# Polymorphism in Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase 1 Gene (*ENPP1/PC-1*) and Early Development of Advanced Diabetic Nephropathy in Type 1 Diabetes

**Luis H. Canani, Daniel P.K. Ng, Adam Smiles, John J. Rogus, James H. Warram, and Andrzej S. Krolewski**



**A polymorphism in the ecto-nucleotide pyrophosphatase/phosphodiesterase 1 gene (*ENPP1*) (previously known as *PC-1*), resulting in an amino acid change from lysine to glutamine at codon 121 (K121Q), is associated with insulin resistance. A small follow-up study of patients with type 1 diabetes and proteinuria found that renal function declines more rapidly in carriers of the Q variant than in noncarriers. To examine this finding further, we conducted a large case-control study and a family-based study. Genomic DNA was obtained from 659 patients: 307 with normal urinary albumin excretion despite diabetes duration of >15 years (control subjects) and 352 with advanced diabetic nephropathy, of whom 200 had persistent proteinuria and 152 had end-stage renal disease (ESRD). Individuals were genotyped for Q and K variants using a previously described protocol. The frequency of Q variant carriers was 21.5% in control subjects, 31.5% in subjects with proteinuria, and 32.2% in subjects with ESRD ($P = 0.012$). In a stratified analysis according to duration of diabetes, the risk of early-onset ESRD for carriers of the Q variant was 2.3 times that for noncarriers (95% CI, 1.2–4.6). The Q variant was not associated with late-onset ESRD. Similar findings were obtained in a family-based study. We conclude that carriers of the Q variant of *ENPP1* are at increased risk for developing ESRD early in the course of type 1 diabetes. *Diabetes* 51:1188–1193, 2002**

There is growing evidence that genetic factors play an important role in the development of diabetic nephropathy (1). Efforts to identify these factors rely primarily on the candidate gene approach, that is, searching for DNA sequence differences (polymorphisms/mutations) in candidate genes and testing them for association with diabetic nephropathy. Candidates include any gene that encodes for a protein involved in a pathway that contributes to the etiology of diabetic nephropathy. Insulin resistance has been implicated in the progression of microvascular complications of diabetes (2) and has also been shown to be a common characteristic of both type 1 and type 2 diabetic patients with increased urinary albumin excretion (3,4). Therefore, candidate genes for insulin resistance may be considered candidates for diabetic nephropathy as well.

A polymorphism in the ecto-nucleotide pyrophosphatase/phosphodiesterase 1 gene (*ENPP1*), previously known as *PC-1*, has been demonstrated to be associated with insulin resistance (5). ENPP1 is one of five cell membrane proteins with an extracellular active site catalyzing the release of nucleoside 5′-monophosphatase from nucleotides and their derivatives. These proteins consist of a short $NH_2$-terminal intracellular domain, a single transmembrane domain, two somatomedin-B–like domains, a catalytic domain, and a COOH-terminal nuclease-like domain. Three of these proteins (ENPP1–3) have been characterized and implicated in various processes, including bone mineralization, signaling by insulin and by nucleotides, and the differentiation and motility of cells (6). However, the precise mechanisms through which the proteins affect these processes are not known.

ENPP1 is by far the best-characterized ENPP. It was first discovered as a surface marker of antibody-secreting B-cells, hence the name plasma-cell differentiation antigen-1 or PC-1 (6). Now, however, it is known that ENPP1 is expressed in various tissues, including muscle, fat, liver, and kidney (7). The physiologic functions of ENPP1 in these tissues have not been described. However, it has been found that overexpression of ENPP1 inhibits insulin receptor tyrosine kinase activity in various cells (8–10) and causes insulin resistance (8,11,12). ENPP1 seems to inhibit insulin signal transduction by interacting directly with the insulin receptor α subunit (12).

The gene encoding for ENPP1 has 25 exons and is located on human chromosome 6q22–23 (5,13). Recently, Pizzuti et al. (5) described a DNA polymorphism in exon 4 that causes an amino acid change from lysine to glutamine at codon 121 (K121Q). This amino acid change is located in the second somatomedin-B–like domain of ENPP1 and may interfere with protein-protein interactions (6). Studies in vitro have shown that the Q variant of *ENPP1* interacts more strongly with the insulin receptor than the K variant

From the Research Division, Joslin Diabetes Center, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Andrzej S. Krolewski, Section on Genetics and Epidemiology, Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: andrzej.krolewski@joslin.harvard.edu.

