Low HDL-cholesterol is not responsible for decreased paraoxonase activity in chronic renal failure

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Running title: Low HDL-C and PON1 in renal failure

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Abstract

Background/Aims: Human paraoxonase-1 (PON1) is responsible for the antioxidant effect of high-density lipoprotein (HDL) by inhibiting low-density lipoprotein oxidation. Previous studies discovered dyslipidemia and decreased PON1 activity in chronic renal failure (CRF). We aimed to determine paraoxonase and arylesterase activity, and phenotypic distribution of the PON1 enzyme, and lipid profile in low and normal HDL-cholesterol (HDL-C) patients with CRF, and renal transplant (TX), compared to primary dyslipidemia (DL).

Methods: 116 CRF (low or normal HDL-C), 52 TX (low or normal HDL-C), and 62 DL patients (low or normal HDL-C) were included. Paraoxonase and arylesterase activities were measured spectrophotometrically. Phenotype was determined using the dual substrate method.

Results: Aryl/HDL-C was significantly higher in low HDL-C patients. Patients with CRF had significantly lower arylesterase activity compared to DL, independent from HDL-C. Paraoxonase activity and PON1/HDL-C did not differ significantly in CRF compared to TX and DL. Phenotypic distribution was similar in patient groups. Low HDL-C CRF patients had significantly lower cholesterol and triglyceride than DL.

Conclusion: Decreased arylesterase activity, correlating with PON1 enzyme protein quantity, is not explicable by decreased HDL-C in CRF. Low HDL-C CRF patients' increased cardiovascular morbidity is not attributable to changes in PON1 activity, or phenotypic distribution.

Introduction

Increased cardiovascular mortality in renal failure can be explained by multiple risk factors, including secondary dyslipidemia. CRF is frequently associated with disturbances of lipoprotein transport, alterations of lipoprotein concentration, and abnormalities of lipid and (apo)protein composition of lipoproteins [1-3].

Previous studies suggested that LDL-cholesterol (LDL-C) does not predict mortality in CVD and renal transplanted patients as well as in patients without renal disease; however, HDL-C remains a significant cardiovascular risk factor. In these patients HDL fails to mature normally as a result of decreased LCAT (lecithin-cholesterol acyltransferase) activity, this and increased clearance results in low HDL-C, mainly comprising of cholesterol ester-poor, triglyceride-rich HDL3, an HDL-subfraction with decreased antioxidant activity [4-6]. The ability of HDL from hemodialysis patients to protect LDL against oxidation in vitro is controversial and may be impaired or unchanged [7,8].

According to previous data, more than half of renal transplant recipients have low HDL-C, which significantly increases the incidence of post-transplant major adverse cardiovascular events [9]. About one third of hemodialysis patients also have low HDL-C [10], and HDL-status is independently related with coronary artery disease [11].

Several studies have shown decreased activity of human paraoxonase-1 (PON1) in CRF patients, particularly on maintenance hemodialysis. The decrease in PON1 activity, hence the reduction in its antioxidant and antiatherogenic properties could be an essential factor for premature atherogenesis [12]. Oxidized LDL is known to possess atherogenic and pro-inflammatory properties, thus PON1 significantly contributes to the atheroprotective effect of HDL [13-15]. Low serum arylesterase/paraoxonase PON1 activity is associated with several risk factors for coronary heart disease, including diabetes, primary dyslipidemia and smoking [16]. Recent studies have indicated that PON1 also possesses lactonase activity [17]. It

hydrolyses homocysteine thiolactone and prevents protein homocysteinylation, a process involved in atherogenesis [18]. Major part of PON1 in serum is associated with HDL particles, and the enzyme's activity is stabilized by ApoA1. HDL-bound PON1 is transported across the plasma membrane of phospholipid expressing cells.

