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Association between moderately oxidized low-density lipoprotein and high-density lipoprotein particle subclass distribution in hemodialyzed and post-renal transplant patients*

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Abstract: Disturbances in the metabolism of lipoprotein profiles and oxidative stress in hemodialyzed (HD) and post-renal transplant (Tx) patients are proatherogenic, but elevated concentrations of plasma high-density lipoprotein (HDL) reduce the risk of cardiovascular disease. We investigated the concentrations of lipid, lipoprotein, HDL particle, oxidized low-density lipoprotein (ox-LDL) and anti-ox-LDL, and paraoxonase-1 (PON-1) activity in HD (n=33) and Tx (n=71) patients who were non-smokers without active inflammatory disease, liver disease, diabetes, or malignancy. HD patients had moderate hypertriglyceridemia, normocholesterolemia, low HDL-C, apolipoprotein A-I (apoA-I) and HDL particle concentrations as well as PON-1 activity, and increased ox-LDL and anti-ox-LDL levels. Tx patients had hypertriglyceridemia, hypercholesterolemia, moderately decreased HDL-C and HDL particle concentrations and PON-1 activity, and moderately increased ox-LDL and anti-ox-LDL levels as compared to the reference, but ox-LDL and anti-ox-LDL levels and PON-1 activity were more disturbed in HD patients. However, in both patient groups, lipid and lipoprotein ratios (total cholesterol (TC)/HDL-C, LDL-C/HDL-C, triglyceride (TG)/HDL-C, HDL-C/non-HDL-C, apoA-I/apoB, HDL-C/apoA-I, TG/HDL) were atherogenic. The Spearman's rank coefficient test showed that the concentration of ox-LDL correlated positively with HDL particle level (R=0.363, P=0.004), and negatively with TC (R=−0.306, P=0.012), LDL-C (R=−0.283, P=0.020), and non-HDL-C (R=−0.263, P=0.030) levels in Tx patients. Multiple stepwise forward regression analysis in Tx patients demonstrated that ox-LDL concentration, as an independent variable, was associated significantly positively with HDL particle level. The results indicated that ox-LDL and decreased PON-1 activity in Tx patients may give rise to more mildly-oxidized HDLs, which are less stable, easily undergo metabolic remodeling, generate a greater number of smaller pre-β-HDL particles, and thus accelerate reverse cholesterol transport, which may be beneficial for Tx patients. Further studies are necessary to confirm this.

Key words: Lipids, Lipoproteins, Paraoxonase-1 (PON-1) activity, Oxidized low-density lipoprotein (ox-LDL), High-density lipoprotein (HDL) particles, Post-renal transplant, Hemodialysis

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1 Introduction

Patients with chronic renal failure on hemodialysis and post-renal transplantation are exposed to several risk factors for atherosclerosis, such as oxidative stress, dyslipidemia, and endothelial dysfunction (Moradi *et al*., 2009). Classic cardiovascular risk factors interact with adverse renal transplant features, such as immunosuppression. In the face of hyperlipidemia, the impact of an episode of acute rejection is more severe than in transplant recipients with normal lipid profiles (Moreno *et al*., 2005). In renal transplantation there is an imbalance between the reactive oxygen species (ROS) and antioxidants, which results