Received for publication 31 July 2001 and accepted in revised form 4 January 2002.

ACR, albumin/creatinine ratio; ESRD, end-stage renal disease; GFR, glomerular filtration rate; IRS, insulin receptor substrate; TDT, transmission disequilibrium test.

and reduces insulin receptor autophosphorylation (5). The Q variant also reduces insulin-induced phosphorylation of insulin receptor substrate (IRS)-1, phosphatidylinositol-3 kinase activity, glycogen synthesis, and cell proliferation (14). Furthermore, human studies demonstrated that Sicilian carriers of the Q variant had lower insulin sensitivity than noncarriers. Similar results were obtained in studies of Finns and Swedes (15) but not of Danes (16).

De Cosmo et al. (17) described an effect of the *ENPP1* polymorphism on the rate of loss of renal function in Caucasians with type 1 diabetes and proteinuria. During 6.5 years of follow-up, the glomerular filtration rate (GFR) declined more rapidly in carriers of the Q variant (QQ/KQ, $n = 22$) than in noncarriers (KK, $n = 55$). The median rates of decline were 7.2 and 3.7 $ml \cdot min^{-1} \cdot year^{-1}$, respectively. With such rapid loss of renal function, diabetic carriers of the Q variant would progress through the stage of proteinuria to end-stage renal disease (ESRD) in fewer years than noncarriers. Therefore, the effect of this variant would be most evident when ESRD develops early in the course of diabetes.

The objective of this study was to investigate the association of advanced diabetic nephropathy with the K121Q polymorphism in *ENPP1*. Specifically, we sought to test whether the Q variant confers an increased risk of the development of ESRD early in the course of type 1 diabetes. Both case-control and family-based (transmission disequilibrium test [TDT]) study designs were used.

## RESEARCH DESIGN AND METHODS

**Study groups.** Individuals with type 1 diabetes were recruited for the study from among patients attending the Joslin Clinic in the decade 1991–2000. Diabetes was classified as type 1 if it was diagnosed before age 30 years and if continuous treatment with insulin began within 1 year of diagnosis. During 1991–1993, we enrolled a 50% sample of Joslin Clinic attendees with type 1 diabetes in the age range 15–44 years ($n = 1{,}598$) in the Natural History of Microalbuminuria Study (18). On the basis of multiple measurements of the albumin/creatinine ratio (ACR) in random urine specimens during a 2-year observation period, patients were classified as having normoalbuminuria ($n = 1{,}080$), persistent microalbuminuria ($n = 312$), or advanced nephropathy (persistent proteinuria or ESRD; $n = 206$) (18).

As unrelated control subjects for genetic studies on diabetic nephropathy, we selected Caucasian patients with normoalbuminuria and type 1 diabetes duration ≥15 years. Among the 1,080 normoalbuminuric participants in the Natural History of Microalbuminuria Study, 420 met these additional criteria, and we examined 307 (73%) of them.

As unrelated cases for the genetic studies on diabetic nephropathy, we selected Caucasian patients with persistent proteinuria or ESRD. We were able to enroll and examine 152 of the 206 patients with advanced nephropathy identified in the Natural History Study described above. In addition, we enrolled and examined 201 (86%) of 235 Caucasian patients with type 1 diabetes and proteinuria or ESRD attending the clinic between 1994 and 2000 who had not been included in the 50% random sample screened in 1991–1993 for the Natural History Study. The two groups of patients did not differ with regard to sex, age at examination, duration of diabetes, or values of $HbA_{1c}$, total serum cholesterol, or serum triglycerides. Therefore, we pooled the two groups, bringing the total number of unrelated subjects with advanced diabetic nephropathy to 352.

For the family-based association study, both parents of 174 patients and both parents of 78 control subjects were examined.

**Examination of study participants.** After consenting to participate in the study, each subject had a standardized physical examination and provided a diabetes history (diagnosis, treatment, and complications). Each individual provided a blood sample for biochemical measurements and DNA extraction. For the family-based study, parents provided blood samples for DNA extraction. The Committee on Human Subjects of the Joslin Diabetes Center approved the protocol and informed consent procedures.

