Pronatriodilatin Gene Polymorphisms, Microvascular Permeability, and Diabetic Nephropathy in Type 1 Diabetes Mellitus

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Abstract. Approximately 30% of diabetic patients develop nephropathy, the appearance of which is partially under genetic control. Atrial natriuretic peptide (ANP) has associated physiologic effects on the kidney. This study was conducted to examine the relationship between a newly identified and known polymorphism at the pronatriodilatin (PND) gene locus and renal involvement in type 1 diabetic subjects. Of 454 type 1 diabetic patients (219 men, 235 women), 323 showed no sign of nephropathy, 79 had incipient renal involvement, and 52 established nephropathy; 58 healthy control subjects were examined for comparison. Allele frequencies (C^{708} *versus* T^{708}) were: 0.95 and 0.05 in normoalbuminuric patients, respectively; 0.88 and 0.12 in microalbuminuric patients; 0.96 and 0.04 both in those with overt nephropathy and in healthy control subjects ($P = 0.011$). Patients with incipient nephropathy were in disequilibrium compared with the total diabetic cohort ($P = 0.02$). In the same populations, an additional genotype for *Sca*I polymorphism of the PND gene was tested. The A^1 and A^2 allele frequencies were: 0.21 and 0.79 in normoalbuminuric patients; 0.13 and 0.87 in microalbuminuric patients; 0.06 and 0.94 in type 1 diabetic subjects with overt nephropathy; and 0.20 and 0.80 in healthy control subjects, respectively ($P < 0.0001$). A subset of 55 normotensive patients with type 1 diabetes, well matched for clinical features, plasma ANP levels, and microvascular permeability to macromolecules, was investigated on the basis of the C^{708}/T and A^2/A^1 polymorphisms. Both transcapillary escape rate of albumin (TER_{alb}) and plasma ANP levels were significantly lower in patients with the T^{708} than with C^{708} allele, as well as in the A^1 than in A^2 allele (TER_{alb}: T^{708} *versus* C^{708}: 5.5 ± 1.7 *versus* 7.8 ± 2.0%/h, $P = 0.0001$; plasma ANP levels: 8.3 ± 3.9 *versus* 15.3 ± 7.7 pg/ml, $P = 0.0003$; A^1 *versus* A^2: 6.05 ± 2.2 *versus* 7.3 ± 2.1%/h, $P = 0.044$; 8.53 ± 4.6 *versus* 14.5 ± 7.4 pg/ml, $P = 0.0024$, respectively). Thus, in a large ethnically homogeneous cohort of diabetic subjects, our data show: (*1*) a significant association of C^{708}/T polymorphism with microalbuminuria in long-term diabetes and with both lower plasma ANP levels and widespread albumin leakage; and (*2*) a strong association between *Sca*I polymorphism and both diabetic nephropathy and plasma ANP concentrations. These results suggest a possible role of PND gene in conferring protection from nephropathy and microvascular damage in type 1 diabetes.

About 30% of patients with type 1 diabetes mellitus develop clinical nephropathy that is followed by end-stage renal failure, as well as excess cardiovascular disease and death (1). The incidence of this complication sharply declines during the third decade with type 1 diabetes (2,3).

Both of these observations and the elevated concordance in renal destiny among siblings with type 1 diabetes (4,5) are consistent with the hypothesis that genetic factors might influence the development of nephropathy (6).

The pathogenesis of diabetic nephropathy remains largely unknown. Nonetheless, several genes have recently been proposed to explain the susceptibility to this complication (7–9). In particular, because a predisposition to hypertension may reflect a risk factor for kidney disease in patients with type 1 diabetes (10–12), the genes involved in the control of BP have been more extensively studied (13). However, the findings are controversial and the corresponding relative risk is very low (14–21).

Atrial natriuretic peptide (ANP) plays a central role in the regulation of BP, sodium homeostasis, and vascular permeability (22–24), and significant associations between DNA poly-

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morphisms at the pronatriodilatin (PND) locus and hypertension have been reported (25–35). Moreover, ANP could be involved in the regulation of GFR (36–38), and hyperfiltration seems to participate in the pathogenesis of glomerular injury. Serum ANP levels have also been reported to be elevated in diabetic patients with micro- and macroalbuminuria (39,40), and salt sensitivity may be a characteristic of these patients (41,42). These observations suggest that the PND gene may be involved in the genetic susceptibility to diabetic nephropathy. Therefore, we examined the relationship between a diallelic polymorphism recently identified in the PND gene with microalbuminuria and overt nephropathy in an ethnically homogeneous cohort of patients with type 1 diabetes. In the same cohort of diabetic patients, additional genotypings have been performed, using known polymorphisms of the PND gene. Finally, in a subset of normotensive patients with type 1 diabetes, we also investigated the relationship of these genetic variants with plasma ANP levels and microvascular permeability to macromolecules.

Materials and Methods

Patient Selection

We examined a cohort of 454 patients with type 1 diabetes of Caucasian origin (219 men, 235 women) attending our diabetic clinic as inpatients or outpatients. All subjects were older than 18 yr, and age at the diagnosis of diabetes was less than 31 yr. The diagnosis of type 1 diabetes was based on the continuous need for insulin treatment since the onset of the disease (allowing for a short remission of up to 6 mo during the first year) and at least two daily injections of insulin. The duration of diabetes was always longer than 1 yr. Pregnant women and patients unrepresentative of the regional ethnic group were excluded.

An age-matched group of 58 nondiabetic healthy individuals (19 men, 39 women) of Caucasian origin was also considered for control purposes. Both cohorts enrolled in the study were carefully recruited to avoid selection bias and geographic differences that may have introduced undue unrecognized stratification in our populations. Using the EURODIAB (43) Family Study questionnaire, we obtained information about geographic origin of the families (at least three generations) of each subject. Only subjects with family origin in Tuscany were recruited for the study. The study protocol was approved by the local ethics committee, and all participants gave informed consent for genetic studies.

Clinical Variables

The clinical variables for the assessment of renal disease were urinary albumin excretion, plasma creatinine, and treatment for renal failure. Urinary albumin excretion rate was measured in at least three 24-h urine collections obtained over a period of 6 mo, after excluding urinary tract infection. Mean urinary albumin excretion rates <20 μg/min defined normoalbuminuria (NA). A diagnosis of microalbuminuria (μA) was based on the persistence of albumin excretion rates between 20 and 200 μg/min. Finally, the clinical diagnosis of overt nephropathy was based on the coexistence of persistent macroalbuminuria (MA; AER $\geq$ 200 μg/min) and either background or proliferative retinopathy, in the absence of clinical or laboratory evidence of other causes of increased urinary albumin excretion. Patients with persistent proteinuria within 5 yr of the onset of type 1 diabetes were not included in the study. About 10% of normoalbuminuric patients were hypertensive, and 80% of them were treated with antihypertensive agents (angiotensin-converting enzyme [ACE] inhibitors or angiotensin II receptor antagonists in monotherapy). About 80% of microalbuminuric and 10% of macroalbuminuric patients were on monotherapy (ACE inhibitors, angiotensin II receptor antagonists, or calcium channel blockers); the large majority of macroalbuminuric patients required adjunctive treatments (α- or β-blockers or clonidine and/or diuretics). Any treatment with ACE inhibitors, angiotensin II receptor antagonists, or calcium channel blockers was withdrawn for at least 10 d before collecting urine. In these patients, three urine collections were obtained over a 2-wk period. However, other antihypertensive medication (α- or β-blockers or clonidine and/or diuretics) was allowed, to preserve BP control.

