Thesis for the degree of Doctor of Philosophy (Ph.D.)

Factors determining the activity of human paraoxonase-1

in chronic renal insufficiency,

and after renal transplantation

By Éva Varga, M.D.

Supervisor: Prof. György Paragh, M.D.

University of Debrecen

Doctoral School of Health Sciences

Debrecen, 2012

Factors determining the activity of human paraoxonase-1 in chronic renal insufficiency, and after renal transplantation

Thesis for the degree of Doctor of Philosophy (Ph.D.)

By Éva Varga, M.D.

Supervisor: Prof. György Paragh, M.D.

Doctoral School of Health Sciences
(Doctoral Program of the „Prevention and Control of
Metabolic and Endocrine Diseases")

Examination Committee:
      Head: Dr. …………………………….
      Members: Dr. …………………………….
                     Dr. …………………………….

The Examination takes place at …, Medical and Health Science Center, University of Debrecen
      201… . ………………… … .

Defense Committee:
      Head: Dr. …………………………….
      Reviewers: Dr. …………………………….
                     Dr. …………………………….
      Members: Dr. …………………………….
                     Dr. …………………………….
                     Dr. …………………………….
                     Dr. …………………………….

The Dissertation's Defense takes place at the lecture hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
      201… . ………………… … .

Introduction

The life expectancy of patients suffering from chronic renal insufficiency (CRI) is nowadays determined not by the underlying disease, but mainly by cardiovascular complications resulting from early atherosclerosis. Cardiovascular morbidity and mortality of patients on maintenance hemodialysis and after renal transplantation significantly exceeds that of the general population, which renders the study of cardiovascular risk factors and defence mechanisms especially important in chronic renal insufficiency.

Atherosclerosis

Atherosclerosis and the consequential acute myocardial infarction (AMI), peripheral vascular disease and stroke are leading causes of morbidity and mortality. Previous studies demonstrated the pathogenic effect of dyslipidemia, especially elevated low density lipoprotein-cholesterol (LDL-C), hypertension, smoking, diabetes mellitus, obesity, and lately of hyperhomocysteinemia as well as various genetic factors.

Changes in other, non-LDL components of the lipid profile, above all hypertriglyceridemia, decreased high density lipoprotein-cholesterol (HDL-C), qualitative changes of LDL and HDL particles, elevated small dense LDL (sdLDL), as well as altered distribution of HDL-subpopulations and decreased activities of HDL-bound antioxidant enzymes paricles also contribute to the atherosclerotic process.

As a result of the proatherogenic lipid profile, LDL is transferred into the vascular wall, and oxidatized by reactive oxigen species and enzymatic processes, leading to the expression of adhesion molecules by enothelial cells and the recruitment of monocytes and T-cells into the intima. Macrophages phagocyte oxidized LDL (oxLDL) and damaged cells to form foam cells, and release cytokines, then they form lipid deposits that increase the activity of inflammatory cells and fibroblasts. OxLDL activates the migration of smooth muscle cells into the inima and their proliferation, resulting in the formation of a fibromuscular plaque, which inhibits

the bood flow by obstructing the vascular lumen. The rupture of unstable plaques consisting of an inflammatory lipid core leads to an acute vascular catastrophe. The accumulation and oxidation of lipids, as well as the increased activity of enzymes and cytokines cause the plaque's fibrous cap to diminish, which results in instability. The accumulation of oxidized LDL in the plaque, as well as the decreased antioxidant capacity of HDL play a crutial role in the process.

Chronic renal insufficiency and cardiovascular risk

Independently from age one of the most serious complications and the leading cause of mortality in chronic renal insufficiency is ischemic heart disease (IHD). Cardiovascular mortality occuring before and after the initiation of hemodialysis therapy far exceeds that of the general population. This is explained by the increased incidence of hypertension, diabetes mellitus, central obesity, dyslipidemia, hyperhomocysteinemia, and altered leptin production. Specific factors in renal disease are the changes in serum calcium and phosphate metabolism, hypoalbuminemia, coagulation disorders, and the increased systemic inflammation and oxidative stress resulting from the underlying disease.

One of the most important risk factors is secondary dyslipidemia, which is characterized by the qualitative and quantitative changes of lipoproteins, including disturbances of their synthesis, metabolism and transport. About one third of patients suffering from chronic renal insufficiency have high serum triglyceride, and about one third have high total cholesterol. CRI patients belong to the group with the highest cardiovascular risk, and many of them lack sufficient lipid-lowering therapy.