**Diagnosis of diabetic nephropathy.** The diabetic nephropathy status of each patient was determined on the basis of questionnaires, medical records of the Joslin Clinic (supplemented with records of other physicians if necessary), and measurements of the ACR. Methods for measuring the ACR have been described previously (18). Patients were classified as control subjects ($n = 307$) if they had diabetes duration ≥15 years and ACR <17 mg/g (men) or <25 mg/g (women) in at least two of the three most recent urine specimens. Patients with microalbuminuria or intermittent proteinuria were excluded from the study. Patients were considered case subjects ($n = 352$) if they had persistent proteinuria or if they had ESRD due to diabetic nephropathy. Persistent proteinuria was defined as two of three successive urinalyses positive by reagent strip (≥2+ on Multistix; Bayer, Elkhart, IN) or as ACR ≥250 mg/g (men) or ≥355 mg/g (women). At the time of the first examination (1992–2000), subjects were divided into those with persistent proteinuria ($n = 260$) and those with ESRD ($n = 92$). Patients with proteinuria were followed until the end of July 2001, by which time ESRD had developed in 60 of them, i.e., renal dialysis had been initiated. These patients were considered incident case subjects of ESRD. The individuals who had begun dialysis or received a renal transplant before the first examination were considered prevalent case subjects of ESRD. For prevalent or incident cases of ESRD, the year when dialysis began was used to calculate the duration of diabetes. In total, we had 152 subjects with ESRD (92 prevalent and 60 incident).

**Genotyping protocol.** The DNA polymorphism in exon 4 of *ENPP1* has been described previously (5). A transversion from A to C at the first position of codon 121, resulting in an amino acid change from lysine to glutamine (K121Q), was genotyped with a previously described protocol (5). PCR was performed using the described conditions and primers: forward 5′-CTGTGT TCACTTTGGACATGTTG-3′ and reverse 5′-GACGTTGGAAGATACCAGGTTG-3′. PCR products were digested by the restriction enzyme *Ava*II and separated on agarose gel. The allele encoding the K variant appeared as a single fragment of 238 bp, and the allele encoding the Q variant appeared as two fragments of 148 and 90 bp each.

Q carriers (KQ and QQ) were analyzed together, given that both genotypes were previously described to be associated with faster decline in GFR (17).

**Analytic methods.** The data from this study were analyzed first as a case-control study. The characteristics of the case and control subjects were compared by $\chi^2$ test or Fisher's exact test for frequencies and one-way ANOVA for continuous variables. Odds ratios (ORs) and 95% CIs for persistent proteinuria and ESRD were estimated for carriers of the Q variant (19). Logistic regression analysis and Breslow-Day test were performed in SAS (version 8.02 for Windows; SAS Institute, Cary, NC).

Transmission of the risk variant from heterozygous parents to offspring was assessed by TDT (20). Based on an a priori hypothesis that the Q variant confers risk for diabetic nephropathy, a one-sided exact test based on binomial distribution was used to test the transmission of the risk allele. We ascertained the total number of heterozygous parents (i.e., the number of Bernoulli trials) and the total number of these parents transmitting the Q allele by direct counting and calculated statistical significance using the standard Excel spreadsheet function (BINOMDIST).

## RESULTS

The data were analyzed first as a case-control study. Comparisons were made between the one group of control subjects and two groups of case subjects, proteinuric and ESRD. The control group consisted of individuals with long-duration type 1 diabetes and normal urinary albumin excretion (normoalbuminuria). Proteinuric subjects had proteinuria when they were enrolled in the study and did not progress to ESRD during follow-up. ESRD subjects were subgrouped according to whether they had ESRD when they were enrolled in the study (prevalent case subjects, $n = 92$) or developed ESRD during follow-up (incident case subjects, $n = 60$). For most of the prevalent case subjects, ESRD had developed in the decade 1983–1993, before ACE inhibition was considered standard treatment for diabetic nephropathy. For incident subjects, ESRD developed between 1993 and 2001, and 48% of them had been treated with ACE inhibitors from the time they were enrolled. Otherwise, the two ESRD groups had similar clinical characteristics and were combined. Characteristics of the three study groups are summarized in Table 1. Age at examination was similar in all three groups, whereas duration of diabetes was slightly longer for case subjects than for control subjects. Both groups of case