Urinary albumin concentration was measured by a single-antibody RIA (Albumina DA; Techno-Genetics, Milan, Italy) on 2-ml aliquots stored at -20°C. This assay has a sensitivity of 0.5 μg/ml and an interassay coefficient of variation within the working range (2 to 80 μg/ml) of less than 10%.

Sitting arterial BP (Korotkov phase I-V) was taken twice with approximation to 2 mmHg after a 10-min rest, and the arithmetic mean of readings was recorded. In agreement with the Joint National Committee criteria (JNC-VI), hypertension was defined by BP levels higher than 140 (systolic) and/or 90 mmHg (diastolic) on at least three different visits over the 3 mo preceding the study, or by treatment with antihypertensive medication (44).

The presence and severity of retinopathy was evaluated by two-field retinal photography (disc-macula-temporal and disc-nasal) in each eye (45°Canon CRC4–45 nuclear magnetic resonance camera) after pharmacologic mydriasis. Retinopathy was graded as absent, background, or proliferative. The presence of new vessels, fibrous proliferation, preretinal or vitreous hemorrhages, or photocoagulation scars defined the proliferative stage of retinopathy.

Glycated hemoglobin (HbA_{1c}) was measured by HPLC (Diamat; Bio-Rad, Richmond, CA; the normal range in our laboratory is 4.1 to 6.1%), and serum creatinine was determined by a standard laboratory technique. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2).

Finally, plasma ANP levels and transcapillary escape rate of albumin (TER_{alb}) were measured in a subset of normotensive patients ($n = 55$) with type 1 diabetes. All patients were on a diet with a controlled daily sodium intake of 120 mmol during the week before the study. Two consecutive 24-h urine collections were performed the day before the study to evaluate sodium excretion. None of these patients was taking any drug other than insulin. On the morning of the study, patients with type 1 diabetes omitted their normal insulin treatment. Peripheral blood was drawn between 8:30 and 9:00 a.m., after at least 30 min of rest in the supine position, with the patients in a fasting state. Plasma ANP concentration was determined by an immunoradiometric assay method as described previously (45). The assay has a sensitivity of 2 pg/ml, and the interassay coefficient of variation within the working range (5 to 2000 pg/ml) was between 10 and 4%.

TER_{alb}, defined as the fraction of the intravascular mass of albumin going through the vascular bed per unit time, was measured as described in detail elsewhere (46). Briefly, a cannula was inserted into the antecubital vein of each arm, and the first blood sample was drawn after 30 min of rest in the supine position.

The TER_{alb} was measured by an intravenous bolus injection of 6 to 8 μCi (222 to 296 kBq) of freshly purified ^{125}I-labeled human serum albumin (SARI-125-A-2; Sorin Biomedica, Vercelli, Italy). From the opposite cannula, 8 ml of blood was drawn after discarding the first 2 ml at 10, 15, 20, 30, 40, 45, 50, 55, and 60 min after the injection.

Possible thyroid uptake of radioactive iodide resulting from the catabolism of labeled albumin was blocked by administration of Lugol's solution.

Plasma radioactivity (1 ml in duplicate) was measured in a well-typed gamma counter (Cobra 5000; Packard, Downers Grove, IL). Counting time for each sample was 40 min with less than 0.20% error. Plasma glucose, serum albumin, and hematocrit levels (Coulter Counter S5; Coulter Electronics, Bedfordshire, United Kingdom) were determined in each sample, and the plasma radioactivity was expressed taking into account the small changes in plasma volume occurring during the study.

Transcapillary escape rate (%/h) was compiled from the slope of the linear regression equation of the ^{125}I curve from 10 to 60 min. This measurement was accepted only if the correlation coefficient of the time–plasma radioactivity curve exceeded 0.85.

Genetic Study

Leukocytes were isolated from peripheral blood, and DNA was extracted by standard techniques (47).

Detection of the C^{708}/T polymorphism

The gene encoding for PND, located on the short arm of chromosome 1 (48), contains three exons separated by two introns.

Step 1: Analysis to Detect New Point Mutations. As an initial study, a fragment of 640 bp extending from exon I to exon II was amplified by PCR and analyzed to detect unknown point mutations.

PCR Cycling Conditions. The primers used to amplify this fragment, selected by the "OLIGO" Program (Med Probe, Oslo, Norway), were: sense primer 5′-AGACAG-AGCAGCAAGCAGTG-3′, complementary to nucleotides 527 to 544; antisense primer 5′-CATTTC-CATCCCCAGTTCC-3′, complementary to nucleotides 1148–1166 of the published sequence (48). Primers were synthesized on a Gene Assembler (Pharmacia-Biotech, Uppsala, Sweden). Amplification of template DNA (50 ng) was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer Corp., Norwalk, CT) programmed to allow a two-step cycle: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 45 s; the last cycle had extension for 20 min at 72°C. The reaction was performed in a final volume of 50 μl, containing 5 pmol of each primer, 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM of dNTP, and 2.5 U of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany).

To detect unknown point mutations within the 640-bp PCR product, single-strand conformation polymorphism (SSCP) analysis was performed in a randomly selected sample of only 100 patients with insulin-dependent diabetes mellitus. Because SSCP has higher sensitivity in the detection of point mutations when DNA fragments are in the range of 100 to 300 bp (49), PCR products (640 bp) were incubated with the *Ava*II restriction enzyme (Sigma-Aldrich, St. Louis, MO). *Ava*II digestion produced three fragments of 298, 116, and 226 bp, respectively.

SSCP Conditions. Six microliters of digested samples was denatured by mixing with 2 μl of a stop solution (95% formaldehyde, 20 mM ethylenediaminetetra-acetic acid, 0.05% bromphenol blue, and 0.05% xylene cyanol), heating at 95°C for 5 min, and quenching on ice. Strand separation was carried out using electrophoresis through nondenaturing 6% polyacrylamide slab gels at 20°C (20 W constant for 2 h), and was visualized by silver staining.

Six of 100 samples showed a similar running pattern that differed from the others, and therefore were sequenced by the dideoxy chain termination method.

