Universidade Federal do Rio Grande do Sul
Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

ESTUDO DO EFEITO DO POLIMORFISMO K121Q NO GENE *ENPP-1* NA EXPRESSÃO DESTA PROTEÍNA EM CÉLULAS RENAIS

Dissertação de Mestrado

Denise Alves Sortica

Porto Alegre, março de 2013

Universidade Federal do Rio Grande do Sul
Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

ESTUDO DO EFEITO DO POLIMORFISMO K121Q NO GENE *ENPP-1* NA EXPRESSÃO DESTA PROTEÍNA EM CÉLULAS RENAIS

Denise Alves Sortica

Orientadores: Prof. Dr. Luis Henrique Canani

Prof[a]. Dr[a]. Daisy Crispim Moreira

Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Mestre em Endocrinologia.

Porto Alegre, março de 2013.

Dedico essa dissertação aos meus pais, meus exemplos de vida.

“Chegou a tua vez, oh! Natureza!
Eu desafio agora essa grandeza,
Perante a qual meus olhos se extasiam...
Eu desafio, desta cova escura,
No histerismo danado da tortura
Todos os monstros que os teus peitos riam!”

Augusto dos Anjos

AGRADECIMENTOS

Aos meus orientadores Dr. Luís Henrique Canani e Dr[a]. Daisy Crispim, por acreditarem em mim e me dar oportunidade desde a época de iniciação científica, por me apresentar à ciência e por todos os ensinamentos, pela impecável orientação e por ser um exemplo de profissional.

Ao Dr. Jorge Luiz Gross, Dr. Milton Berger, Dra. Maria Isabel Edelweiss, Dra. Claudete Zanatta, Mariana P. Carpena, Bianca de Souza, Letícia A. Brondani, Fabiana Góes Nogueira, Taís Assmann e Guilherme Zaffari pelas fundamentais colaborações que enriqueceram este trabalho.

Aos colegas e amigos do laboratório do Serviço de Endocrinologia pelo incentivo, troca de conhecimentos e valiosas conversas, propiciando um ótimo ambiente de trabalho.

Aos meus pais Antonio e Nilza por sempre me incentivarem a buscar e acreditar nos meus sonhos e pelas sábias lições de amor, honestidade e perseverança.

A minha irmã Ana Helena e ao meu neto Joaquim por existirem na minha vida.

Ao meu filho preferido Paulo Roberto Aymone Filho.

A todos os meus amigos que me apoiaram em minhas decisões e participaram indiretamente na realização desse trabalho, especialmente à Ana Paula Bouças, Bianca Souza, Marisa Teresinha, Alessandra Zucatti, Leticia Follmann, Amanda Brito pela amizade, pelos momentos divertidos e pelas palavras de incentivo. Por fim, agradeço a todos que de alguma forma também contribuíram para a realização deste trabalho.

Esta dissertação de mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de um artigo de revisão e um artigo original sobre o tema da dissertação.

- Artigo de revisão: "The role of ecto-nucleotide pyrophosphatase/phosphodiesterase1 in diabetic nephropathy" (publicado na Revista Arq Bras Endocrinol Metab).

- Artigo original: "The K121Q polymorphism in the Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase 1 (*ENPP1*) gene is not associated with changes of *ENPP1* gene expression in human kidney"..

SUMÁRIO

LISTA DE ABREVIATURAS

3' UTR	3' untranslated region
Akt2	Serine-threonine kinase 2
AU	Arbitrary units
cDNA	Complementary DNA
CI	Confidence interval
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DM	Diabetes mellitus
DN	Diabetic nephropathy
ENPP1	Ecto-nucleotide pyrophosphatase/phosphodiesterase 1
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
HOMA	Homeostasis model assessment
HPLC	High performance liquid chromatography
HWE	Hardy-Weinberg equilibrium
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
mRNA	Messenger RNA
ND	Nefropatia diabética

OR	Odds ratio
PI3K	Phosphatidylinositol 3 kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate;
PKA	Protein kinase A
PTP-1 β	Protein tyrosine phosphatase 1 β
RI	Resistencia à insulina
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SD	Standard deviation
SE	Standard error
SHIP	Src homology 2 domain containing inositol 5 phosphatase
shRNA	Short hairpin RNA
SNP	Single nucleotide polymorphism
TDT	Transmission disequilibrium test
TNF	Tumor necrosis factor
UAE	Uriny albumin excretion

RESUMO

A Nefropatia Diabética (ND) é uma complicação crônica do Diabetes Mellitus (DM) que atinge em torno de 30% destes indivíduos, sendo responsável por mais de um terço dos novos casos de insuficiência renal em indivíduos no início do programa de diálise. Esta complicação é também associada a um aumento significativo da mortalidade nesses pacientes.

Estudos epidemiológicos e de agregação familial têm demonstrado que além dos conhecidos fatores ambientais, ND é uma doença multifatorial com um componente genético. Grandes esforços têm sido feitos para identificar estes genes, mas os resultados ainda são inconsistentes com diferentes genes associados a um efeito pequeno em populações específicas. A identificação de tais genes que permitem a detecção de indivíduos de alto risco para desenvolver ND pode permitir-nos ter uma melhor compreensão da sua fisiopatologia.

A ENPP1 (ecto-nucleotide pyrophosphatase / phosphodiesterase 1) é uma proteína expressa na membrana celular de vários tecidos, incluindo os rins. Constatou-se que níveis aumentados de expressão da ENPP1 inibem a atividade tirosina-quinase do receptor da insulina em vários tipos celulares, causando resistência à insulina. A expressão aumentada de ENPP1 inibe a sinalização da insulina em vários tipos celulares in vitro, parecendo estar fortemente associada ao receptor da insulina na superfície celular. O polimorfismo K121Q (A/C) do gene *ENNP1* está associado com resistência à insulina e com o desenvolvimento da ND em diferentes populações.

Apesar do papel da proteína ENPP1 na patogênese de DN ser de importância vital, ainda são desconhecidos os efeitos do polimorfismo K121Q na expressão deste gene em tecido de rim humano.

No presente estudo, analisamos a expressão gênica e protéica da ENPP1 em biópsias de tecido renal humano de acordo com os diferentes genótipos do polimorfismo K121Q.

Foram incluídos 107 pacientes submetidos à nefrectomia terapêutica para se obter a amostra esperada em cada grupo. Após os critérios de exclusão, 57 amostras (9 Q/Q, 27 Q/K, 20 K/K) foram elegíveis para o estudo e técnica de imunohistoquímica a fim de analisar a expressão protéica da ENPP1. Posteriormente foram selecionadas 34 amostras (9 Q/Q, 12 Q/K, 13 K/K) para investigar a expressão do gene *ENPP1*, através da técnica de PCR em tempo real.

A expressão gênica da ENPP1 no rim não diferiu significativamente entre os portadores do alelo Q *vs.* K/K (QQ/KQ vs. K/K; 1.0 (0.1 – 1.6) *vs*. 0.72 (0.1 – 2.1) AU (unidades arbitrárias), respectivamente; P = 0.483). Da mesma forma, a expressão protéica da ENPP1 não diferiu em portadores (K/Q + Q/Q) quando comparados a não portadores (K/K) do genótipo de risco (2.86 ± 0.32 vs. 2.78 ± 0.35 AU; P = 0.417).

Em conclusão, nosso resultado sugere que o polimorfismo K121Q não está associado a mudanças de expressão gênica ou protéica no rim humano.

INTRODUÇÃO

A Nefropatia Diabética (ND) é uma complicação crônica do Diabetes Mellitus (DM) que atinge em torno de 30% destes indivíduos, sendo responsável por mais de um terço dos novos casos de insuficiência renal em indivíduos no início do programa de diálise. Esta complicação é também associada a um aumento significativo da mortalidade nesses pacientes (1).

Os tratamentos em busca do melhor controle glicêmico e da pressão arterial, associado ao uso de inibidores do sistema renina-angiotensina, tem retardado o surgimento e progressão para ND terminal nos últimos anos (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm_5443a2.htm), porém uma grande proporção de pacientes com DM ainda desenvolve essa complicação.

Estudos de agregação familiar mostram uma importante concordância para o desenvolvimento de ND em algumas famílias e reforçam a hipótese de que existem fatores genéticos envolvidos na sua patogênese (2, 3). Indivíduos com DM que possuem familiares diabéticos com ND mostram risco aumentado de desenvolver esta complicação em comparação com indivíduos com DM sem familiares com ND (2, 4, 5, 6). Acredita-se que os indivíduos com DM tipo 2 que não desenvolvem esta complicação nos primeiros 10 a 15 anos após o início da doença parecem estar protegidos geneticamente (5).

Dados epidemiológicos também indicam suscetibilidade genética para o desenvolvimento da ND uma vez que existe um pico de incidência entre 15 a 20 anos após o diagnóstico de DM, seguido por um rápido declínio, resultando em uma incidência cumulativa de menos de 30% (7, 8). Se a ND fosse causada apenas pela hiperglicemia, sua incidência aumentaria progressivamente ao longo do tempo e a maioria dos pacientes com DM desenvolveriam doença renal, similarmente ao que

acontece com a retinopatia diabética (9). A complexidade da ND depende do efeito de muitas variáveis genéticas atuando sinergicamente e aditivamente uma com a outra e com fatores ambientais (10).

Neste sentido, há vários métodos de se pesquisar o papel de genes na suscetibilidade para a ND (11, 12, 13, 14). Uma estratégia frequentemente utilizada é a do gene candidato (15, 16). Grandes esforços têm sido feitos para identificar estes genes, mas os resultados ainda são inconsistentes com diferentes genes associados a um efeito pequeno em populações específicas. A identificação de tais genes que permitem a detecção de indivíduos de alto risco para desenvolver ND pode permitir-nos ter uma melhor compreensão da sua fisiopatologia.

A ENPP1 (ectonucleotídeo pirofosfatase/fosfodiesterase 1) é uma proteína expressa na membrana celular de vários tecidos, incluindo os rins. Constatou-se que níveis aumentados de expressão da ENPP1 inibem a atividade tirosina-quinase do receptor da insulina em vários tipos celulares, causando resistência à ação da insulina (RI). A expressão aumentada de ENPP1 inibe a sinalização da insulina em vários tipos celulares *in vitro*, parecendo estar fortemente associada ao receptor da insulina na superfície celular (17).

Dados publicados por Pizzuti *et al*. descreveram um polimorfismo no éxon 4 do gene *ENPP1*, o qual causa a troca do aminoácido lisina (K) para glutamina (Q) no códon 121 (K121Q) (18). Essa troca de aminoácido está localizada no segundo domínio *somatomedin-B-like* da ENPP1 e pode interferir com as interações proteína-proteína. Estudos *in vitro* demonstraram que a variante Q do gene *ENPP1* interage mais fortemente com o receptor da insulina do que a variante K e reduz a autofosforilação do receptor da insulina, indicando que o polimorfismo K121Q (A/C) do gene *ENNP1* está associado com RI e com o desenvolvimento da ND em diferentes populações (18).

Desta forma, o gene *ENPP1* torna-se um possível gene candidato para o desenvolvimento da ND. Apesar do papel da proteína ENPP1 na patogênese da ND parecer ser relevante, ainda são desconhecidos os efeitos do polimorfismo K121Q na expressão deste gene em tecido renal humano.

O trabalho desenvolvido nesta dissertação teve como objetivo revisar e aprofundar nosso conhecimento sobre o papel da ENPP1 na ND (artigo de revisão), com foco no efeito do polimorfismo K121Q do gene *ENPP1* em tecido renal humano (artigo original).