TABLE 1
Clinical characteristics according to renal status

| | Control subjects | Proteinuria subjects | ESRD* subjects | *P* |
|---|---|---|---|---|
| *n* | 307 | 200 | 152 | |
| Sex (% male) | 49.2 | 55.5 | 50.0 | 0.36 |
| Age at examination (years) | 36 ± 7.8 | 36 ± 8.0 | 37 ± 5.4 | 0.19 |
| Diabetes duration (years) | 24 ± 6.9 | 25 ± 8.0 | 26 ± 8.0 | 0.03 |
| $HbA_{1c}$ (%) | 8.1 ± 1.3 | 9.14 ± 1.7 | 9.00 ± 1.8 | <0.001 |
| Creatinine (mg/dl) | 0.9 ± 0.2 | 1.2 ± 0.5 | —† | <0.001 |
| Treated hypertension (%) | 7.8 | 54.5 | 65.8 | <0.001 |
| ACE inhibition (%) | 5.2 | 47.0 | 28.9 | <0.001 |
| Cholesterol (mg/dl) | 192 ± 40 | 234 ± 67 | 238 ± 82 | <0.001 |
| HDL cholesterol (mg/dl) | 55 ± 17 | 52 ± 17 | 52 ± 19 | 0.33 |
| Triglycerides (mg/dl)‡ | 111 ± 59 | 177 ± 134 | 195 ± 120 | <0.001 |
| Q carrier frequency (%) | 21.5 | 31.5 | 32.2 | 0.01 |
| Q allele frequency (%) | 12.1 | 17.0 | 17.8 | 0.03 |

Data are means ± SD or %. *The ESRD group consisted of 92 prevalent and 60 incidence case subjects of ESRD. †Of ESRD patients, 68.7% had a kidney transplant at the time of enrollment. ‡Significance test performed on log-transformed data.

subjects were more frequently treated with antihypertensive medication (including ACE inhibitors) and had higher values of $HbA_{1c}$, cholesterol, and triglycerides than the control group.

*ENPP1* genotypes were in Hardy-Weinberg equilibrium in each of the study groups. However, the prevalence of Q carriers was different in the three groups: 21.5% in control subjects (2.3% QQ), 31.5% in subjects with proteinuria (2.5% QQ), and 32.2% in subjects with ESRD (3.3% QQ) (2 degrees of freedom [df], $P = 0.012$) (Table 1). The frequency of the *Q* allele paralleled the frequency of carriers (2 df, $P = 0.026$). The frequency of carriers of the Q variant was similar in prevalent (30.4%) and incident (35%) patients of ESRD. Expressed as ORs, these frequencies indicate that carriers of the Q variant of *ENPP1* have a higher risk of advanced nephropathy than noncarriers: OR 1.6 for proteinuria and 1.7 for ESRD (95% CI 1.2–2.5, if the two groups of case subjects are combined).

To examine the hypothesis that the Q variant is associated with the early development of advanced diabetic nephropathy in type 1 diabetes, we divided each of the study groups at the median duration of diabetes, 24 years (Table 2). Among subjects with diabetes duration <24 years, carriers of the Q variant had a significantly higher overall risk of advanced diabetic nephropathy (proteinuria and ESRD combined) than noncarriers: OR 1.9 (95% CI 1.1–3.3). Moreover, in agreement with our hypothesis, the OR for ESRD was 2.3 (95% CI 1.2–4.4) and only 1.6 for persistent proteinuria, the latter being nonsignificant (95% CI 0.9–2.9). Among subjects with diabetes duration ≥24 years, carriers of the Q variant had a higher overall risk of advanced diabetic nephropathy than noncarriers, but the OR was not significantly different from 1: OR 1.6 (95% CI 0.9–2.7). However, their risk of persistent proteinuria was significantly increased (OR 1.9, 95% CI 1.1–3.3), whereas their risk of ESRD was not (OR 1.3, 95% CI 0.7–2.4). With the sample sizes available, the OR for ESRD in subjects with diabetes duration <24 years was not significantly different from that in subjects with duration ≥24 years (Breslow-Day test). Similarly, when we used a multiple logistic model with duration of diabetes as a continuous variable instead of as dichotomous, the interaction between the presence of the Q variant and diabetes duration was still not statistically significant (data not shown).

