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Role of the Endothelin-1 Gene Locus for Renal Impairment in the General Nondiabetic Population

SARA-JOAN PINTO-SIETSMA,* STEFAN-MARTIN HERRMANN,‡ KLAUS SCHMIDT-PETERSEN,‡ TIANHUA NIU,‖ HANS L. HILLEGE,† WILBERT M.T . JANSSEN,*† DICK DE ZEEUW,*† PAUL DE JONG,* and REINHOLD KREUTZ‡§

**Department of Internal Medicine, Division of Nephrology, Academic Hospital Groningen, and †Department of Clinical Pharmacology, University Groningen, Groningen, the Netherlands; Departments of ‡Clinical Pharmacology and §Medicine IV Nephrology, Benjamin Franklin Medical Center, Berlin, Germany; and ‖Division of Preventive Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.*

Abstract. A decreased GFR in the range of mild renal insufficiency and an increased urinary albumin excretion (UAE) rate in the range of microalbuminuria are important cardiovascular risk factors. Endothelin-1 (ET-1) has been suggested to be a major disease promoting factor in renal disease. The role of the *ET-1* gene locus (EDN1) for renal function in the general nondiabetic population was evaluated. To explore the overall relevance of EDN1, two suitable single-nucleotide polymorphisms, EDN1 K198N and EDN1 T-1370G, were selected, and haplotype analysis was performed. Determined were genotypes in 7291 nondiabetic subjects from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study. Genetic analysis was related to UAE and GFR as continuous variables and to microalbuminuria and diminished filtration as dichotomous traits. In a logistic regression analysis, no significant higher risk for increased UAE, microalbuminuria, decreased GFR, or diminished filtration could be observed for either single-nucleotide polymorphism separately. Haplotype analysis revealed that individuals with the homozygous G-N haplotype (compound EDN1 −1370GG/198NN genotype) have a lower GFR than the remaining subjects ($P < 0.05$) and exhibit a significant higher risk for the presence of a diminished filtration (relative risk, 2.4; 95% confidence interval, 1.07 to 5.33; $P < 0.05$). Further analysis demonstrated no association between this haplotype and UAE or plasma ET-1 levels. Although a functional relevance of the EDN1 G-N haplotype itself remains unclear, the data demonstrate that genetic variation at the EDN1 locus has a significant effect on glomerular filtration but not on UAE in the general nondiabetic population.

Mild renal insufficiency (defined by serum creatinine concentrations above 1.4 or 1.5 mg/dl or a calculated GFR below 60 ml/min) and elevated urinary albumin excretion (UAE) rates in the range of microalbuminuria represent independent risk factors for cardiovascular events in patients with hypertension, diabetes, or preexisting cardiovascular disease (1,2). This has been highlighted in recent large prospective clinical trials (1,2). It is important to point out that both mild renal insufficiency and microalbuminuria represent quantitative clinical phenotypes such as hypertension, hypercholesterolemia, or obesity that are arbitrarily defined on the basis of a quantitative cutoff value. Like other quantitative phenotypes, microalbuminuria and mild renal insufficiency are probably influenced by environmental and genetic factors and thus belong to the category of complex disorders (3). The nature of complex diseases makes it difficult to identify contributing genes (3,4). For the dissection of the genetic basis of mild renal insufficiency and microalbuminuria, it may therefore be helpful to consider these phenotypes as continuous quantitative traits, in addition to dichotomous clinical classifications. Genetic segregation analysis of UAE in family members with type 2 diabetes has indeed recently demonstrated that levels of UAE are determined by a mixture of genes with large and small effects (5). In this study, UAE was analyzed as a continuous quantitative trait, and diabetes was found as an important modifier for UAE levels.

