

Serum cystatin C is a determinant of paraoxonase activity in hemodialyzed and renal transplanted patients

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Abstract. *Background:* Human paraoxonase-1 (PON1) inhibits LDL-oxidation and atherogenesis, and possesses lactonase activity. Decreased PON1 activity was found in hemodialyzed and renal transplanted patients. Cystatin C plays a protective role in atherosclerosis, and is a new, sensitive marker of renal function. The relationship between these two markers in renal failure has not been investigated.

Aims: The goal of this study was to clarify the relationship between PON1 activity, cystatin C and homocysteine in chronic renal failure. We also determined the levels of oxidatively modified LDL (oxLDL) and thiobarbituric acid reactive substances (TBARS) to characterize lipid peroxidation.

Patients and methods: 74 hemodialyzed (HD), 171 renal transplanted patients (TRX), and 110 healthy controls (C) were involved in the study. PON1 activity and TBARS levels were measured spectrophotometrically. OxLDL level was determined with sandwich ELISA.

Results: There was a negative correlation between PON1 activity and cystatin C level. Homocysteine level correlated negatively with PON1 activity, and positively with cystatin C level. OxLDL and TBARS levels were significantly higher in the HD and TRX groups compared to C.

Conclusions: Cystatin C may be a good predictive factor not only for homocysteine levels but for the antioxidant status in patients with renal failure and renal transplantation.

Keywords: Cystatin C, homocysteine, paraoxonase, renal failure, oxidative stress

1. Introduction

Human paraoxonase-1 (PON1) is a calcium-dependent esterase exclusively bound to apolipoprotein A-I and J containing high-density lipoprotein (HDL) particles [1]. Previous studies have indicated that PON1

can prevent low density lipoprotein (LDL) oxidation by hydrolyzing lipid peroxides in lipoproteins, deoxygenate homocysteine thiolactone and therefore protect against the development of atherosclerosis [2–4]. The enzyme's activity is reduced in patients with enhanced atherosclerosis, in disorders of lipoprotein metabolism, in renal insufficiency and after renal transplantation, caused by elevated oxidative stress and disturbances of apolipoprotein metabolism [5]. PON1 has newly been discovered to possess lactonase activity, and hydrolyze various lactones, thiolactones and cyclic carbonate esters. Homocysteine thiolactone, which occurs in hu-

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man and rodent plasma, is a natural substrate of PON1, and the enzyme's likely natural function is to detoxify homocysteine thiolactone and, therefore, to minimize protein-*N*-homocysteinylation [2].

Previous studies showed that serum creatinine is not an optimal marker of glomerular filtration rate (GFR). Cystatin C is a product of a housekeeping gene, a low molecular weight basic protein produced by all nuclear cells at a constant rate, freely filtered, and not secreted or metabolized after tubular reabsorption [6]. Therefore Cystatin C is a more sensitive marker of GFR than serum creatinine in adults including renal transplant recipients and patients with chronic renal failure [7,8]. Cystatin C has been shown to have a protective role in atherogenesis. It works as an inhibitor of cysteine proteases of the cathepsin family. These enzymes are known to degrade extracellular matrix constituents of the vascular wall including elastin and collagen [9]. Cystatin C deficiency results in the development of larger atherosclerotic lesions with increased macrophage content in apolipoprotein E-deficient mice [10].

Previous clinical studies have shown that homocysteine is a risk factor for cardiovascular and cerebrovascular diseases and an independent predictor of mortality in the general population as well as in patients with chronic renal failure, particularly patients on hemodialysis [11]. Homocysteine is known to induce unfolded protein response, oxidative stress and the production of pro-inflammatory factors [12]. The metabolic conversion of homocysteine by methionyl-tRNA synthetase to homocysteine thiolactone leads to a reaction with lysine residues in proteins, damaging their structures and impairing their physiological activities [13]. Induction of an autoimmune response explains why relatively small amounts of *N*-homocysteinylation occurring in the human organism can be detrimental. Protein targets for the modification by Hcy-thiolactone in human blood include fibrinogen, low-density lipoprotein, and high-density lipoprotein [13,14]. Some studies proved that PON1 protects against protein homocysteinylation via its homocysteine thiolactone hydrolase activity [2].