REFERÊNCIAS

1. Bloomgarden ZT 2008 Diabetic nephropathy. Diabetes Care 31:823-827

2 Canani LH, Gerchman F, Gross JL 1999 Familial clustering of diabetic nephropathy in Brazilian type 2 diabetic patients. Diabetes 48:909-913

3. Yajima Y, Nakajima S 1997 [Familial clustering and genetics of diabetic nephropathy]. Nippon Rinsho 55 Suppl:795-801

4. Seaquist ER, Goetz FC, Rich S, Barbosa J 1989 Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. N Engl J Med 320:1161-1165

5. Krolewski AS 1999 Genetics of diabetic nephropathy: evidence for major and minor gene effects. Kidney Int 55:1582-1596

6. Quinn M, Angelico MC, Warram JH, Krolewski AS 1996 Familial factors determine the development of diabetic nephropathy in patients with IDDM. Diabetologia 39:940-945

7. Krolewski AS, Warram JH, Christlieb AR, Busick EJ, Kahn CR 1985 The changing natural history of nephropathy in type I diabetes. Am J Med 78:785-794

8. Krolewski AS, Warram JH, Rand LI, Christlieb AR, Busick EJ, Kahn CR 1986 Risk of proliferative diabetic retinopathy in juvenile-onset type I diabetes: a 40-yr follow-up study. Diabetes Care 9:443-452

9. Klein R, Klein BE, Moss SE, Davis MD, DeMets DL 1984 The Wisconsin epidemiologic study of diabetic retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. Arch Ophthalmol 102:520-526

10. Martini S, Eichinger F, Nair V, Kretzler M 2008 Defining human diabetic nephropathy on the molecular level: integration of transcriptomic profiles with biological knowledge. Rev Endocr Metab Disord 9:267-274

11. Conway BR, Savage DA, Maxwell AP 2006 Identifying genes for diabetic nephropathy--current difficulties and future directions. Nephrol Dial Transplant 21:3012-3017

12. Lim SC, Liu JJ, Low HQ, Morgenthaler NG, Li Y, Yeoh LY, Wu YS, Goh SK, Chionh CY, Tan SH, Kon YC, Soon PC, Bee YM, Subramaniam T, Sum CF, Chia KS 2009 Microarray analysis of multiple candidate genes and associated plasma proteins for nephropathy secondary to type 2 diabetes among Chinese individuals. Diabetologia 52:1343-1351

13. Pezzolesi MG, Poznik GD, Mychaleckyj JC, Paterson AD, Barati MT, Klein JB, Ng DP, Placha G, Canani LH, Bochenski J, Waggott D, Merchant ML, Krolewski B, Mirea L, Wanic K, Katavetin P, Kure M, Wolkow P, Dunn JS, Smiles A, Walker WH, Boright AP, Bull SB, Doria A, Rogus JJ, Rich SS, Warram JH, Krolewski AS 2009 Genome-wide association scan for diabetic nephropathy susceptibility genes in type 1 diabetes. Diabetes 58:1403-1410

14. He B, Osterholm AM, Hoverfalt A, Forsblom C, Hjorleifsdottir EE, Nilsson AS, Parkkonen M, Pitkaniemi J, Hreidarsson A, Sarti C, McKnight AJ, Maxwell AP, Tuomilehto J, Groop PH, Tryggvason K 2009 Association of genetic variants at 3q22 with nephropathy in patients with type 1 diabetes mellitus. Am J Hum Genet 84:5-13

15. Freedman BI, Bostrom M, Daeihagh P, Bowden DW 2007 Genetic factors in diabetic nephropathy. Clin J Am Soc Nephrol 2:1306-1316

16. Susztak K, Sharma K, Schiffer M, McCue P, Ciccone E, Bottinger EP 2003 Genomic strategies for diabetic nephropathy. J Am Soc Nephrol 14:S271-278

17. Goldfine ID, Maddux BA, Youngren JF, Reaven G, Accili D, Trischitta **V**, *et al.* The role of membrane glycoprotein plasma cell antigen 1/ectonucleotide

pyrophosphatase phosphodiesterase 1 in the pathogenesis of insulin resistance and related abnormalities. Endocr Rev2008 Feb; 29 (1):62-75.

18. Pizzuti A, Frittitta L, Argiolas A, Baratta R, Goldfine ID, Bozzali M**,** *et al*. A polymorphism (K121Q) of the human glycoprotein PC-1 gene coding region is strongly associated with insulin resistance. Diabetes1999 Sep; 48 (9):1881-4.

PARTE I

Artigo de revisão

THE ROLE OF ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE 1 IN DIABETIC NEPHROPATHY

O PAPEL DA ECTONUCLEOTÍDEO PIROFOSFATASE/FOSFODIESTERASE 1 (ENPP1) NA NEFROPATIA DIABÉTICA

THE ROLE OF ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE 1 IN DIABETIC NEPHROPATHY

Denise Alves Sortica, Daisy Crispim, Guilherme Pozueco Zaffari, Rogério Friedman, Luis Henrique Canani

Endocrinology Division, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul. Porto Alegre, RS, Brazil. Emails: denisefarm@hotmail.com(LAB).

*** Correspondence:** Dr. Luis Henrique Canani. Rua Ramiro Barcelos 2350; prédio 12; 4° andar. CEP 90035-003. Porto Alegre, RS, Brazil. Phone number: +55 51 33598318. Fax number: 55 5133598777. E-mail: luishenriquecanani@gmail.com

Artigo publicado nos Arquivos Brasileiros de Endocrinologia e Metabologia, 2011, vol. 55.

ABSTRACT

The increased prevalence of diabetes mellitus has caused a rise in the occurrence of its chronic complications, such as diabetic nephropathy (DN), which is associated with elevated morbidity and mortality. Familial aggregation studies have demonstrated that besides the known environmental risk factors, DN has a major genetic component. Therefore, it is necessary to identify genes associated with risk for or protection against DN. Ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is expressed in several tissues, including the kidneys. Increased levels of ENPP1 expression inhibit tyrosine-kinase activity of the insulin receptor in several cell types, leading to insulin resistance. K121Q polymorphism of the *ENPP1* gene seems to be associated with insulin resistance and DN development. The elucidation of genetic factors and their associations will provide better understanding of the pathogenesis of DN and, may consequently, lead to a more effective approach to prevention and treatment.

Keywords: ENPP1; diabetic nephropathy; diabetes mellitus; DNA polymorphisms; chronic renal disease; insulin resistance

INTRODUCTION

Diabetes mellitus (DM) is characterized by chronic hyperglycemia resulting from defects in both insulin secretion and action (1). Depending on the intensity and time of exposure to hyperglycemia, structural lesions can occur in the vascular endothelium and neuronal tissue, leading to the onset of diabetic chronic complications and, ultimately, causing dysfunction and failure of several organs and tissues. These complications can be divided into microangiopathic and macroangiopathic, and they are the most common causes of morbidity and mortality in diabetic patients. Among the most important diabetic chronic complications is diabetic nephropathy (DN) (1).

DIABETIC NEPHROPATHY (DN)

Chronic kidney disease (CKD) is defined as kidney damage resulting from structural or functional abnormalities of the kidneys, or by glomerular filtration rate (GFR) < 60 ml/min/1.73 m2, with or without structural kidney damage, for a period of 3 months or more, independent of cause (2). DN is the main cause of kidney disease in patients entering dialysis programs (2). Traditionally, DN is characterized by physiopathological changes resulting from the diabetic milieu, which begin with glomerular hyperfiltration and kidney hypertrophy, and tend to progress to proteinuria and progressive GFR reduction (2). Hyperglycemia, elevated blood pressure levels and genetic predisposition are major risk factors for DN (2, 3). In addition, elevated levels of serum lipids, smoking, and the amount and source of dietary protein also appear to be risk factors for developing DN (2).

DN incipient stage is characterized by a small increase in urinary protein excretion, mainly albumin. This small amount of protein (known as microalbuminuria) is not detected by total urinary protein measurements. Only very sensitive tests (immunoassays or HPLC

techniques) are able to detect these small amounts of albumin in urine. The alteration may be present at any time in DM, but is more frequently detected after at least five years of disease evolution. The evolution of microalbuminuria is very variable. Without intervention in patients with type 1 DM, microalbuminuria can evolve to clinical proteinuria (or macroalbuminuria) over a period of approximately 10 years. It is only then that conventional urine tests begin to detect the alteration. During microalbuminuria stage, no change in GFR is expected, but a progressive decline in GFR occurs in the proteinuric phase, which may ultimately evolve to end-stage renal disease (ESRD) within another 10 years (4). However, more recent knowledge suggests that this progression is not so well-defined. About one third of subjects with microalbuminuria will spontaneously regress to normoalbuminuria, and approximately the same proportion will remain stable in the microalbuminuria stage (5). It is also known that a proportion of subjects will present reduced GFR in the absence of increased urinary albuminuria (6).

DN affects around 30% of diabetic patients and is responsible for over a third of new cases of kidney failure in individuals entering dialysis programs. It is associated with a major increase in mortality, mainly from cardiovascular causes (7). Bruno and Gross followed up 111 diabetic patients beginning dialysis at different centers for an average 3.6 years, and observed that DN was the primary kidney disease in 61% of the cases (8). The mean survival rate in the 1st, 2nd and 3rd year of follow-up was 69%, 51% and 28%, respectively. Macrovascular complications were the main predictors of mortality in this cohort, and cardiovascular disease was the main cause of death (63% of the cases) (8).

Between 25 and 40% of type 1 DM patients develop DN after 25 years of disease, and DN is the main cause of death in these patients (9). The cumulative incidence of microalbuminuria in type 1 DM patients was 12.6% in a 7.3 years follow-up, according to

The European Diabetes (EURODIAB) Prospective Complications Study Group (10). Proteinuria or macroalbuminuria occur in 15% to 40% of type 1 DM patients, with a peak incidence at 15-20 years of DM (11). In type 2 DM, up to 33% of patients presented DN after an 18-year follow-up, in a Danish study (12). In patients with type 2 DM, the prevalence of DN varies from 20% to 50%, depending on the ethnic origin (12). The anual incidence of proteinuria was 2.0%, and the prevalence after 10 years of type 2 DM diagnosis was 25% in the UK Prospective Diabetes Study (UKPDS) cohorts (13). The prevalence of proteinuria can vary between 5% to 20% (13).

GENETICS OF THE DIABETIC NEPHROPATHY

Familial aggregation studies show major agreement for the development of DN in some families, strengthening the hypothesis that there are genetic factors involved in its pathogenesis (14). Diabetic patients who have relatives with both DM and DN present a significantly greater risk of developing renal disease compared with diabetic patients who have no relatives with this complication (14, 15). Epidemiological data indicate that there is a genetic susceptibility to the development of DN, because the incidence peaks between 15 and 20 years after the diagnosis of DM, and then declines rapidly, resulting in a cumulative incidence of less than 30% (16). If DN were caused only by hyperglycemia, its incidence would increase progressively over time, and most diabetic patients would develop renal disease, similar to what happens with diabetic retinopathy (17).

Furthermore, patients with DM and DN present a family history of arterial hypertension and cardiovascular disease (CVD) more often than diabetic patients without DN (18). The presence of microalbuminuria is a strong predictor of death by CVD, possibly even stronger than it predicts the development of more severe forms of DN (12, 19).

Therefore, it is necessary to identify genes that may predispose to the development and progression of kidney disease, as well as genes that may be associated with protection against this complication.

It has been estimated that the human genome contains about 25,000 genes and more than 10 million genetic polymorphisms (20). The development of a complex disease such as DN depends on the effect of many genetic variables acting synergistically and additively with each other and with environmental factors (21).

There are several ways of studying the role of genes in relation to DN susceptibility (20). An often used strategy is the candidate gene approach (22). Among the different studies that have evaluated the role of genetics in DN using this approach, we will focus on those related to gene *ENPP1*.

ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE 1 (ENPP1) PROTEIN AND INSULIN RESISTANCE

ENPP1 (ecto-nucleotide pyrophosphatase/phosphodiesterase 1) is one of the five cell-membrane proteins containing an active extracellular site which catalyzes the release of nucleoside-5-phosphatase from nucleotides and their products. These proteins consist of a short terminal NH2 intracellular domain, a single transmembrane domain, two somatomedin-B-like domains, and one COOH-terminal nuclease-like domain (23). ENPP1 is a 230-260 kDa homodimer, and its reduced form has a molecular size of 115-135 kDa, depending on the cell type (24). Human ENPP1 has 873 aminoacids (24).

It is known that ENPP1 is expressed in several tissues, including skeletal tissue, adipose tissue, and liver and kidneys (24). However, the physiological functions of ENPP1 in these tissues have not yet been fully described. It is also expressed, in smaller amounts, in

pancreatic islets, brain, heart, placenta, lungs, epididymis, salivary glands, chondrocytes, lymphocytes, and fibroblasts (24). Increased ENPP1 expression inhibits the tyrosine kinase activity of the insulin receptor in several cells (25, 26), and causes insulin resistance (25, 27, 28).

Several studies have demonstrated the in vitro and *in vivo* action of ENPP1. Increased expression of ENPP1 inhibits insulin signaling in several cell types *in vitro* (26, 29, 30), and appears to be physically associated with the insulin receptor on the cell surface (26) (Figure 1).

ENPP1 seems to play a role in glucose metabolism impairment *in vivo*. Insulin resistant rodents and humans present high levels of expression of this protein (27, 28, 30, 31). Furthermore, genetically modified mice with increased ENPP1 expression in the liver and skeletal muscle show high levels of glucose and insulin, a lower degree of oral glucose tolerance, and a lower uptake of glucose in muscle (32). On the other hand, using a short hairpin RNA (shRNA) linked to an adenoviral vector to nearly completely block of ENPP1 expression in the liver of db/db mice reduced postprandial plasma glucose levels by about 30%, fasting plasma glucose levels by 25%, and significantly improved the oral glucose tolerance rate (33).

The pioneer study of Maddux and cols. was the first to describe a rare case of extreme insulin resistance in a patient whose marked inhibition of the insulin receptor function was due to increased ENPP1 expression (25). The deleterious effects of increased ENPP1 expression on the insulin receptor was confirmed by all subsequent studies performed on human cells (10, 26, 34, 35). Results from preclinical models involving insulin resistant, non-obese individuals without DM (thus avoiding the interference of obesity or metabolic defects resulting from hyperglycemia in ENPP1 expression), enabled the elaboration of the

hypothesis that the increased expression of this protein in insulin resistant humans is an early and intrinsic defect, instead of the result of secondary metabolic alterations associated with the insulin resistance state (27, 28, 31).