Sequencing Conditions. A total of 0.1 pmol of amplicons as template in the two-step cycle temperature sequencing reactions, the thermo-Sequenase fluorescein-labeled cycle sequencing kit (Amersham Life Science, Buckinghamshire, United Kingdom) and 1 pmol of fluorescein-labeled sense primer were used, according to the manufacturer's protocol. After heating at 94°C for 2 min, the termination reactions were subject to 28-cycle sequencing reactions under the following conditions: denaturation at 94°C for 20 s, annealing-extension at 55°C for 45 s. Subsequently, half of the final reaction volume of the formamide loading dye was added to each reaction. Finally, reaction products were loaded on a denaturing 6% acrylamide gel in the Automatic Laser Fluorescence DNA Sequencer (Pharmacia Biotech), and the sequence was automatically detected. Direct sequencing showed a new point mutation at position 708 inside the intron 1 of PND gene. The point mutation encodes a thymidine instead of a cytosine (C^{708}/T) (EMBL Nucleotide Sequence Database, accession no. Y11073).

This point mutation introduces a new polymorphic restriction site for *Bst*XI restriction enzyme in the PND gene.

Step 2: Screening for C^{708}/T Polymorphism in a Cohort of Type 1 Diabetic Patients and in Healthy Control Groups. The allele and genotype frequencies of the C^{708}/T point mutation were evaluated in a cohort of 454 unrelated patients with type 1 diabetes and in 58 age-matched nondiabetic healthy control subjects by restriction fragment length polymorphism (RFLP) analysis.

RFLP Conditions. Fifteen microliters of amplicons (640 bp) was incubated at 55°C with 3 μl of dedicated buffer 10× and 5 U *Bst*XI enzyme in a total volume of 30 μl. Digested products were size-separated by electrophoresis through nondenaturing 10% polyacrylamide slab gels and visualized by silver staining.

In samples homozygous for the polymorphism (C^{708}/C), *Bst*XI identified a single restriction site inside the PCR products, and gave origin to two fragments of 442 and 198 bp. If the new mutation was present, two restriction sites were identified: *Bst*XI produced three fragments of 262, 198, and 180 bp in patients homozygous for the polymorphism (T^{708}/T) and four fragments in heterozygote patients (C^{708}/T). In this case, fragments were of 442, 262, 198, and 180 bp, respectively.

Screening for Known Polymorphisms in the Same Cohort of Type 1 Diabetic Patients and in Healthy Control Groups. Hpa*II Polymorphism, PCR-RLFP Conditions.* An amplicon of 1344 bp, derived from nucleotides 946 to 2289 of the published sequence (48), was amplified using the sense primer RSP1: 5′-GGAAGTCAGC-CCAGCCCAGAGAGAT-3′ from nucleotides 946 to 970 and the antisense primer RSP2: 5′-GCAGTCTGTCCCTAGGCCCA-3′ from nucleotides 2270 to 2289 of the published sequence (48). The PCR method was performed as described elsewhere (28). One-fifth of the PCR product was digested with *Hpa*II, size-separated on a 10% polyacrylamide gel, and silver stained.

A unique two-allele polymorphism in intron 2 was identified by *Hpa*II restriction enzyme producing fragment sizes 666, 193, and 172 bp (constant fragments) and 313 (H^1) and/or 282 (H^2) bp.

Sca*I Polymorphism, PCR-RLFP Conditions*

A fragment of 133 bp was amplified in the region overlapping intron 2 and the 3′ flanking region (48), using the sense primer 5′-GGCACACTCATACATGAAGCTGACTTTT-3′ from nucleotides 2158 to 2185 and the antisense primer 5′-GCAGTCTGTC-CCTAGGCCCA-3′ from nucleotides 2330 to 2349. The PCR method was performed as described elsewhere (27). A single, two-allele polymorphism was detected by digestion with *Sca*I and by size

separation of PCR products on a 15% polyacrylamide gel, and stained with silver. In the presence of the polymorphic site (allele A^2), two fragments corresponding to sizes 77 and 56 bp were generated; in the absence of the site, a fragment of 133 bp was observed (allele A^1). The loss of that site caused by mutation leads to an extension of the human ANP by two additional arginines, so that the original peptide of 28 amino acids is extended to 30 amino acids.

Statistical Analyses

Data are presented as arithmetic means (± SD). AER and serum creatinine were not normally distributed and values are given as medians and 95% confidence intervals (95% CI). Normally distributed, continuous variables were compared among groups using one-way ANOVA and, if differences were found, using Scheffe *F* test. Non-normally distributed continuous variables were compared using the Kruskall–Wallis test and Mann–Whitney *U* test. The sign test was used to verify the symmetry of the distributions. Categorical variables were compared by contingency table analysis. The relationship between variables was assessed by Spearman rank correlation. Multivariate stepwise regression analysis was carried out to evaluate the independence of the relationships (AER was log-transformed before inclusion in the analysis). ANOVA for repeated measures (one grouping factor and one trial factor) was applied to verify the constancy of variables such as plasma glucose, serum albumin, and hematocrit over time during the TER_{alb} evaluation. Statistical significance was considered at the conventional 5% level.

Genetic data are presented according to genotype, while allele frequencies were calculated. The Fisher exact test was used to analyze genotype distribution in different populations. Allele and genotype frequencies among patients and control subjects were addressed by the χ^2 test using the values estimated by the Hardy–Weinberg equilibrium association. Odds ratios were calculated to measure the association of the ANP genotype and various phenotypes (nephropathy, retinopathy) with the effect of the mutated allele assumed as dominant (score 0 for wild type and score 1 for heterozygote and mutated homozygote combined), and a more stringent *P* value was used to define significance. Furthermore, the association analysis was repeated after the whole diabetic cohort was partitioned by duration of diabetes.

Results

Clinical Characteristics of Patients

The clinical features of patients according to AER levels are shown in Table 1. Normo- (NA, $n = 323$) and microalbuminuric patients (μA, $n = 79$) were younger than subjects with overt nephropathy (MA, $n = 52$) and control subjects (C, $n = 58$); NA patients were older at the onset and had a shorter duration of diabetes than the other groups.