In hemodialyzed patients the ratio of small dense LDL-pariticles is increased. The increased serum triglyceride (TG) is probably the result of the increased TG-content of very low density lipoproteins (VLDL's), and the increased amount of VLDL-remnants and intermediate density lipoproteins (IDL's). OxLDL and lipoprotein(a) (Lp(a)) is also increased in serum. These components of the atherogenic dyslipidemia are also atherogenic by themselves. The dynamics of

cholesterol transport between lipoprotein particles from peripheral cells towards the site of catabolism are altered as well. As a result, the mechanism of atherosclerosis can be different in chronic renal insufficiency, and the real benefits of lipid-lowering therapy needs further investigation.

Chronic renal insufficiency and HDL

In chronic renal insufficiency LDL-C is not such a good predictor of cardiovascular morbidity and mortality, as in case of normal renal function; in contrast, HDL remains a significant CV rick factor in these patients as well. The antiatherogenic role of HDL is partly explained by its role in reverse cholesterol transport, during which it transfers the cholesterol accumulated in peripheral tissues to the liver for excretion. Discoid HDL is produced in the liver and small intestine. It contains phospholipids and proteins (including apolipoproteins like ApoA1 and other, non-structural proteins). The cholesterol from the extrahepatic tissues is esterified by lecithin-cholesterol acyltransferase (LCAT), and then it is stored in the core of HDL. Afterwards some of the cholesterol is transported by cholesterol ester transfer protein (CETP) to lipoproteins with lower density, another part to the liver. CETP, LCAT and platelet activating factor acetylhydrolase (PAFAH) are all antioxidants of the HDL-particle, but the enzyme with the most important antioxidant activity is human paraoxonase-1 (PON1).

Intact CETP an hepatic lipase (HL) activity leads to low HDL-C with low risk of atherosclerosis, while ApoA1-deficiency, as well as low LCAT activity result in low HDL-C and ischemic heart disease. Decreased CETP acitivity leads to high serum HDL, (but increased incidence of ischemic heart disease), while increased LCAT activity inhibits the progress of ischemic heart disease by increasing HDL. Serum HDL correlates with age, gender, physical acitivity, oral anticoncipients, triglyceride and alcohol consumption, smoking, and body mass index (BMI). HDL is decreased is familiar combined hyperlipidemia, familiar dyslipidemia, familiar hypoalpha lipoproteinemia and Tangier disease.

The maturation of HDL is disturbed in CRI. The result of decreased LCAT and increased CETP activity is an accelerated removal of HDL-C from the circulation. The remaining HDL3 is poor in cholesterol ester, however, becaure of decreased hepatic lipase (HL) activity, rich in triglyceride, and demonstrates lower antioxidant capacity. The antioxidant activity of HDL from dialyzed patiens on LDL might be preserved or impaired. Earlier studies found low serum HDL-C in about one third of dialyzed patients.

More than half of transplanted patients have low HDL-C, and the incidence of cardiovascular complications increase after renal transplantation. The HDL-status of dialyzed patients is an independent risk factor of coronary disease.

Chronic renal insufficiency and human paraoxonase-1

Human paraoxonase-1 (PON-1) is responsible for the majority of serum paraoxonase activity. Most of the enzyme proteins are bound to HDL. The enzyme's concentration is higher in the HDL3 subfraction than in HDL2. Decreased antioxidant activity of PON1 leads to accelerated atherosclerosis. Through the hydrolysis of lipid peroxides PON1 inhibits LDL-oxidation, and the inhibition of the atherogenic and proinflammatoric activity of LDL contributes to the atheroprotective effect of HDL.

PON1 has lately been discovered to have lactonase activity as well, and through the hydrolysis of homocysteine thiolactone it inhibits protein homocysteinylation, and thus atherogenesis. PON1's antiatherogenic capacity is probably not determined by its activity on the artificial substrates paraoxon (paraoxonase activity, PON), or phenylacetate (arylesterase, Aryl), but on the oxidized lipids and lactones of the organism. However, its PON activity correlates with the enzyme's antioxidant activity, and its Aryl activity is proportional to the quantity of PON1 enzyme proteins.

PON1's activity is influenced by the constitution of food, serum total cholesterol, BMI, insulin resistance, inflammation, smoking, organophosphate-exposition, pregnancy and lactation. Fenofibrate and simvastatin increase, some other statins

decrease the quantity of PON1-mRNA. Decreased arylesterase and paraoxonase activity of PON1 are characteristics of various conditions associated with accelerated atherosclerosis (stroke, peripheral vascular disease, chronic hepatitis, diabetes mellitus, primary dyslipidemia, smoking), in which the disturbance of lipoprotein metabolism and increased oxidative stress play an important role. PON1's activity on paraoxon was lower in patients who suffered AMI, however, there was no significant correlation with its activity on other substrates, or the enzyme protein's concentration. PON activity was a risk factor of acute myocardial infarction that was independent from HDL-concentration.