THE ASSOCIATION BETWEEN POLYMORPHISMS IN THE ENPP1 GENE AND INSULIN RESISTANCE, TYPE 2 DIABETES MELLITUS, OR DIABETIC NEPHROPATHY

The gene that encodes ENPP1 has 25 exons and is located on chromosome 6q22-23 (36, 37) (Figure 2). This gene is regulated by glucocorticoids, agents that lead to an increase in cAMP, protein kinase C activator, phorbol myristate acetate, growth factors such as fibroblast growth factor, and cytokines, including IL-1β and TNF (revised by (24). Curiously, it appears that insulin levels regulate ENPP1 expression in humans (38). However, treating cells with insulin changes the location of ENPP1 from the intracellular region to the plasma membrane (39).

In 1999, Pizzuti and cols. described a polymorphism in exon 4 of the gene *ENPP1*, which causes the change of the amino acid lysine (K) to glutamine (Q) in codon 121 (K121Q; rs1044498) (36). This amino acid change is located in the second ENPP1 somatomedin-B-like domain, and may interfere with protein-protein interactions (23). Non-diabetic Sicilians carrying the Q variant show less insulin sensitivity than non-carriers (36). Pizzuti and cols. also demonstrated that the Q variant of this polymorphism interacts more strongly with the insulin receptor than the K variant, reducing the autophosphorylation of this receptor (36). Q variant also reduces insulin-induced phosphorylation of the insulin receptor substrate 1 (IRS1), the kinase activity of phosphatidilinositol-3, glycogen synthesis and cell proliferation (35). Other

studies confirmed the association between the K121Q polymorphism and greater insulin resistance in different populations (40-43); however, a study performed in a Danish population did not find any association between this polymorphism and markers of insulin resistance or type 2 DM (44).

It is not clear whether there is a difference in the risk for type 2 DM among subjects who are heterozygous or homozygous for the Q allele of K121Q polymorphism. In fact, the low proportion of Q/Q homozygous individuals observed in most studies (approximately 2%-3% of the general population) does not enable appropriately testing of different genetic models (dominant, additive or recessive), due to the lack of statistical power (45).

Three meta-analyses, published in 2005 and 2006, analyzed case and control studies, and reported that although the results were not homogeneous between the studies, including thousands of adults from different ethnic groups, individuals carrying the Q allele of K121Q polymorphism have an approximately 20% increased risk of developing type 2 DM (46-48).

Certainly, the K121Q polymorphism has been the most studied polymorphism in the *ENPP1* gene. Table 1 summarizes the main results of the studies that evaluated the association between the K121Q polymorphism, and DN or related characteristics.

De Cosmo and cols. reported that K121Q polymorphism has an effect on the rate of kidney function loss in Caucasians with type 1 DM and proteinuria. During the 6.5 years of follow-up, GFR declined faster in Q allele carriers (QQ/KQ) than in non-carriers (49). The mean decline rates were 7.2 and 3.7 ml.min-1.year-1, respectively. With this rapid loss of kidney function, diabetic patients carrying the Q allele would progress from the proteinuria stage to ESRD in fewer years than non-carriers. Furthermore, the effects of this variant were more evident when ESRD developed earlier in the course of type 1 DM (49).

Canani and cols., in both a case-control and afamily study, investigated the association between advanced DN in patients with type 1 DM and K121Q polymorphism frequencies. The authors reported that the Q variant was observed in 21.5% of the control group (without DN), 31.5% of the cases with proteinuria, and 32.2% of the cases with ESRD ($p = 0.012$). After stratification according to DM duration, the risk of developing ESRD earlier, for carriers of Q variant, was 2.3 times higher than the risk for non-carriers (CI 95% 1.2 - 4.6). However, Q variant was not associated with late development of ESRD (50). Similar results were found in the familial study, using the transmission disequilibrium test.

Recently, Wu and cols. also reported the association between the Q variant and increased risk of DN in type 2 DM patients from Taiwan (51). Likewise, De Cosmo and cols., studying patients with type 2 DM from Gargano and Padua (Italy), and from Boston (USA), demonstrated that Q allele carriers from Gargano and Boston presented a greater risk of having a lower GFR (OR = 1.69; CI 95%: 1.1 - 2.6 and OR = 1.50; CI 95%: 1.0 - 2.2; respectively) than non-carriers (52). In the Padua population, results obtained showed the same trend, but did not reach formal statistical significance (OR = 1.77; CI 95%: 0.7 - 4.5). The same group analyzed a sample of type 2 DM patients with abnormal (20 - 5416 μg/min) albumin excretion rate, and observed that patients carrying the Q allele presented more severe DN (53).

However, not all studies agree on the role of this polymorphism on DN, suggesting that its effect may depend on the ethnic group. In fact, the Q (risk) allele frequency varies greatly according to the ethnic group. Q allele frequency among Brazilians of African ancestry is much higher than among those of European ancestry (54). Keene and cols. studied the K121Q polymorphism in a sample of African-Americans with type 2 DM, but did not find a statistically significant association between this polymorphism and ESRD. These authors

studied 48 other polymorphisms spread throughout *ENPP1* gene, and nine of them showed an association with ESRD (55). Leitao and cols. did not find an association between K121Q polymorphism and type 2 DM or its complications (DN or diabetic retinopathy), in a cross-sectional study performed in a Brazilian sample constituted of different ethnic groups (54). In a prospective, 10-year follow-up study of 30 normoalbuminuric and normotensive type 1 DM patients from southern Brazil, de Azevedo and cols., did not find an association between K121Q polymorphism, and the development of new cases of DN or diminished GFR (56). Jacobsen and cols. did not observe any association between the K121Q polymorphism and DN progression in Danish type 1 DM patients (57).

The molecular mechanisms that mediate the association between the Q variant and the development of advanced stages of DN may only be hypothesized. As previously mentioned, ENPP1 is expressed in several tissues and cell types, including mesangial and endothelial cells in the kidneys (50). These cells show progressive pathological changes throughout the evolution from normality to overt diabetic nephropathy, and it is tempting to hypothesize that ENPP1 plays a role in kidney tissue damage in DN.

In vitro studies demonstrated that the ENPP1 Q variant interacts more strongly with the insulin receptor than the K variant. Therefore, cells carrying the Q variant show reduced insulin receptor self-phosphorylation (36), reduced induction of the insulin receptor substrate (IRS)-1 phosphorylation, phosphatidyli nositol 3-kinase activity, glycogen synthesis, and cell proliferation (58). Humans carrying the Q allele have greater insulin resistance and hyperinsulinemia than non-carriers (40). Hyperinsulinemia can stimulate the renal reabsorption of sodium, leading to volume expansion, increased sympathetic-adrenergic activity, and increased expression of angiotensin type II receptor, which hamper peripheral vasodilatation (59). Increased circulating volume and reduced peripheral vasodilatation

eventually predispose to increased blood pressure and loss of the physiological nocturnal blood pressure dipping (59, 60), both acknowledged DN risk factors.

OTHER POLYMORPHISMS IN *ENPP1* GENE ASSOCIATED WITH DIABETES MELLITUS

Meyer and cols. described that a risk haplotype, constituted by three *ENPP1* gene polymorphisms, was associated with type 2 DM in adults and children (total OR for adults + children = 1.58; CI 95% 1.18 - 2.1). This haplotype includes the Q allele of the K121Q polymorphism and two other polymorphisms in the non-encoding region (IVS20delT in the promoter region and A>G+1044TGA in the 3'UTR region), which may possibly be involved in *ENPP1* gene expression (61). Frittitta and cols. reported the association between a haplotype constituted by three polymorphisms in the 3´UTR *ENPP1* region (G2897A, G2906C and C2948T, also called haplotype ACT) and insulin resistance and type 2 DM (OR for type 2 DM = 1.69; CI 95% 1.01 - 2.83) (58).

Bochenski and cols. investigated the association between seven polymorphisms and some haplotypes from a disequilibrium linkage block containing the K121Q polymorphism and the occurrence of type 2 DM in a Polish population, controlling for obesity. In the total sample, neither type 2 DM nor obesity were significantly associated with any of the seven polymorphisms. However, in obese subjects, the K121Q polymorphism was associated with type 2 DM (OR = 1.6; CI 95% 1.0 - 2.6) (62). Furthermore, the T allele of polymorphism rs997509 in intron 1 was also associated with type 2 DM (OR = 4.7; CI 95% 1.3 - 13.9). Interestingly, the haplotype constituted by both rs997509 T and 121Q alleles was the only haplotype associated with risk for type 2 DM (OR = 4.2; CI 95% 1.3 - 13.5). Santoro and cols., studying lean and obese children, observed that those who were carriers of the rs997509

T allele of the *ENPP1* gene presented higher levels of plasma insulin and HOMA-IR (homeostasis model assessment – insulin resistance) and lower insulin sensitivity index compared with children who were homozygous for the most common allele (63). A similar observation was made for the Q variant of the K121 polymorphism. Besides, children carrying the rare rs997509 T allele were at higher risk of developing metabolic syndrome and impaired glucose tolerance than children who were homozygous for the common allele. Evaluating the combined effects of polymorphisms rs997509 and K121Q, which are in strong disequilibrium linkage, the authors demonstrated that the effect on insulin sensitivity was due to the presence of the rs997509 T allele and not due to the K121Q polymorphism (63).

Keene and cols. genotyped 48 polymorphisms in the *ENPP1* gene in African-American type 2 DM patients with ESRD (cases) or without any degree of DN (controls). Nine polymorphisms were associated with ESRD in one or more inheritance models; however, K121Q polymorphism was not one of them. The most significant associations with CKD were observed for rs7754586 and rs1974201 polymorphisms, located in the 3´UTR region or in intron 24, respectively. Furthermore, an association with CKD was found for 13 haplotypes constituted by 2 or more polymorphisms located on the 3´UTR region or intron 24 of the *ENPP1* gene (55).

CONCLUSION

To date, the molecular mechanisms responsible for the association between the ENPP1 121Q variant and the development of advanced stages of DN may only be hypothesized. Not all studies agree on the role of the K121Q polymorphism in the develpment of DN, suggesting that its effect may depend on the ethnic group. Besides, evaluating the combined effects of ENPP1 rs997509 and K121Q polymorphisms, which are in strong

disequilibrium linkage, some studies demonstrated that the effect on insulin sensitivity was due to the presence of the rs997509 T allele and not due to the presence of the K121Q polymorphism. Further studies are needed to clarify these relationships.

Statement: The authors declare that there are no conflicts of interests.

REFERENCES

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care2009; 32 (1):62.

2. Gross JL, *et al*. Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes Care2005;28 (1):164-76.

3. Carpena MP, Rados DV, Sortica DA, Souza BM, Reis AF, Canani LH, et al. Genetics of diabetic nephropathy. Arq Bras Endocrinol Metabol2010 Mar; 54 (3):253-61.

4. Marshall SM. Recent advances in diabetic nephropathy. Postgrad Med J2004;80 (949):624-33.

5. Caramori ML, Fioretto P, Mauer M. The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? Diabetes2000 Sep; 49 (9):1399-408.

6. Kramer CK, Leitao CB, Pinto LC, Silveiro SP, Gross JL, Canani LH. Clinical and laboratory profile of patients with type 2 diabetes with low glomerular filtration rate and normoalbuminuria. Diabetes Care2007 Aug; 30 (8):1998-2000.

7. Bloomgarden ZT. Diabetic nephropathy. Diabetes Care2008 Apr ;31 (4):823-7.

8. Bruno RM, Gross JL. Prognostic factors in Brazilian diabetic patients starting dialysis: a 3.6-year follow-up study. J Diabetes Complications2000 Sep-Oct;14(5):266-71.

9. Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogensen CE, Parving HH. Diabetic nephropathy. Diabetes Care2003 Jan; 26 Suppl 1:S94-8.

10. Grupe A, et al. Inhibition of insulin receptor phosphorylation by PC-1 is not mediated by the hydrolysis of adenosine triphosphate or the generation of adenosine. J Biol Chem1995;270 (38):22085-8.

11. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR. Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int2003 Jan; 63 (1):225-32.

12. Young BA, Maynard C, Boyko EJ. Racial differences in diabetic nephropathy, cardiovascular disease, and mortality in a national population of veterans. Diabetes Care2003 Aug;26(8):2392-9.

13. Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T. Diabetic nephropathy in Type 1 (insulin-dependent) diabetes: an epidemiological study. Diabetologia1983 Dec; 25 (6):496-501.

14. Canani LH, Gerchman F, Gross JL. Familial clustering of diabetic nephropathy in Brazilian type 2 diabetic patients. Diabetes1999 Apr; 48 (4):909-13.

15. Krolewski AS. Genetics of diabetic nephropathy: evidence for major and minor gene effects. Kidney Int1999 Apr; 55 (4):1582-96.

16. Krolewski AS, Warram JH, Rand LI, Christlieb AR, Busick EJ, Kahn CR. Risk of proliferative diabetic retinopathy in juvenile-onset type I diabetes: a 40-yr follow-up study. Diabetes Care1986 Sep-Oct;9 (5):443-52.

17. Klein R, Klein BE, Moss SE, Davis MD, DeMets DL. The Wisconsin epidemiologic study of diabetic retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. Arch Ophthalmol1984 Apr; 102 (4):520-6.