In the group of NA patients, 159 subjects had a duration of diabetes less than 15 yr, and 164 subjects showed a duration of disease more than 15 yr. NA patients with long diabetes

Table 1. Clinical characteristics of type 1 patients (stratified by AER levels) and nondiabetic healthy control subjects[a]

Characteristic	Type 1 Diabetic Patients			Healthy Control Subjects ($n = 58$)	>*P* Value[b]
	Normoalbuminuria ≤20 ($n = 323$)	AER (μg/min) Microalbuminuria 20 to 200 ($n = 79$)	Macroalbuminuria ≥200 ($n = 52$)		
Gender (M/F)	160/163	32/47	27/25	19/39	NS
Age (yr)	33.6 ± 10.2[c]	33.5 ± 8.7[c]	37.9 ± 8.6	36.9 ± 7.6	0.02
Age at onset of diabetes (yr)	17.9 ± 9.1[d]	15.0 ± 9.3	13.4 ± 7.5		0.006
Diabetes duration (yr)	17.0 ± 10.0[e]	21.1 ± 9.0	27.4 ± 7.4		0.0001
BMI (kg/m^2)	23.2 ± 2.8	23.2 ± 2.7	24.5 ± 2.7	25.8 ± 3.5[f]	0.0001
SBP (mmHg)	121 ± 15[e]	128 ± 15	142 ± 21[g]	124 ± 17	0.0001
DBP (mmHg)	77 ± 10[e]	81 ± 110	89 ± 14[g]	81 ± 8	0.0001
Serum creatinine (mg/dl)[h]	0.87 (0.84 to 0.90)	0.91 (0.86 to 0.97)	1.8[g] (0.80 to 2.90)	0.88 0.84–0.90	0.0001
HbA_{1c} (%)	7.9 ± 1.4	8.0 ± 1.7	8.9 ± 1.4[f]	5.2 ± 0.8	0.03
Prevalence of hypertension (%)	10.6	20.4	84.6		$X^2 = 47.4$, 0.0001
Prevalence of retinopathy (absent/background/proliferative) (%)	64.4/19.5/16.1	40.8/30.6/28.6	0/16.7/83.3		$X^2 = 58.2$, 0.0001
AER (μg/min)[h]	10.7 (6.6 to 17.9)	51.1 (38.6 to 63.5)	473.2 (387.6 to 713.1)		0.0001

[a] AER, albumin excretion rate; BMI, body mass index; SPB, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1c}, glycated hemoglobin.
[b] One-way ANOVA.
[c] $P < 0.05$ *versus* groups 3 and 4.
[d] $P < 0.05$ *versus* groups 2 and 3.
[e] $P < 0.001$ *versus* groups 2 and 3.
[f] $P < 0.001$ *versus* groups 1 and 2.
[g] $P < 0.001$ *versus* other groups.
[h] Kruskal—Wallis and Mann—Whitney *U* tests, values expressed as median (95% confidence interval).

duration (LDNA) (65 men and 99 women) were older than patients with short diabetes duration (SDNA) and with μA (38.3 ± 10.1 *versus* 28.5 ± 7.5 and 33.5 ± 8.7 yr, $P < 0.001$ and $P < 0.05$, respectively). LDNA had a diabetes duration longer than μA patients (24.6 ± 7.4 *versus* 21.1 ± 9.0 yr, $P < 0.001$), and they had an age at onset of diabetes less than SDNA (15.0 ± 9.1 *versus* 21.1 ± 7.6 yr, $P < 0.001$). BMI in LDNA was significantly higher than SDNA subjects (23.8 ± 3.0 *versus* 22.6 ± 2.5 kg/m^2, $P < 0.001$), but lower than healthy control group (23.8 ± 3.0 *versus* 25.8 ± 3.5 kg/m^2, $P < 0.001$). Systolic BP was higher in LDNA than in SDNA subjects (124.1 ± 17 *versus* 116.7 ± 12.6 mmHg, $P < 0.001$). Finally, LDNA subjects had diastolic BP levels (77.9 ± 9.9 mmHg), serum creatinine concentrations (0.80 mg/dl, 0.80 to 0.86 mg/dl), and HbA_{1c} levels (7.9 ± 1.3%) comparable to those of SDNA patients. Prevalence of hypertension in LDNA was 15.9% and prevalence of retinopathy, evaluated as absent, background, and proliferative, was 41.8, 28.7, and 29.5%, respectively. Patients with microalbuminuria had intermediate diabetes duration.

Patients with overt nephropathy had higher systolic and diastolic BP, serum creatinine, and HbA_{1c} levels compared with both NA groups and μA subjects; in these patients, the prevalence of hypertension was also significantly higher than in the other groups. All of the patients with MA had diabetic retinopathy, and the prevalence of proliferative retinopathy was significantly higher when compared with other groups. Patients with retinopathy (background and proliferative, $n = 194$) were older (37.2 ± 9.8 *versus* 30.0 ± 8.8 yr), younger at onset of diabetes (14.4 ± 8.8 *versus* 18.3 ± 8.5 yr), had longer diabetes duration (24.4 ± 8.4 *versus* 12.9 ± 7.6 yr), and higher systolic and diastolic BP values (128/81 ± 20/12 *versus* 117/75 ± 13/9 mmHg) ($P < 0.0001$) than patients with no sign of retinopathy. Prevalence of raised AER levels was 32 and 4%, respectively ($P < 0.0001$).

Frequencies of Alleles and Genotypes and Association Between Genotypes and Phenotypes for C^{708}/T Polymorphism

Among the whole cohort with type 1 diabetes, the C^{708} and T^{708} alleles had frequencies of 0.94 (95% CI, 0.92 to 0.955) and 0.06 (95% CI, 0.045 to 0.077), respectively. The genotype frequencies are consistent with the Hardy–Weinberg equilibrium ($\chi^2 = 0.26$, 1 *df*, $P = 0.88$). The frequencies of alleles among the control subjects did not differ from diabetic patients ($C^{708} = 0.96$, 95% CI, 0.90 to 0.98; $T^{708} = 0.04$, 95% CI, 0.011 to 0.091), and genotype distribution was in agreement with the frequencies predicted by the Hardy–Weinberg equilibrium ($\chi^2 = 0.07$, 1 *df*, $P = 0.96$).

Allele and genotype distributions of the PND polymorphism in type 1 diabetic patients were estimated separately in NA, μA, and MA patients (Table 2). There was no Hardy–Weinberg disequilibrium either in NA subjects or in patients with μA and MA, indicating that the distribution over these pheno-

Table 2. Allele frequency and genotype distribution of the C^{708}/T and *Sca*I polymorphisms of the PND gene in 454 type 1 diabetic patients with or without nephropathy and 58 healthy control subjects[a]

Category	Type 1 Diabetic Patients			Healthy Control Subjects	*P* Value[b]
	Normoalbuminuria ≤20	AER (μg/min) Microalbuminuria 20 to 200	Macroalbuminuria ≥200		
Patients genotypes	$n = 323$	$n = 79$	$n = 52$	$n = 58$	
$C^{708}C$	292 (0.91)	61 (0.77)	48 (0.92)	54 (0.93)	0.012
$C^{708}T$	31 (0.09)	17 (0.22)	4 (0.08)	4 (0.07)	
$T^{708}T$	0	1 (0.01)	0		
Chromosome alleles	$n = 646$	$n = 158$	$n = 104$	$n = 116$	
C^{708}	614 (0.95)	139 (0.88)	100 (0.96)	112 (0.96)	0.011
T^{708}	32 (0.05)	19 (0.12)	4 (0.04)	4 (0.04)	
Patients genotypes	$n = 323$	$n = 79$	$n = 52$	$n = 58$	
A^2A^2	193 (0.60)	61 (0.77)	46 (0.88)	36 (0.62)	<0.0001
A^2A^1	124 (0.38)	16 (0.21)	6 (0.12)	21 (0.36)	
A^1A^1	6 (0.02)	2 (0.02)	0	1 (0.02)	
Chromosome alleles	$n = 646$	$n = 158$	$n = 104$	$n = 116$	
A^2	510 (0.79)	138 (0.87)	98 (0.94)	93 (0.80)	<0.0001
A^1	136 (0.21)	20 (0.13)	6 (0.06)	23 (0.20)	