Previously a positive correlation between cystatin C and homocysteine was found in elderly subjects [15] and in hemodialyzed patients [16]. Cystatin C level has been shown to reflect subtle decreases in renal function that independently predicts total homocysteine level among stable renal transplant recipients [17]. A negative correlation has been found between PON1 activity and homocysteine level in patients with coronary artery disease [18]. However, the relationship between cystatin C and PON1 activity has not been investigated.

The aim of our study was to examine the relationship between cystatin C, paraoxonase activity and homocysteine. We hypothesized that there is a correlation between cystatin C representing renal function, antioxidant PON1 paraoxonase activity, and the level of prooxidant homocysteine.

2. Patients and methods

2.1. Study population

Two hundred and forty-five patients with renal failure, or transplant, and one hundred and ten controls were enrolled. Patients with renal insufficiency had 4 hour sessions of hemodialysis therapy three times per week. The mean dialysis time was 26.1 ± 10.7 months. The optimal dry weight of patients was determined using bioimpedance (BIODYNAMICS 310 appliance), then based on this the rate of ultrafiltration was calculated. Transplanted patients received combined immunosuppressive therapy (cyclosporin, azathioprine, methylprednisolone). Post-transplant time was 31.6 ± 11.3 months. Serum cyclosporin (Sandimmun-Neoral) concentration (therapeutic level 80–300 ng/ml) was measured with the help of monoclonal antibodies (FPIA-immunoassay-Abbott), in the first month daily, during the next five months weekly, from the 6th to the 12th month every second week, after the first year monthly. In the beginning we applied 20 mg corticosteroid per day, then we lowered the dose gradually by 5 mg. We employed azathioprine (Imuran) 1–2 mg/kg per os daily while checking the blood count regularly.

We excluded patients with diabetes mellitus, or increased fasting glucose level; alcoholism, liver disease, or elevated liver enzymes; recent myocardial infarct; endocrine diseases; pregnancy, lactation; patients receiving chemotherapy, or lipid-lowering medication; and smokers. Patients gave a written, informed consent to participate, and the study was performed according to the requirements of the Ethical Committee of the Medical and Health Science Center, University of Debrecen, as well as the Code of Ethics of the World Medical Association.

2.2. Blood sampling

After 12 hours of fasting, 10 ml venous blood sample was taken between 7.30 and 8.00 in the morning before dialysis. Lipid parameters, homocysteine, cystatin C and TBARS concentrations were determined on fresh sera. The sera for paraoxonase activity measurements were kept at -70°C before analysis.

2.3. Analysis of paraoxonase activity

PON1 activity was measured as previously described by Eckerson [19]. Briefly, we set up the following enzymatic reaction using paraoxon (O,O-diethyl-O-p-nitrophenylphosphate, Sigma) as substrate, and the generation of 4-nitrophenol was measured spectrophotometrically. 50 μ l serum was dissolved in 1 ml Tris/HCl buffer (100 mmol/l, pH = 8.0) containing 2 mmol/l CaCl_2 and 5.5 mmol/l paraoxon. We measured the absorbance at 412 nm (25 °C), using a Hewlett-Packard 8453 UV-visible spectrophotometer. Enzyme activity was calculated using the molar extinction coefficient $17100 \text{ M}^{-1}\text{cm}^{-1}$. One unit of PON1 activity is defined as 1 nmol of 4-nitrophenol formed per minute under the assay conditions mentioned above.

2.4. Paraoxonase phenotyping

The phenotype distribution of PON1 was determined by the dual substrate method [20]. The genetic polymorphism at codon 192 Q->R is responsible for the presence of two isotypes: A (low activity) and B (high activity). The ratio of the hydrolysis of paraoxon in the presence of 1 M NaCl (salt-stimulated PON1 activity) to the hydrolysis of phenylacetate was used to assign individuals to one of the three possible (AA, AB, BB) phenotypes. Cut-off values between phenotypes were as follows: type AA, ratio < 3.0; type AB, ratio 3.0–7.0; and type BB, ratio > 7.0. AA represents low; AB intermediate; and BB high enzyme activity.

2.5. Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARs) were measured spectrophotometrically. 300 μ l of plasma was added to 600 μ l of thiobarbituric acid reagent. The samples were heated at 100°C for 15 min, and after cooling TBARs were extracted with 3 ml of n-butanol. The absorbance was recorded at 532 nm in a Hewlett Packard 8453 UV-Visible spectrophotometer and the results were calculated ($\text{EC} = 1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) [21].