18. Canani LH, Gerchman F, Gross JL. Increased familial history of arterial hypertension, coronary heart disease, and renal disease in Brazilian type 2 diabetic patients with diabetic nephropathy. Diabetes Care1998 Sep; 21 (9):1545-50.

19. Roglic G, Colhoun HM, Stevens LK, Lemkes HH, Manes C, Fuller JH. Parental history of hypertension and parental history of diabetes and microvascular complications in insulin-dependent diabetes mellitus: the EURODIAB IDDM Complications Study. Diabet Med1998 May; 15 (5):418-26.

20. Conway BR, Savage DA, Maxwell AP. Identifying genes for diabetic nephropathy--current difficulties and future directions. Nephrol Dial Transplant2006 Nov; 21 (11):3012-7.

21. Martini S, Eichinger F, Nair V, Kretzler M. Defining human diabetic nephropathy on the molecular level: integration of transcriptomic profiles with biological knowledge. Rev Endocr Metab Disord2008 Dec;9(4):267-74.

22. Freedman BI, Bostrom M, Daeihagh P, Bowden DW. Genetic factors in diabetic nephropathy. Clin J Am Soc Nephrol2007 Nov; 2 (6):1306-16.

23. Bollen M, Gijsbers R, Ceulemans H, Stalmans W, Stefan C. Nucleotide pyrophosphatases/phosphodiesterases on the move. Crit Rev Biochem Mol Biol2000; 35 (6):393-432.

24. Goldfine ID, Maddux BA, Youngren JF, Reaven G, Accili D, Trischitta V, et al. The role of membrane glycoprotein plasma cell antigen 1/ectonucleotide pyrophosphatase phosphodiesterase 1 in the pathogenesis of insulin resistance and related abnormalities. Endocr Rev2008 Feb; 29 (1):62-75.

25. Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, et al. Membrane glycoprotein PC-1 and insulin resistance in non-insulin-dependent diabetes mellitus. Nature1995 Feb 2; 373 (6513):448-51.

26. Maddux BA, Goldfine ID. Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor alpha-subunit. Diabetes2000 Jan; 49 (1):13-9.

27. Frittitta L, Youngren JF, Sbraccia P, D'Adamo M, Buongiorno A, Vigneri R, et al. Increased adipose tissue PC-1 protein content, but not tumour necrosis factor-alpha gene expression, is associated with a reduction of both whole body insulin sensitivity and insulin receptor tyrosine-kinase activity. Diabetologia1997 Mar; 40 (3):282-9.

28. Frittitta L, Youngren J, Vigneri R, Maddux BA, Trischitta V, Goldfine ID. PC-1 content in skeletal muscle of non-obese, non-diabetic subjects: relationship to insulin receptor tyrosine kinase and whole body insulin sensitivity. Diabetologia1996 Oct; 39 (10):1190-5.

29. Kumakura S, Maddux BA, Sung CK. Overexpression of membrane glycoprotein PC-1 can influence insulin action at a post-receptor site. J Cell Biochem1998 Mar 1; 68 (3):366-77.

30. Youngren JF, Maddux BA, Sasson S, Sbraccia P, Tapscott EB, Swanson MS, et al. Skeletal muscle content of membrane glycoprotein PC-1 in obesity. Relationship to muscle glucose transport. Diabetes1996 Oct; 45 (10):1324-8.

31. Frittitta L, Spampinato D, Solini A, Nosadini R, Goldfine ID, Vigneri R, et al. Elevated PC-1 content in cultured skin fibroblasts correlates with decreased in vivo and in vitro insulin action in nondiabetic subjects: evidence that PC-1 may be an intrinsic factor in impaired insulin receptor signaling. Diabetes1998 Jul; 47 (7):1095-100.

32. Maddux BA, Chang YN, Accili D, McGuinness OP, Youngren JF, Goldfine ID. Overexpression of the insulin receptor inhibitor PC-1/ENPP1 induces insulin resistance and hyperglycemia. Am J Physiol Endocrinol Metab2006 Apr; 290 (4):E746-9.

33. Zhou HH, Chin CN, Wu M, Ni W, Quan S, Liu F, et al. Suppression of PC-1/ENPP-1 expression improves insulin sensitivity in vitro and in vivo. Eur J Pharmacol2009 Aug 15; 616 (1-3):346-52.

34. Belfiore A, Costantino A, Frasca F, Pandini G, Mineo R, Vigneri P, *et al*. Overexpression of membrane glycoprotein PC-1 in MDA-MB231 breast cancer cells is associated with inhibition of insulin receptor tyrosine kinase activity. Mol Endocrinol1996 Nov; 10 (11):1318-26.

35. Costanzo BV, Trischitta V, Di Paola R, Spampinato D, Pizzuti A, Vigneri R, *et al*. The Q allele variant (GLN121) of membrane glycoprotein PC-1 interacts with the insulin receptor and inhibits insulin signaling more effectively than the common K allele variant (LYS121). Diabetes2001 Apr; 50 (4):831-6.

36. Pizzuti A, Frittitta L, Argiolas A, Baratta R, Goldfine ID, Bozzali M, et al. A polymorphism (K121Q) of the human glycoprotein PC-1 gene coding region is strongly associated with insulin resistance. Diabetes1999 Sep; 48 (9):1881-4.

37. Buckley MF, Loveland KA, McKinstry WJ, Garson OM, Goding JW. Plasma cell membrane glycoprotein PC-1. cDNA cloning of the human molecule, amino acid sequence, and chromosomal location. J Biol Chem1990 Oct 15; 265 (29):17506-11.

38. Frittitta L, Sbraccia P, Costanzo BV, Tassi V, D'Adamo M, Spampinato D, et al. High insulin levels do not influence PC-1 gene expression and protein content in human muscle tissue and hepatoma cells. Diabetes Metab Res Rev2000 Jan-Feb;16 (1):26-32.

39. Menzaghi C, Di Paola R, Baj G, Funaro A, Arnulfo A, Ercolino T, et al. Insulin modulates PC-1 processing and recruitment in cultured human cells. Am J Physiol Endocrinol Metab2003 Mar; 284 (3):E514-20.

40. Gu HF, Almgren P, Lindholm E, Frittitta L, Pizzuti A, Trischitta V, et al. Association between the human glycoprotein PC-1 gene and elevated glucose and insulin levels in a paired-sibling analysis. Diabetes2000 Sep; 49 (9):1601-3.

41. Abate N, Chandalia M. The impact of ethnicity on type 2 diabetes. J Diabetes Complications2003 Jan-Feb; 17 (1):39-58.

42. Frittitta L, Baratta R, Spampinato D, Di Paola R, Pizzuti A, Vigneri R, et al. The Q121 PC-1 variant and obesity have additive and independent effects in causing insulin resistance. J Clin Endocrinol Metab2001 Dec; 86 (12):5888-91.

43. Kubaszek A, Pihlajamaki J, Karhapaa P, Vauhkonen I, Laakso M. The K121Q polymorphism of the PC-1 gene is associated with insulin resistance but not with dyslipidemia. Diabetes Care2003 Feb; 26 (2):464-7.

44. Rasmussen SK, Urhammer SA, Pizzuti A, Echwald SM, Ekstrom CT, Hansen L, et al. The K121Q variant of the human PC-1 gene is not associated with insulin resistance or type 2 diabetes among Danish Caucasians. Diabetes2000 Sep; 49 (9):1608-11.

45. Abate N, Chandalia M, Di Paola R, Foster DW, Grundy SM, Trischitta V. Mechanisms of disease: Ectonucleotide pyrophosphatase phosphodiesterase 1 as a 'gatekeeper' of insulin receptors. Nat Clin Pract Endocrinol Metab2006 Dec; 2 (12):694-701.

46. Abate N, Chandalia M, Satija P, Adams-Huet B, Grundy SM, Sandeep S, et al. ENPP1/PC-1 K121Q polymorphism and genetic susceptibility to type 2 diabetes. Diabetes2005 Apr; 54 (4):1207-13.

47. Bacci S, Ludovico O, Prudente S, Zhang YY, Di Paola R, Mangiacotti D, *et al*. The K121Q polymorphism of the ENPP1/PC-1 gene is associated with insulin resistance/atherogenic phenotypes, including earlier onset of type 2 diabetes and myocardial infarction. Diabetes2005 Oct; 54 (10):3021-5.

48. Grarup N, Urhammer SA, Ek J, Albrechtsen A, Glumer C, Borch-Johnsen K, et al. Studies of the relationship between the ENPP1 K121Q polymorphism and type 2 diabetes, insulin resistance and obesity in 7,333 Danish white subjects. Diabetologia2006 Sep; 49(9):2097-104.

49. De Cosmo S, Argiolas A, Miscio G, Thomas S, Piras GP, Trevisan R, et al. A PC-1 amino acid variant (K121Q) is associated with faster progression of renal disease in patients with type 1 diabetes and albuminuria. Diabetes2000 Mar; 49(3):521-4.

50. Canani LH, Ng DP, Smiles A, Rogus JJ, Warram JH, Krolewski AS. Polymorphism in ecto-nucleotide pyrophosphatase/phosphodiesterase 1 gene (ENPP1/PC-1) and early development of advanced diabetic nephropathy in type 1 diabetes. Diabetes2002 Apr; 51 (4):1188-93.

51. Wu LS, Hsieh CH, Pei D, Hung YJ, Kuo SW, Lin E. Association and interaction analyses of genetic variants in ADIPOQ, ENPP1, GHSR, PPARgamma and TCF7L2 genes for diabetic nephropathy in a Taiwanese population with type 2 diabetes. Nephrol Dial Transplant2009 Nov; 24 (11):3360-6.

52. De Cosmo S, Minenna A, Zhang YY, Thompson R, Miscio G, Vedovato M, et al. Association of the Q121 variant of ENPP1 gene with decreased kidney function among patients with type 2 diabetes. Am J Kidney Dis2009 Feb; 53 (2):273-80.

53. De Cosmo S, Trevisan R, Dalla Vestra M, Vedovato M, Argiolas A, Solini A, et al. PC-1 amino acid variant Q121 is associated with a lower glomerular filtration rate in type 2 diabetic patients with abnormal albumin excretion rates. Diabetes Care2003 Oct; 26 (10):2898-902.

54. Leitao CB, Nabinger GB, Krahe AL, Bolson PB, Gerchman F, Friedman R, et al. The role of K121Q ENPP1 polymorphism in diabetes mellitus and its complications. Braz J Med Biol Res2008 Mar; 41 (3):229-34.

55. Keene KL, Mychaleckyj JC, Smith SG, Leak TS, Perlegas PS, Langefeld CD, et al. Association of the distal region of the ectonucleotide pyrophosphatase/phosphodiesterase 1 gene with type 2 diabetes in an African-American population enriched for nephropathy. Diabetes2008 Apr; 57(4):1057-62.

56. de Azevedo MJ, Dalmaz CA, Caramori ML, Pecis M, Esteves JF, Maia AL, et al. ACE and PC-1 gene polymorphisms in normoalbuminuric Type 1 diabetic patients: a 10-year prospective study. J Diabetes Complications2002 Jul-Aug; 16 (4):255-62.

57. Jacobsen P, Grarup N, Tarnow L, Parving HH, Pedersen O. PC-1 amino acid variant (K121Q) has no impact on progression of diabetic nephropathy in type 1 diabetic patients. Nephrol Dial Transplant2002 Aug; 17 (8):1408-12.

58. Frittitta L, Ercolino T, Bozzali M, Argiolas A, Graci S, Santagati MG, et al. A cluster of three single nucleotide polymorphisms in the 3'-untranslated region of human glycoprotein PC-1 gene stabilizes PC-1 mRNA and is associated with increased PC-1 protein content and insulin resistance-related abnormalities. Diabetes2001 Aug; 50 (8):1952-5.

59. Abate N. Obesity and cardiovascular disease. Pathogenetic role of the metabolic syndrome and therapeutic implications. J Diabetes Complications2000 May-Jun; 14 (3):154-74.

60. McFarlane SI, Banerji M, Sowers JR. Insulin resistance and cardiovascular disease. J Clin Endocrinol Metab2001 Feb; 86 (2):713-8.

61. Meyre D, Bouatia-Naji N, Tounian A, Samson C, Lecoeur C, Vatin V, et al. Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. Nat Genet2005 Aug; 37 (8):863-7.

62. Bochenski J, Placha G, Wanic K, Malecki M, Sieradzki J, Warram JH, et al. New polymorphism of ENPP1 (PC-1) is associated with increased risk of type 2 diabetes among obese individuals. Diabetes2006 Sep;55 (9):2626-30.

63. Santoro N, Cirillo G, Lepore MG, Palma A, Amato A, Savarese P, et al. Effect of the rs997509 polymorphism on the association between ectonucleotide pyrophosphatase phosphodiesterase 1 and metabolic syndrome and impaired glucose tolerance in childhood obesity. J Clin Endocrinol Metab2009 Jan; 94 (1):300-5.