[a] PND, pronatriodilatin. Other abbreviations as in Table 1.
[b] Fisher exact test.

types was as could be expected for a diallelic trait with such an allelic frequency. There were no significant differences in the allele frequencies between NA and MA patients (odds ratio for $C^{708}/T+T^{708}/T$ *versus* C^{708}/C was 0.78, 95% CI, 0.26 to 2.32, $\chi^2 = 0.192$, 1 *df*, $P = 0.66$). Consistently, no differences were observed between these groups and healthy control subjects (odds ratio for $C^{708}/T+T^{708}/T$ *versus* C^{708}/C were 0.69, 95% CI, 0.24 to 2.06, in NA *versus* C, $\chi^2 = 0.43$, 1 *df*, $P = 0.51$; and 0.89, 95% CI, 0.21 to 3.75, in MA *versus* C, $\chi^2 = 0.026$, 1 *df*, $P = 0.87$).

However, the group of patients with μA was in disequilibrium compared with the allelic frequencies observed within the total group of diabetic patients ($\chi^2 = 7.8$; 2 *df*, $P = 0.02$). Significant differences were observed in the allele frequencies and genotype distribution between μA subjects and the other groups (Table 2). The odds ratio of the $C^{708}/T+T^{708}/T$ genotype compared with the C^{708}/C genotype for microalbuminuria *versus* normoalbuminuria was 2.78, 95% CI, 1.46 to 5.29, $\chi^2 = 10.31$, 1 *df*, $P = 0.0013$. Allele frequencies and genotype distributions were reevaluated in the type 1 diabetes cohort with more than 15 yr diabetes duration (Table 3). No differences were observed between SDNA ($n = 159$) and LDNA ($n = 164$) patients (odds ratio 1.04, 95% CI, 0.49 to 2.18).

The exposure odds ratio of the $C^{708}/T+T^{708}/T$ genotype compared with the C^{708}/C genotype comparing μA and NA patients both with long diabetes duration (≥15 yr) rose to 3.44 ($\chi^2 = 10.64$, 1 *df*, $P = 0.001$). No significant associations were seen between the PND genotypes and diabetic retinopathy (odds ratio of the $C^{708}/T+T^{708}/T$ genotype *versus* the C^{708}/C genotype for absence *versus* presence of retinopathy was 1.76, 95% CI, 0.89 to 3.47, NS).

Allele and Genotype Frequencies and Association Between Genotypes and Phenotypes for Hpa*II Polymorphism*

The H^1 and H^2 alleles were similarly distributed in type 1 diabetic patients as well as in healthy control groups (H^1 *versus* $H^{2:}$ 0.02 *versus* 0.98 and 0.02 *versus* 0.98, respectively). Both cohorts were in Hardy–Weinberg equilibrium.

Genotype polymorphism was detected as either H^2/H^2 and H^2/H^1, because no H^1/H^1 was observed. No differences were observed in the allele and genotype distributions over the groups of NA, μA, and MA patients and healthy control subjects (H^2: 0.98, 0.97, 0.98, and 0.98; H^1: 0.02, 0.03, 0.02, and 0.02, NS). Allele frequencies were superimposable in type 1 diabetic patients with long diabetes duration selected on the basis of AER concentrations.

Frequencies of Alleles and Genotypes and Association Between Genotypes and Phenotypes for Sca*I Polymorphism*

Among the whole cohort with type 1 diabetes, the A^1 and A^2 alleles had frequencies of 0.18 (95% CI, 0.15 to 0.20) and 0.82 (95% CI, 0.79 to 0.84), respectively. The genotype frequencies are consistent with the Hardy–Weinberg equilibrium ($\chi^2 = 4.27$, 1 *df*, $P = 0.12$). The frequencies of alleles among the control subjects did not differ from diabetic patients (A^1 0.20, 95% CI, 0.13 to 0.28; A^2 0.80, 95% CI, 0.71 to 0.86), and genotype distribution was in agreement with the frequencies predicted by the Hardy–Weinberg equilibrium ($\chi^2 = 1.12$, 1 *df*, $P = 0.57$).

Allele and genotype distributions of the *Sca*I polymorphism were determined separately in type 1 diabetic patients with NA, μA, and MA and in healthy control subjects (Table 2).

A significant difference in the distribution of the A^1 and A^2 alleles over the groups was observed ($P < 0.0001$). Genotype distribution was significantly different between NA and MA patients (odds ratio 0.194, 95% CI, 0.08 to 0.46, $\chi^2 = 15.99$, 1 *df*, $P = 0.0001$). Consistently, differences were observed between NA and μA groups (odds ratio 0.48, 95% CI, 0.24 to 0.77, $\chi^2 = 8.32$, 1 *df*, $P = 0.0039$); no difference was found between μA and MA subjects (odds ratio 0.44, 95% CI, 0.16 to 1.2, $\chi^2 = 2.65$, 1 *df*, $P = 0.1$).

Allele frequencies and genotype distributions were reevaluated in the type 1 diabetic cohort with more than 15 yr diabetes duration (Table 3), and a difference in the distribution of A^1 and A^2 allele was observed in the groups ($P = 0.001$). The exposure odds ratio of the $A^1/A^1+A^1/A^2$ genotype *versus* the A^2/A^2 genotype comparing NA and MA patients both with long diabetes duration (≥15 yr) was 0.23 (95% CI, 0.09 to 0.58, $\chi^2 = 10.91$, 1 *df*, $P = 0.001$); odds ratio between NA and μA subjects with long duration of disease was 0.455 (95% CI, 0.22 to 0.90, $\chi^2 = 5.13$, 1 *df*, $P = 0.023$). Odds ratio between μA and MA patients with a diabetes duration of more than 15 yr was 0.51 (95% CI, 0.18 to 1.48, $\chi^2 = 1.54$, 1 *df*, $P = 0.21$).

A stepwise regression analysis including several clinical and biochemical features (gender, age, diabetes duration, BMI, presence of hypertension [as dummy variable], HbA_{1c}, the C^{708}/T and A^1/A^2 genotypes) compared to the presence of diabetic nephropathy, considered as the dependent variable, was carried out. Hypertension, the A^1/A^2 genotype, diabetes duration, and age were significantly and independently associated with kidney disease in the diabetic cohort as a whole (step 1: $r = 0.32$, *F* test 24.2; step 2: multiple $r = -0.38$, *F* test 10.46; step 3: $r = 0.40$, *F* test 11.21; step 4: $r = -0.43$, *F* test 8.7).