After the original characterization of endothelin-1 (ET-1) by Yanagisawa *et al.* (6), this 21–amino acid peptide was believed to play an important role primarily in the regulation of vascular tone and BP by virtue of its strong vasoconstricting potency. Subsequently, the spectrum of biologic functions attributed to the endothelin system has been significantly extended beyond the originally documented vasoconstricting role of ET-1 (7). A case in point represents its role in chronic renal disease because several studies documented that renal synthesis of ET-1 is increased either in animal or in human chronic nephropathies (8,9). The question remains, however, whether ET-1 is primarily involved in renal disease, or whether it is only a secondary

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Correspondence to Dr. Reinhold Kreutz, Department of Clinical Pharmacology, Medicin IV Nephrology, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany. Phone: +49-30-8445-2280; Fax: +49-30-8445-4482; E-mail: Kreutz@medizin.fu-berlin.de
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modulator promoting progression after disease initiation. Genetic studies are very helpful in dissecting primary from secondary disease effects both in experimental models and in clinical studies. Indeed, transgenic animals overexpressing the human ET-1 promoter (10) or the human endothelin-2 (*ET-2*) (11) gene develop glomerulosclerosis and display reduced GFR and proteinuria, whereas BP remain unchanged. More recently, the *ET-1* gene locus has been linked to increased albuminuria in the Munich Wistar Frömter genetic rat model of spontaneous albuminuria (12), thus pointing to a potential genetic involvement of this locus for the development of albuminuria.

Whether or not ET-1 exerts an effect on renal function in individuals without documented renal disease or diabetes is unclear. We therefore investigated the relevance of the *ET-1* gene locus (EDN1) for glomerular filtration and microalbuminuria in a large prospective nondiabetic population-based cohort obtained from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) Study (13). We first set out to test the overall relevance of EDN1 by taking into account all polymorphisms previously identified at this locus (http://genecanvas.idf.inserm.fr). Second, for reasons of allele frequencies, for reasons of pairwise linkage disequilibrium coefficients among EDN1 polymorphisms, and with respect to their potential functionality, we selected two single-nucleotide polymorphisms (SNP): EDN1 T-1370G and K198N (Figure 1). Third, we performed haplotype analysis with these SNP and tested whether EDN1 is associated with variation in GFR or UAE in the PREVEND Study cohort (13).

Materials and Methods

Study Population

This investigation is part of the ongoing PREVEND Study, in the city of Groningen, the Netherlands. All inhabitants, aged 28 to 75 yr (n = 85,421), were asked to send in a morning urine sample and to fill out a short questionnaire on demographics and cardiovascular history. Subjects with insulin-dependent diabetes mellitus or pregnant women were excluded from participation in this screening program. In total, 11,163 subjects were invited to the outpatient clinic, 8592 of whom completed the screening program. The study cohort was drawn from this screening program. Details of this protocol have been described elsewhere (13).

The screening program in the outpatient clinic consisted of two consecutive visits. At both visits, BP was measured with an automatic device (13). Subjects were asked to collect 24-h urine on two consecutive days in the week before the second visit. Measurements of urinary volume and albumin and creatinine concentrations were performed on each collection. At the second visit, blood was drawn after an overnight fast for determination of plasma glucose, serum creatinine, and cholesterol (13).