PON1's expression and activity is partly controlled by its molecular variability. The most important polymorphisms are at the 192nd and 55th amino acid position (PON1-192, Glu: Q, Arg: R genotype, and PON1-55, Leu: L, Met: M genotype). The allozymes are characterized by different substrate specificity and they inhibit the process of LDL-oxidation in different stages. These polymorphisms are the main determinants of the enzyme's activity, and independent risk factors of accelerated atherosclerosis.

In individuals with normal renal function the allelic frequencies of the QR-genotypes can be estimated with the so-called dual substrate method, moreover, the enzyme's AB-phenotype was a better predictor of cardiovascular events, than its QR-genotype. The dual substrate method is used worldwide to estimate allelic frequencies in clinical studies, but only a few former studies described the discordance between phenotypes and genotypes, and there is even less sufficient data concerning patients with decreased PON1 activity. So far we are not aware of another study examining simultaneously the distribution of genotypes and phenotypes in chronic renal insufficiency, or after renal transplantation. In healthy individuals former research discovered 7.2% discordance between PON1's genotype and phenotype.

Chronic renal insufficiency and cystatin C

The metabolism of cystatin C decreases even in the early phase of CRI, rendering it more accurate in estimating renal function compared to creatinine, and consequently demonstrating a positive correlation with the incidence of cardiovascular disease. However, it stabilizes atherosclerotic plaques and protects from atherogenesis by inhibiting cysteine proteases of the cathepsin family, which catabolize several components of the extracellular matrix in the vascular wall. In ApoE knock out mice the deficiency of cystatin C led to the formation of large atherosclerotic plaques with increased macrophage content.

Chronic renal insufficiency and homocysteine

Homocysteine is an independent risk factor of atherosclerotic morbidity and mortality in the general population, as well as in chronic renal insufficiency. Homocysteine increases oxidative stress by forming reactive homocysteine thiolactone, which impairs protein structure and leads to apoptosis. PON1 is protective against protein homocysteinylation through its homocysteine thiolactonase activity. In hemodialyzed patients researchers discovered a positive correlation between cystatin C and homocysteine. Cystatin C was a good marker of even moderate renal impairment, and it was an independent predictor of serum total homocysteine after renal transplantation. Negative correlation was found between PON1's activity and serum homocysteine in patients with coronary disease; however, no former study examined the relationship between cystatin C and PON1.

Study designs

The purpose of our study was to analyze proatherogenic and antiatherogenic factors in patients suffering from chronic renal insufficiency and after renal transplantation. We evaluated parameters that are influenced by renal function, participate in oxidative processes, and influence various steps of atherosclerosis. We looked for markers to adequately characterize chronic renal failure patients' cardiovascular risk state. Former studies performed in renal disease discovered decreased PON1 acitivity, which they attributed to decreased serum HDL concentration, as HDL is the main serum carrier of PON1. The study was based on our former observation that serum HDL and PON1 phenotypic distribution are important, but not the only determinants of the enzyme's activity. The secondary dyslipidemia in chronic renal failure, which is characterized by elevations in triglyceride and/or total cholesterol, sometimes accompanied by decreased HDL-C, is similar to that of some patients with primary dyslipidemia, so we compared out patients to primary dyslipidemic controls as well to eliminate the effect of hereditary and lifestyle factors.

- As no previous comprehensive study examined PON1's activity, genotypic and phenotypic distribution simultaneously in the general population, one of our aims was to estimate the frequency of genotypes, phenotypes and paraoxonase activity of human paraoxonase-1, HDL's most important antioxidant enzyme. We wanted to determine if there is a difference between the general population's distribution and that of patients with chronic renal failure, which would imply that there is a relationship between PON1's genotype or phenotype, and renal failure.

- We compared PON1's phenotype and genotype of patients with chronic renal insufficiency (CRI), or renal transplant (TX) with that of a control group representing the general population (C), to find out if there is discordance between phenotypes and genotypes in renal failure and after transplantation, which conditions demonstrated decreased PON activity in our previous studies.

We expected to find similar discordance in our patients to that of the general population.

- We examined how the concentration of the proxidant homocysteine, oxLDL and thiobarbituric acid reactive substances (TBARS) change in our patients. We expected to find higher levels of prooxidants in both patient groups.

- We studied the relationship between cystatin C, PON activity and homocysteine in CRI, TX patients and controls. We expected to find correlation in renal failure and after transplantation between cystatin C representing the kidney function, antioxidant PON1's paraoxonase activity and prooxidant homocysteine.

- We also intended to determine the importance of the studied parameters during the progress of atherosclerosis in chronic renal insufficiency, and to decide if it is advisable to monitor either of them in the future.