2.6. Measurement of oxidized LDL

Oxidized LDL (oxLDL) was measured with a sandwich ELISA technique (WAK-chemie Medical GmbH, Germany). We incubated plasma samples anticoagulated with ethylene-diamine-tetraacetic acid (EDTA) with microtiter strips covered by monoclonal mouse anti-oxLDL antibodies. After removing non-reactive plasma components, we added buffer-containing anti-apolipoprotein B antibodies conjugated with peroxidase, which bound to the oxLDL fixed to the solid phase. After the removal of unbound conjugates with washing, tetramethyl-benzidine (TMB) was added as a chromogenic substrate. The concentration of oxLDL was detected spectrophotometrically after the enzyme-catalyzed color change. Plasma concentration of oxLDL was determined with a reference to a standard curve constructed with purified oxLDL [22].

2.7. Measurement of homocysteine, cystatin C and lipid parameters

Homocysteine, cystatin C and lipid parameters were determined at the General Chemistry Laboratory of the University.

Fasting plasma tHcy concentration was determined by an enzyme-linked immunoassay using an automated fluorescence polarization analyser (FPIA, IMX System, Abbott Diagnostics, Roma, Italy). This fluorescence polarization immunoassay is based on the highly selective enzymatic conversion of homocysteine to *S*-adenosyl-L-homocysteine, which is then recognized by a monoclonal antibody.

Plasma cystatin C measurements were performed with a latex enhanced reagent (N Latex Cystatin C, Dade Behring, Deerfield, IL, USA) using a Behring BN ProSpec analyzer (Dade Behring) and calibrators from Dade Behring. The method was a fully automated particle-enhanced nephelometric immunoassay.

Serum cholesterol and triglyceride levels were measured with enzymatic, colorimetric tests (GPO-PAP, Modular P-800 Analyzer, Roche/Hitachi), while HDL-cholesterol was assessed with a homogenous, enzymatic, colorimetric assay (Roche HDL-C plus 3rd generation). LDL-cholesterol was measured with a homogenous, enzymatic, colorimetric assay (Roche LDL-C plus 2nd generation).

The tests were performed according to the recommendation of the manufacturer.

Table 1
Characteristics and demographic parameters of the study population

	Control	Hemodialyzed	Transplanted
Number of subjects	110	74	171
Age (years)	53.2 ± 9.3	61.8 ± 9.2	41.5 ± 9.3
BMI (kg/m ²)	22.5 ± 2.5	23.8 ± 4.3	26.1 ± 4.5
Cholesterol (mmol/l)	4.71 ± 0.89	5.45 ± 1.49**	6.67 ± 1.72*
Triglyceride (mmol/l) (median quartiles)	1.04 (0.84–1.52)	2.48 (2.03–3.17)*	2.05 (1.2–2.8)*
HDL-C (mmol/l)	1.43 ± 0.31	1.06 ± 0.29*	1.37 ± 0.43
LDL-C mmol/l	2.6 ± 0.6	3.39 ± 1.09*	4.18 ± 1.2*
ApoA1 (g/l)	1.82 ± 0.25	1.17 ± 0.26*	1.53 ± 0.35*
ApoB (g/l)	1.14 ± 0.29	1.39 ± 0.57*	1.46 ± 0.48*
ApoB/ApoA1	0.63	1.19	0.95
Creatinine (mmol/l)	81.2 ± 13.1	795 ± 121*	146 ± 49*
Uric acid (mmol/l)	149 ± 68	426 ± 89*	366 ± 117*
Cystatin C (mg/l)	1.25 ± 0.15	5.38 ± 1.27*	2.29 ± 0.61*
Homocysteine (μmol/l)	10.8 ± 2.45	23.07 ± 4.63*	15.58 ± 5.06*
TBARS (μmol/l)	0.06 ± 0.01	0.16 ± 0.07*	0.12 ± 0.04*
oxLDL (U/l)	40 ± 9.4	108.6 ± 18.6*	77.2 ± 13.45*
paraoxonase activity (U/l)	188 ± 62	64.6 ± 16.2**	110.1 ± 23.6**

* $p < 0.001$, ** $p < 0.01$.