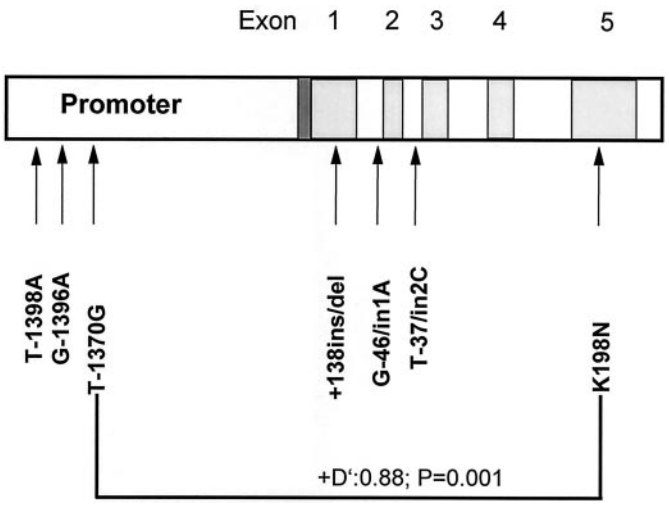

Figure 1. Positions of polymorphisms in the endothelin-1 (*ET-1*) gene. Pairwise linkage disequilibrium (+D') data are given for *ET-1* gene locus (EDN1) T-1360G and K198N. Further details on allele frequencies and pairwise linkage disequilibrium data of all polymorphisms at EDN1 are available at http://genecanvas.idf.inserm.fr/.

Data Handling and Definitions

For the analysis presented here, 479 subjects were excluded because of erythrocyturia or leukocyturia or treatment for overt proteinuria due to renal disease. In addition, 301 subjects were excluded because they had non–insulin-dependent diabetes mellitus, and 492 subjects were excluded because of missing data. Altogether, 7366 subjects were eligible for this analysis. Microalbuminuria was defined according to the established definition of 30 to 300 mg/24 h. Creatinine clearance was defined as the mean of the two measurements based on 24-h urinary creatinine excretions divided by plasma creatinine and corrected for body surface area (ml/min·1.73 m^{-2}). A diminished filtration was defined as a creatinine clearance below more than two times the SD of the mean creatinine clearance of a group of nondiabetic subjects with an UAE of 0 to 15 mg/24 h (13). The mean creatinine clearance with the two times SD borders was obtained by means of a linear regression analysis adjusted for age and gender. Hypertension, obesity, hypercholesterolemia, and smoking status were defined as reported (13).

Laboratory Methods

Urinary albumin concentration was determined by nephelometry with a threshold of 2.3 mg/L and intra- and interassay coefficients of variation of ≤4.3% and ≤4.4%, respectively (Dade Behring Diagnostic, Marburg, Germany). UAE is given as the mean of the two 24-h urine excretions. Plasma glucose, serum cholesterol, and serum and urinary creatinine were determined by Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY). Urinary leukocyte and erythrocyte measurements were done by Nephur-test+leuco sticks (Boehringer Mannheim, Mannheim, Germany).

DNA amplification by PCR was performed with 100 ng of DNA in a total volume of 50 μl containing 10 mmol/L Tris-HCl (pH 9), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml BSA, 200 μmol/L dNTPs, 25 pmol of each primer, and 0.2 U *Taq* polymerase as reported (14). A total of 7366 individuals participating in the PREVEND Study were genotyped for EDN1 T-1370G and K198N; allele-specific oligonucleotides were used (14). The oligonucleotide sequences used for PCR and allele-specific oligonucleotides are available at http://genecanvas.idf.inserm.fr.

In a subgroup of 181 homozygous G-N haplotype carriers and a matched control group of 362 homozygous T-K haplotype carriers, plasma ET-1 levels were measured as previously reported (15). The control group was matched for age, gender, obesity, smoking, and creatinine clearance.

Statistical Analyses

All calculations were performed by SPSS version 9.0 (SPSS, Chicago, IL) software. Continuous data are reported as mean ± SD. In case of skewed distribution, the median with 25th and 75th percentile was presented. Comparisons of different variables among the different genotypes or haplotypes were performed by χ^2 analysis or ANOVA. Differences between the ET-1 plasma levels were assessed by *t* test. All *P* values are two-tailed. $P < 0.05$ was considered statistically significant.

The linear regression model used to define elevated and diminished filtration was done in the following way: first, from the entire study population, two control (normal) subgroups were selected, consisting of either male or female subjects without diabetes with a UAE of 0 to 15 mg/24 h. Second, in a linear regression model with creatinine clearance corrected for body surface area as the dependent variable and age as the independent variable, we constructed the regression line for creatinine clearance for men and women separately. Third, because each of these two subgroups showed a normal distribution concerning creatinine clearance corrected for body surface area, we used the 2× SD borders, or the lowest 2.5%, of the total population distribution of this regression line of creatinine clearance to define diminished filtration. We used this method because we found it more appropriate to generate our own control group for renal function and correct it for albumin excretion because we found in an earlier study that this influences renal function (13).