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in oxidative stress (Moreno *et al*., 2005). The studies suggest that paraoxonase (PON) prevents low-density lipoprotein (LDL) oxidation by hydrolyzing lipid peroxides (Draganov *et al*., 2005). Oxidized LDL (ox-LDL), together with risk factors, damages the endothelium of the arterial wall and impairs lipoprotein metabolism in hemodialyzed (HD) and post-renal transplant (Tx) patients, as well as characteristic abnormalities such as hypertriglyceridemia and decreased high-density lipoprotein (HDL) cholesterol (Chen *et al*., 2005; Kimak *et al*., 2010). HDL possesses antioxidant, anti-inflammatory, antithrombotic and endovascular properties. HDL inhibits LDL oxidation and interferes with a process that initiates atherosclerosis. Hydrolysis of oxidized lipids by HDL contributes to an overall anti-inflammatory effect as ox-LDL molecules are pro-inflammatory (Joy and Hegele, 2008). HDL particles are highly heterogeneous in their size, structure, metabolism, and biological function (Joy and Hegele, 2008). Ultracentrifugation subdivides HDL into HDL_2 and HDL_3 subclasses according to density. HDL_2 consists of larger, less dense cholesterol ester-enriched particles compared with the smaller, dense HDL_3 particles. HDL_2 seems to be more atheroprotective than HDL_3 (Joy and Hegele, 2008). The α-HDL subfractions, which are defined by electrophoretic mobility, can be subdivided into four categories: α-1 (large, spherical) to α-4 (small, discoidal) HDLs. Subfractions α-1 and α-4 HDLs contain apolipoprotein A-I (apoA-I) without apoA-II but α-2 and α-3 contain apoA-I and apoA-II. Three other HDL subfractions which are defined by electrophoretic mobility are pre-α, pre-β-1 (small, discoidal HDL) and pre-β-2 (large HDL), which all contain apoA-I but not apoA-II (Joy and Hegele, 2008). However, methodologies for HDL subclassification testing are not generally available at present.

The aim of the present study was to investigate the concentrations of lipids, lipoproteins, leptin, ox-LDL, anti-ox-LDL antibodies, and HDL particles, lipid and lipoprotein ratios, and PON-1 activity in HD and Tx patients in comparison to healthy subjects. This analysis may inform about the association between ox-LDL and HDL particle concentrations and subclass distributions in HD and Tx patients with chronic renal failure (CRF), and provides a better understanding of diagnostic and therapeutic methods used to decrease mortality rates.

2 Materials and methods

2.1 Participants

Serum levels of lipid, lipoprotein, leptin, ox-LDL, anti-ox-LDL antibody and HDL particles and PON-1 activity were determined in 33 HD and 71 Tx patients and 89 healthy individuals as reference group. We investigated HD and Tx patients who were non-smokers without active inflammatory disease, liver disease, malignancy, or hypertension. They received vitamins, phosphate binders, calcitriol, and erythropoietin (EPO) (n=23). The patients had hemodialysis for 3.5–4.0 h three times a week. The dialyses were performed using low flux polysulfone membranes (Fresenius F-6, 40 μm thick of 1.3 m^2 surface; F6 polysulfone UF 5.5, Fresenius, St. Wendel GmbH, Frankfurt, Germany).

The Tx patients received prednisone and cyclosporine A (CsA), or prednisone and prograf. Tx patients without statin therapy had moderate hypercholesterolemia and hypertriglyceridemia. Tx patients with hypercholesterolemia and hypertriglyceridemia received atorvastatin or simvastatin. Hypertension occurred in approximately 50% patients. Hypertensive patients were using anti-hypertensive medications of either calcium channel blockers or angiotensin converting enzyme antagonists, angiotensin II receptor subtype-1 (AT1) blockers and α-blockers, but no diuretics were in each studied group. No cardiac incidence occurred in Tx patients. The control group consisted of 89 subjects chosen from among apparently normolipidemic people as described previously (Kimak *et al.*, 2010). The study was carried out in accordance with the guidelines of the Ethics Committee of the Medical University of Lublin, Poland.

2.2 Detections of lipid, lipoprotein, and HDL particle concentrations

Lipids, lipoproteins, and routine laboratory parameters were obtained in serum after a 14-h overnight fast. Blood was taken from veins into commercial tubes. Serum was immediately separated and stored in aliquots at −80 °C until use. Routine laboratory parameters (the levels of urea, uric acid, creatinine, total protein, and albumin) and lipids and lipoproteins (apoA, apoB) were determined by use of the Hitachi 902 analyzer, and hemoglobin was determined by

using ADVIA analyzer, as described previously (Kimak *et al*., 2010). ApoA-I and apoB were measured using the turbidimetric methods (Roche kits). The measurement of HDL particle concentration was made by using the enzyme-linked immunosorbent assay (ELISA) method in our laboratory. The method was based on specific direct immunological reaction between purified chicken anti-human HDL antibodies and human HDL (GenWay Biotech Inc., USA), as described previously (Kimak *et al*., 2010).