The extent of disequilibrium in pairwise combinations of alleles at the ANP locus was estimated by means of the maximum likelihood from the frequency of diploid genotypes. The *Sca*I and the *Bst*XI sites are in positive linkage disequilibrium in the coupling phase with a *P* value for the χ^2 test of zero disequilibrium < 0.001. No significant associations between the A^1/A^2 genotype and diabetic retinopathy were observed.

Association Between PND Gene Polymorphisms and Plasma ANP Concentrations and TER_{alb}

Plasma ANP concentrations, urinary sodium excretion, and TER_{alb} were measured in a subset of 55 normotensive type 1 diabetic patients selected on the basis of C^{708}/T genotype (C^{708}/C, $n = 31$; C^{708}/T, $n = 24$) (Table 4). Thirty-three type 1 diabetic patients (C^{708}/C, $n = 19$; C^{708}/T, $n = 14$) had AER in the range of normoalbuminuria and 22 patients (C^{708}/C, $n = 12$; C^{708}/T, $n = 10$) had AER in the range of microalbumin-

Table 3. Allele frequency and genotype distribution of C^{708}/T and *Sca*I polymorphisms of the PND gene in type 1 diabetic patients with or without nephropathy[a]

Category	Type 1 Diabetic Patients			*P* Value[b]
	Normoalbuminuria ≤20	AER (μg/min) Microalbuminuria 20 to 200	Macroalbuminuria ≥200	
Patients genotypes	*n* = 164	*n* = 60	*n* = 48	
$C^{708}C$	148 (0.90)	43 (0.72)	44 (0.92)	0.003
$C^{708}T$	16 (0.10)	16 (0.27)	4 (0.08)	
$T^{708}T$	0	1 (0.001)	0	
Chromosome alleles	*n* = 328	*n* = 118	*n* = 96	
C^{708}	312 (0.95)	102 (0.86)	92 (0.96)	0.003
T^{708}	16 (0.05)	18 (0.14)	4 (0.04)	
Patients genotypes	*n* = 164	*n* = 60	*n* = 48	
A^2A^2	102 (0.62)	47 (0.78)	42 (0.88)	0.002
A^2A^1	59 (0.36)	11 (0.19)	6 (0.12)	
A^1A^1	3 (0.02)	2 (0.03)	0	
Chromosome alleles	*n* = 328	*n* = 118	*n* = 96	
A^2	253 (0.80)	103 (0.87)	90 (0.94)	0.001
A^1	65 (0.20)	15 (0.13)	6 (0.06)	

[a] Only patients with diabetes duration ≥15 yr are included. Abbreviations as in Tables 1 and 2.
[b] Fisher exact test.

Table 4. Clinical characteristics of 55 normotensive patients with type 1 diabetes by C^{708}/T polymorphism and *Sca*I at the atrial natriuretic peptide gene[a]

Characteristic	C^{708}/C (*n* = 31)	C^{708}/T (*n* = 24)	*P* Value[b]	A^2/A^2 (*n* = 33)	A^2/A^1 (*n* = 22)	*P* Value[b]
Gender (M/F)	14/17	11/13	NS	15/18	10/12	NS
Age (yr)	36 ± 7	37 ± 9	NS	36 ± 8	39 ± 8	NS
Diabetes duration (yr)	18 ± 7	19 ± 8	NS	18 ± 7	20 ± 8	NS
BMI (kg/m^2)	23.5 ± 1.8	22.6 ± 1.7	NS	23.4 ± 1.7	23.0 ± 1.7	NS
SBP (mmHg)	124 ± 9	119 ± 15	NS	121 ± 18	124 ± 8	NS
DBP (mmHg)	77 ± 6	77 ± 6	NS	77 ± 6	78 ± 6	NS
Serum creatinine (mg/dl)	0.87 (0.84 to 0.90)	0.91 (0.86 to 0.97)	NS	0.86 (0.84 to 0.91)	0.90 (0.86 to 0.96)	NS
HbA_{1c} (%)	8.3 ± 1.0	8.2 ± 1.1	NS	8.3 ± 1.0	8.1 ± 1.4	NS
HbA_{1c} (%), mean of previous 2 yr	8.3 ± 1.3	8.3 ± 1.4	NS	8.4 ± 1.3	8.2 ± 1.4	NS
Fasting plasma glucose (mg/dl)	206 ± 80	212 ± 76	NS	209 ± 80	203 ± 60	NS
Plasma volume (ml)	2674 ± 333	2539 ± 519	NS	2719 ± 449	2452 ± 363	NS
Urinary sodium excretion (mmol/24 h)	120 ± 10	122 ± 13	NS	122 ± 9	121 ± 12	NS

[a] Abbreviations as in Table 1.
[b] Two-tailed *t* test.

uria. As expected (50), TER_{alb} was higher in μA patients than in NA subjects (7.7 ± 1.9 *versus* 6.2 ± 2.2%/h, $P = 0.0092$).

No difference in plasma ANP levels was observed between NA and μA patients (12.1 ± 7.5 *versus* 12.6 ± 6.9 pg/ml, NS). In the whole cohort, plasma ANP concentrations were significantly higher in C^{708}/C compared with C^{708}/T (15.3 ± 7.7

versus 8.3 ± 3.9 pg/ml, $P = 0.0003$) (Figure 1a). The difference in plasma ANP concentrations between C^{708}/C and C^{708}/T genotypes also persisted when NA and μA patients were analyzed separately (NA: 15.1 ± 7.6 *versus* 7.8 ± 4.9 pg/ml, $P = 0.005$; μA: 15.6 ± 8.2 *versus* 9.0 ± 2.1 pg/ml, $P = 0.03$; (Figure 1b).

TER_{alb} was also higher in C^{708}/C than in C^{708}/T patients (7.8 ± 2.0 *versus* 5.5 ± 1.7%/h, $P = 0.0001$) (Figure 2a). A significant difference in TER_{alb} between C^{708}/C and C^{708}/T genotypes also persisted when NA and μA patients were analyzed separately (NA: 7.3 ± 1.9 *versus* 4.7 ± 1.5%/h, $P = 0.0003$; μA: 8.6 ± 1.9 *versus* 6.7 ± 1.3%/h, $P = 0.015$) (Figure 2b).

In the same subset of type 1 diabetic patients, *Sca*I polymorphism (A^2/A^1: 22 subjects, A^2/A^2: 33 subjects) was also evaluated in relation to plasma ANP levels and TER_{alb} values. No differences were observed between patients with A^2/A^1 and A^2/A^2 genotype for gender, age, diabetes duration, BP levels, fasting plasma glucose, HbA_{1c}, previous 2-yr mean HbA_{1c}, and 24-h urine sodium excretion in patients with A^2/A^2 and A^2/A^1 genotypes (Table 4).