- We examined whether the decrease of HDL has a different effect on the quantity of PON1's enzyme proteins, and on its arylesterase activity and/or antioxidant capacity characterized by its paraoxonase activity in chronic renal insufficiency (CRI) compared to primary dyslipidemia (DL). As patients with low HDL-cholesterol suffering from chronic renal failure or after renal transplantation have an even higher cardiovascular risk compared to those with primary dyslipidemia, we expected arylesterase, or paraoxonase activity, phenotypic distribution, and/or the lipid profile to differ between low LDL-C patients in CRI, TX, and DL.

Patients and methods

Patients

In the first part of our study we included 117 patients with chronic renal insufficiency, 146 with a renal transplant, and 1185 control individuals. In the second part we examined 116 patients with CRI, 52 with TX, and 60 with DL (divided into low HDL-C (male: <1 mmol/L, female: <1.3 mmol/L) and normal HDL-C (male: >1 mmol/L, female: >1.3 mmol/L) subgroups). In the third part we compared 74 CRI and 171 TX patients to 110 controls.

CRI patients underwent 4 hrs of hemodialysis therapy 3 times per week, transplanted patients received combined immunosuppressive therapy (cyclosporin, or tacrolimus, azathioprine, or mycophenolate mofetil, and methylprednisolone) after cadaver kidney transplantation.

In the first part inclusion criteria were age between 21-70 yrs, pysiological level of the most important inflammatory parameters, and normal Lp(a). We excluded from all parts patients with diabetes mellitus, or increased fasting glucose, liver disease, alcohol or drug dependency, biliary stones, recent myocardial infarction, endocrine diseases, pregnancy, or lactation, HIV positivity, severe mental retardation, smoking, as well as patients receiving anti-tumor chemotherapy, and from the first part those with TG>4.5 mmol/L, or patients receiving anticoagulant therapy.

Blood sampling

After 12 hrs of fasting 10 ml of venous blood sample was acquired between 7:30 and 8:00 AM, in CRI patients in the morning of dialysis. From the samples lipid parameters, liver enzymes, renal function tests, and inflammatory parameters were determined in the Central Laboratory of the Kenézy Hospital and in the Department of Laboratory Medicine of the University of Debrecen. From the sera we measured PON1's activity in our Research Laboratory.

Lipid parameters

Serum total cholesterol and triglyceride were determined with enzymatic, colorimetric tests, HDL-C with a homogeneous, enzymatic method (HDL plus 3rd generation, Modular P-800 analyzer, Roche Ltd.), the ratio of LDL-C was calculated with the Friedewald equation in the first and second part, and measured with a direct, enzymatic method in the third, apolipoproteins were determined with an immune-turbidimetric method (Tina-Quant ApoA and ApoB Version 2, Roche Ltd.).

Paraoxonase and arylesterase activity

To determine PON1's paraoxonase (PON) activity we measured the conversion of paraoxon to 4-nitrophenol using a Hewlett-Packard 8453 UV-visible spectrophotometer. One unit (1 U) of PON activity equals to 1 µmol 4-nitrophenol formed per minute.

To evaluate PON1's arylesterase (Aryl) activity we measured the breakdown of phenylacetate. One unit (1 U) of Aryl activity equals to 1 µmol phenylacetate catabolized per minute.

Phenotypic and genotypic distribution

We measured PON1's phenotypic and genotypic distribution with the dual substrate method. Depending on the PON1-192QR polymorphism two allozymes are synthesized: A (low activity) and B (high activity). Based on the ratio of the enzyme's paraoxonase activity in the presence of 1 mmol/L NaCl (salt-stimulated), and its arylesterase activity, we identify individuals with AA (low PON1 activity), AB (intermediate PON1 activity), and BB (high PON1 activity).

We determined the PON1-55 and the PON1-192 polymorphisms using a PCR method combining fluorescence resonance energy transfer (FRET) and melting point analysis (Light Cycler real-time technology). We performed the amplification of the PON1 gene's regions surrounding the PON1-55 and PON1-192

polymorphisms resulting in 151- and 138-bp amplicons (School of Public Health, TIB Molbiol Co. and Roche).

Thiobarbituric acid reactive substances and oxidized LDL

We measured the amount of TBARS with a spectrophotometric method (Hewlett Packard 8453 spectrophotometer), and oxLDL with a sandwich ELISA (enzyme-linked immunosorbent) assay (WAK-Chemie Medical GmBH).

Homocysteine and cystatin C

Homocysteine and cystatin C was measured in the Department of Laboratory Medicine, and in the Central Laboratory of the Kenézy Hospital (fluorescence polarization immunoassay (FPIA, Abbott), and immunoturbidimetric assay (PETIA, Bühlmann)).