PON1 expression and activity is partly controlled by its molecular variation [19]. Out of the polymorphisms, especially Q192R seems to determine antioxidant activity, here RR means an 8-fold increase in activity compared to QQ. PON1-Q and PON1-R may act on different substrates generated during LDL oxidation and may possess different sensitivities to the action of peroxides formed during oxidation. These differences may contribute to the divergence in the possible antiatherosclerotic roles of the PON1 allozymes [20]. Previous studies have shown that the dual substrate method using the ratio of PON1's paraoxonase activity in the presence of NaCl and its arylesterase activity could estimate the frequencies of Q192R genotypes in patients with preserved renal function, although our previous study implied that this correlation is lower in CRF patients compared to healthy individuals. The enzyme's phenotype was a better predictor of cardiovascular risk than its genotype. [21].

Some previous studies suggested that the decrease in PON1 activity could be the result of lower HDL concentrations in CRF patients, given that HDL is the main serum carrier of PON1 [22]. Our previous studies suggested that HDL concentration and phenotypic distribution may be important, but not the only determining factors [23]. Chronic renal disease and low HDL-C both increase the prevalence of cardiovascular disease, and in case of both conditions, the risk is even higher [24].

We hypothesized that in renal disease and in dyslipidemia the decrease in HDL-C level has a different effect on the quantity of PON1's enzyme proteins, thus on the correlating arylesterase activity and/or on its antioxidant capacity, characterized by its paraoxonase activity. As low HDL-C ESRD patients and renal transplant recipients have a cardiovascular

risk even higher than low HDL-C patients with primary dyslipidemia, we expected them to have lower arylesterase and paraoxonase activities than the dyslipidemic ones. The aim of our study was to determine if PON1 enzyme's paraoxonase and arylesterase activity, its phenotypic distribution, or the lipid profile differs in normal and low HDL-C patients in CRF, renal transplant recipients (TX), or patients with primary dyslipidemia (DL).

Materials and methods

Two hundred and thirty patients with renal failure, transplant, or dyslipidemia were enrolled, who had either low HDL-C (male: <1 g/L, female: <1.3 g/L), or normal HDL-C (male: >1 g/L, female: >1.3 g/L). We excluded patients with diabetes mellitus, or increased fasting glucose level, alcoholism, liver disease, or elevated liver enzymes, fresh myocardial infarct; endocrine diseases, pregnancy, lactation, patients receiving chemotherapy, or lipid-lowering medication. Patients with renal failure had 4 hour sessions of hemodialysis three times per week. The mean time on maintenance hemodialysis was 47±29 months. Transplanted patients received combined immunosuppressive therapy (cyclosporine or tacrolimus, azathioprine or mycophenolate mofetil and methylprednisolone) after cadaver kidney allotransplantation. The average post-transplant time was 68±47 months. Demographic data are shown in **Table 1**. Informed consents were taken from all patients after explaining the nature and the purpose of the study. The Ethical Committee of the University of Debrecen approved the study.

Blood sampling

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

Lipid measurements

Serum cholesterol and triglyceride levels were measured using enzymatic, colorimetric tests (GPO-PAP, Modular P-800 Analyzer, Roche/Hitachi), while HDL-C was assessed by homogenous, enzymatic, colorimetric assay (Roche HDL plus 3rd generation). LDL-cholesterol fraction was calculated indirectly using the Friedewald equation [25]. Apolipoprotein examination was performed by immuno-turbidimetric assay Tina-Quant ApoA (Version 2, Roche), Tina-Quant ApoB (Version 2, Roche).

Measurement of serum paraoxonase activity

PON1 activity was measured as previously described. 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 followed spectrophotometrically: 50 µl 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 Hewlett-Packard 8453 UV-visible spectrophotometer. Enzyme activity was calculated using the molar extinction coefficient 17100 $mol^{-1}cm^{-1}$. One unit of PON1 activity is defined as 1 nmol of 4-nitrophenol formed per minute under the assay conditions mentioned above [26].

Measurement of serum arylesterase activity

Arylesterase activity was measured spectrophotometrically as previously described. Briefly, the assay contained 1 mmol phenylacetate (Sigma) in 20 mmol Tris/HCl, pH=8.0. The reaction was started by the addition of the serum, and then the absorbance was monitored at 270 nm. Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. Enzyme activity was calculated using the molar extinction coefficient 1310 $mol^{-1}cm^{-1}$. Arylesterase activity is expressed in U/ml; 1 U is defined as 1µmol phenylacetate hydrolyzed per minute [27].