The family association study confirmed the results of the case-control study (Table 3). The allele that encodes the Q variant was transmitted from heterozygous parents to index subjects with ESRD significantly more often than the expected 50% (30 of 47, 64%; $P = 0.040$). When stratified by diabetes duration (<24 and ≥24 years), transmission of the Q variant was significantly increased only among those with an early onset of ESRD (16 of 23, 70%; $P = 0.047$). Among those with diabetes of longer duration (≥24 years), transmission of the Q variant was not different from the expected 50% (14 of 24; $P = 0.270$). Transmission of the Q variant to index subjects with

TABLE 2
Risk of advanced diabetic nephropathy in carriers of the Q variant of *ENPP1* according to duration of type 1 diabetes

| | Renal status | | |
|---|---|---|---|
| | Control subjects | Proteinuria subjects | ESRD subjects |
| Diabetes duration <24 years | | | |
| Noncarriers of Q variant, (*n*) | 112 | 82 | 48 |
| Carriers of Q variant (*n*) | 27 | 32 | 27 |
| Odds ratio | 1.0 | 1.6 | 2.3 |
| 95% CI | Reference | 0.9–2.9 | 1.2–4.4 |
| Diabetes duration ≥24 years | | | |
| Noncarriers of Q variant, (*n*) | 129 | 55 | 55 |
| Carriers of Q variant (*n*) | 39 | 31 | 22 |
| Odds ratio | 1.0 | 1.9 | 1.3 |
| 95% C.I. | Reference | 1.1–3.3 | 0.7–2.4 |

TABLE 3
Transmission of the Q variant of *ENPP1* from heterozygous parents to offspring according to renal status

| Renal status of offspring | Heterozygous parents | Transmission of Q variant | | |
|---|---|---|---|---|
| | | Transmitted | Not transmitted | *P* |
| ESRD | 47 | 30 (64) | 17 | 0.04 |
| Proteinuria | 47 | 26 (55) | 21 | 0.28 |
| Control | 43 | 21 (49) | 22 | 0.50 |

Data are *n* or *n* (%).

proteinuria was close to the expected (45%; $P = 0.280$) and did not differ when stratified by duration of diabetes (data not shown). Transmission of the Q variant to index cases with normoalbuminuria was close to the expected (21 of 43, 49%; $P = 0.500$).

In the group of control and case subjects with proteinuria, carriers of the Q variant (QQ or KQ) did not differ from noncarriers (KK) with regard to BMI, systemic blood pressure, lipid profile, $HbA_{1c}$, or frequency of treatment with ACE inhibitors (data not shown).

## DISCUSSION

In this study, we demonstrated an association between the Q variant of *ENPP1* and the presence of advanced diabetic nephropathy, diagnosed as persistent proteinuria or ESRD. Moreover, the association was strongest if ESRD developed early in the course of diabetes (<24 years' duration), as would be predicted if the Q variant accelerates the rate of progression through the stages of diabetic nephropathy. Thus, while we do not have a measure of the rate of decline of GFR in our subjects during the stage of proteinuria, this pattern of association is consistent with the accelerated decline in renal function reported by De Cosmo et al. (17) in carriers of the Q variant. In a 6.5-year follow-up study of individuals with type 1 diabetes and proteinuria, the GFR rate declined faster in carriers of the Q variant than in noncarriers, 7.2 ml · $min^{-1}$ · $year^{-1}$ compared with 3.7 ml · $min^{-1}$ · $year^{-1}$ (17). Although their patients were not followed to the onset of ESRD, one would expect that the faster decline in GFR would bring carriers of the Q variant to renal failure earlier in the course of diabetes than noncarriers. Together, the two studies provide complementary evidence that individuals with type 1 diabetes and the Q variant of *ENPP1* are at high risk of advanced diabetic nephropathy and the early development of ESRD. This conclusion, however, is not supported by the recent study published by Tarnow et al. (21). The negative results of that study are most likely due to an insufficient number of cases with ESRD and too-small study groups to detect an OR as low as 1.7.

Because case-control findings may be biased by unrecognized population stratification, a family-based approach, the TDT, can be used to examine the association of the Q variant with diabetic nephropathy. For this test, DNA from both parents is examined, and only those families with at least one heterozygous parent are used in the analysis. Excess transmission of a variant beyond the expected 50% confirms its role as a risk allele. In agreement with the results of the case-control study, transmission of the allele that encodes the Q variant to index subjects with early-onset ESRD exceeds the expected 50%. In contrast, transmission of the Q variant from heterozygous parents to subjects with persistent proteinuria is consistent with the expected 50%. Thus, if the Q variant does have an impact on the risk of proteinuria, the effect is too weak to be detected in our relatively small number of TDT families.