2.3 Detections of leptin, ox-LDL and anti-ox-LDL antibody concentrations and PON-1 activity

Serum leptin was measured by ELISA kit (DRG Instruments GmbH, DRG International Inc., Marburg, Germany). Quantitative determinations of human ox-LDL and IgG autoantibody against ox-LDL in serum were made by using ELISA kits (Biomedica GmbH, Wienna, Austria). PON-1 activity was determined using 1.2 mmol/L paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphate; Sigma Chemical, St. Louis, MO, USA) as the substrate. The PON-1 activity was measured by the modified Furlong *et al*. (1989)'s method from the initial velocity of *p*-nitrophenol production at 37 °C, and its increased absorbance at 405 nm was recorded by an autoanalyzer (Cobas-Mira Plus, Roche Diagnostics, Switzerland). Serum was added to basal assay mixture containing 100 mmol/L Tris-HCl buffer (pH 8.5) with paraoxon and 1 mmol/L $CaCl_2$. The PON-1 activity of 1 U/L was defined as 1 μmol of *p*-nitrophenol hydrolyzed per minute.

2.4 Statistical analysis

The data were expressed as medians and minimum to maximum. A statistical analysis of our results was performed using the non-parametric Kurskal-Wallis test for comparison of HD vs. Tx patients and to the reference group.

The relations between concentration of ox-LDL and clinical parameters, lipid, lipoprotein and HDL particle levels, lipid and lipoprotein ratios, and PON-1 activity were examined by Spearman's correlation analysis. The variables with skewed distribution were log10 transformed. However, the values in Tables 1 and 2 are presented as non-transformed data. Multivariance regression analysis was used to investigate the relationships between the concentration of ox-LDL and lipid, lipoprotein, HDL particle and anti-ox-LDL-antibody levels, and PON-1 activity. In the model of multiple stepwise forward regression analysis, ox-LDL was selected as the independent variable, and for each dependent variable, parameters were calculated according to the equation: $y=\beta_0+\beta_1x_1+\beta_2x_2+\ldots+\beta_nx_n$ (Stanisz, 2000). The relationship between the independent and dependent variables is expressed by the coefficient of multiple regression (β), which gives information about the relationship between the independent ox-LDL and the dependent variables. After adjustment for age and gender, multiple stepwise forward regression analyses were performed in HD and Tx patients with CRF. Dependent variables which were highly correlated to apoA-I and apoB were removed from the model until the best-fitting model with the maximum adjusted multiple R^2 was achieved.

The statistical significance of all variables was established at $P<0.05$, and statistical analysis was performed using the STATISTICA program (StatSoft, Krakow, Poland).

3 Results

3.1 Basic information of subjects

Table 1 presents the results of the clinical and laboratory parameters of HD and Tx patients and the reference group.

3.2 Concentrations of serum lipid, lipoprotein, HDL particle, ox-LDL and anti-ox-LDL antibody, PON-1 activity, and lipid and lipoprotein ratios

Table 2 shows that HD patients had moderate hypertriglyceridemia and normocholesterolemia, and low HDL-C, apoA-I and HDL particle concentrations and PON-1 activity, and increased ox-LDL and anti-ox-LDL levels in comparison to the reference group. Tx patients had hypertriglyceridemia and hypercholesterolemia and moderately decreased HDL-C and HDL particle concentrations and PON-1 activity, and moderately increased ox-LDL and anti-ox-LDL levels, as compared to the reference group. However, ox-LDL and anti-ox-LDL levels and PON-1 activity were more disturbed in HD patients. In both HD and Tx patients lipid and lipoprotein ratios (total cholesterol (TC)/HDL-C, LDL-C/HDL-C, triglyceride (TG)/

HDL-C, HDL-C/non-HDL-C, apoA-I/apoB, HDL-C/apoA-I, and TG/HDL) were atherogenic.