Determination of phenotypic distribution

The phenotype distribution of PON1 was determined by the dual substrate method. 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 mol 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 [26].

Statistical analysis

Statistical evaluation was performed with the SPSS program. First we checked if parametric tests are applicable using the Kolmogoroff-Smirnoff normality test, and Levene's test for the equality of standard deviations. As most parameters were not normally distributed, especially in the low HDL-C subpopulation, we used the non-parametric Kruskal-Wallis test to check the dependence of the parameters on the presence of renal failure or transplantation rather than ANOVA. As distributions are non-Gaussian in most of the groups, averages and SD-s were not suitable for presenting central value and scatter of data, so we used medians, upper and lower quartiles to represent the groups in our tables and figures. To check the differences between the patient groups' gender, smoking, and phenotype, we used Pearson's χ^2 test.

Results

Arylesterase activity

Arylesterase activity, which significantly correlates with the amount of PON1 enzyme proteins, significantly depended on the presence of renal failure or transplant in the normal ($p<0.05$) as well as in the low HDL-C group ($p<0.005$) **(Figure 1A, Table 1)**. Arylesterase

activity was lower in CRF than in dyslipidemia, transplanted patients were in between. As PON1 is associated with HDL, we tested if HDL-C concentration influences arylesterase activity. The arylesterase/HDL-C ratio also depended significantly on the presence of renal failure or transplant, both in the normal ($p<0.005$), and in the low HDL-C group ($p<0.05$). The Aryl/HDL-C ratio was significantly higher in low HDL-C patients in all three groups compared to patients with normal HDL-C ($p<0.05$) **(Figure 1B, Table 1)**.

Paraoxonase activity

In contrast to arylesterase activity, paraoxonase activity and the PON/HDL-C ratio did not depend on the presence of renal failure or transplant (neither in the normal, nor in the low HDL-C group) **(Figure 2A and B, Table 1)**. There was no significant difference in paraoxonase activity between low and normal HDL-C patients **(Figure 2A, Table 1)**; however, low HDL-C CRF patients had significantly higher paraoxonase/HDL-C ratios than normal HDL-C CRF patients **(Figure 2B, Table 1,** $p<0.05$). There was no significant difference in PON activity between the low and normal HDL-C groups with DL, nor with TX.

Phenotypic determination

Phenotypic distribution did not differ sigificantly between the groups **(Table 2)**.

Lipid parameters

In patients with normal HDL-C, LDL-C and total cholesterol depended significantly on the presence of renal failure or transplant ($p<0.005$), while in case of low HDL-C the difference was not significant. Trigliceride depended significantly on kidney state in both the normal ($p<0.05$) and low ($p<0.005$) HDL-C groups, with lower Tg in CRF. We saw a tendency of low HDL-C patients to have less favorable triglyceride levels in all three patient groups. There was a significant difference in ApoB, as well as in ApoA1, with the lowest ApoB and

ApoA1 levels in CRF ($p<0.05$). ApoA1 values of low HDL-C CRF, TX and DL patient groups were lower than those of the corresponding normal HDL-C groups **(Table 1)**.