Table 1. Studies on the association between the K121Q polymorphism in the *ENPP1* gene and diabetic nephropathy or associated characteristics

Study Population	Results
Thai population with type 2 DM (51)	Association between the Q/Q genotype and increased risk for DN (OR = 1.85; CI 95% = 1.17 – 2.92).
African-American population with type 2 DM (55)	No association with DN.
Caucasians with type 1 DM and proteinuria (49)	Association between the Q (KQ/QQ) allele and decline of GFR (OR = 5.7; CI 95% = 4.1 – 7.2).
Caucasians with type 1 DM (50)	Association between the Q allele and increased risk for ESRD (OR = 2.3; CI 95% = 1.2 – 4.6).
Italian and American populations with type 2 DM (52)	Association between the Q allele and GFR reduction in patients from Gargano (OR = 1.69; CI 95% = 1.1 - 2.6) and Boston (OR = 1.50; 95% = 1.0 – 2.2).
Danish population with type 1 DM (57)	No association with DN.
Southern Brazilian population with type 1 DM (56)	No association with DN.

Type 2 DM = type 2 diabetes mellitus; DN = diabetic nephropathy; ESRD = end-stage kidney disease; OR = odds ratio; CI = confidence interval; GFR = glomerular filtration rate.

Figura 1

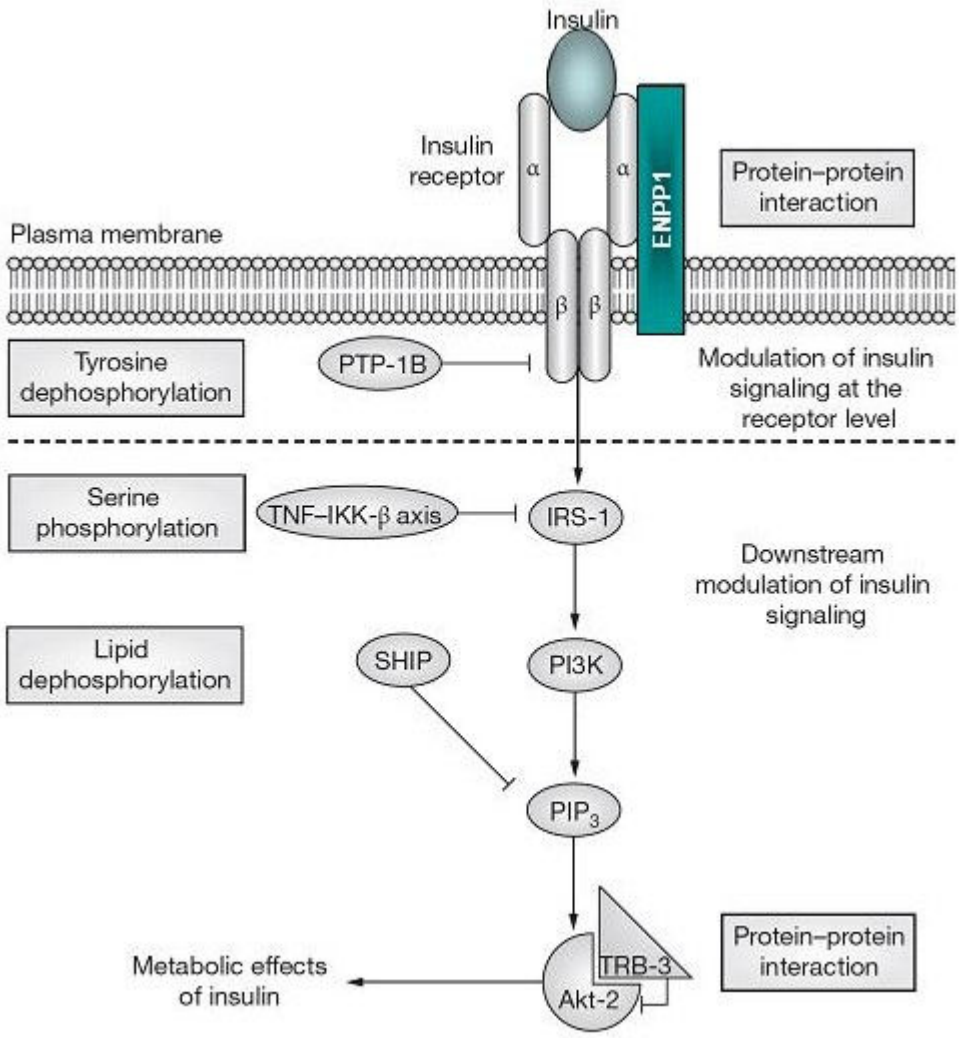

Figure 1. Systematic representation of insulin inhibitors signaling pathway, with in vivo established function of insulin resistance. After binding to the α-subunit of its receptor, insulin stimulates the signaling cascade events. The most important of these events are the tyrosine residue autophosphorylation of the insulin receptor β-subunits and of IRS-1, and also the subsequent PI3K and Akt-2 activation. Several negative modulators of insulin signaling were described as main determinants of insulin resistance in humans; these include the ENPP1, for which both post-translational and translocation to the cell surface processes are augmented by insulin signaling. The inhibitory effects of these modulators may be mediated by several mechanisms: 1) protein-protein interaction with signaling

molecules, mediated by ENPP1 with the insulin receptor and by TRB-3 with Akt-2; 2) serin residue phosphorylation of the IRS-2, mediated by TNF and IKK-β; 3) dephosphorylation of proteins with tyrosine residues phosphorylated, mediated by PTP-1B, which acts as a phosphatase in the insulin receptor; and 4) dephosphorylation of lipid substrates, mediated by SHIP, which hydrolizes the products of PI3K via its 5'-phosphatase activity. Abbreviations: Akt-2, serine-threonine kinase 2 (also known as B kinase protein); ENPP1, ecto-nucleotide pyrophosphatase / phosphodiesterase 1; IKK-β, IkB kinase β; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; PI3K, phosphatidylinositol 3 kinase; PIP3, phosphatidylinositol 3,4,5-triphosphate; PTP-1B, protein tyrosine phosphatase 1B; TNF, tumor necrosis factor; SHIP, Src homology 2 domain containing inositol 5 phosphatase; TRB-3, mammalian tribbles homolog 3. Modified from reference Abate *et al*. (45).

Figura 2

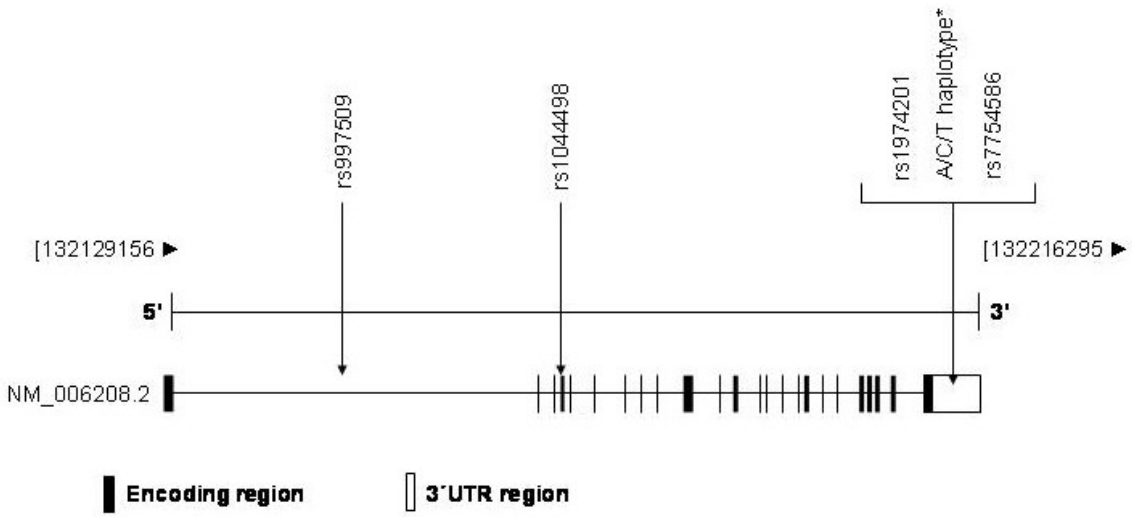

Figure 2. Map of the *ENPP1* gene in chromosome 6q22-23. The 25 exons (boxes) are numbered from left to right according to the transcription region. The black boxes constitute the encoding regions and the white box represents the 3`UTR region. Vertical arrows show the polymorphic sites associated with type 2 diabetes mellitus or diabetic nephropathy. Figure adapted from http://www.nbci.nlm.nih.gov/gene/5167. *A/C/T haplotype is constituted by 62897A, G2906C and C2948T ENPP1 polymorphisms.

PARTE II

Artigo Original

THE K121Q POLYMORPHISM IN THE ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE 1 (*ENPP1*) GENE IS NOT ASSOCIATED WITH CHANGES OF *ENPP1* GENE EXPRESSION IN HUMAN KIDNEY".

O POLIMORFISMO K121Q NO GENE ECTO-NUCLEOTIDEO PIROFOSFATASE/ FOSFODIESTERASE 1 (*ENPP1*) NÃO ESTÁ ASSOCIADO COM MUDANÇAS DA EXPRESSÃO DO GENE NO RIM HUMANO

The K121Q polymorphism in the Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase 1 (*ENPP1*) gene is not associated with changes of *ENPP1* gene expression in human kidney".

Short title: *ENPP1* gene expression on human kidney.

Denise Alves Sortica [4], Daisy Crispim[1,4], Guilherme Pozzueco Zaffari[4], Letícia A. Brondani[4], Fabiana Góes Nogueira [4], Mariana P. Carpena[4], Maria Isabel Edelweiss[2,4], Milton Berger[3,4], Luís H. Canani[1,4]

1 - Endocrinology Division, Hospital de Clínicas de Porto Alegre, RS, Brazil

2 - Pathology Service, Hospital de Clínicas de Porto Alegre, RS, Brazil

3 - Urology Service, Hospital de Clínicas de Porto Alegre, RS, Brazil

4 –Universidade Federal do Rio Grande do Sul. Porto Alegre, RS, Brazil.

Corresponding author:

Dr. Luis Henrique Canani. Rua Ramiro Barcelos 2350; prédio 12; 4° andar. CEP 90035-003. Porto Alegre, RS, Brazil. Phone number: +55 51 33598318. Fax number: 55 5133598777. E-mail: luishenriquecanani@gmail.com

Word count: 3592

Number of tables and figures: Table – 1 and Figure – 2

ABSTRACT

Objective **-** Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) is expressed in several tissues, including the kidneys. Increased levels of ENPP1 expression inhibit the tyrosine-kinase activity of the insulin receptor in several cell types, then leading to insulin resistance and/or diabetes mellitus (DM). The K121Q polymorphism of the *ENPP1* gene seems to be associated with DM and with the development of diabetic nephropathy (DN). However, a little is known about the effect of this polymorphism in the human kidney. In this study, the expression of *ENPP1* gene and protein in human kidney according to the K121Q polymorphism (rs1044498) was evaluated.

Research design and methods – A cross-sectional study was performed and included 107 consecutive patients undergoing therapeutic nephrectomy to obtain the expected sample in each group. According to the exclusion criteria patients with diabetes mellitus and tumor mass were not enrolled in this study. Among the 107 patients, 57 samples (9 Q/Q, 27 Q/K, 20 K/K) were eligible to the study and immunohistochemistry technique was used to analyze the *ENPP1* protein expression. Then 34 samples (9 Q/Q, 12 Q/K, 13 K/K) were selected to investigate the *ENPP1* gene expression by RT-qPCR.

Results – *ENPP1* cDNA concentrations in kidney samples did not differ significantly neither among K121Q genotypes nor between the presence or absence of the Q allele (QQ/KQ vs. K/K; 1.0 (0.1 – 1.6) *vs*. 0.72 (0.1 – 2.1) AU (arbitrary units); P = 0.483). In agreement with the gene expression data, ENPP1 protein levels were similar in Q allele carriers when compared with the K/K genotype group (2.86 ± 0.32 vs. 2.78 ± 0.35 AU; P = 0.417), after controlling for covariates.

Conclusions – This study suggest that the K121Q polymorphism is not associated with *ENPP1* gene and protein expression in the human kidney.

Keywords: *ENPP1* gene expression, K121Q polymorphism, human kidney, diabetic nephropathy.

INTRODUCTION

Epidemiological and familial aggregation studies have demonstrated that, besides the known environmental risk factors, DN is a multifactorial disease with a genetic component (1, 2). Great efforts have been made to identify the genes associated with DN, but results are still inconsistent with different genes associated to a small effect in specific populations. The identification of these genes could allow the detection of high risk individuals to develop DN and might allow us to have a better understanding of its pathophysiology.