Genetic and Haplotype Analysis

The Hardy-Weinberg equilibrium was tested by the Pearson χ^2 test. The heterozygosity and polymorphism information content were calculated by the computer program POLYMORPHISM, version 2.2 (16). The haplotype frequencies were estimated by the computer program PL-EM (17), which implemented the partition-ligation strategy (18) by the expectation-maximization framework. Individual haplotype phase for T-1370G and K198N was inferred by PL-EM (18). Logistic regression analysis was performed to test the independent association of the homozygous G-N haplotype—that is, individuals carrying the compound EDN1 −1370GG/198NN genotype, with either microalbuminuria or diminished glomerular filtration in a recessive model. Relative risk (RR) was calculated with individuals homozygous for the T-K haplotype as the reference group. Crude age- and gender- adjusted and confounder adjusted risk ratios were calculated. We adjusted for the following potential confounders: age, gender, smoking, hypertension, and obesity as defined in this study.

Results

Genotypes were available for 7291 individuals, and their distributions were within Hardy-Weinberg equilibrium for both SNP. The allele frequencies for both EDN1 SNP observed in our population are close to the previously published allele frequencies in the ECTIM Study (19), the frequencies for the minor alleles −1370G and 198N being 0.19 and 0.23, respectively, in the ECTIM Study (19) compared with 0.17 and 0.22, respectively, in the study presented here. The heterozygosity was 0.28 and 0.34, and the polymorphism information content was 0.24 and 0.28 for T-1370G and K198N, respectively.

The data for the separate analysis for each SNP in relation to diminished filtration are listed in Table 1. As a result, in the crude analysis, no significant association with diminished filtration was found for either variant, although both SNP showed a higher RR for a diminished filtration, which did not reach statistical significance. This effect remained after adjustment for age and gender and other confounders (Table 1). Concerning microalbuminuria status, neither a trend nor a significant effect was observed (data not shown).

Subsequently, haplotype analysis was performed, and the data for renal filtration and microalbuminuria status according to haplotypes are presented in Table 2. Both the G-N and T-N haplotypes were significantly associated with renal filtration status ($P < 0.0001$, respectively); there was no significant association of any haplotype with microalbuminuria status. Thereafter, in the logistic regression analysis, only the compound homozygous G-N haplotype but not the T-N haplotypes showed a significant effect on renal filtration status. Therefore, only the data for the G-N haplotype were further analyzed and are presented in Tables 3 and 4.

With respect to our entire study population, 181 individuals were carriers of the homozygous G-N haplotype (Table 3), and among these carriers, obesity and diminished filtration were more common and creatinine clearance lower compared with the remaining genotype carriers ($P < 0.05$). The minimum creatinine clearance in the nine carriers of the homozygous G-N haplotype with diminished filtration was 31.23 ml/min·1.73 m^2, with a mean of 48.01 ml/min·1.73 m^2 and a maximum of 61.75 ml/min·1.73 m^2.

Table 1. Crude and adjusted RR for diminished filtration according to EDN1 T-1370G and K198N polymorphisms based on single-SNP analysis[a]

Category		Diminished Filtration		
		Crude RR	Age and Gender RR	Confounder RR
EDN1 T-1370G	(T/T)	1.0	1.0	1.0
	(T/G)	1.1 (0.78 to 1.50)	1.1 (0.77 to 1.48)	0.9 (0.57 to 1.32)
	(G/G)	1.8 (0.88 to 3.51)	1.8 (0.88 to 3.52)	2.1 (0.92 to 4.57)
EDN1 K198N	(K/K)	1.0	1.0	1.0
	(K/N)	0.8 (0.60 to 1.15)	0.8 (0.60 to 1.15)	0.7 (0.49 to 1.09)
	(N/N)	1.6 (0.92 to 2.77)	1.6 (0.90 to 2.70)	1.5 (0.75 to 2.91)

[a] RR indicates relative risk; EDN1, endothelin-1; and SNP, single nucleotide polymorphism.