Discussion

Based on the observation that PON1 activity is decreased with the increased severity of renal failure [28], previous studies suggested that decreased HDL and ApoA1 levels could be the most important cause of low PON1 enzyme protein quantity and activity in CRF patients [29], and the low enzyme activity is responsible for the increased oxidation of LDL by lipid peroxidation, thereby contributing to the accelerated development of atherosclerosis in CRF. Our results of arylesterase activity imply that the level of PON1 enzyme proteins decrease in kidney disease, but this reduction is not significantly dependent on HDL-C level, so the decreased antioxidant defense cannot be attributed to dyslipidemia with low HDL **(Figure 1B and 2B)**. We measured a higher arylesterase activity in TX patients compared to CRF, which indicates that transplantation had a positive effect on enzyme synthesis. Possible explanations for the decrease in PON1 activity in CRF patients may be unfavorable uremic environment due to the retention of uremic toxins, as well as advanced glycation endproducts (AGEs), free adducts and peptides, as well as protein homocysteinylation [30-32]. Since PON1 is sensitive to oxidants and is inactivated by oxidized lipids and homocysteine-thiolactone [33], increased oxidative damage may decrease serum PON1 activity and impair the antioxidant activity of HDL in CRF. In the present study the enzyme's paraoxonase activity did not significantly depend on the presence of renal disease when compared to dyslipidemic controls, suggesting that in spite of less enzyme proteins, the antioxidant response does not decrease significantly when compared to dyslipidemic patients. Low HDL-C renal patients' significantly lower paraoxonase activity compared to normal HDL-C renal patients was significantly higher when corrected to HDL-C, implying that the increase in HDL could help CRF patients' antioxidant defense. The changes in apolipoprotein quantities implied a structural difference of

lipoprotein particles in renal disease compared to primary dyslipidemia, which may be caused by a reduced protein synthesis. Previous studies found that the serum PON1 activity was significantly reduced in uremic patients, and altered HDL subfraction is likely to be the main cause of the decreased PON1 activity [22], as the enzyme's concentration is higher in HDL3 than in HDL2 [13]. CRF patients' decreased total cholesterol level could also result in a higher paraoxonase activity/HDL-C ratio. The data suggest alternative antioxidant pathways (e.g. lecithin:cholesterol acyltransferase) that catabolize prooxidants in low HDL-C renal failure patients compared to dyslipidemic ones. Previous studies also suggested the possibility that in spite of lower PON1 activity, the ability of HDL to protect isolated LDL against mild oxidation is not abnormally low in hemodialysis patients [32], while others found it impaired [8]. The limitations of our study can be the differences in immunosuppressive medication, as well as the differences in age and BMI, so our results may also be influenced by these factors. Although Schiavon et al. found the prevalence of the B allele higher in renal failure, we, in accordance with other studies, found no significant difference in phenotypic distribution, so we cannot attribute the increased atherosclerosis of renal patients to this factor [27].

Many research groups have compared renal patients with healthy controls [7,8,12,22,27,34-39], but only our group used dyslipidemic ones [40]. In the present study PON1's paraoxonase activity did not differ significantly, because we studied low HDL-C patients suffering from primary dyslipidemia as „controls".

Decreased arylesterase activity of low HDL-C renal patients compared to dyslipidemic ones is a novel result. Many studies found a decrease compared to healthy subjects, however, this was the first time that dyslipidemic controls were used. When corrected to HDL-C, the alterations were not univocal in previous studies. In our study, the decrease in arylesterase activity remained significant also when corrected to HDL-C.

In the present study we found no significant difference in PON1's paraoxonase activity in renal failure compared to primary dyslipidemia, so this does not explain their higher cardiovascular risk compared to dyslipidemic patients. Increased high sensitive C-reactive protein (HS-CRP), abnormal lipoprotein profile, unfavorable HDL- and LDL-subgroup distribution, and increased oxidative stress linked to uremia may contribute to increased cardiovascular risk in people undergoing hemodialysis [41,42]. Patients on maintenance hemodialysis suffer from hyperhomocysteinemia, and PON1 has been discovered to have thiolactonase activity, so decreased PON1 activity in patients on hemodialysis may augment protein homocysteinylation, which can lead to earlier atherogenesis compared to dyslipidemic individuals with normal or only moderately elevated homocysteine levels [43,44]. Enhancement or maintenance of the PON1 activity may prevent the development of CVD and its consequences in patients on hemodialysis [45]. Therapy that restores impaired HDL level as well as its function, thus raising paraoxonase activity, and possibly influence lipoprotein quality and quantity, correct HDL distribution, increase LDL size and decrease the quantity of triglyceride-rich remnants seems to be important for CRF patients. Non-pharmacological measures, e.g. nutrition, or abandoning smoking, as well as pharmacological therapy, e.g. statins and fibrates are available to improve PON1 activity and dyslipidemia in CRF and TX patients [23,46-48].