In 1999, Pizzuti *et al.* reported that the K121Q (rs1044498) polymorphism located in the exon 4 of the pyrophosphatase/phosphodiesterase 1 (*ENPP1*) gene was associated with insulin resistance (IR) in non-diabetic Sicilians (3). ENPP1 is one of the five cell membrane proteins containing an active extracellular site that catalyzes the release of nucleoside 5´phosphatase from nucleotides and their products. These proteins consist of a short terminal NH_2 intracellular domain, a single transmembrane domain, two somatomedin-B-like domains, a catalytic domain, and a COOH-terminal nuclease-like domain (2, 4). It is well known that ENPP1 is expressed in several tissues and cell types, including skeletal tissue, adipose tissue, liver, and kidney mesangial and endothelial cells (5-9). Although the physiological functions of ENPP1 in these tissues have not been fully described yet (10), it has been reported that ENPP1 overexpression directly inhibits the tyrosine kinase activity of the insulin receptor-α subunit in several cell types (10-12) and, consequently, causes IR (13-17).

Gene candidates for IR can also be considered as DN candidates genes, because IR is a common characteristic of patients with type 1 and type 2 DM who present increased urine albumin excretion (UAE) (18, 19). In fact, De Cosmo *et al*. demonstrated that the Q allele of the K121Q polymorphism is associated with a faster progression of DN in proteinuric patients

with type 2 DM (20). Besides, using a case-control and family study, we also showed that this genetic variant is associated with earlier development of end-stage renal disease in type 1 DM subjects (5). This association was confirmed using transmission disequilibrium test (TDT), which indicated that the data was not due to population stratification (5). Reinforcing these observations, De Cosmo *et al*. reported that the K121Q polymorphism also has an effect on the rate of loss of renal function in Caucasians with type 1 DM and proteinuria, during 6.5 years of follow-up (21).

Taking this into account, in the present study, for the first time, we evaluated the effect of the K121Q polymorphism on *ENPP1* gene expression in human kidney tissue biopsies.

PATIENTS AND METHODS

Samples

A standard questionnaire was used to collect information regarding age, gender, presence of arterial hypertension and smoking. The protocol was approved by the Hospital ethical committees, and all patients gave their written informed consent.

A cross-sectional study was performed and included 107 consecutive non-diabetic patients undergoing therapeutic nephrectomy suggested by the urologist over the study period of 18 months. Peripheral blood samples were collected from each subject for DNA extraction and genotyping of the K121Q polymorphism. Following genotyping, subjects were divided into groups according to the presence of the Q allele: 64 K/K, 33 K/Q and 10 Q/Q. Most of subjects had the kidney removed due to the presence of tumor (n = 79). After removal of the kidney, a biopsy was made by the surgeon and divided in aliquots for *ENPP1* mRNA expression analyses and evaluation of ENPP1 protein by immunohistochemistry technique. According to the inclusion criteria, kidney samples were normal tissue without visible tumors

at optical microscopy. Fifty-seven kidney samples (9 Q/Q, 28 Q/K, 20 K/K) were eligible to the study. Immunohistochemistry technique to analyze the ENPP1 protein expression was performed in this 57 samples (20 K/K, 28 K/Q and 9 Q/Q) and the gene expression was performed in 34 samples (9 Q/Q, 12 Q/K, 13 K/K).

Genotyping

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. Genotyping of the K121Q (A/C) polymorphism (rs1044498) in exon 4 was performed using primers and probes contained in the Human Custom TaqMan Genotyping Assay 40x (Life Technologies, Foster City, CA). Reactions were conducted in 96-well plates, in a total 5 μl volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Life Technologies) and Custom TaqMan Genotyping Assay 1x. Primer and probe sequences used for genotyping this polymorphism are: 5-AGCCTCTGTGCCTGTTCAG-3' (forward), 5'-ACACACAGAACTGTAGTTGATGCA-3' (reverse), 5'-AGTCGCCCTTGTCCTT-3' (VIC), and 5'-TCGCCCTGGTCCTT-3'(FAM).

RNA isolation

Kidney tissue biopsies were homogenized in phenol-guanidine isothiocyanate (Invitrogen Life Technologies, Carlsbad, CA). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 x g) at 4°C. RNA pellet was washed twice with 75% ethanol and ressuspended in 10-50 μl of diethylpyrocarbonate treated water. Concentrations of isolated RNAs were assessed using NANODROP 2000 spectrophotometer (Thermo Scientific Inc., DE, USA). Only total RNA samples which achieved adequate purity rations (A260/A280 = 1.9 - 2.1) were used for subsequent analyses (22). In addition, RNA integrity

and purity was also checked on agarose gel containng GelRed Nucleic Acid Gel Sain (Biotium Inc, Hayward, CA). The mean RNA concentraction (± SD) isolated was 16.84 ± 31.41 µg/250mg kidney.

Immunohistochemistry for ENPP1 protein in human kidney

ENPP1 protein distributions and intensities were determined by immunohistochemistry in formalin-fixed, paraffin-embedded kidney sections. An anti-ENPP1 goat polyclonal antibody (Abcam, Cambridge, MA) was used to detect ENPP1 protein expression in human kidney tissue. Immunohistochemistry analyses were performed on 4-µm kidney sections as described previously (23). The routine immunohistochemistry technique comprised deparaffinization and rehydration, antigenic recovery, inactivation of endogenous peroxidase and blocking of nonspecific reactions. Slides were incubated with primary antibody and then incubated with a biotinylated secondary antibody, streptavidin horseradish peroxidase conjugate (LSA; Dako Cytomation, Inc Carpinteria, CA), and diaminobenzidine tetrahydrochloride (Kit DAB Dako Cytomation, Inc). Quantification of the ENPP1 protein was performed by digital analysis using the Image Pro Plus software version 4.5 (Media Cybernetics, Bethesda, MD). Images were visualized through a Zeiss microscope (model AXIOSKOP-40: Carl Zeiss Oberkochen, Germany) and captured using the Cool Snap-Pro CS (Media Cybernetics) camera. Two independent researchers analyzed the intensity of brownish-colored immunostaining in pixels in 10 fields from each slide, achieving a Pearson´s correlations between their results of r^2 = 0.92 (P = 0.0001). We used the mean number of pixels (in logarithmic scale) identified by both researchers to quantify ENPP1 in each sample.

Quantification of ENPP1 gene expression by Real-time PCR

Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of 5µg of RNA into cDNA was carried out using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), following the manufacturer's protocol for oligo (dT) method.

RT-qPCR experiments were performed in a Vii™ 7 Fast Real-Time PCR System Thermal Cycler with Vii™ 7 Ruo Software (Life Technologies). Experiments were performed by monitoring in real time the increase in fluorescence of the FAM dye. *ENPP1* and *β-actin* gene expressions were performed using primers and probes contained in Taqman Gene Expression Assays (HS01054040_m1 for *ENPP1* and HS99999903_m1 for *β-Actin*). PCR reactions were performed using 5 µl TaqMan Gene Expression Assay 20x for *ENPP1* or *β-Actin* (Life Technologies), 5 µl TaqMan Fast Universal PCR Master Mix 2x (Life Technologies) and 1 µl of cDNA (5µg/µl), in a total volume of 10 µl. Each sample was assayed in triplicate and a negative control was included in each experiment. The thermocycling conditions for these genes were as follows: an initial cycle of 95°C for 10min, followed by 50 cycles of 95°C for 15s and 60°C for 1min.

Quantification of the *ENPP1* mRNA was performed using the relative standard curve method and the *β-Actin* gene as the reference gene (22). Relative standard curves were generated for both target and reference genes by preparing serial dilutions of the same cDNA sample with a known relative quantity, the samples were analyzed to make sure that the standard curves for the target and reference genes were agreement. Then relative amounts of each *ENPP1* cDNA sample were obtained by normalizing their signals by those of *β-Actin*, and are presented as AU.

Statistical analyses

For the expression studies it was estimated the needed of 10 kidney samples in each genotype group. Considering the prevalence of the minor allele, around 100 subjects would have to be evaluated to find the calculated number in each group. Allele frequencies were determined by gene counting, and departures from the Hardy-Weinberg equilibrium (HWE) were verified using the χ^2 test. Allele and genotype frequencies were compared between groups using the χ^2 test. Clinical and laboratory characteristics, cDNA abundance and ENPP1 protein expression were compared between groups using the unpaired Student's *t* test or the χ^2 test, as appropriate. Variables with normal distribution are presented as mean ± SD (Standard deviation) or percentage. Variables with skewed distribution were log-transformed before analyses and are presented as median (minimum – maximum values) or mean ± 2 SD in logarithmic scale.

Pearson's correlation test was used to assess correlations between different quantitative variables. Multiple linear regression analyses were performed with *ENPP1* gene expression or protein (logarithmic) as dependent variables and age and sex as independent variables. Since previous reports showed that the subjects carrying the Q allele (KQ/QQ) were at higher risk, the data was presented both by genotype and by the presence of the risk allele. Results for which $P < 0.05$ were considered statistically significant. These statistical analyses were performed using SPSS 18.0 (Chicago, IL, USA).

RESULTS

Sample description

The main clinical characteristics of the 57 subjects included in the immunohistochemistry evaluation were as follows: mean age was 57.6 ± 13.8 years, males comprised 40.4% of the

sample, 61.4% of all patients had arterial hypertension and 37.8% were smokers. The K121Q genotypes were in HW equilibrium (P >0.05).

ENPP1 protein concentrations

ENPP1 protein concentration for the entire kidney tissue group was 2.83 ± 0.33 pixels. No significant difference was observed when ENPP1 protein immunoreactivity was analyzed by sex (men: 2.77 ± 0.35 *vs*. women: 2.88 ± 0.32 pixels, in logarithmic scale; P = 0.236), hypertension status (normotensives: 2.86 ± 0.34 *vs*. hypertensives: 2.84 ± 0.33 pixels; P = 0.903) or smoking habits (nonsmokers: 2.85 ± 0.32 *vs*. smokers: 2.89 ± 0.29 pixels; P = 0.663). ENPP1 protein concentrations did not correlate with age (r = 0.057; P = 0.684) or with *ENPP1* cDNA concentrations (r = 0.150, P = 0.485).

ENPP1 protein concentrations in kidney samples broken down according to the presence of the K121Q polymorphism are depicted in **Table 1.** ENPP1 protein levels were similar in the different K121Q polymorphism genotypes (Q/Q 2.93 ± 0.27, K/Q 2.84 ± 0.34 and K/K 2.79 ± 0.36; pixels P = 0.542). In the same way, when analyzed according to the presence of the minor allele, those with the Q allele (QQ/KQ) had similar protein quantities of those with the K/K genotype (Figure 1, P = 0.417). Even after controlling for age and sex, the ENPP1 concentrations was similar among those with or without the Q allele (P = 0.212). Moreover, ENPP1 protein immunoreactivity was exclusive in the tubules of the kidney.

ENPP1 gene expression

Thirty four samples were used to analyze *ENPP1* gene expression. The mean age of this group was 54.8 ± 12.1 years, males comprised 38.2% of the sample, 56.0% of all patients had arterial hypertension and 37.0% were smokers.

ENPP1 cDNA concentration for the whole kidney tissue group was 0.99 (0.06 – 2.13) AU. No significant difference was observed when *ENPP1* gene expression was analyzed by hypertensive status (hypertensives: 0.88 (0.10 – 2.13) *vs.* non-hypertensives 1.004 (0.11 – 1.58) AU; P = 0.745) or smoking status (smokers: 1.01 (0.12 – 2.13) *vs*. nonsmokers: 0.99 (0.06 - 1.58) AU, in logarithmic scale; P = 0.476). However, females had higher *ENPP1* gene expression as compared to males [females: 1.02 (0.06 – 2.13) *vs*. males: 0.67 (0.10 – 1.02) AU; P = 0.008]. *ENPP1* gene expression did not correlate with age (r= -0.015; P = 0.934).

ENPP1 cDNA concentration were similar in the different K121Q polymorphism genotypes [Q/Q 1.0 (0.10 - 1.34), K/Q 0.88 (0.06 - 1.58) and K/K 0.72 (0.10 - 2.13) AU; P = 0.689]. In the same way, when analyzed according to the presence of the minor allele, those with the Q allele (QQ/KQ) had similar cDNA quantity of those with the K/K genotype (Figure 2; P = 0.483]. After controlling for age and sex in the multiple linear regression analysis, presence of the Q allele remained not associated with *ENPP1* gene expression (P = 0.502). However, sex remained independently associated with *ENPP1* gene expression (Beta = -0.559; P = 0.007).

DISCUSSION

In 1999, Pizzuti *et al.* described a polymorphism in exon 4 of the gene *ENPP1*, which causes the change of the amino acid lysine (K) to glutamine (Q) in codon 121 (K121Q; rs1044498). This amino acid change is located in the second *ENPP1* somatomedin-B-like domain, and may interfere with protein-protein interactions (3). Pizzuti *et al.* also demonstrated that the Q variant of this polymorphism interacts more strongly with the insulin receptor than the K variant, reducing the autophosphorylation of this receptor (3). Q variant also reduces insulin-

induced phosphorylation of the insulin receptor substrate 1 (IRS1), the kinase activity of phosphatidilinositol-3, glycogen synthesis and cell proliferation and showed a cause-effect relationship between the Q carrying genotype and the IR phenotype (24). Other studies confirmed the association between the K121Q polymorphism and greater IR and type 2 DM in different populations (25-28), but not all (29, 30).