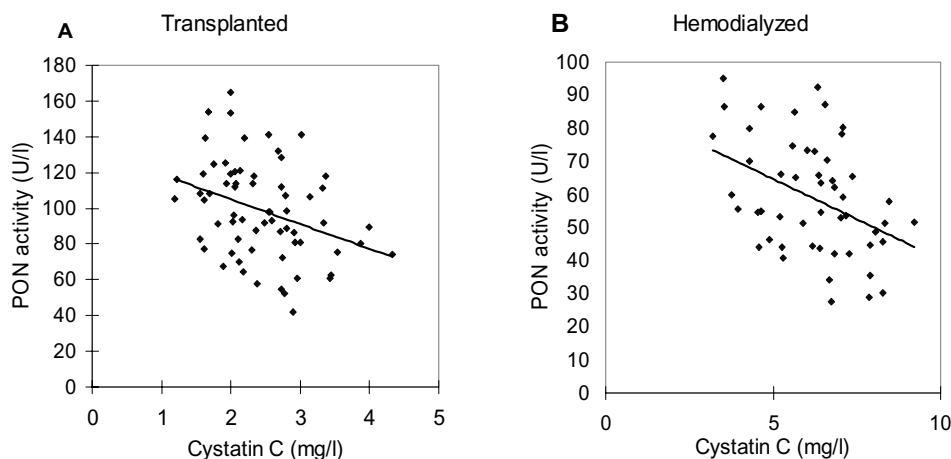


Fig. 1. Correlation between serum cystatin C and PON1 activity in transplanted (A) and hemodialyzed (B) patients. A: $r = -0.34$, $p < 0.05$, B: $r = -0.42$, $p < 0.05$.

2.8. Statistical methods

Statistical evaluation was performed with the SASTM for WindowsTM 6.12 program (SAS Institute, Cary NC 275313 USA). Data were expressed as means ± S.D. in case of normal distribution and medians and quartiles in case of non-normal distribution. Normality of the distribution was tested with the Kolmogorov-Smirnov test. Parameters with a skewed distribution were transformed logarithmically to normalize their distribution. The unpaired *t*-test was used to compare groups, $p < 0.05$ was considered to be significant. Correlations between continuous variables were assessed using Spearman's regression analysis. Multiple regression analysis

(using the backward stepwise elimination procedure) was performed to determine which variables were the best predictors of PON1 activity.

3. Results

Demographic data, laboratory parameters and characteristics of the study population are shown in Table 1.

We found a negative correlation between cystatin C level and paraoxonase activity in both hemodialyzed and transplanted patients (HD: $r = -0.42$, $p < 0.05$; TRX: $r = -0.34$, $p < 0.05$) (Fig. 1A, B). There was a negative correlation between homocysteine level and

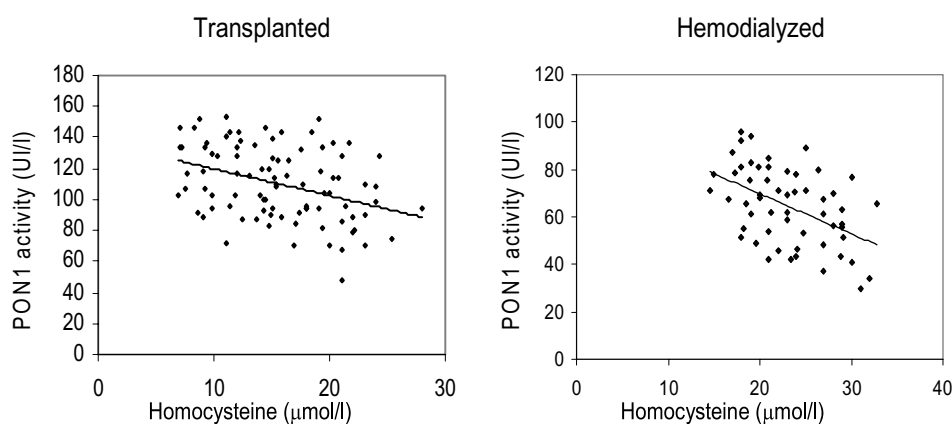


Fig. 2. Correlation between serum homocysteine and PON1 activity in transplanted (A) and hemodialyzed (B) patients. A: $r = -0.37$, $p < 0.05$; B: $r = -0.48$, $p < 0.05$.