We conclude that the increased cardiovascular mortality of low HDL-C CRF patients cannot be explained by the decreased antioxidant activity of PON1 resulting from the low HDL level, although in CRF the PON1 enzyme level is decreased even compared to primary dyslipidemic patients. Qualitative and quantitative differences between the lipid profiles, potential interactions, as well as the altered lipoprotein metabolism justifies a therapeutic approach different from primary dyslipidemia. It is especially important to distinguish subgroups where the expected therapeutic benefits of lipid lowering exceed the risks. Still, the present paper

may suggest that in low HDL-C CRF patients lipid-lowering therapy may be especially important, as it may improve the protection of the antioxidant PON1 enzyme, however, it also draws attention to the need to investigate the effect of lipid lowering drugs in other large studies of subgroups of kidney patients with different kinds of dyslipidemia, and to specify the advantages and disadvantages of lipid-lowering therapy.

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There is no conflict of interest.

Abbreviations

ApoA: apolipoprotein A

ApoB: apolipoprotein B

Aryl: arylesterase activity

CRF: chronic renal insufficiency (patient)

CVD: cardiovascular disease

HCI: higher confidence interval

HDL(-C): high density lipoprotein(-cholesterol)

DL: dyslipidemic

HS-CRP: high sensitive C-reactive protein

LCAT: lecithin-cholesterol acyltransferase

LCI: lower confidence interval

LDL(-C): low density lipoprotetin(-cholesterol)

Lp(a): lipoprotein(a)

PON: paraoxonase (activity)

PON1: human paraoxonase-1 enzyme(-protein)

TX: transplanted

VLDL: very low density lipoprotein

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Tables

Table 1. Characteristics and demographic parameters of the study population

		Normal HDL-C (median and quartiles)		Low HDL-C (median and quartiles)	
Age (years)	**DL**	**49.8** (44.2-61.0)		**47.7** (38.3-56.0)	
	CRF	**63.8** (49.2-71.1)	***	**58.3** (45.3-70.6)	***
	TX	**46.2** (32.4-56.2)		**45.6** (35.7-53.2)	
BMI (kg/m^2)	**DL**	**28.1** (25.5-31.4)		**26.7** (25.1-34.0)	
	CRF	**23.0** (20.6-25.8)	***	**23.4** (20.8-26.1)	**
	TX	**25.9** (23.8-28.0)		**25.9** (not sufficient)	
Triglyceride (mmol/L)	**DL**	**1.4** (1.0-3.6)		**3.6** (2.0-5.0)	
	CRF	**1.3** (0.9-1.9)	*	**1.9** (1.5-2.8)	***
	TX	**1.9** (1.3-2.6)		**2.3** (1.6-3.7)	
Cholesterol (mmol/L)	**DL**	**5.1** (4.1-6.1)		**5.5** (4.3-6.5)	
	CRF	**5.5** (4.5-6.6)	***	**4.9** (4.1-5.8)	
	TX	**6.5** (5.4-7.8)		**5.1** (4.5-6.9)	
HDL-C (mmol/L)	**DL**	**1.2** (1.1-1.3)		**0.9** (0.9-1.0)	
	CRF	**1.5** (1.2-1.7)	***	**0.9** (0.8-1.1)	
	TX	**1.4** (1.2-1.6)		**1.0** (0.9-1.1)	
LDL-C (mmol/L)	**DL**	**3.2** (2.4-3.8)		**3.0** (2.4-3.6)	
	CRF	**3.2** (2.0-4.0)	***	**3.0** (2.3-3.4)	
	TX	**4.3** (3.3-5.1)		**3.2** (2.9-4.2)	
PON/HDL-C (U/umol)	**DL**	**80.9** (47.0-139.4)		**83.5** (57.5-164.8)	
	CRF	**67.8** (49.3-98.4)		**88.4** (53.7-127.9) †	
	TX	**82.1** (49.6-107.5)		**74.7** (48.9-188.2)	
PON/APOA1 (U/mg)	**DL**	**68.9** (44.5-121.9)		**70.9** (43.4-141.1)	
	CRF	**63.6** (53.0-97.0)		**70.8** 43.3-107.3)	
	TX	**71.6** (48.4-98.6)		**59.3** (37.8-132.3)	
PON (U/mL)	**DL**	**101.4** (59.9-155.0)		**71.6** (46.5-159.9)	
	CRF	**103.0** (71.1-122.3)		**83.0** (45.1-122.2)	
	TX	**108.4** (74.5-153.2)		**71.0** (51.8-182.6)	
Aryl/HDL-C (U/umol)	**DL**	**78.7** (63.3-94.7)		**100.1** (68.9-124.7) †	
	CRF	**50.9** (34.3-68.2)	***	**75.0** (54.8-95.5) †††	*
	TX	**59.2** (47.6-76.5)		**74.9** (59.8-134.7) †	
Aryl/ApoA1 (U/mg)	**DL**	**67.1** (50.9-80.7)		**65.8** (43.6-95.2)	
	CRF	**50.2** (40.4-64.9)		**62.0** (43.4-78.6)	
	TX	**54.9** (41.2-67.2)		**56.5** (49.8-83.3)	
Arylesterase (U/mL)	**DL**	**86.7** (63.9-115.0)		**83.9** (63.4-122.4)	
	CRF	**76.5** (55.7-86.0)	*	**65.5** (54.8-85.7)	***
	TX	**84.7** (74.6-106.8)		**77.6** (64.1-105.9)	
ApoA1 (g/L)	**DL**	**1.47** (1.34-1.53)		**1.29** (1.17-1.35)	
	CRF	**1.40** (1.21-1.70)	*	**1.13** (1.01-1.26)	***
	TX	**1.57** (1.36-1.92)		**1.30** (1.19-1.45)	
ApoB (g/L)	**DL**	**1.15** (0.80-1.35)		**1.24** (0.99-1.40)	
	CRF	**0.98** (0.71-1.43)	*	**0.98** (0.80-1.27)	*
	TX	**1.25** (0.95-1.73)		**1.11** (0.79-1.71)	