The K121Q polymorphism also was reported as being associated with DN in some populations (5, 31, 32). Wu *et al*. demonstrated significant association of this polymorphism in the *ENPP1* gene on the risk of DN in type 2 DM (adjusted OR = 1.85; 95% CI = 1.17-2.92; P = 0.0032) (31). De Cosmo *et al.* described an effect of the *ENPP1* polymorphism on the rate of loss of renal function in Caucasians with type 1 and type 2 DM and proteinuria (21). As already commented, Canani *et al*. in both a case-control and a family study, reported the association between the Q allele of the K121Q polymorphism and advanced DN in type 1 DM patients (5). However, other studies were not able to demonstrate any association between this polymorphism and DN (33-35).

The molecular mechanisms that mediate the association between the Q variant and the development of advanced stages of DN may only be hypothesized. As previously mentioned, ENPP1 is expressed in several tissues and cell types, including mesangial and endothelial cells in the kidneys (5). These cells show progressive pathological changes throughout the evolution from normality to overt DN and it is tempting to hypothesize that ENPP1 plays a role in kidney tissue damage in DN. *In vitro* studies demonstrated that the ENPP1 Q variant interacts more strongly with the insulin receptor than the K variant. Therefore, cells carrying the Q variant show reduced insulin receptor self-phosphorylation (3), reduced induction of the IRS1 phosphorylation, phosphatidylinositol 3-kinase activity, glycogen synthesis, and cell proliferation (24). Humans carrying the Q allele have greater IR and hyperinsulinemia than

non-carriers (26). Hyperinsulinemia can stimulate the renal reabsorption of sodium, leading to volume expansion, increased sympathetic-adrenergic activity, and increased expression of angiotensin type II receptor, which hamper peripheral vasodilatation (36). Increased circulating volume and reduced peripheral vasodilatation eventually predispose to increased blood pressure and loss of the physiological nocturnal blood pressure dipping (36, 37), both presumptive DN risk factors (5, 37, 38).

Taking into account there is not data regarding the effect of the K121Q polymorphism on *ENPP1* gene expression in human kidney, in the present study we evaluated whether this polymorphism is associated with changes in *ENPP1* cDNA and protein concentrations in human kidney biopsy samples. Our data showed that this polymorphism is not associated with any significant changes in either *ENPP1* cDNA levels or ENPP1 protein immunoreactivity in kidney samples of non-diabetic subjects. These data are conflicting with the data of Costanzo *et al*. who showed the Q allele has increased *ENPP1* expression and stronger inhibitory activity on insulin receptor function and insulin action than the more common K allele in fibroblasts cell culture (39). This is likely a consequence of the intrinsic characteristics of the molecule, which more strongly interacts with the insulin receptor. Maddux *et al*. produced transgenic mice overexpressing the potent Q allele of human ENPP1 in muscle and liver. This study demonstrated that ENPP1 plays an important role in IR, hyperglycemia, as well as overexpression of human ENPP1 in insulin sensitive tissues (40). Pizzuti *et al.* showed that the insulin receptor autophosphorylation was reduced ($P < 0.01$) in cultured skin fibroblasts from K/Q versus K/K subjects. These results suggest a cause-effect relationship between the Q carrying genotype and the IR phenotype. However, the protein content was similar in both genotypes (K/Q and K/K) (3).

The lack of the association of the Q allele with the *ENPP1* gene expression showed in the present study can be explained in different ways. It is possible that the *ENPP1* K121Q polymorphism might be in linkage disequilibrium with other mutations in the *ENPP1* gene. Accordingly, Santoro *et al*. showed that the rs997509 polymorphism in the *ENPP1* gene predispose obese children to metabolic syndrome and impaired glucose tolerance (41). One possibility is that the T allele of rs997509 could be functional and responsible for the effect of the risk variant 121Q. If this is the case, the Q121 variant would be a silent marker. Despite being located in the 3'end of intron 1, the rs997509 single nucleotide polymorphism (SNP) is in a region that may contain a regulatory element, as suggested by the Five Way Regulatory Track of the University of California Santa Cruz genome browser (42). We evaluated the K121Q since this polymorphism is the most frequently associated with DN in the literature. Besides, the SNP rs997509 has a low allele frequency, accounting for only 10% and it would limit the effect as a DN candidate gene. Then, studies analyzing if K121Q and rs997509 polymorphisms are in linkage disequilibrium are necessary to confirm this hypothesis. Another hypothesis is that the K121Q polymorphism might cause changes in the protein activity, but not necessarily would change ENPP1 expression. As already commented, some studies in culture cells (16, 18, 42, 45, 46, 47) and in transgenic mice (40) showed that the ENPP1 activity play a role in the downregulation of insulin sensitivity, rather than gene or protein expression. Unfortunately, we did not performed activity assays in a lineage of kidney cells.

As a limitation of this study, we could include the sample size, that possibly was not large sufficient to show a small effect of this polymorphism in the ENPP1 expression. However, it is clear the limitation to have access to human kidney tissue, and the local ethics committee recommends that we should include the smallest sample size that allowed us to

evaluate a moderate effect. We could also include as a limitation, the fact that the kidney tissue was selected from non-diabetic subjects. Regarding the non-diabetic origin of the biopsies, we could argue that if we consider that the K121Q has a major effect on the development of DN through IR, we would see an effect even in non-diabetic subjects. On the other hand, if the effect of the polymorphism is in the progression of DN, we could see the effect only in the presence of DM. Furthermore, some studies about this polymorphism showed that the risk allele was associated with an advantaged progression of the renal loss of function and/or the diagnostic for early end stage renal disease (ESRD).

Finally and taking all together these data suggested that the K121Q polymorphism is not associated with the *ENPP1* gene and protein expressions in the human kidney. However, additional studies investigating the association of the K121Q polymorphism with the ENPP1 activity in a human lineage of kidney cells and also studies analyzing *ENPP1* mRNA and protein in the kidney of patients with DM are needed to clarify the role of this polymorphism in the risk to DN.

REFERENCES

1. Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes care. 2005 Jan;28(1):164-76.

2. Sortica DA, Crispim D, Zaffari GP, Friedman R, Canani LH. The role of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 in diabetic nephropathy. Arq Bras Endocrinol Metabol. 2011 Dec;55(9):677-85.

3. Pizzuti A, Frittitta L, Argiolas A, Baratta R, Goldfine ID, Bozzali M, et al. A polymorphism (K121Q) of the human glycoprotein PC-1 gene coding region is strongly associated with insulin resistance. Diabetes. 1999 Sep;48(9):1881-4.

4. Bollen M, Gijsbers R, Ceulemans H, Stalmans W, Stefan C. Nucleotide pyrophosphatases/phosphodiesterases on the move. Crit Rev Biochem Mol Biol. 2000;35(6):393-432.

5. Canani LH, Ng DP, Smiles A, Rogus JJ, Warram JH, Krolewski AS. Polymorphism in ecto-nucleotide pyrophosphatase/phosphodiesterase 1 gene (ENPP1/PC-1) and early development of advanced diabetic nephropathy in type 1 diabetes. Diabetes. 2002 Apr;51(4):1188-93.

6. Harahap AR, Goding JW. Distribution of the murine plasma cell antigen PC-1 in non-lymphoid tissues. J Immunol. 1988 Oct 1;141(7):2317-20.

7. Huang R, Rosenbach M, Vaughn R, Provvedini D, Rebbe N, Hickman S, et al. Expression of the murine plasma cell nucleotide pyrophosphohydrolase PC-1 is shared by human liver, bone, and cartilage cells. Regulation of PC-1 expression in osteosarcoma cells by transforming growth factor-beta. J Clin Invest. 1994 Aug;94(2):560-7.

8. Jin-Hua P, Goding JW, Nakamura H, Sano K. Molecular cloning and chromosomal localization of PD-Ibeta (PDNP3), a new member of the human phosphodiesterase I genes. Genomics. 1997 Oct 15;45(2):412-5.

9. Pan W, Ciociola E, Saraf M, Tumurbaatar B, Tuvdendorj D, Prasad S, et al. Metabolic consequences of ENPP1 overexpression in adipose tissue. Am J Physiol Endocrinol Metab. 2011 Nov;301(5):E901-11.

10. Goldfine ID, Maddux BA, Youngren JF, Reaven G, Accili D, Trischitta V, et al. The role of membrane glycoprotein plasma cell antigen 1/ectonucleotide pyrophosphatase phosphodiesterase 1 in the pathogenesis of insulin resistance and related abnormalities. Endocr Rev. 2008 Feb;29(1):62-75.

11. Maddux BA, Goldfine ID. Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor alpha-subunit. Diabetes. 2000 Jan;49(1):13-9.

12. Zhou HH, Chin CN, Wu M, Ni W, Quan S, Liu F, et al. Suppression of PC-1/ENPP-1 expression improves insulin sensitivity in vitro and in vivo. Eur J Pharmacol. 2009 Aug 15;616(1-3):346-52.

13. Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, et al. Membrane glycoprotein PC-1 and insulin resistance in non-insulin-dependent diabetes mellitus. Nature. 1995 Feb 2;373(6513):448-51.

14. Frittitta L, Youngren J, Vigneri R, Maddux BA, Trischitta V, Goldfine ID. PC-1 content in skeletal muscle of non-obese, non-diabetic subjects: relationship to insulin receptor tyrosine kinase and whole body insulin sensitivity. Diabetologia. 1996 Oct;39(10):1190-5.

15. Frittitta L, Youngren JF, Sbraccia P, D'Adamo M, Buongiorno A, Vigneri R, et al. Increased adipose tissue PC-1 protein content, but not tumour necrosis factor-alpha gene

expression, is associated with a reduction of both whole body insulin sensitivity and insulin receptor tyrosine-kinase activity. Diabetologia. 1997 Mar;40(3):282-9.

16. Di Paola R, Caporarello N, Marucci A, Dimatteo C, Iadicicco C, Del Guerra S, et al. ENPP1 affects insulin action and secretion: evidences from in vitro studies. PLoS One.6(5):e19462.

17. Prudente S, Morini E, Trischitta V. Insulin signaling regulating genes: effect on T2DM and cardiovascular risk. Nat Rev Endocrinol. 2009 Dec;5(12):682-93.

18. (Yip J eaL, 1993 Yip, J., Mattock MB, Morocutti A, Sethi M, Trevisan R, Viberti G. Insulin resistance in insulin-dependent diabetic patients with microalbuminuria. Lancet. 1993 Oct 9;342(8876):883-7.

19. Groop L, Ekstrand A, Forsblom C, Widen E, Groop PH, Teppo AM, et al. Insulin resistance, hypertension and microalbuminuria in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia. 1993 Jul;36(7):642-7.

20. De Cosmo S, Trevisan R, Dalla Vestra M, Vedovato M, Argiolas A, Solini A, et al. PC-1 amino acid variant Q121 is associated with a lower glomerular filtration rate in type 2 diabetic patients with abnormal albumin excretion rates. Diabetes Care. 2003 Oct;26(10):2898-902.

21. De Cosmo S, Argiolas A, Miscio G, Thomas S, Piras GP, Trevisan R, et al. A PC-1 amino acid variant (K121Q) is associated with faster progression of renal disease in patients with type 1 diabetes and albuminuria. Diabetes. 2000 Mar;49(3):521-4.

22. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

23. Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. Am J Clin Pathol. 1981 Jun;75(6):816-21.

24. Frittitta L, Ercolino T, Bozzali M, Argiolas A, Graci S, Santagati MG, et al. A cluster of three single nucleotide polymorphisms in the 3'-untranslated region of human glycoprotein PC-1 gene stabilizes PC-1 mRNA and is associated with increased PC-1 protein content and insulin resistance-related abnormalities. Diabetes. 2001 Aug;50(8):1952-5.

25. Frittitta L, Baratta R, Spampinato D, Di Paola R, Pizzuti A, Vigneri R, et al. The Q121 PC-1 variant and obesity have additive and independent effects in causing insulin resistance. J Clin Endocrinol Metab. 2001 Dec;86(12):5888-91.

26. Gu HF, Almgren P, Lindholm E, Frittitta L, Pizzuti A, Trischitta V, et al. Association between the human glycoprotein PC-1 gene and elevated glucose and insulin levels in a paired-sibling analysis. Diabetes. 2000 Sep;49(9):1601-3.

27. Abate N, Chandalia M. The impact of ethnicity on type 2 diabetes. J Diabetes Complications. 2003 Jan-Feb;17(1):39-58.

28. Kubaszek A, Pihlajamaki J, Karhapaa P, Vauhkonen I, Laakso M. The K121Q polymorphism of the PC-1 gene is associated with insulin resistance but not with dyslipidemia. Diabetes care. 2003 Feb;26(2):464-7.

29. Shi X, Wang L, Jin F, Sun J, Sun L, Tang L, et al. The ENPP1 K121Q polymorphism is not associated with type 2 diabetes in northern Chinese. Acta Diabetol. Dec;48(4):303-10.

30. Mtiraoui N, Turki A, Nemr R, Echtay A, Izzidi I, Al-Zaben GS, et al. Contribution of common variants of ENPP1, IGF2BP2, KCNJ11, MLXIPL, PPARgamma, SLC30A8 and TCF7L2 to the risk of type 2 diabetes in Lebanese and Tunisian Arabs. Diabetes Metab. Nov;38(5):444-9.