3.3 Relationships between concentration of ox-LDL and lipid, lipoprotein, anti-ox-LDL antibody and HDL particle levels, and PON-1 activity in HD and Tx patients

Correlation between variables was calculated by non-parametric Spearman's rank coefficient test. In Tx patients, the concentration of ox-LDL was positively correlated with HDL particle level (R=0.363, P=0.004) and negatively correlated with TC (R=−0.306, P=0.012), LDL-C (R=−0.283, P=0.020), and non-HDL-C (R=−0.263, P=0.030) levels.

Multiple stepwise forward regression analyses in Tx patients demonstrated that ox-LDL concentration, as an independent variable, was associated positively with HDL particle level (R^2=0.328, β=0.359, P=0.0009). HD patients did not show significant correlation between ox-LDL and other parameters.

Table 1 Clinical and routine laboratory parameters of HD and Tx patients and the reference group

Parameters	Value[a]		
	HD patients n=33	Tx patients with dyslipidemia n=71	Reference group[b] n=89
Age (year)	55 (30–78)	45 (19–72)	46 (22–69)
Sex	17 M, 16 F	36 M, 35 F	44 M, 45 F
BMI (kg/m^2)	22 (19–30)	25 (19–30)*	21.5 (18.5–25.3)
Leptin (ng/ml)	15.0 (4.5–157.0)*	23.8 (4.5–169.0)*	10.0 (2.9–14.5)
Urea (mmol/L)	22.80 (10.30–36.30)***	7.69 (3.27–22.20)**	4.40 (2.54–6.42)
Creatinine (mmol/L)	822 (592–1281)***	129 (70–315)**	75 (62–102)
Total protein (g/L)	68 (52–81)***	72 (59–85)	73 (71–78)
Albumin (g/L)	38 (31–43)***	42 (32–49)	45 (42–48)
Hemoglobin (mmol/L)	7.19 (4.51–8.39)***	8.70 (6.45–10.32)	9.00 (8.38–10.12)

[a] Values are expressed as median (min–max); [b] From Kimak *et al*. (2010). HD patients: hemodialyzed patients; Tx patients: post-renal transplant patients; BMI: body mass index; M: male; F: female. * P<0.05, ** P<0.01, *** P<0.001, vs. the reference group

Table 2 Lipid, lipoprotein, ox-LDL, anti-ox-LDL antibody and HDL particle concentrations, lipid and lipoprotein ratios, and PON-1 activity in HD and Tx patients and the reference group

Parameters	Value[a]		
	HD patients n=33	Tx patients with dyslipidemia n=71	Reference group[b] n=89
TG (mmol/L)	1.33 (0.69–3.03)***	1.79 (0.80–3.67)***†	0.93 (0.40–1.70)
TC (mmol/L)	4.51 (2.85–6.94)	5.82 (4.11–10.30)*†	4.58 (3.14–5.31)
LDL-C (mmol/L)	2.82 (1.32–4.92)	3.65 (2.56–7.36)*†	2.69 (1.32–4.22)
HDL-C (mmol/L)	0.90 (0.62–1.32)***	1.25 (0.88–2.12)*†	1.48 (1.24–2.12)
Non-HDL-C (mmol/L)	3.57 (1.94–6.06)*	4.48 (2.54–8.49)**†	3.13 (1.81–3.50)
HDL (mg/L)	13.9 (4.8–30.9)**	20.7 (13.8–33.8)	23.6 (18.5–38.1)
ApoA-I (mg/L)	1330 (880–1600)***	1590 (900–1980)†	1610 (1380–1860)
ApoB (mg/L)	750 (490–1740)	840 (560–1450)	740 (600–1180)
PON-1 activity (U/L)	78 (45–299)**	115 (52–491)*†	142 (81–569)
Ox-LDL (ng/ml)	253 (77–1050)**	189 (67–960)*†	153 (108–242)
OLAB (mU/ml)	462 (89–850)**	225 (40–850)*†	175 (45–350)
TC/HDL-C	4.76 (2.49–8.80)***	4.74 (2.17–9.08)***	2.96 (1.20–4.41)
LDL-C/HDL-C	3.07 (1.28–6.30)***	2.98 (1.28–6.32)***	1.81 (0.80–3.46)
TG/HDL-C	3.15 (1.13–9.60)***	3.13 (0.85–9.50)***	1.21 (0.52–2.61)
ApoA-I/apoB	1.65 (0.86–2.61)**	1.92 (0.99–3.20)	2.31 (1.56–2.57)
HDL-C/non-HDL-C	0.27 (0.13–0.56)***	0.28 (0.12–0.65)***	0.50 (0.44–0.78)
HDL-C/apoA-I	0.27 (0.19–0.42)***	0.29 (0.19–0.39)***	0.35 (0.29–0.37)
TG/HDL	84.40 (29.00–348.00)**	73.00 (35.00–188.00)**	36.00 (28.00–51.00)

