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Research Article

Diabetic nephropathy is a glomerular disease due to uncontrolled diabetes and genetic factors. It can be caused by glomerular hypertension produced by capillary vasodilation, due to diabetes, against constitutional glomerular resistance. As angiotensin II increases glomerular pressure, we studied the relationship between genetic polymorphisms in the renin-angiotensin system-angiotensin I converting enzyme (ACE), angiotensinogen (AGT), and angiotensin II, subtype 1, receptor-and the renal involvement of insulin-dependent diabetic subjects with proliferative retinopathy: those exposed to the risk of nephropathy due to diabetes. Of 494 subjects recruited in 17 centers in France and Belgium (GENEDIAB Study), 157 (32%) had no nephropathy, 104 (21%) incipient (microalbuminuria), 126 (25 %) established (proteinuria), and 107 (22%) advanced (plasma creatinine \geq 150 micromol/liter or renal replacement therapy) nephropathy. The severity of renal involvement was associated with ACE insertion/deletion (I/D) polymorphism: χ^2 for trend 5.135, $P = 0.023$; adjusted odds ratio attributable to the D allele 1.889 (95% CI 1.209-2.952, $P = 0.0052$). Renal involvement was not directly linked to other polymorphisms. However, ACE I-D and AGT M235T polymorphisms interacted significantly ($P = 0.0166$): in subjects with ACE ID and DD genotypes, renal involvement increased from the AGT MM to TT genotypes. Thus, genetic determinants that affect renal angiotensin II and kinin productions are risk factors for the progression of glomerular disease in uncontrolled insulin-dependent diabetic [...]

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Contribution of Genetic Polymorphism in the Renin–Angiotensin System to the Development of Renal Complications in Insulin-dependent Diabetes

Génétique de la Néphropathie Diabétique (GENEDIAB) Study Group

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Abstract

Diabetic nephropathy is a glomerular disease due to uncontrolled diabetes and genetic factors. It can be caused by glomerular hypertension produced by capillary vasodilation, due to diabetes, against constitutional glomerular resistance. As angiotensin II increases glomerular pressure, we studied the relationship between genetic polymorphisms in the renin-angiotensin system—angiotensin I converting enzyme (ACE), angiotensinogen (AGT), and angiotensin II, subtype 1, receptor—and the renal involvement of insulin-dependent diabetic subjects with proliferative retinopathy: those exposed to the risk of nephropathy due to diabetes. Of 494 subjects recruited in 17 centers in France and Belgium (GENEDIAB Study), 157 (32%) had no nephropathy, 104 (21%) incipient (microalbuminuria), 126 (25%) established (proteinuria), and 107 (22%) advanced (plasma creatinine ≥ 150 $\mu\text{mol/liter}$ or renal replacement therapy) nephropathy. The severity of renal involvement was associated with ACE insertion/deletion (I/D) polymorphism: χ^2 for trend 5.135, $P = 0.023$; adjusted odds ratio attributable to the D allele 1.889 (95% CI 1.209–2.952, $P = 0.0052$). Renal involvement was not directly linked to other polymorphisms. However, ACE I-D and AGT M235T polymorphisms interacted significantly ($P = 0.0166$): in subjects with ACE ID and DD genotypes, renal involvement increased from the AGT MM to TT genotypes. Thus, genetic determinants that affect renal angiotensin II and kinin productions are risk factors for the progression of glomerular disease in uncontrolled insulin-dependent diabetic patients. (*J. Clin. Invest.* 1997. 99:1585–1595.) Key words: angiotensin I converting enzyme • angiotensinogen • diabetes mellitus • diabetic nephropathy • glomerular disease

Introduction

Diabetic nephropathy is a glomerular disease that accounts for most of the reduced life expectancy of insulin-dependent diabetic patients (1). The main causes of premature death in insulin-dependent subjects with diabetic nephropathy are cardiovascular events (primarily coronary heart disease), and not renal failure (2, 3). The development of diabetic nephropathy depends mostly on the duration of diabetes and on how well it is controlled, as demonstrated by studies on animals (4) and cohort (5) and intervention (6) studies in humans. However, diabetic nephropathy develops in only a fraction of insulin-dependent diabetic subjects, while nearly all of them can develop other manifestations of diabetic microangiopathy like diabetic retinopathy (2). The fact that some insulin-dependent diabetic subjects are protected against diabetic nephropathy provides the impetus to search for nonglycemic factors that modulate the risk of renal complications in diabetes. Such factors can be genetically transmitted, because several studies indicate a familial clustering of diabetic nephropathy, together with cardiovascular disease (7–9).