Statistical methods

In the first part of our study, PON1-55 and PON1-192 allele frequencies were calculated by gene counting, and we checked the validity of the Hardy-Weinberg equilibrium model using χ^2 test. In the second and third studies we used the Kolmogoroff-Smirnoff normality test to check whether parametric tests are applicable, and Levene's test for the equality of variances. Since most parameters were not normally distributed in the second study, we applied the nonparametric Kruskal-Wallis test to check the dependence of the parameters on the presence of renal failure or transplant, medians and quartiles to describe the non-Gaussian data groups, and Pearson's χ^2 test to compare gender, smoking, and phenotype. In the third study, the unpaired t-test and Spearman's regression analysis was used to compare groups and continuous variables. Lipid parameters with a skewed distribution were transformed logarithmically to normalize their distribution. Multiple regression analysis was performed to determine which variables were the best predictors of PON1 activity.

Results

Genotype and phenotype of human paraoxonase-1 in the general population and chronic renal insufficiency

LDL-C, HDL-C, ApoA1 and ApoB was significantly higher in transplanted patients compared to CRI patients ($p<0.005$).

The allelic frequencies were similar to our previous findings and other studies in case of genotype and phenotype as well, and the results were in in accordance with the Hardy-Weinberg equilibrium. There was no significant difference between the distributions of our patient groups.

The difference between phenotypic and genotypic distributions exceeded our expectations. The discordance was 29.58% (413/1396) in the whole studied population, 30.9% (350/1133) in controls, 28.2% (33/117) in CRI, 20.55% (30/146) in TX. In the patient gorups we observed the greatest discordance in case of AB- and BB-phenotypes (20.51% in CRI and 17.1% in TX), while in controls the greatest difference was in case of AA- (18.1%) and AB-phenotypes (12.8%). In the whole studied population the genotypic differences were as follows: 5,37% in cas of Gln192 (PON-192-QQ), 17.12% in case of Gln192Arg (PON-192-QR), and 7.1% in case of Arg192 (PON-192-RR).

PON and salt stimulated PON activity did not differ significantly in CRI and TX compared to C. Arylesterase activity was significantly lower in CRI compared to controls ($p<0.001$).

Low HDL and human paraoxonase-1 in chronic renal insufficiency and primary dyslipidemia

Arylesterase activity, which is propotionate to PON1 enzyme protein quantity, showed a significant correlation with the presence of chronic renal insufficiency, or renal transplantation both in the normal ($p<0.05$) and in the low HDL group ($p<0.005$). Arylesterase activity was significantly lower in CRI than in dyslipidemia, TX patients were between the other two groups. The

arylesterase/HDL-C ratio (Aryl/HDL-C) also correlated significantly with the presence of chronic renal insufficiency, or renal transplantation both in the normal (p<0.005) and in the low HDL group (p<0.05). Aryl/HDL-C was significantly higher in low HDL-C patients than in case of normal HDL-C (p<0.05).

In contrast to arylesterase activity paraoxonase activity (PON) and the PON/HDL-C ratio did not depend significantly on the presence of chronic renal insufficiency, or renal transplantation (neither in the normal, nor in the low HDL group). There was no significant difference between the paraoxonase activity of the low and the normal HDL-C groups, however, in low HDL-C CRI patients the paraoxonase/HDL-C ratio was significantly higher, than in normal HDL-C CRI patients (p<0,05). There was no significant difference between the paraoxonase activity of the low and normal HDL-C groups in case of DL and TX groups.

Phenotypic distributions did not differ significantly between the patient groups.

In normal HDL-C patients LDL-C and total cholesterol depended significantly on the presence of chronic renal insufficiency, or renal transplantation (p<0.005), while in low HDL-C patients the difference was not significant. Triglyceride correlated significantly with the presence of chronic renal insufficiency, or renal transplantation both in case of normal (p<0.05), as well as low HDL-C (p<0.005) esetén, with lower TG values in CRI. In case of low HDL-C TG tended to be better in all three patient groups. There was a significant difference in ApoB and ApoA1 as well, both parameters were lowest in CRI (p<0.05). In the low HDL-C patient groups ApoA1 was significantly lower than the corresponding normal HDL-C gorups.

Cystatin C, homocysteine and human paraoxonase-1 in chronic renal insufficiency

We discovered a negative correlation between serum cystatin C and paraoxonase activity both in CRI, and in TX patients (CRI: r=–0.42, p<0.05; TX: r=–0.34, p<0.05). Homocysteine and PON activity correlated negatively in both patient groups (CRI: r=–0.48, p<0.05; TX: r=–0.37, p<0.05). Cystatin C correlated

positively with homocysteine in case of both patient groups (CRI: r=0.53, p<0.05; TX: r=0.32, p<0.05).