[a] Values are expressed as median (min–max); [b] From Kimak *et al*. (2010). HD patients: hemodialyzed patients; Tx patients: post-renal transplant patients; TG: triglyceride; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; Apo: apolipoprotein; PON-1: paraoxonase-1; Ox-LDL: oxidized LDL; OLAB: anti-ox-LDL antibody. * P<0.05, ** P<0.01, *** P<0.001, vs. the reference group; † P<0.05, vs. HD patients

4 Discussion

Ox-LDL plays an important role in atherogenesis. International studies reported that HDL lipoprotein correlates inversely with the incidence of cardiovascular disease because HDL lipoproteins have anti-oxidative and anti-atherogenic properties. Moreover, the ability of HDL to protect against the oxidative modification of LDL has been demonstrated (Sviridov *et al*., 2008; Sakuma *et al*., 2010).

In the present study we investigated Tx patients with hypertriglyceridemia and hypercholesterolemia, and moderately decreased HDL-C and HDL particle concentrations and PON-1 activity, as well as moderately increased ox-LDL and anti-ox-LDL levels. These abnormalities, however, were more disturbed in HD patients, which is in agreement with other researches (Krishnaswamy *et al*., 2006; An *et al*., 2009; Samouilidou *et al*., 2010). Reductions of HDL-C and apoA-I concentrations and PON-1 activity, and an increase oxidative stress were linked to uremia which can contribute to increasing cardiovascular disease risk in HD patients (Rizzo *et al*., 2009; Prakash *et al*., 2010). It was demonstrated that ox-LDL can play an important role in progression of atherosclerosis in renal transplantation (Cofan *et al*., 2005). The authors reported that immunosuppressive therapy seems to be the main factor that influences the post-transplant lipidemic profile and oxidative stress in Tx patients. Conversion from cyclosporine to tacrolimus in stable renal transplant recipients resulted in a more favorable lipid profile and lower in vivo LDL oxidation, and might reduce anti-ox-LDL antibody titers in vivo (Cofan *et al*., 2005).

Previously we demonstrated that Tx patients have disturbed concentration, composition, and metabolism of TG-rich lipoprotein and HDL particles (Kimak *et al*., 2008; 2010). HDL particles transport lesser amounts of HDL-C but more TG as indicated by increased TG/HDL-C and TG/HDL particle ratios; HDL particles are hypercatabolized and removed from circulation, and therefore concentration of HDL particles in serum was decreased (Kimak *et al*., 2010). In the present study, Spearman's correlation test indicated that ox-LDL concentration was positively correlated with HDL particle levels, and negatively correlated with TC, LDL-C, and non-HDL-C in Tx patients with higher HDL-C, TC, and TG levels. Multiple stepwise forward regression analyses in Tx patients demonstrated that ox-LDL concentration, as independent variable, was associated positively correlated with HDL particle level.