Table 2. Frequencies for EDN1 haplotypes consisting of T-1370G and K198N polymorphisms among subjects according to renal filtration status and microalbuminuria status[a]

Haplotype	Renal Filtration Status		Microalbuminuria Status	
	Nomal	Diminished	Nomal	Microalbuminuria
T-K	0.7503 (0.0036)	0.7443 (0.0222)	0.7538 (0.0041)	0.7373 (0.0103)
G-N	0.0746 (0.0022)	0.1816 (0.0196)[b]	0.0716 (0.0024)	0.0727 (0.0061)
T-N	0.1602 (0.0031)	0.0566 (0.0118)[b]	0.1633 (0.0035)	0.1727 (0.0089)
G-K	0.0149 (0.0010)	0.0175 (0.0067)	0.0114 (0.0010)	0.0174 (0.0031)

[a] EDN1 indicates endothelin-1.
[b] $P < 0.0001$.

Table 3. Population characteristics of the homozygous EDN1 1370G/198N haplotype carriers versus the remaining haplotype carriers in the PREVEND Study[a]

Category	Remaining Haplotype Carriers	Homozygous G-N Haplotype Carriers
n (%)	7110 (97.5)	181 (2.5)
Male gender (%)	50.8	50.8
Age (yr)	49 ± 12	49 ± 13
Minimal waist circumference (cm)	87.9 ± 13	88.4 ± 13
Body mass index (kg/m^2)	25.9 ± 4.1	26.0 ± 4.1
Obesity (%)	13.9	19.2[b]
Systolic BP (mmHg)	128 ± 20	129 ± 19
Diastolic BP (mmHg)	74 ± 10	75 ± 10
Hypertension (%)	15.8	18.9
Glucose, mg/dl (mmol/L)	84.68 ± 10.81 (4.7 ± 0.6)	84.68 ± 12.61 (4.7 ± 0.7)
Cholesterol, mg/dl (mmol/L)	216.22 ± 42.47 (5.6 ± 1.1)	216.22 ± 38.61 (5.6 ± 1.0)
Hypercholesterolemia (%)	24.6	26.4
Smoking (%)	53.8	44.9
Urinary albumin excretion (mg/24 h)	8.9 (6.2 to 15.7)	8.5 (5.8 to 17.3)
Microalbuminuria (%)	11.0	14.9
Serum creatinine, mg/dl (μmol/L)	0.95 ± 0.19 (84 ± 17)	0.95 ± 0.18 (84 ± 16)
Creatinine clearance, ml/min · 1.73 m^2	92.9 ± 20.8	89.8 ± 21.1[b]
Diminished filtration (%)	2.5	5.1[b]

[a] EDN1 indicates endothelin-1; PREVEND, Prevention of Renal and Vascular End-Stage Disease.
[b] $P < 0.05$.

As shown in Table 4, the effect of the G-N haplotype in the logistic regression analysis is consistent with a recessive model (*i.e.,* two copies of the EDN1 G-N haplotype are needed to have an adverse effect on renal filtration). Therefore, we also conducted the analysis by comparing G-N haplotype carriers *versus* all other haplotype carriers. The results were as follows: the crude RR was 2.1 (95% confidence interval [CI], 1.05 to 4.17), the age- and gender-adjusted RR was 2.1 (95% CI, 1.05 to 4.17), and the confounder-adjusted RR was 2.5 (95% CI, 1.14 to 5.63). No significant higher risk for microalbuminuria could be observed (Table 4).