Cystatin C, which represents the severity of renal impirment, was significantly increased in CRI and TX compared to C. PON activity was significantly lower in dialyzed patients compared to controls, and to a lesser degree to transplanted patients as well. Homocysteine was elevated in both patient groups. Serum homocysteine in CRI patients significantly exceeded that of both controls and TX patients, and the homocysteine of transplanted ones was significantly higher than that of controls.

Serum triglyceride, total cholesterol, TG, LDL-C, ApoB, creatinine and uric acid was significantly higher, HDL-C and ApoA1 was significantly lower in CRI compared to C, TX was between the other two groups. TBARS and oxLDL, which characterize oxidative processes, were significantly higher in CRI and TX patients compared to C.

Discussion

Genotype and phenotype of human paraoxonase-1 in the general population and chronic renal insufficiency

Previous studies found the allelic frequencies of PON1-192 in healthy individuals as follows: QQ: 51.7%, QR: 40.6%, RR: 7.7%; and for PON1-55: LL: 49.3%, LM: 38.6%, MM: 12.1%.

- Genotypic distribution was similar in our Hungarian control group as previously described.

The role of PON1 in the atherosclerotic process polymorphisms is contradictious. The enzyme's phenotype, PON activity and concentration are better predictor of vascular disease than the PON1-192, or PON1-55 genotype.

- A previous publication reported 7.2% discordance between genotypic and phenotypic distributions of healthy individuals, so the great discordance in all three of our studied groups is an unexpected result.

Presumably the role of environmental (alimentary, geographical and pharmacological) factors determining PON activity is so strong that the phenotypic distribution doesn't represent the PON1-192 allelic distribution.

Hemodialysis is accompanied by decreased antioxidant defense and the production of reactive oxygen species. PON1 inhibits the oxidative modification of LDL through the hydrolysis of lipid peroxides; however, its paraoxonase and arylesterase activity significantly decreases in CRI. Previous research concerning the PON1-status of patients with chronic renal insufficiency or renal transplant has been relatively scarce.

- We found no difference of the PON/HDL, or the Aryl/HDL ratios between dialyzed patients and controls, neither in PON1's phenotypic distribution.

In our previous study not only PON activity, but the PON1/HDL ratio also turned out to be significantly lower in CRI, while phenotypic distribution did not differ significantly from healthy controls. Some other groups described similar, others reported significantly lower values of PON1's paraoxonase, salt-stimulated PON,

and arylesterase activity, as well as the more frequent occurrence of the B allele in dialyzed patients. Most researchers measured the activity of PON1 without phenotyping or genotyping.

- In the present study we found no significant difference of PON, or salt-stimulated PON activity in either of the patient groups compared to controls, while the arylesterase activity was significantly lower in CRI compared to C (-22%, $p<0.001$).

The control group we used in this study represented the general adult population in the distribution of age and gender. Previous studies found strong correlation between Aryl activity and the plasma concentration of PON1. In CRI the synthesis of the enzyme can decrease because of altered regulation.

- In the present study there was no significant difference between the PON, salt-stimulated PON, or Aryl activity of the transplanted and control groups.

Consequently, the increasing cardiovascular mortality after renal transplantation might be caused by factors independent from PON1 status, e.g. permanent immunosuppressive treatment, or post-transplant dyslipidemia. In previous studies the main predictors of PON activity were PON1-192, PON1-107, and PON1-55 genotype, and to a lesser degree, alcohol consumption, smoking, and serum HDL concentration. In the present study various factors modifying PON1 phenotype could be present in the control group (dyslipidemia, alcohol consumption, and smoking). In the CRI and TX groups the metabolic changes resulting from the underlying disease might produce different phenotypic distribution even in case of similar genetic background. In future studies simultaneous determination of phenotype and genotype is necessary.

Low HDL and human paraoxonase-1 in chronic renal insufficiency and primary dyslipidemia

As PON1 activity decreases with the severity of renal impairment, the question arose if low HDL and ApoA1 account for the decreased quantity and paraoxonase activity of PON1 enzyme proteins in chronic renal insufficiency. Decreased

antioxidant activity and the consequent LDL-oxidation may contribute to the accelerated atherosclerosis in CRI.

- In the present study the decreased arylesterase activity we observed in CRI compared to controls means that although the quantity of PON1 enzyme proteins decrease, this does not depend significantly on HDL-C, so the decreased antioxidant defense cannot solely be attributed to dyslipidemia with low serum HDL.

- We observed higher arylesterase activity in TX patients compared to CRI, which implies that transplantation can favorably affect enzyme synthesis.

The decreased enzyme activity observed in CRI is explicable by the retention of uremic toxins, glycation end-products, free radicals and modified peptides, as well as increased protein homocysteinylation. Oxidized lipids and homocysteine thiolactone can inactivate PON1, resulting in decreased serum paraoxonase activity and impaired antioxidant capacity of HDL.