These results indicate that ox-LDL and decreased PON-1 activity in Tx patients may give rise to more mildly-oxidized HDLs, which are less stable, more easily undergo metabolic remodeling, generate a greater number of smaller pre-β-HDL particles, and thus accelerate reverse cholesterol transport, which may be beneficial for Tx patients. Recent Pirillo *et al*. (2007) hypothesized that mild oxidative modification of HDL, which leads to the formation of pre-β-HDL migrating particles, might be considered an anti-atherogenic process enhancing reverse cholesterol transport, and thus impairing intracellular lipid deposition in peripheral cells. However, when oxidation proceeds further, HDLs lose their cholesterol effluxing properties. A low degree of oxidation is needed for steady balance of lipid metabolism. There were observations that oxidation of lipid-bound apoA-I significantly decreases HDL stability (Gao *et al*., 2008); destabilized apoA-I can be readily released from HDL, producing a pool of lipid-free/lipid-poor apoA-I that exhibits a pre-β electrophoretic mobility. An extensive HDL_3 oxidation leads to an increased particle size with a reduced ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux. In hypercholesterolemic subjects, the plasma level of pre-β-HDL is increased. In patients with low HDL cholesterol (hypertriglyceridemic patients), cholesterol efflux from J774 cells overexpressing ABCA1 significantly increases, when compared to normolipidemic controls (Pirillo *et al*., 2007).

Gao *et al*. (2008) reported that oxidation may alter HDL functions not only by chemically-modifying proteins and lipids but also by altering the rate of HDL remodeling and/or shifting the population distribution among HDL subclasses. Mild oxidation in vivo may enhance HDL functions in reverse cholesterol transport, but extensive oxidation would inhibit protein distribution and lipoprotein remodeling, which would impair reverse cholesterol transport.

Guha *et al*. (2008) suggested that moderate structural disorder accelerates metabolic remodeling of HDL, which may benefit HDL functions in cholesterol removal. Therefore, HDL stability must be delicately balanced to maintain the structural integrity

of the lipoprotein assembly and ensure structural specificity necessary for HDL interactions with its metabolic partners, yet enable efficient efflux and processing of cell cholesterol and rapid HDL remodeling and turnover at key junctures of reverse cholesterol transport.

Our in vivo results and Spearman's correlation analysis as well as multiple stepwise forward regression analyses confirmed previous studies which indicated that mild oxidative stress accelerates remodeling of HDL particles and enhances HDL functions in reverse cholesterol transport.

Samouilidou *et al*. (2010) reported that in HD patients, high serum levels of ox-LDL are associated with low HDL_2-C subclass levels. This might suggest that oxidative stress affects the HDL subclass which is more related to the protecting activity of HDL-C, contributing to atherosclerosis development. Sakuma *et al*. (2010) suggested that HDL_2 can inhibit further oxidative modification of partially-oxidized LDL by interrupting the chain oxidation reaction after lipid hydroperoxide (LOOH) formation in a concentration-dependent manner. The observation of Shuhei *et al*. (2010) highlighted the notion that the distribution of HDL subpopulations has important implications for the potential of HDL as an antioxidant source. They reported that subjects with low HDL-C displayed marked changes in their HDL compositions and subclass distributions. Larger HDL_2 particles, as well as HDL mean particle sizes, are reduced in subjects with low HDL-C. Small, dense HDL_3 particle subclasses have more capacity to protect LDL against oxidation than large, light HDL_2 particles. The HDL_3 particle subclasses, from the low HDL-C subjects, contained more TG with less cholesterol esters than those from the control group, and possessed a higher PON-1 activity as compared to HDL_2 particles. The resistance of HDL_3 to oxidation is higher than that of HDL_2 in vivo and is affected by HDL lipid/apolipoprotein composition, HDL-associated proteins other than apolipoproteins, subclass distribution, and systemic inflammation. Therefore, the low susceptibility of HDL to oxidation in the low HDL-C subjects may be due to the compositional changes specifically in the HDL_2 subclass. Our HD patients had low HDL-C and apoA-I concentrations, low PON-1 activity, and significant compositional changes in HDL particles. In these patients, we did not observe a significant relationship between ox-LDL concentration and other parameters in Spearman's correlation analysis as well as multiple stepwise forward regressions, so future studies are needed in a larger group.

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