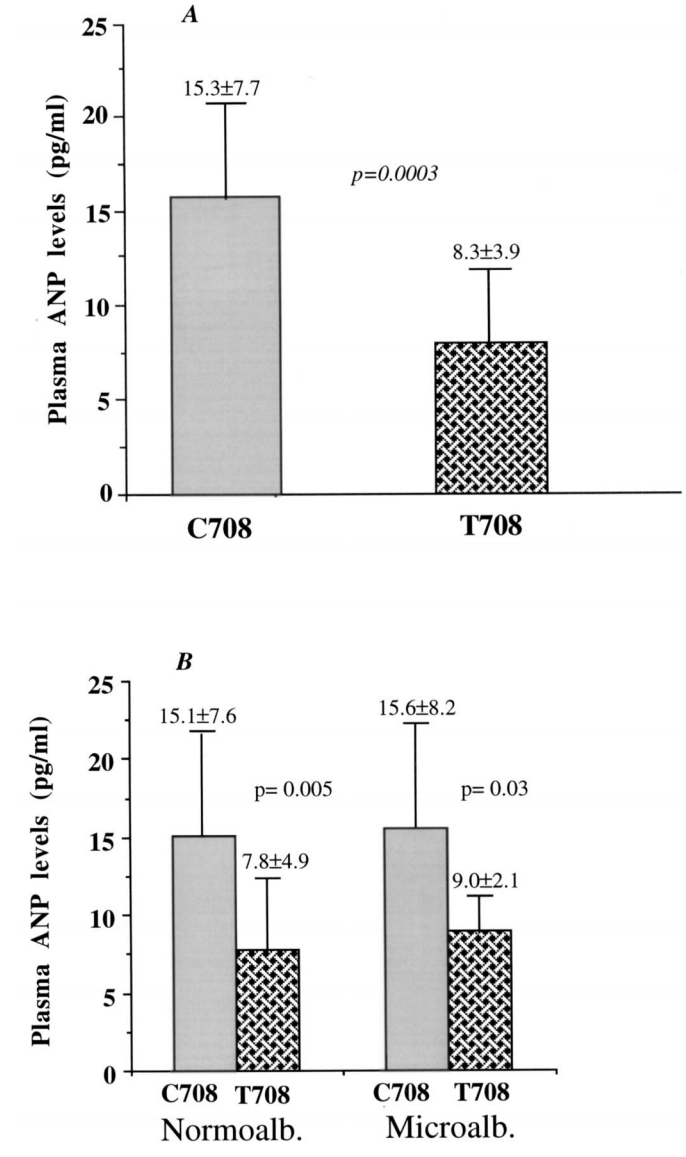

Figure 1. Fasting plasma atrial natriuretic peptide (ANP) levels in normotensive patients with type 1 diabetes by C^{708}/T genotype (A). Plasma ANP concentrations by C^{708}/T genotype and albumin excretion rate (AER) levels (B).

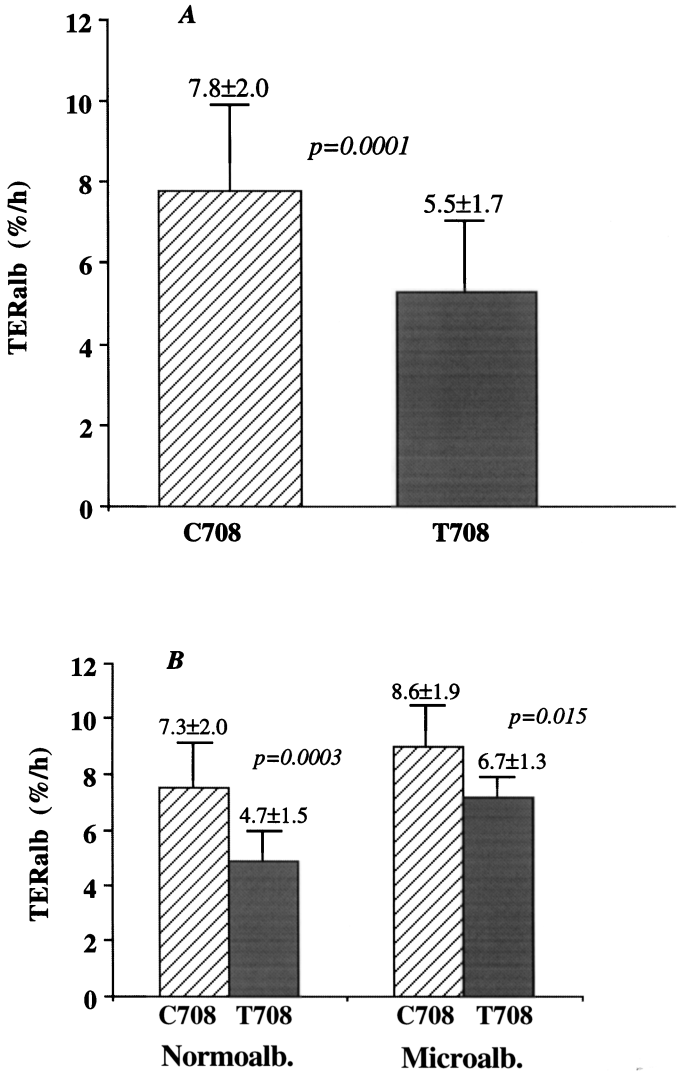

Figure 2. Transcapillary escape rate of intravenously injected radiolabeled albumin (TER_{alb}) by C^{708}/T genotype in normotensive patients with type 1 diabetes (A); TER_{alb} by C^{708}/T genotype and AER levels (B).

Plasma ANP levels were significantly higher in patients with A^2/A^2 genotype than in patients with A^2/A^1 genotype (14.5 ± 7.4 *versus* 8.5 ± 4.7 pg/ml, $P = 0.0024$). Plasma ANP levels were also higher in NA patients with A^2/A^2 genotype ($n = 19$) compared with A^2/A^1 genotype ($n = 14$) (14.6 ± 7.6 *versus* 8.4 ± 5.7 pg/ml, $P = 0.018$), but only slightly higher in μA subjects with A^2/A^2 genotype ($n = 13$) than in A^2/A^1 genotype ($n = 9$) (14.3 ± 7.4 *versus* 8.7 ± 2.7, $P = 0.06$).

TER_{alb} was increased in patients with A^2/A^2 genotype ($n = 33$) compared to patients with A^2/A^1 genotype ($n = 22$) (7.3 ± 2.1 *versus* 6.05 ± 2.2%/h, $P = 0.044$). The difference on the

basis of genotype persisted in NA patients (A^2/A^2 *versus* A^2/A^1, 6.83 ± 1.9 *versus* 5.3 ± 2.2%/h, $P = 0.04$), but disappeared in the group of μA patients (8.1 ± 2.2 *versus* 7.5 ± 1.2, P = NS).