Our findings can be interpreted as supporting two alternative disease models. The first invokes two effects of the Q variant: *1*) carriers of Q have a moderately increased risk of developing persistent proteinuria, regardless of the duration of diabetes, and *2*) the Q variant interacts in some people with a factor to accelerate progression of proteinuria to ESRD. Curiously, that factor is relatively isolated to individuals with short-duration diabetes. This model attributes the negative TDT analysis in late-onset proteinuria to a lack of power (type 2 error). The second model is simpler and, therefore, considered more likely: the Q variant accelerates the natural history of diabetic nephropathy (earlier onset and faster progression through each stage), so the association is strongest in patients with the earliest onset of ESRD. This model attributes the association of the Q variant with late-onset proteinuria in the case-control study to type 1 error and accepts the negative TDT analysis as truth rather than type 2 error. With the available evidence, distinction between these two models is not possible. Much larger groups for case-control comparisons and a much larger set of families, with both parents examined for TDT analysis, will be required.

The molecular mechanisms accounting for the association between the Q variant of *ENPP1* and the development of advanced stages of diabetic nephropathy can only be hypothesized. *ENPP1* is expressed in various tissues, including muscle, fat, liver, and kidney (7). Recently, we demonstrated that this gene is also expressed in human mesangial and endothelial cells (L.H.C., D.P.K.N., A.S.K., unpublished data). This pattern of expression is compatible with the localization of diabetic nephropathy disease processes. The DNA sequence difference in exon 4 of *ENPP1* results in an amino acid change from lysine to glutamine at codon 121 (K121Q). In a recent review, Bollen et al. (6), using a different transcriptional start site, suggested that the actual position of the polymorphism is at codon 173 (K173Q). This amino acid change is located in the second somatomedin-B–like domain of *ENPP1*, and it seems to interfere with the homodimerization of ENPP1 and interactions between ENPP1 and other proteins (6).

For example, studies in vitro show that the Q variant of *ENPP1* interacts more strongly with the insulin receptor than the common K variant. Because of this, cells with the Q variant have reduced insulin receptor autophosphorylation (5). The Q variant also reduces insulin-induced phosphorylation of the insulin receptor substrate (IRS)-1, phosphatidylinositol 3-kinase activity, glycogen synthesis, and cellular proliferation (22). In humans, carriers of the Q variant are more insulin resistant and hyperinsulinemic than noncarriers (15). Hyperinsulinemia may stimulate renal sodium reabsorption, leading to volume expansion, enhanced sympathetic adrenergic activity, and upregulation of the angiotensin II type 1 receptor, causing impaired peripheral vasodilation (23). Volume overload and impaired peripheral vasodilation may predispose to blood pressure elevation and decrease nocturnal dipping of the

blood pressure (23,24). These are known phenotypic characteristics of ensuing diabetic nephropathy. However, in agreement with De Cosmo et al. (17), carriers and noncarriers of the Q variant in our study did not differ with regard to phenotypic characteristics of the insulin resistance syndrome, such as BMI, blood pressure, lipid profile, or $HbA_{1c}$ values. However, because we did not measure insulin sensitivity directly, we cannot exclude the possibility that the early development of ESRD in carriers of the Q variant might be accounted for by factors associated with insulin resistance.

Similar to the insulin receptor, ENPP1 may be a binding partner of integrins, which may be implicated in the pathologic processes involved in progression of diabetic nephropathy. Integrins are extracellular-matrix proteins involved in cell adhesion (25). Hyperglycemia is reported to influence expression of integrins in mesangial cells (26), and alterations in expression of these molecules has been hypothesized to be involved in the pathogenesis of diabetic nephropathy (27). The Q variant of *ENPP1* may interfere with this putative process, leading in some unknown way to increased risk of diabetic nephropathy.

Also noteworthy is the cysteine richness of somatomedin-B–like domains, which may be involved in the dimerization of ENPPs via disulfide bonds (6). However, this has not been studied, and the impact of the Q variant on structure and function of dimerized ENPP1 is unknown. ENPP1 does regulate the concentration of pharmacologically active extracellular compounds such as adenosine and its derivatives, and they affect cell motility (28). Other possible functions of ENPP1 are associated with sulfation of glycosaminoglycans and regulation of cellular proliferation through modulation of local concentrations of ATP. Any of these functions may be relevant to the development of diabetic nephropathy in patients with type 1 diabetes.

Finally, we cannot exclude the possibility that *ENPP1* polymorphism is in linkage disequilibrium with flanking DNA sequence differences that may be responsible for susceptibility to diabetic nephropathy. Screening of 25 exons of this gene has not yielded any DNA sequence differences beyond the polymorphism in exon 4 that was investigated in this report (17). A relatively infrequent haplotype (6.8% in the general population) in the 3′ end of the gene is associated with insulin resistance (22). This sequence difference was not examined by us, but confounding of our findings by this haplotype is unlikely because it is in linkage equilibrium with the polymorphism in exon 4 (22).