- In the present study the enzyme's PON activity did not depend significantly on the presence of chronic renal insufficiency, or transplant compared to dyslipidemic controls, which implies that in spite of the decreased quantity of enzyme proteins the antioxidant defense was not significantly lower in kidney disease compared to primary dyslipidemia.

- In CRI the low HDL-C patients' decreased paraoxonase activity compared to normal HDL-C CRI patients turned out to be significantly higher when normalized to HDL-C, which implies that increasing HDL in chronic renal insufficiency can improve the antioxidant capacity.

- Qualitative and quantitative changes of apolipoproteins (e.g. ApoA1) indicates a structural difference in lipoprotein particles among CRI, TX and DL groups, which can be the result of altered protein synthesis and metabolism.

The different distribution of HDL subfractions can influence PON1 activity (its concentration in HDL3>in HDL2). Alternative antioxidant pathways (e.g. LCAT)

can have a more important role in the inactivation of prooxidants in case of low HDL.

- A previous study reported the incidence of the B allele to be more frequent in CRI, however, our present results, in concurrence with other publications, found no significant difference among the patient groups' phenotypic distribution.

Low HDL CRI patients' decreased Aryl activity compared to dyslipidemic controls is a novel result, which is still significant when corrected to HDL-C. We found no significant difference between PON1's paraoxonase activity between CRI and DL, so this cannot explain the even higher cardiovascular morbidity of dialyzed patients compared to primary dyslipidemia. Higher CRP, atherogenic lipid profile, unfavorable distribution of HDL and LDL subpopulations, as well as increased oxidative stress all contribute to the increased cardiovascular risk.

Because of PON1's decreased thiolactonase activity protein homocysteinylation increases in CRI, and this accelerates atherosclerosis compared to dialyzed patients with near normal homocysteine.

Increasing PON1 activity can delay the progress of renal impairment, and its complications. Therapeutic interventions that restore HDL's physiological concentration, function and the distribution of subfractions, increasing PON1 activity, LDL size, and decreasing the amount of triglyceride-rich remnants, are of considerable importance in chronic renal failure. Proper nutrition, cessation of smoking, statins, fibrates, ezetimibe and nicotinic acid can be favorable. In CRI the differences of the underlying disease, lipid profile, potential interactions, and altered lipoprotein metabolism justify a different therapeutic approach. It is especially important to define subclasses with very high cardiovascular risk, where therapeutic benefits are likely to exceed the risk of side effects.

Cystatin C, homocysteine and human paraoxonase-1 in chronic renal insufficiency

In CRI and after TX cystatin C correlates with cardiovascular mortality and the severity of renal impairment, which leads to the decreased renal elimination of homocysteine.

- In the present study we found elevated cystatin C and homocysteine in chronic renal failure similarly to previous results, while the elevation was less severe in transplanted patients compared to controls.

- We found negative correlation between serum cystatin C and homocysteine similarly to previous results.

The metabolism of homocysteine is impaired in CRF because of the decreased activity of metabolic enzymes caused by oxidative stress, homocysteinylation, and decreased cofactor vitamin B12, B6 and folic acid. Some studies described inverse correlation between homocysteine and PON1's arylesterase activity, others found no significant correlation. They proposed that the negative effect of homocysteine on PON1's activity is only prominent in case of pathologically increased serum homocysteine.

- In the present study we found negative correlation between PON1's paraoxonase activity and serum homocysteine, which supports our previous observations.

Another group reported that the determinants of PON1's homocysteine thiolactonase activity were total homocysteine, age, and total cholesterol, but not HDL. They found that PON1's homocysteine thiolactonase activity correlated with its paraoxonase activity. PON1's homocysteinylation results in decreased enzyme activity and the HDL-associated proteins' (also PON1's) increased sensitivity to oxidative damage and further homocysteinylation, consequential accumulation of endogen lactones (e.g. homocysteine thiolactone), which forms homocysteine-thiolactone-LDL-aggregates with LDL, and these promote foam cell formation in atherosclerotic plaques.

- Our results support the initial hypothesis about the correlation of PON activity and cystatin C.

The relationship is explicable by the increased oxidative stress and homocysteinylation that leads to decreased PON1 activity and oxidative modification of HDL. Decreasing renal function leads to higher cystatin C, which, however, can only partly inhibit the accelerating atherosclerotic process.

- Elevated TBARS and oxLDL in chronic renal failure implies increasing oxidative damage, which is characterized by increased serum concentration of the prooxidant homocysteine, decreased activity of the antioxidant PON1, and elevated cystatin C resulting from impaired renal function. Renal transplantation can only partly restore these changes. Our results are in concordance with previous findings.