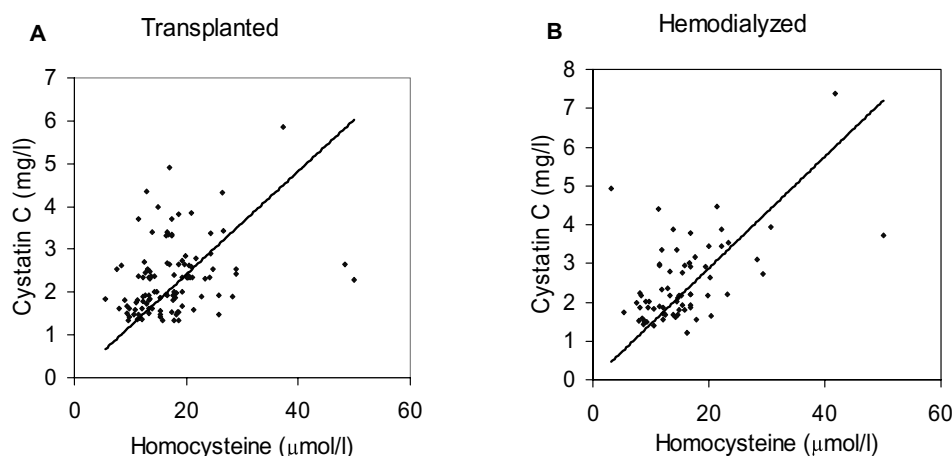


Fig. 3. Correlation between serum homocysteine and cystatin C levels in transplanted (A) and hemodialyzed (B) patients. A: $r = 0.32$, $p < 0.05$; B: $r = 0.53$, $p < 0.05$.

paraoxonase activity in both patient groups (HD: $r = -0.48$, $p < 0.05$; TRX: $r = -0.37$, $p < 0.05$) (Fig. 2A, B). Cystatin C correlated positively with homocysteine in both patient groups (HD: $r = 0.53$, $p < 0.05$; TRX: $r = 0.32$, $p < 0.05$) (Fig. 3A, B).

The allelic frequencies found in the study population using the phenotypic determination are shown in Table 2. The allelic frequencies were in accordance with the results of our previous studies and the literature, following the Hardy-Weinberg equilibrium ($p < 0.05$). PON1 phenotype distribution of the two patient groups and the controls did not differ significantly ($p = 0.1005$).

The level of renal marker cystatin C was significantly higher in renal failure and in transplanted patients compared to controls. PON1 activity was significantly decreased in renal failure compared to con-

trols, and to a lesser degree in transplanted patients as well. The level of homocysteine was also elevated in both patient groups. Homocysteine in hemodialyzed patients was higher than both controls and transplanted patients, while in case of transplantation the level was only moderately, but still significantly elevated compared to healthy individuals (Table 1).

Serum cholesterol, triglyceride, LDL-C, apolipoprotein B (ApoB), creatinine and uric acid were significantly higher, HDL-C and apolipoprotein A1 (ApoA1) were significantly lower in renal failure compared to controls, transplanted patients were between the other two groups. TBARS and oxLDL, that characterize oxidative processes, were significantly higher in hemodialyzed and transplanted patients compared to controls (Table 1.).

Table 2
Paraoxonase phenotype distribution and allelic frequencies in the study population

	Control (<i>n</i> = 110)		Hemodialized (<i>n</i> = 74)		Transplanted (<i>n</i> = 171)	
	n	%	n	%	n	%
AA	69	63	48	65	102	60
AB	35	32	24	32	64	37
BB	6	5	2	3	5	3
A	173	79	120	81	268	78
B	47	21	28	19	74	22

Table 3
Multiple regression analysis for PON1 activity as a dependent variable

	Hemodialized (<i>n</i> = 74)		Transplanted (<i>n</i> = 171)	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Cystatin C	8.258	0.007	4.488	0.044
Age	0.797	0.379	0.525	0.475
BMI	0.199	0.740	0.578	0.635
Cholesterol	2.404	0.131	1.358	0.721
Triglyceride	0.181	0.673	0.062	0.804
HDL-c	0.002	0.961	1.434	0.242
LDL-c	1.974	0.170	0.276	0.604
Creatinine	0.227	0.637	0.027	0.870
Homocysteine	0.212	0.730	0.137	0.715
TBARS	1.418	0.323	1.245	0.418
oxLDL	0.137	0.599	0.713	0.637

F-to-remove values are indicated in bold and *F*-to-enter values are shown in normal letters.