31. Wu LS, Hsieh CH, Pei D, Hung YJ, Kuo SW, Lin E. Association and interaction analyses of genetic variants in ADIPOQ, ENPP1, GHSR, PPARgamma and TCF7L2 genes for diabetic nephropathy in a Taiwanese population with type 2 diabetes. Nephrol Dial Transplant. 2009 Nov;24(11):3360-6.

32. De Cosmo S, Minenna A, Zhang YY, Thompson R, Miscio G, Vedovato M, et al. Association of the Q121 variant of ENPP1 gene with decreased kidney function among patients with type 2 diabetes. Am J Kidney Dis. 2009 Feb;53(2):273-80.

33. Keene KL MJ, Smith SG, Leak TS, Perlegas PS, Langefeld, CD ea. Association of the distal region of the ectonucleotide pyrophosphatase/phosphodiesterase 1 gene with type 2 diabetes in an African-American population enriched for nephropathy. Diabetes. 2008;57(4):1057-62.

34. Jacobsen P GN, Tarnow L, Parving HH, Pedersen O. PC-1 amino acid variant (K121Q) has no impact on progression of diabetic nephropathy in type 1 diabetic patients. Nephrol Dial Transplant. 2002;17(8):1408-12.

35. de Azevedo MJ DC, Caramori ML, Pecis M, Esteves JF, Maia AL, et al. ACE and PC-1 gene polymorphisms in normoalbuminuric Type 1 diabetic patients: a 10-year prospective study. . J Diabetes Complications. 2002;16(4):255-62.

36. Abate N. Obesity and cardiovascular disease. Pathogenetic role of the metabolic syndrome and therapeutic implications. J Diabetes Complications. 2000 May-Jun;14(3):154-74.

37. McFarlane SI, Banerji M, Sowers JR. Insulin resistance and cardiovascular disease. J Clin Endocrinol Metab. 2001 Feb;86(2):713-8.

38. De Cosmo S, Menzaghi C, Prudente S, Trischitta V. Role of insulin resistance in kidney dysfunction: insights into the mechanism and epidemiological evidence. Nephrol Dial Transplant. Jan;28(1):29-36.

39. Costanzo BV, Trischitta V, Di Paola R, Spampinato D, Pizzuti A, Vigneri R, et al. The Q allele variant (GLN121) of membrane glycoprotein PC-1 interacts with the insulin receptor and inhibits insulin signaling more effectively than the common K allele variant (LYS121). Diabetes. 2001 Apr;50(4):831-6.

40. Maddux BA, Chang YN, Accili D, McGuinness OP, Youngren JF, Goldfine ID. Overexpression of the insulin receptor inhibitor PC-1/ENPP1 induces insulin resistance and hyperglycemia. Am J Physiol Endocrinol Metab. 2006 Apr;290(4):E746-9.

41. Santoro N CG, Lepore MG, Palma A, Amato A, Savarese P, et al. Effect of the rs997509 polymorphism on the association between ectonucleotide pyrophosphatase phosphodiesterase 1 and metabolic syndrome and impaired glucose tolerance in childhood obesity. J Clin Endocrinol Metab. 2009;94(1):300-5.

42. Bejerano G, Siepel AC, Kent WJ, Haussler D. Computational screening of conserved genomic DNA in search of functional noncoding elements. Nat Methods. 2005 Jul;2(7):535-45.

Table 1. Clinical characteristics and *ENPP1* gene and protein relative quantification in human kidney samples according to the K121Q polymorphism

	Q/Q(n = 9)	**K/Q(n = 28)**	**K/K(n = 20)**	**P**
Age	57.5 ± 14.1	56.8 ± 15.0	60.3 ± 9.3	0.799
Gender (male %)	32.2	46.4	40.0	0.636
Hypertension status (%)	66.7	42.1	78.9	0.063
Smoker status (%)	33.3	33.3	44.4	0.753
ENPP1 protein (pixels)	2.93 ± 0.27	2.84 ± 0.34	2.79 ± 0.36	0.544
ENPP1 gene expression (AU) *	1.0 (0.10 – 1.34)	0.88 (0.06 – 1.58)	0.72 (0.10 – 2.13)	0.689

* n = 34 (9 for the Q/Q genotype, 12 for the K/Q and 13 for the K/K genotype).

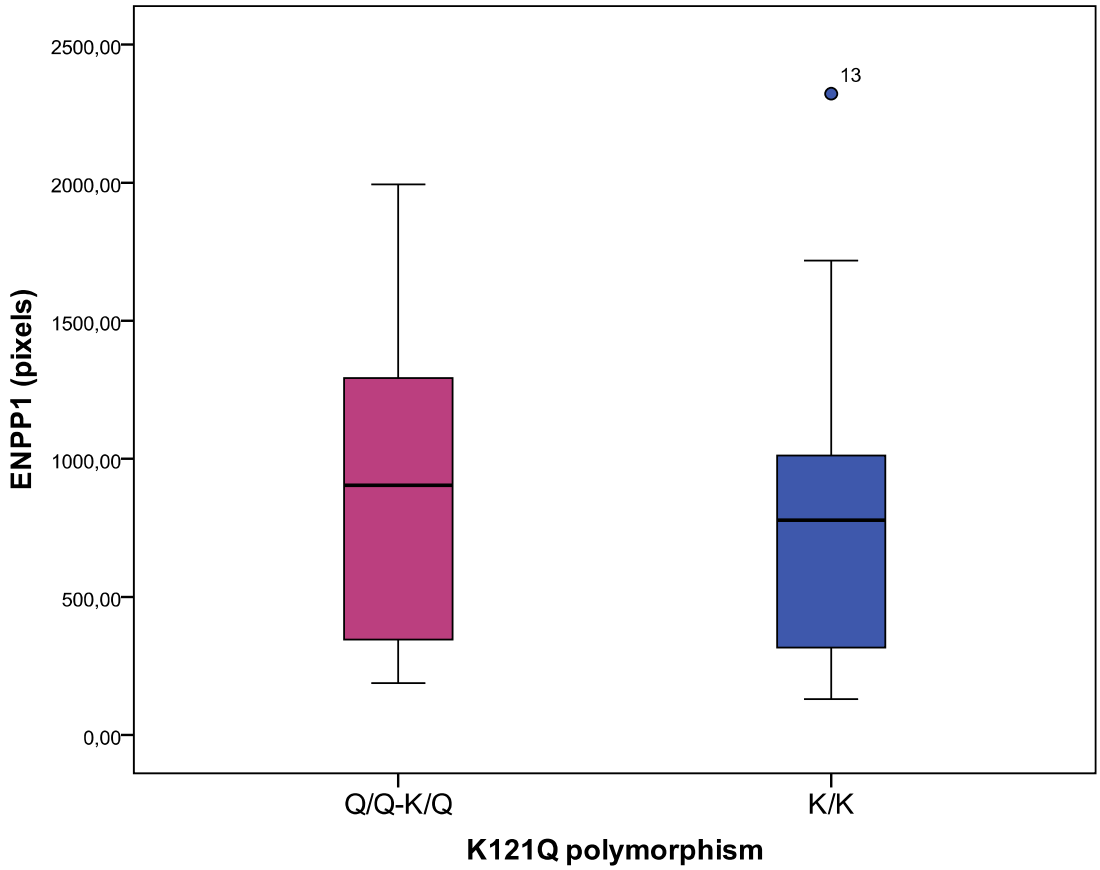

Figure 1. ENPP1 protein expression in human kidney samples. ENPP1 protein expression in samples stratified according to the presence of the Q allele (K/K *vs*. Q/K+Q/Q). P = 0.417 (Student's t-test). Values are represented as pixels and are presented as median (95% CI for mean).

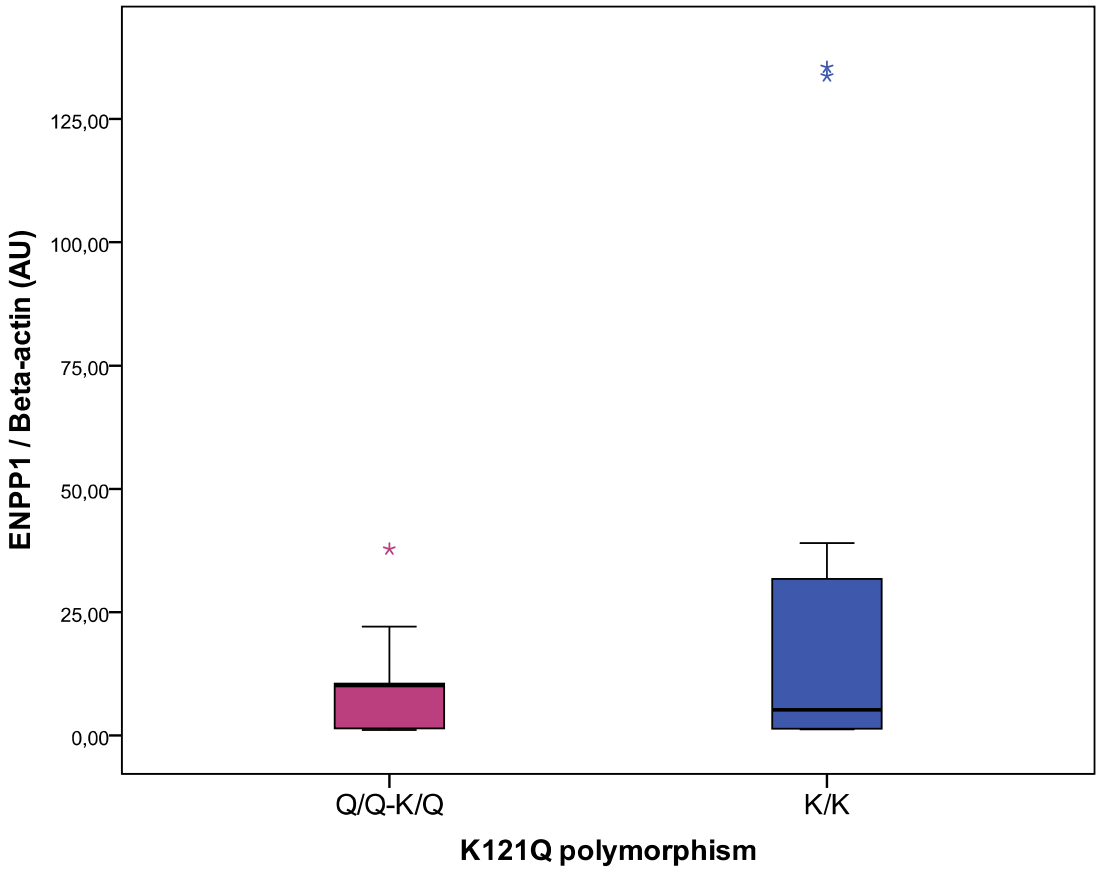

Figure 2. *ENPP1* mRNA expression in human kidney samples. *ENPP1* gene expression in samples stratified according to the presence of the Q allele (K/K *vs*. Q/K+Q/Q). P = 0.483 (Student's t-test). Values are represented as pixels and are presented as median (95% CI for mean).

CONCLUSÕES

O presente estudo investigou pioneiramente a expressão gênica e protéica do polimorfismo K121Q no gene *ENPP1* em rim humano, uma vez que este gene mostrou-se frequentemente associado à ND na literatura. Entretanto, nossos dados sugerem que o polimorfismo K121Q não está associado com modificação na expressão gênica, uma vez que as quantidades de cDNA da ENPP1 não diferiram significativamente entre os portadores do alelo de risco K/Q + Q/Q vs. K/K. Da mesma forma, a expressão protéica da ENPP1 não mostrou diferença em portadores (K/Q + Q/Q) quando comparados a não portadores (K/K) do genótipo de risco.

A ausência de associação tanto na expressão gênica quanto protéica pode ser explicada de forma alternativa à ausência de efeito do polimorfismo, pelo tipo de tecido analisado, que não foram de indivíduos com DM. Uma segunda hipótese, ainda que pouco provável, está relacionada com o desequilíbrio de ligação do polimorfismo K121Q com outras variantes do gene *ENPP1*. Ainda, acredita-se que este polimorfismo pode também influenciar mudanças na atividade enzimática desta proteína, mas não necessariamente alterações na expressão gênica da ENPP1. Por fim, alguns dos estudos que avaliaram a associação deste polimorfismo, mostraram que o alelo de risco está associado com progressão mais rápida de perda de função renal e/ou com diagnóstico de doença renal terminal mais precoce. Desta forma, talvez o polimorfismo seja importante uma vez que a ND está instalada, sendo determinante de sua evolução prognóstico.

PERSPECTIVAS

A partir dos achados deste estudo, torna-se necessário a investigação do polimorfismo K121Q com a atividade da ENPP1 em linhagem de células renais humanas, bem como estudos de RNAm e expressão protéica da ENPP1 em tecido renal de pacientes com DM em diferentes estágios de doença renal. Além disso, iremos avaliar o efeito deste polimorfismo na taxa de rejeição de rins transplantados, uma vez que o polimorfismo pode estar associado à progressão da doença renal.