The role of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 in diabetic nephropathy

Papel da ectonucleotídeo pirofosfatase/fosfodiesterase 1 (ENPP1) na nefropatia diabética

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SUMMARY

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. Arq Bras Endocrinol Metab. 2011;55(9):677-85

Keywords

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

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SUMÁRIO

A crescente prevalência do diabetes melito tem causado aumento na ocorrência das suas complicações crônicas, como a nefropatia diabética (ND), a qual está associada com elevada morbidade e mortalidade. Estudos de agregação familiar demonstram que a ND tem um importante componente genético, além dos conhecidos fatores de risco ambientais. Portanto, existe a necessidade de se identificarem genes associados ao risco ou proteção à ND. A *ectonucleotide pyrophosphatase/phosphodiesterase 1* (ENPP1) é expressa em vários tecidos, incluindo nos rins. Foi encontrado que níveis aumentados de expressão da ENPP1 inibem a atividade tirosino-quinase do receptor da insulina em vários tipos celulares, causando resistência à insulina. O polimorfismo K121Q do gene *ENNP1* parece estar associado com resistência à insulina e com o desenvolvimento da ND. A elucidação dos fatores genéticos e de suas associações permitirá um melhor entendimento da patogênese da ND e, consequentemente, poderemos ter uma abordagem mais efetiva em sua prevenção e tratamento. Arq Bras Endocrinol Metab. 2011;55(9):677-85

Descritores

ENPP1; nefropatia diabética; diabetes melito; polimorfismos de DNA; doença renal crônica; resistência à insulina

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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 m^2, 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 (8) 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. 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 cardiovas-

cular 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 NH_2 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. (25) 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. 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 NEPHROPHATY

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-α (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).

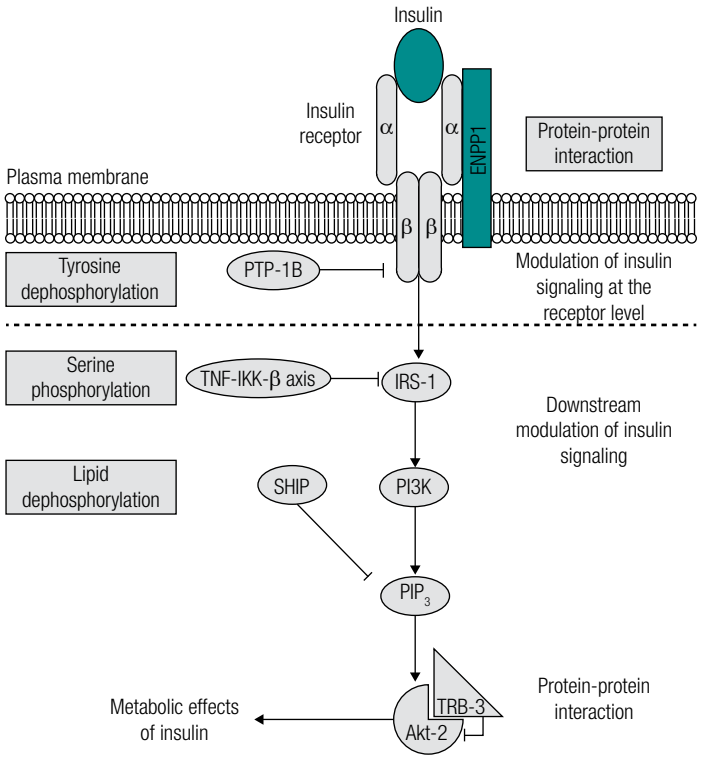

Figure 1. Systematic representation of insulin inhibitors signaling pathway with *in vivo* established function of insulin resistance. After binding to the α-subunit of its receptor, insulin stimulates the signal cascade events. The most important of these events are the tyrosine residue autophosphorylation of the insulin receptor β-subunits and IRS-1, and 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 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 in the insulin receptor, and by TRB-3 in Akt-2; 2) serine residue phosphorylation of the IRS-2, mediated by TNF-α 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; PIP_3: phosphatidylinositol 3,4,5-triphosphate; PTP-1B: protein tyrosine phosphatase 1B; TNF-α: tumor necrosis factor; SHIP: Src homology 2 domain containing inositol 5 phosphatase; TRB-3: mammalian tribbles homolog 3. Modified from Abate and cols. (45).

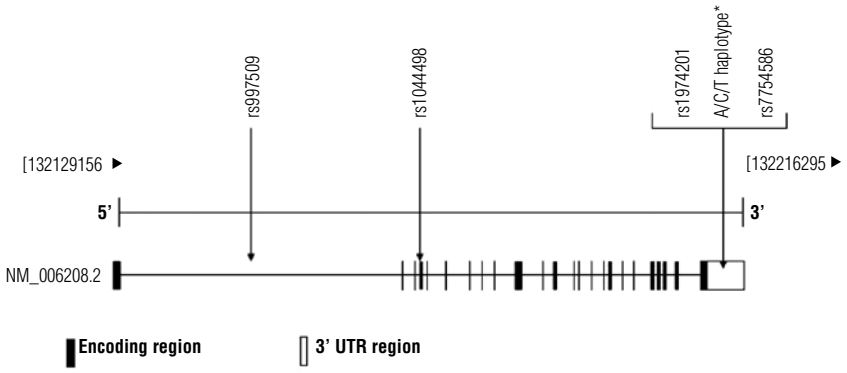

Figure 2. Map of the *ENPP1* gene in chromosome 6q22-23. The 25 exons (boxes) are numbered from left to right according to the transcription region. The black boxes constitute the encoding regions, and the white box represents the 3'UTR region. Vertical arrows show the polymorphic sites associated with 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.

In 1999, Pizzuti and cols. (36) 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 (23). Non-diabetic Sicilians carrying the Q variant show less insulin sensitivity than non-carriers (36). Pizzuti and cols. (36) 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. 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. (49) 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. 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).

Table 1. Studies on the association between K121Q polymorphism in *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.

Canani and cols. (50), in both a case-control and a family 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. Similar results were found in the familial study, using the transmission disequilibrium test.

Recently, Wu and cols. (51) also reported the association between the Q variant and increased risk of DN in type 2 DM patients from Taiwan. Likewise, De Cosmo and cols. (52), 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. 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 (53) 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.

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. (55) 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. Leitao and cols. (54) 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. In a prospective, 10-year follow-up study of 30 normoalbuminuric and normotensive type 1 DM patients from southern Brazil, de Azevedo and cols. (56), did not find an association between K121Q polymorphism, and the development of new cases of DN or diminished GFR. Jacobsen and cols. (57) did not observe any association between the K121Q polymorphism and DN progression in Danish type 1 DM patients.

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, phosphatidylinositol 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*

Meyre and cols. (61) 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. Frittitta and cols. (58) 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).

Bochenski and cols. (62) 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 controlled 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). Furthermore, 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. (63), studying lean and obese children, observed that those who were carriers of the rs997509 T allele of *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. A similar observation was made for the Q variant of 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. (55) 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 two or more polymorphisms located on the 3'UTR region or intron 24 of the *ENPP1* gene.

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 development 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.

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