Furthermore, a positive linear correlation was found between plasma ANP concentrations and TER_{alb} in the whole cohort ($r = 0.37$, $P = 0.0068$). Finally, a stepwise regression analysis including several clinical and biochemical features, such as age, diabetes duration, BMI, systolic BP, diastolic BP, HbA_{1c}, previous 2-yr mean HbA_{1c}, log-transformed AER (or presence of microalbuminuria), and the C^{708}/T and *Sca*I polymorphisms, *versus* TER_{alb}, considered as the dependent variable, was performed. The C^{708}/T polymorphism and AER levels (or presence of microalbuminuria, as dummy variable), but no other variables, were significantly and independently associated with albumin leakage in the whole diabetic cohort (step1: $r = -0.49$, F test 20.05; step 2: multiple $r = 0.63$, F test 11.79).

Discussion

This study addresses PND genotypes and diabetic complications. The first finding was the detection of a previously unknown point mutation inside the first intron of the gene encoding for the human PND peptide (51). This mutation was detected by SSCP of multiple fragments obtained by restriction digestion of a large (640-bp) PCR product (49), and was confirmed by direct DNA sequencing. It is located in position 708 of the published PND sequence and encodes a thymidine instead of a cytosine.

The frequency distribution of this polymorphism was evaluated by restriction enzyme length polymorphism in a cohort of 454 type 1 diabetic patients and a group of nondiabetic healthy control subjects of Caucasian origin.

In the total group of diabetic patients studied, the frequency of the C^{708}/T genotype was about 12%. In the group with type 1 diabetes, the T^{708} allele was more common in patients with microalbuminuria, although not in patients with overt nephropathy, thereby failing to show an association of the corresponding chromosomal region with this long-term complication of diabetes.

It is thought that microalbuminuria may predict established nephropathy with a positive predictive value greater than 80% (52,53), but this has recently been questioned in patients with duration of diabetes longer than 15 yr (54). This heterogeneity in the chance for progression is supported by recent epidemiologic findings. After 30 yr of diabetes, the prevalence of nephropathy levels off at 30%, whereas the prevalence of microalbuminuria and diabetic nephropathy is 58%. This 28% excess of patients with persistent microalbuminuria after 30 yr of diabetes suggests that many patients with microalbuminuria may never progress to overt proteinuria and only about 50% do so (43,55). In our study, the T^{708} allele was apparently more common among patients with microalbuminuria and long-term diabetes. Only a longitudinal observation will establish whether this subset of patients will eventually show any progression to overt nephropathy. In the present cross-sectional study, there were stringent criteria to define renal disease and any chance that this may be due to diabetes; it is therefore unlikely that the detection of an association among patients with microalbuminuria rather than patients with proteinuria may reflect an inaccurate definition of reference phenotypes. Furthermore, genotype and allele frequencies did not differ significantly in patients with nephropathy compared with control subjects, short-duration (<15 yr) normoalbuminuric patients who still had a full risk of developing kidney disease, and long-standing normoalbuminuric subjects, whose risk of developing nephropathy is expected to be relatively low (52,53). Patients with microalbuminuria are in disequilibrium with respect to all diabetic cohorts, and the exposure odds ratio of the C^{708}/T *versus* C^{708}/C genotype is higher than in patients with normoalbuminuria. It is unlikely that this could be due to an unrecognized stratification of our population because both cohorts of type 1 diabetic patients and healthy control subjects came from the same geographic location and were probably homogeneous. So, we suggest that it could be likely related to the presence of microalbuminuria, which has also been considered to be an independent risk factor of microvascular damage in type 1 diabetes (54). It is tempting to speculate that the mutated allele might theoretically be involved in conferring protection from the progression of microvascular damage and also glomerular impairment.

ANP seems to be involved in the regulation of GFR and in the genesis of hyperfiltration (36–38), and some studies have demonstrated that ANP infusion increases renal and vascular permeability to proteins in diabetes (24,56,57).

Plasma ANP concentrations and microvascular permeability to albumin have been evaluated in a selected subset of strictly normotensive type 1 diabetic patients, to avoid confounding factors such as any, even slight, increase in BP. As described in other studies (24,47), our findings showed a positive correlation between plasma ANP levels and TER_{alb}, supporting a role for ANP in the regulation of microvascular permeability. Furthermore, we observed that normotensive type 1 diabetic patients with the T^{708} allele had significantly lower plasma ANP concentrations and TER_{alb} values than patients with the C^{708} allele, independently of the presence of normo- or microalbuminuria. Because functional endothelial injury is likely to have a role in the pathogenesis of microvascular complications and because TER_{alb} is a reasonable, although not perfect, method for assessing endothelial function *in vivo* in humans (58), these findings seem to support the hypothesis of a protective role for the recently identified PND gene variant in the progression of microvascular damage.

Moreover, data about additional polymorphisms in the PND gene have been obtained. Interestingly, although the *Hpa*II polymorphism does not seem to play a role in the development of microvascular complications, the *Sca*I polymorphism shows a strong association with diabetic nephropathy.

Indeed, in type 1 diabetes, the A^1 allele was relatively more frequent in normoalbuminuric patients with short- and long-term diabetes duration and progressively decreased in micro- and macroalbuminuric patients, showing an association of the corresponding chromosomal region with this long-term complication of diabetes. Furthermore, stepwise regression showed

that the *Sca*I polymorphism, hypertension, diabetes duration, and age are independently related to diabetic nephropathy. These findings, taken as a whole, suggest an important protective role for the A^1 allele in the development and progression of kidney damage.

The A^1 allele has been reported to cause the loss of the regular stop codon, leading to an extension of the human natriuretic peptide by two additional arginines, *i.e.*, the peptide of 28 amino acids is extended to 30 amino acids (27). Lower plasma ANP levels have been found in normotensive subjects with type 1 diabetes with the A^1 allele, independently of normo- and microalbuminuria, but a weak association has been found between the *Sca*I polymorphism and microvascular permeability, measured by TER_{alb}.

Because raised ANP levels have been described in patients with micro- and macroalbuminuria (39,40), our data could suggest that the *Sca*I polymorphism may play a major role particularly in the regulation of GFR and the genesis of hyperfiltration (36–38).

In conclusion, in type 1 diabetes, the C^{708}/T PND gene variant is associated on the one hand with microalbuminuria and on the other with decreased plasma ANP levels and microvascular permeability. The A^1 allele of *Sca*I polymorphism, related to lower plasma ANP concentrations, seems to play a major role in the susceptibility to diabetic nephropathy, conferring protection from the development of this complication. Whether these phenomena are associated with reduced risk of progression to kidney disease and/or microvascular complications remains to be seen in conjunction with renal function evaluation as well as prospective follow-up studies of microalbuminuria and extrarenal diseases.

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