To test the potential functionality of the G-N haplotype, we used a nested case-control design by matching the 181 individuals carrying the G-N haplotype in a 1:2 ratio with 362 control subjects carrying the corresponding T-K haplotype (*i.e.,* compound EDN1 1370TT/198KK genotype) and measured plasma ET-1 levels. No significant difference in ET-1 plasma levels between these groups (2.1 ± 2.7 fmol/ml *versus* 2.3 ± 2.6 fmol/ml, respectively) was observed.

Discussion

The goal of the study presented here was to evaluate the overall role of the EDN1 gene locus for abnormal glomerular filtration and increased UAE in the general nondiabetic population. To this end, we selected two suitable SNP at EDN1 and investigated their relation to renal function in the PREVEND Study, a large population-based study cohort (13).

EDN1 represents a strong candidate as a susceptibility locus for the manifestation and progression of chronic nephropathies. ET-1 causes vasoconstriction of renal blood vessels and contraction of mesangial cells, and it stimulates glomerular cell proliferation and extracellular matrix deposition (7). Neverthe-

Table 4. RR for diminished filtration and microalbuminuria according to carrier status of EDN1 haplotypes consisting of T-1370G and K198N polymorphisms in the PREVEND Study[a]

Category	Crude RR	Age and gender RR	Confounder RR
Diminished filtration			
remaining haplotype carriers	1.0	1.0	1.0
heterozygous G-N haplotype carriers	1.0 (0.70 to 1.38)	1.0 (0.69 to 1.37)	0.8 (0.51 to 1.22)
homozygous G-N haplotype carriers	2.1 (1.04 to 4.18)[b]	2.1 (1.04 to 4.17)[b]	2.4 (1.07 to 5.33)
Microalbuminuria			
remaining haplotype carriers	1.0	1.0	1.0
heterozygous G-N haplotype carriers	0.9 (0.77 to 1.09)	0.9 (0.76 to 1.08)	0.9 (0.74 to 1.19)
homozygous G-N haplotype carriers	1.3 (0.88 to 2.04)	1.3 (0.84 to 2.04)	0.9 (0.42 to 1.77)

[a] RR indicates relative risk; EDN1, endothelin-1; and PREVEND, Prevention of Renal and Vascular End-Stage Disease.
[b] $P < 0.0001$.

less, linkage analysis in 168 multiplex African American families failed to provide an association between EDN1 and all-cause end-stage renal disease (20). Animal studies, on the other hand, point to an independent role of endothelins for the initiation of renal disease phenotypes (10–12), but it is unclear whether EDN1 plays a role for the development of mild forms of renal insufficiency or microalbuminuria in humans.

One important finding of the analysis presented here is that a subgroup of individuals of our study population carrying the homozygous G-N haplotype more commonly showed obesity, diminished filtration, and lower creatinine clearance compared with the remaining genotype carriers. No effect could be observed for UAE or microalbuminuria, suggesting that this EDN1 haplotype does not contribute to genetic variation of UAE in people without diabetes.

The EDN1 K198N polymorphism results in a Lys/Asn amino acid change at codon 198, which might affect conversion of preproendothelin and thereby influence biosynthesis of ET-1. The EDN1 T-1370G polymorphism is located in the 5' flanking promoter region of the gene and may thus be involved in its differential transcriptional regulation (Figure 1). Thus, although a functional relevance of either EDN1 K198N or EDN1 T-1370G cannot be excluded on theoretical grounds, experimental data to support this assumption are still lacking. Nevertheless, our rationale for selecting these two polymorphisms was additionally on the basis of their suitability for genetic analysis as a result of their allele frequencies and linkage disequilibrium coefficient. Hence, from the previously obtained data for polymorphisms at EDN1, it was inferred that EDN1 T-1370G and K198N are fairly frequent and display a linkage disequilibrium coefficient of +0.88, which was lower compared with other possible combinations between SNP with similar frequency (http://genecanvas.idf.inserm.fr.). Thus, the haplotype analysis was considered to be a reasonable strategy to evaluate the overall role of genetic variation at EDN1 in the nondiabetic population. In that respect, one of our major findings is that even if two polymorphisms are still fairly associated with one another (linkage disequilibrium of 0.88 between EDN1 T-1360G and K198N in the analysis presented here), the haplotype analysis might help to detect genotype-phenotype associations if the population is relatively large (in the analysis presented here, >7000 individuals). Our data are also compatible with the hypothesis that a yet unidentified molecular variant in linkage disequilibrium with the G-N haplotype is responsible for our finding.