Studying the renin–angiotensin system is of special interest in insulin-dependent diabetes. Experimental (10) and clinical (11) studies suggest that an increase in intraglomerular capillary pressure, a well documented effect of angiotensin II (12), can cause diabetic glomerulosclerosis. Some components of the renin–angiotensin system, angiotensin I converting enzyme (ACE)¹ and angiotensinogen (AGT), display a genetic polymorphism in their circulatory or cellular levels (13–15), which can provide a basis for a relationship between constitutive activity of the renin–angiotensin system and the development of vascular or renal damage in individual subjects. Genomic markers for these polymorphisms have been identified (15–17). An insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene is strongly associated with the plasma and cellular ACE levels (14, 17). Several studies suggest that this polymorphism is an independent risk factor for myocardial infarction and coronary artery disease (18–24). Associations have also been described between AGT gene polymorphism, plasma AGT levels (15), and essential hypertension (15, 16). Polymorphisms have also been described in the gene encoding subtype

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1. Abbreviations used in this paper: ACE, angiotensin I converting enzyme; ACEI, angiotensin I converting enzyme inhibitor; AGT, angiotensinogen; AT1R, subtype 1 of the angiotensin II receptor; I/D, insertion/deletion.

1 of the angiotensin II receptor (AT1R), but these mutations have not yet been linked to any biological phenotype (25).

We and another group reported that the polymorphism of the ACE gene is associated with diabetic nephropathy in single-center case-control studies (26, 27), but this association has since been disputed (28–34). We have now undertaken a large-scale, multicenter study on insulin-dependent diabetic subjects at risk of kidney complications due to long-term exposure to hyperglycemia, i.e., those who have developed proliferative diabetic retinopathy, a severe complication of diabetic microangiopathy, to test the relationship between genetic factors and renal involvement in insulin-dependent diabetes. This study, called GENEDIAB (GENétique de la NEphropathie DIABétique) was conducted prospectively over one year. The degree of renal involvement of the patients was classified according to the genetic polymorphism of ACE, AGT, and AT1R. This study allowed to test the association of the ACE gene polymorphism with the presence of the disease and its evolution, establishing that the ACE gene is involved in both the susceptibility to the disease and its progression toward renal failure. Regarding other genes of the renin–angiotensin system, the AGT and AT1R polymorphisms were not contributive alone, but we found an interaction between the ACE I/D and AGT M235T polymorphisms that can account for the degree of renal involvement in the patients studied.

Methods

Study protocol

PATIENT SELECTION

A cross-sectional study was conducted prospectively in 17 diabetic clinics in France and Belgium (see list in Acknowledgments) from May 1994 to April 1995. All inpatients and outpatients attending these clinics were asked to participate in the study if they fulfilled two criteria: (a) insulin-dependent diabetes before the age of 35 yr, and (b) past or present proliferative diabetic retinopathy. In France, 20% of patients with insulin-dependent diabetes attending diabetic clinics (35) display proliferative retinopathy. Exclusion criteria were terminal cancer and personal disability. Patients gave their written consents to participate in the study after appropriate information. Within each clinic, the mean acceptance rate of this protocol was 86% (range 59–100%) by eligible patients. The study protocol was approved by the Ethics Committee of the Centre Hospitalier Universitaire of Angers (Angers, France).

Insulin-dependent diabetes was defined according to World Health Organization criteria (36); subjects who started insulin > 1 yr after diagnosis of diabetes were excluded. Proliferative diabetic retinopathy was documented by a description of the history of diabetic retinal disease made by a specialized ophthalmologist within each center. The calendar year of the first retinal proliferation was reported. For 76 subjects who first underwent laser panphotocoagulation because of severe preproliferative lesions, the calendar year of laser panphotocoagulation was taken as the year of the first retinal proliferation (37). Subjects given focal laser treatment were excluded. The time to retinopathy onset was calculated as the calendar year of the first retinal proliferation minus the calendar year of insulin-dependent diabetes onset. Control subjects were 346 age- and sex-matched healthy persons participating in a prospective study in the French general population (38).

COLLECTION OF PATIENT CHARACTERISTICS

The following information was collected anonymously:

Demographic data. Age, sex, ethnicity (Caucasian/Black/Asian).