By multiple stepwise logistic regression analysis, only Cystatin C was significantly and independently associated with PON1 paraoxonase activity in hemodialyzed and transplanted patients (Table 3).

4. Discussion

Cystatin C, an endogenous low-molecular-weight marker of glomerular filtration rate, has recently been shown to be associated with future cardiovascular disease in healthy elderly populations and patients with documented atherosclerosis in a dose-dependent manner that possibly reflects a very early stage of chronic renal dysfunction [23]. Increased cystatin C levels in the hemodialyzed and even in the transplanted group can be imputed to a reduced renal function, which also leads to a decreased renal elimination of homocysteine. Similarly to others [15,16], we found a positive correlation between cystatin C and homocysteine levels. The metabolism of homocysteine is impeded as well, as metabolizing enzymes are damaged by the increased oxidative stress, partly caused by reactive products of homocysteine, and the enzyme activities are reduced because of the changed metabolism of cofactor vitamins: vitamin B12, B6 and folic acid [24].

Some studies found an inverse correlation between homocysteine and PON1's arylesterase activity [25], while others found no significant correlation between homocysteine and either PON1 activity or concentration, although the persons in the highest quartile had significantly lower PON1 activities than the remaining participants [26]. They proposed that the negative effect of homocysteine on PON1 activity may be seen only in situations with increased homocysteine levels. In this study we found a negative correlation between PON1 activity and homocysteine level. These results support the previous observations.

Lacinski et al. investigated PON1's homocysteine-thiolactonase activity as well, where one of the determinants found was total homocysteine, the others being age and total cholesterol, but not HDL. They also found a strong correlation between the hydrolytic activities of PON1 towards homocysteine thiolactone and paraoxon [3]. A decreased activity of the homocysteinylated PON1 can lead to an increased susceptibility of the proteins in HDL (e.g. PON1) to further homocysteinylated [27]. This may alter the conformation of the PON1 enzyme protein, and result in a reduced lactonase activity on endogenous lactones, e.g. homocysteine thiolactone. Homocysteine thiolactone forms homocysteine thiolactone-LDL-aggregates with the increased amount of LDL particles in chronic re-

nal failure, which augments the phagocytotic activity of macrophages, and leads to an increased formation of foam cells in the atherosclerotic plaques [28]. The homocysteinylation of LDL particles increases their susceptibility to oxidation, induces the production of anti-homocysteinyllysine antibodies, and accelerates their uptake to macrophages [29,30].

Our results support the initial hypothesis of associated PON1 activity and cystatin C level. This finding can be explained in part with the increased oxidative stress observed in renal failure leading to the reduction of PON1 activity and the oxidative modification and enhanced metabolism of HDL particles. On the other hand, hyperhomocysteinemia is associated with impaired renal function, which is characterized by the decreased cystatin C levels that may cause the homocysteinylation of HDL structure proteins or the PON1 protein itself, resulting in decreased PON1 activity.

In hemodialyzed patients elevated TBARS and oxLDL levels imply increased oxidative damage, which are accompanied by a high prooxidant homocysteine level, decreased paraoxonase activity, and increased cystatin C reflecting deteriorating kidney functions. Transplantation cannot completely normalize this atherogenic state of parameters. These changes can be attributed to renal failure, dialysis therapy, and can partly be considered as results of the unfavorable lipid parameters that may partly be attributed to the primary disease and in part to the effect of medication [31] as well as other factors yet unknown. Our results are in consistence with the previous observations.

It must be noted that there are some limitations of our study. Measuring the lactonase activity of PON1 could provide further information about the role of hyperhomocysteinemia in these patients.

The negative correlation between PON1 activities and serum cystatin C levels in hemodialyzed and renal transplanted patients are novel findings. Based on these results cystatin C may be a good predictive factor not only for homocysteine level but for the antioxidant status in these patients. Therefore the measurement of serum cystatin C level can be recommended in future studies on hemodialyzed and transplanted patients. Further studies are needed to clarify the role of cystatin C in atherogenesis.

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