidant activity of lupus patients was significantly lower and its value was best predicted by PON1 arylesterase activity and the value of ESR, while closely correlated with other inflammatory parameters. Our observations emphasize the importance of PON1 activity and inflammation in the alterations of HDL antioxidant status of SLE patients. This implies, that in SLE the considerable cardiovascular risk accompany inflammation, primarily affecting young patients with active disease. In addition to investigating traditional lipoprotein classes, new biomarkers such as HDL antioxidant capacity, HDL subpopulations and PON1 activity, can provide important supplementary information on the cardiovascular risk of SLE patients and may also play a role in monitoring the effectiveness of anti-inflammatory treatment.

SUMMARY

There are several non-standardized tests for the measurement of HDL antioxidant property, but because of alterations of reactions the results gained by their use are diverse, sometimes contradictory. The method, we developed for testing HDL antioxidant capacity during the hem-induced LDL oxidation is suitable for evaluation of a number of HDL samples with highly different antioxidant activity, healthy and dysfunctional HDLs and their subpopulations included. With the use of hemin, a physiological pro-oxidant, the test conditions approximate better in vivo reactions than previous techniques, and since our method is standardized the results are reproducible.

Henceforth we studied the traditional and disease specific risk factors of increased cardiovascular mortality in patients with SLE. The centre of our interest was the relationship between inflammation and HDL antioxidant capacity and HDL subpopulations. In our young patients with SLE, presence of disease specific risk agents was predominant. Concentrations of all the examined disease markers and disease activity (SLEDAI) were elevated and were related with higher oxLDL level, decreased total HDL antioxidant capacity and HDL-C level, the latter affected equally all HDL subfractions. HDL antioxidant capacity and concentration of small dense HDL correlated positively with arylesterase activity of PON1, main antioxidant enzyme of HDL. Independent predictors of total-HDL antioxidant capacity were PON1 arylesterase activity and erythrocyte sedimentation rate. Our results suggest that systemic inflammation present in SLE not just decrease the amount of HDL, but damages its antioxidant function, impairing its anti-atherogenic effect, thus contribute to the elevated cardiovascular risk of patients. Therefore HDL antioxidant capacity, subpopulation distribution and PON1 activity can serve as new biomarkers of more specified cardiovascular risk estimation in patients with SLE.

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