Clinical characteristics. Weight (in kg); height (in m; body mass index was calculated as weight \times height²); systolic and diastolic blood

pressure (BP; mean of two consecutive measurements made in supine position with a mercury manometer to the nearest 2 mmHg).

Insulin-dependent diabetes. Calendar year of onset (diabetes duration was calculated from the calendar year of data collection minus the calendar year of diabetes onset); current treatment type (1, 2, 3, or 4 daily insulin injections or insulin pump) and doses (expressed as IU/Kg body wt/d); current glycated hemoglobin value measured locally by HPLC (39; not obtained in 32 cases because of pancreatic transplantation, hemoglobinopathies, or other sources of interpretation error).

Renal involvement. In addition to BP values, all types of antihypertensive treatment currently taken by the patients; for angiotensin I converting enzyme inhibitors (ACEI), the calendar year of first treatment with ACEI was noted. All available results of urinary albumin measured locally during the past 5 yr were recorded. For patients on renal replacement therapy, the type of treatment and the year of first treatment were recorded.

Cardiovascular complications. Past or present myocardial infarction, cerebral stroke, and lower limb arteriopathy (indicated by a lack of foot pulse, or leg amputation). Current resting electrocardiogram (ECG) was attached for centralized, anonymous reading (see below).

Prescriptions. All current medications were noticed.

CLASSIFICATION OF PATIENTS

A review committee (see composition in Acknowledgments), not aware of patients' origin, reviewed all case records to validate patient selection criteria and to grade the renal involvement of each patient. The review committee worked in three sessions; it reviewed the records of 50 randomly chosen patients at two separate sessions to analyze the variation in grading patients' renal involvement (these 50 patients were graded identically in both sessions). The stages of renal involvement were assessed from patient records and from the urinary albumin and plasma creatinine concentrations at the time of selection (these variables were measured for all patients in a central laboratory, Biochemistry Department, University Hospital, Angers, France). The following stages of renal involvement were used: (a) no nephropathy—normal kidney function (urinary albumin < 30 mg/24 h, 20 μ g/min, or 20 mg/liter) and plasma creatinine < 150 μ mol/liter without antihypertensive treatment); (b) incipient nephropathy—microalbuminuria (30–300 mg/24 h, 20–200 μ g/min, or 20–200 μ g/liter) without antihypertensive treatment, and plasma creatinine < 150 μ mol/liter; (c) established nephropathy—past or present macroalbuminuria (> 300 mg/24 h, 200 μ g/min, or 200 mg/liter) in patients on antihypertensive treatment or macroalbuminuria without antihypertensive treatment, and plasma creatinine < 150 μ mol/liter; (d) advanced nephropathy—past or present macroalbuminuria with or without antihypertensive treatment and plasma creatinine \geq 150 μ mol/liter, or renal replacement therapy.

ASSESSMENT OF CARDIOVASCULAR COMPLICATIONS

The presence or absence of cerebral stroke or lower limb arteriopathy was taken from patient records. The presence or absence of myocardial infarction was documented by the patient records and/or by the presence of pathological Q wave on current ECG. All current ECG were analyzed blindly by an ECG committee (composition in Acknowledgments) using Minnesota code 1.1.1. to 1.3.6. (40) for Q wave identification.

Determinations

38 ml of blood were collected from each patient (21 ml on EDTA, 10 ml on lithium heparin and 7 ml without preservative) in the fasting ($n = 170$) or nonfasting ($n = 324$) state, as well as a random urine sample; they reached the central laboratory (Biochemistry Department, University Hospital) via express mail within 24 h of collection.

A 500- μ g sample of DNA was extracted from a 15-ml blood sample from each patient by the standard method. The genotypes of the components of the renin–angiotensin system were determined using 100 ng DNA for each PCR reaction. The ACE I/D polymorphism was determined by the original method (41) and by two other meth-