To our knowledge, no molecular variant at EDN1 has yet been described resulting in altered ET-1 biosynthesis and/or ET-1 levels. We analyzed ET-1 plasma levels in the two most contrasting genotype groups. We did not find any difference in plasma ET-1 levels between those individuals homozygous for the G-N haplotype compared with individuals homozygous for the T-K haplotype, ruling out an influence of the G-N haplotype on systemic ET-1 levels. This, however, does not exclude the possibility that genetic variation at EDN1 goes along with a variation of ET-1 levels in the kidney or other tissues because it is known that the majority (approximately 80%) of ET-1 is secreted at the abluminal side of the vessel (21). Hence, endothelin is secreted from the endothelial cells toward the vascular wall and acts predominantly on the underlying vascular smooth muscle cells, where its concentration may be several orders of magnitude higher than it is in plasma (21). Therefore, endothelin acts more as an autocrine/paracrine system, rather than a systemic peptide.

Because studies in humans point to a unique susceptibility of renal vessels to ET-1, it is conceivable that increased renal ET-1 levels, probably occurring as a result of a molecular variant at EDN1, may contribute to increased renal vasoconstriction and a decrease in glomerular filtration (21). At the same time, the stimulatory effects of ET-1 on vascular smooth muscle cell proliferation in arteries, mesangial cell, and extracellular matrix proliferation (7) may as well contribute to the development of intrarenal vascular disease and glomerulosclerosis and thereby, taken together with the hemodynamic effects, may impair glomerular filtration over time (21).

Although only about 0.1% of the population in the United States requires dialysis, more than ten times this number—2.5%—have renal impairment sufficient to produce a serum creatinine concentration exceeding about 150 μmol/L (22). Moreover, previous large prospective clinical trials have clearly shown that the presence of mild renal insufficiency

relating to a GFR below 60 ml/min or serum creatinine concentrations above 1.4 or 1.5 mg/dl translates into a significant increase in total mortality and morbidity in patients with hypertension, diabetes, or preexisting cardiovascular disease (1,2). The prevalence of mild renal insufficiency in these studies was 10% to 15% (1,2). Finally, a striking increase in the number of uremic patients and a similar or even greater increase in the number of patients with mild renal insufficiency is envisaged for the next decade (23). This will lead to a substantial increase of cardiovascular disease in the population (22). Thus, analysis of the genetic susceptibility of the development of mild renal insufficiency in the general population appears of considerable clinical importance.

In the study presented here, the carriers of the G-N haplotype tended to be obese. Because it has been shown that obesity is related to renal abnormalities, we questioned whether the effect of EDN1 on renal function was independent of obesity. After adjusting for obesity and additional potential confounders, including age, gender, smoking, and hypertension, the RR associated with carriers of the G-N haplotype for diminished filtration remained significantly elevated (2.4-fold). Thus, the effect of EDN1 on renal function is independent from the presence or absence of obesity. Moreover, the exclusion of subjects whose urine tested positive for protein, leukocytes, or erythrocytes may have confounded the cross-sectional association analysis presented here. When we included those subjects in the analysis, however, the results remained essentially unchanged.

A note of caution should be added when interpreting our data: this is a cross-sectional study with only a single measurement of creatinine clearance. However, we tried to make this single measurement as accurate as possible by taking the average of two 24-h creatinine clearances in our large sample size. The subjects enrolled onto the PREVEND Study are followed for changes in renal function and cardiovascular complications over time. In addition to further independent studies, such data will be needed to test whether or not EDN1 is indeed related to the development of mild renal insufficiency and cardiovascular risk.

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