In hemodialyzed and transplanted patients the correlation between serum PON activity and cystatin C is a novel result. A new aspect is our observation that in renal failure cystatin C is not only a good predictor of homocysteine, but of antioxidant status as well.

- Consequently, we can conclude that the determination of serum cystatin C is advisable in hemodialyzed and renal transplanted patients to characterize renal function and antioxidant status.

Summary

Chronic renal insufficiency (CRI) is one of the most important cardiovascular risk factors. Previously in CRI our research group discovered decreased activity of the antioxidant and antiatherogenic human paraoxonase-1 enzyme (PON1). We aimed to clarify the role of the enzyme's genotype and phenotype in the changes of PON1 activity in CRI compared to the general population. The elimination of cystatin C is decreased in CRI, rendering it an important marker of renal impairment, and consequently correlating positively with cardiovascular events. Cystatin C has been discovered to stabilize atherosclerotic plaques, thus protecting against atherogenesis. Proatherogenic hyperhomocysteinemia is common in CRI, and it induces endothelial dysfunction. We investigated the relationship between cystatin C, homocysteine and PON1. Low HDL is common in CRI, so to determine the effect of HDL on PON1 activity, we compared PON1 activity in CRI to primary dyslipidemia (DL) and low HDL-cholesterol (HDL-C) rather than healthy individuals.

Unexpected discordances between phenotype and genotype were observed. There were no significant differences between patients and controls in genotypes and phenotypes. We concluded that both phenotype and genotype determinations are necessary to estimate PON1 status. The metabolic changes in CRF result in an inverse relationship between PON1 and cystatin C as well as PON1 and homocysteine, and a positive correlation between cystatin C and homocysteine. Thus, cystatin C may be a predictor of both homocysteine and the antioxidant status in CRI and renal transplantation. We saw similar changes of PON1's paraoxonase activity in CRI and in DL, while PON1's arylesterase activity was more seriously impaired in CRI. This implies that the hepatic synthesis of PON1 enzyme proteins and the correlating arylesterase activity is decreased in CRI; however, this is not explicable by decreased HDL-C. The increased cardiovascular morbidity of patients with CRF and low HDL-C is not attributable to changes in PON1 activity, nor to phenotypic distribution.

Candidate: Éva Varga

Neptun ID: JG73XT

Doctoral School: Doctoral School of Health Sciences

List of publications related to the dissertation

1. **Varga, É.**, Seres, I., Harangi, M., Kárpáti, I., Koncsos, P., Sztanek, F., Paragh, G.: Low HDL-cholesterol is not responsible for decreased paraoxonase activity in chronic renal failure.
   Kidney Blood Press. Res. "accepted by publisher", 2011.
   IF:1.5 (2010)

2. Paragh, G., Seres, I., Harangi, M., Pocsai, Z., Asztalos, L., Lőcsey, L., Széles, G., Kardos, L., **Varga, É.**, Kárpáti, I., Ádány, R.: Discordance in human paraoxonase-1 gene between phenotypes and genotypes in chronic kidney disease.
   Nephron Clin. Pract. 113 (1), C46-C53, 2009.
   DOI: http://dx.doi.org/10.1159/000228075
   IF:1.606

3. **Varga, É.**, Seres, I., Harangi, M., Sztanek, F., Asztalos, L., Lőcsey, L., Borbás, B., Szegedi, J., Kárpáti, I., Paragh, G.: Serum cystatin C is a determinant of paraoxonase activity in hemodialyzed and renal transplanted patients.
   Dis. Markers. 26 (3), 141-148, 2009.
   DOI: http://dx.doi.org/10.3233/DMA-2009-0624
   IF:2.026

List of other publications

4. Oláh, A., Asztalos, L., Ivády, G., **Varga, É.**, Kovács, M.Á., Kappelmayer, J., Varga, J.: Monitoring of mycophenolic acid and kidney function during combined immunosuppressive therapy.
Clin. Chem. Lab. Med. 49 (11), 1849-1853, 2011.
DOI: http://dx.doi.org/10.1515/CCLM.2011.678
IF:2.069 (2010)

5. Káplár, M., Paragh, G., Erdei, A., Csongrádi, É., **Varga, É.**, Garai, I., Szabados, L., Galuska, L., Varga, J.: Changes in cerebral blood flow detected by SPECT in type 1 and type 2 diabetic patients.
J. Nucl. Med. 50 (12), 1993-1998, 2009.
DOI: http://dx.doi.org/10.2967/jnumed.109.066068
IF:6.424

6. Paragh G., **Varga É.**, Márk L.: Az LDL-koleszterin jelentősége a kardiovaszkuláris események gyakoriságának csökkentésében.
Kardiovaszk. Prev. Rehab. 1 (2), 26-35, 2009.

Total IF: 13.625

Total IF (publications related to the dissertation): 5.132