To investigate the possibility that our findings could be explained by linkage disequilibrium with DNA sequence difference in flanking genes, 50-kb sequences 5′ and 3′ from the ENPP1 locus were searched in silico (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). No known genes were found in this region. Although three human UniGene EST clusters were identified (Hs.25557, Hs.44230, and Hs.183276), BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) did not reveal any similarity between these clusters and any known gene or protein. However, one gene from the nucleotide pyrophosphatase/phosphodiesterase family, *ENPP3*, is located ~60 kb centromeric to *ENPP1*. *ENPP3* has 25 exons and spans ~60 kb (29). No systematic search for DNA polymorphisms in this gene has been conducted. Further work is needed to exclude the possibility that DNA sequence differences in this gene could be in linkage disequilibrium with the Q variant of *ENPP1*.

Because patients with diabetic nephropathy have increased mortality (30,31), the possibility of spurious findings due to survival bias has to be evaluated, especially when a majority of patients are prevalent case subjects. In our study, this would imply that the excess of Q carriers in subjects with early-onset ESRD was a spurious result due to better survival of Q carriers than KK homozygotes. While definitive exclusion of this possibility is feasible only in a long-term follow-up study, several features of our investigation provide strong arguments against it. First, we studied relatively young individuals with ESRD, in whom mortality is still low. Second, the frequency of Q variant carriers was not higher in prevalence than among incident cases of ESRD, despite their greater possibility to be affected by selective survival. Finally, a survival advantage associated with the Q variant would be most evident in prevalent case subjects of ESRD and long duration of diabetes, where the cumulative effect of mortality would be highest.

## ACKNOWLEDGMENTS

This research was supported by National Institutes of Health Grant DK41526 and DK53534. L.H.C. was supported by a grant from Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (BEX0034/99–1). D.P.K.N. was supported by a grant from Juvenile Diabetes Foundation (3-2000-82).

We are indebted to Lucjan Wyrwicz for his assistance with in silico work.