DL: dyslipidemic patients; CRF: chronic renal failure patients; TX: renal transplant patients
parameters significantly depending on the presence of renal failure or transplant:
* $p<0.05$; ** $p<0.01$; *** $p<0.005$

parameters significantly different from the corresponding normal HDL-C group:
† $p<0.05$; ††† $p<0.005$

Table 2. Paraoxonase phenotype distribution and allelic frequencies in the whole study population and in the chronic renal failure (CRF), dyslipidemic (DL), and transplanted (TX) groups

	Low HDL-C						Normal HDL-C					
	DL		CRF		TX		DL		CRF		TX	
	number	%	number	%	number	%	number	%	number	%	number	%
AA	26	**62**	41	**59**	11	**69**	14	**74**	30	**64**	17	**46**
AB	15	**36**	22	**32**	5	**31**	3	**16**	17	**36**	19	**51**
BB	1	**2**	6	**9**	0	**0**	2	**11**	0	**0**	1	**3**
Sum	42	**100**	69	**100**	16	**100**	19	**100**	47	**100**	37	**100**
A	67	**80**	104	**75**	27	**75**	31	**82**	77	**82**	53	**72**
B	17	**20**	34	**25**	5	**25**	7	**18**	17	**18**	21	**28**
Sum	84	**100**	138	**100**	32	**100**	38	**100**	94	**100**	74	**100**

Figure legends

Figure 1. Differences of serum arylesterase activity (U/ml) (A) and the arylesterase activity/HDL-C ratio (U/μmol) (B).

Figure 2. Differences of serum paraoxonase activity (U/ml) (A) and the paraoxonase activity/HDL-C ratio (U/μmol) (B).

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Dear Professor Paragh,

Thank you for submitting your revised version (REVISION 1) to "Kidney & Blood Pressure Research". We are pleased to inform you that it has now been accepted for publication and passed on to our production department from whom you will hear shortly.

We hope you will continue to submit work from your group to "Kidney & Blood Pressure Research" in the future.

With kind regards,

Professor Vladimir Tesar
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