## REFERENCES

1. Krolewski AS: Genetics of diabetic nephropathy: evidence for major and minor gene effects. *Kidney Int* 55:1582–1596, 1999
2. Suzuki M, Kanazawa A, Shiba M, Kojima H, Harano Y: Insulin resistance in diabetic microangiopathies. *J Diabetes Complications* 14:40–45, 2000
3. Yip J, Mattock MB, Morocutti A, Sethi M, Trevisan R, Viberti G: Insulin resistance in insulin-dependent diabetic patients with microalbuminuria. *Lancet* 342:883–887, 1993
4. Groop L, Ekstrand A, Forsblom C, Widen E, Groop PH, Teppo AM, Eriksson J: Insulin resistance, hypertension and microalbuminuria in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 36:642–647, 1993
5. Pizzuti A, Frittitta L, Argiolas A, Baratta R, Goldfine ID, Bozzali M, Ercolino T, Scarlato G, Iacoviello L, Vigneri R, Tassi V, Trischitta V: A polymorphism (K121Q) of the human glycoprotein PC-1 gene coding region is strongly associated with insulin resistance. *Diabetes* 48:1881–1884, 1999
6. Bollen M, Gijsbers R, Ceulemans H, Stalmans W, Stefan C: Nucleotide pyrophosphatases/phosphodiesterases on the move. *Crit Rev Biochem Mol Biol* 35:393–432, 2000
7. Goding JW, Howard MC: Ecto-enzymes of lymphoid cells. *Immunol Rev* 161:5–10, 1998
8. Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, Spencer S, Grupe A, Henzel W, Stewart TA, et al.: Membrane glycoprotein PC-1 and insulin resistance in non-insulin-dependent diabetes mellitus. *Nature* 373:448–451, 1995
9. Kumakura S, Maddux BA, Sung CK: Overexpression of membrane glycoprotein PC-1 can influence insulin action at a post-receptor site. *J Cell Biochem* 68:366–377, 1998
10. Maddux BA, Goldfine ID: Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor alpha-subunit. *Diabetes* 49:13–19, 2000
11. 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* 39:1190–1195, 1996
12. Frittitta L, Youngren JF, Sbraccia P, D'Adamo M, Buongiorno A, Vigneri R, Goldfine ID, Trischitta V: 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* 40:282–289, 1997
13. 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 Chem* 265:17506–17511, 1990
14. Costanzo BV, Trischitta V, Di Paola R, Spampinato D, Pizzuti A, Vigneri R, Frittitta L: 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* 50:831–836, 2001
15. Gu HF, Almgren P, Lindholm E, Frittitta L, Pizzuti A, Trischitta V, Groop LC: Association between the human glycoprotein PC-1 gene and elevated glucose and insulin levels in a paired-sibling analysis. *Diabetes* 49:1601–1603, 2000
16. Rasmussen SK, Urhammer SA, Pizzuti A, Echwald SM, Ekstrom CT, Hansen L, Hansen T, Borch-Johnsen K, Frittitta L, Trischitta V, Pedersen O: The K121Q variant of the human PC-1 gene is not associated with insulin resistance or type 2 diabetes among Danish Caucasians. *Diabetes* 49:1608–1611, 2000
17. De Cosmo S, Argiolas A, Miscio G, Thomas S, Piras GP, Trevisan R, Perin PC, Bacci S, Zucaro L, Margaglione M, Frittitta L, Pizzuti A, Tassi V, Viberti GC, Trischitta V: A PC-1 amino acid variant (K121Q) is associated with faster progression of renal disease in patients with type 1 diabetes and albuminuria. *Diabetes* 49:521–524, 2000
18. Warram JH, Gearin G, Laffel L, Krolewski AS: Effect of duration of type I diabetes on the prevalence of stages of diabetic nephropathy defined by urinary albumin/creatinine ratio. *J Am Soc Nephrol* 7:930–937, 1996
19. Greenland S, Rothman K, Eds: *Measures of Effect and Measures of Association*. Philadelphia, Lippincott-Raven, 1998, p. 47–64
20. Spielman RS, McGinnis RE, Ewens WJ: Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52:506–516, 1993
21. Tarnow L, Grarup N, Hansen T, Parving HH, Pedersen O: Diabetic microvascular complications are not associated with two polymorphisms in the GLUT-1 and PC-1 genes regulating glucose metabolism in Caucasian type 1 diabetic patients. *Nephrol Dial Transplant* 16:1653–1656, 2001
22. Frittitta L, Ercolino T, Bozzali M, Argiolas A, Graci S, Santagati MG, Spampinato D, Di Paola R, Cisternino C, Tassi V, Vigneri R, Pizzuti A, Trischitta V: 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* 50:1952–1955, 2001
23. Abate N: Obesity and cardiovascular disease: pathogenetic role of the metabolic syndrome and therapeutic implications. *J Diabetes Complications* 14:154–174, 2000
24. McFarlane SI, Banerji M, Sowers JR: Insulin resistance and cardiovascular disease. *J Clin Endocrinol Metab* 86:713–718, 2001
25. Schwartz MA: Integrin signaling revisited. *Trends Cell Biol* 11:466–470, 2001
26. Chen HC, Chen CA, Guh JY, Chang JM, Shin SJ, Lai YH: Altering expression of alpha3beta1 integrin on podocytes of human and rats with diabetes. *Life Sci* 67:2345–2353, 2000
27. Jin DK, Fish AJ, Wayner EA, Mauer M, Setty S, Tsilibary E, Kim Y: Distribution of integrin subunits in human diabetic kidneys. *J Am Soc Nephrol* 7:2636–2645, 1996
28. Goding JW, Terkeltaub R, Maurice M, Deterre P, Sali A, Belli SI: Ecto-phosphodiesterase/pyrophosphatase of lymphocytes and non-lymphoid cells: structure and function of the PC-1 family. *Immunol Rev* 161:11–26, 1998
29. Andoh K, Piao JH, Terashima K, Nakamura H, Sano K: Genomic structure and promoter analysis of the ecto-phosphodiesterase I gene (PDNP3) expressed in glial cells. *Biochim Biophys Acta* 1446:213–224, 1999
30. Warram JH, Laffel LM, Ganda OP, Christlieb AR: Coronary artery disease is the major determinant of excess mortality in patients with insulin-dependent diabetes mellitus and persistent proteinuria. *J Am Soc Nephrol* 3:S104–S110, 1992
31. Rossing P, Hougaard P, Borch-Johnsen K, Parving HH: Predictors of mortality in insulin dependent diabetes: 10 year observational follow up study. *BMJ* 313:779–784, 1996