ods, because it has been reported that the original PCR method can fail to extend the long allele across the insertion in some samples (42). The first method used an allele-specific oligonucleotide (ASO) hybridization technique in which labeled, specific oligonucleotides for the I or D allele were hybridized to a filter containing the corresponding amplified sequence as described by Ludwig et al. (23). The second technique was nested PCR performed in a single tube that directly extended the insertion. The first step of this new method amplified intron 16 across the insertion using flanking primers GIIS (5'-CTC-AAGCAGCCCCCTCACAGGACTG-3') and GAS (5'-GATGTG-GCCATCACATTCGTCAGAT-3') by heating for 1.5 min at 95°C followed by 15 cycles of amplification (95°C, 1 min; 62°C, 1 min; 72°C, 1 min) in 50- μ l buffer containing 2 mM MgCl₂ and 0.25% DMSO, using 2.5 U of GOLDSTAR DNA polymerase (Eurogentec, Seraing, Belgium), and 400-pM primer concentration. The tubes were cooled briefly at 4°C and the primer FYM, corresponding to the insertion sequence (5'-ATC ACG AGG TCA GGA GAT CGA GAC-3') and GIIS were added (each 20 pmol/tube) and PCR continued for an additional 15 cycles in the same conditions. The extension between GAS and GIIS generated a 561-bp PCR product for the I allele, and a 274-bp product for the D allele. Further amplification between FYM and GIIS generated a 376-bp products for the I allele only (Fig. 1). This method relies on prior amplification of intron 16 to increase the sensitivity and specificity of insertion amplification, which is an Alu repeat-type sequence. The first technique was performed in the Molecular Genetics Laboratory (Broussais Hospital, Paris, France), and the second, independently in the Biochemistry Department (University Hospital, Angers). The techniques generated identical results. These results differed in only four cases from the genotypes determined with the single step PCR method (41).

The M235T and T174M variants of the AGT gene and the A1166C and C573T forms of the AT1R receptor gene were determined by the allele-specific oligonucleotide technique (15, 25).

The plasma (collected on lithium heparin) ACE concentration was measured in patients not taking ACEI and in the control subjects by an enzymatic method using Hippuryl-glycyl-glycine as synthetic substrate (43; intra- and interassay coefficient of variation of 5% each). Urinary albumin was measured by nephelometry (44; assay sensitivity 1 mg/liter; intra- and interassay coefficient of variation of 2 and 4%, respectively). The use of a threshold value for albumin concentration of 20 mg/liter to predict persistent micro- or macroalbuminuria was validated earlier (45). Plasma creatinine was measured by a modification of Jaffe's method (46).

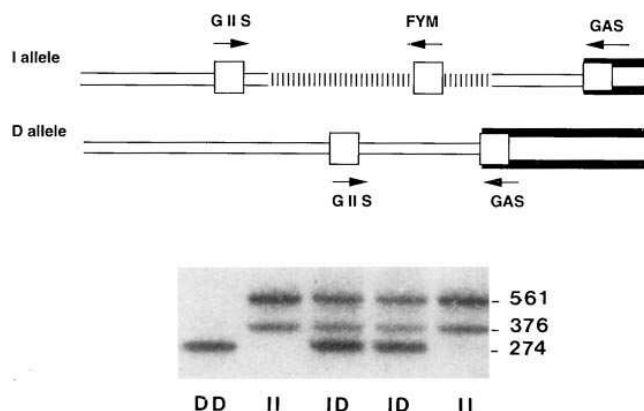


Figure 1. Nested PCR technique for determining ACE gene I/D polymorphism. (Top) Diagram of intron 16 and 5' part of exon 17 with positions of primers used for the first (GIIS and GAS) and the second (GIIS and FYM) round of amplification. Hatched segment is the insertion. (Bottom) Results of genotyping in individuals of the three genotypes. Numbers on the right indicate size (in bp) of amplified fragments.

Statistical analysis

All case record files were read by two independent secretaries and analyzed using the Statview IV.5 program (Abacus Concepts, Inc., Berkeley, CA) running on an Apple II Macintosh. Logistic regressions were determined using the Proc Logistic procedure of SAS software (SAS Statistical Software, version G; SAS Institute Inc., Cary, NC).

Data are presented as means \pm SD, or medians (ranges) when distributions were skewed. Groups were compared using the χ^2 test for trend for categorical variables (47), or parametric (if normally distributed, ANOVA or Student's *t* test) or nonparametric (if not normally distributed, Kruskal-Wallis or Mann-Whitney U tests) tests for continuous variables. Spearman's rank coefficient was used for correlation analysis. Logistic regression analysis was used for calculating adjusted odds ratio, and a polytomous logistic regression model was used for ordinal-scaled outcome variable. The outcome variable was the nephropathy stage. The independent variables were either genotypes and their interaction. Our main hypothesis examined the possible role of ACE I/D polymorphism as a risk factor of nephropathy among diabetic subjects. Because we had no specific hypothesis for the interactions of ACE, AGT, and AT1R polymorphism, only the interaction of ACE I/D with each of the four other tested genotypes was analyzed. The population attributable risk was calculated from the prevalence of diabetic nephropathy (obtained by grouping stages 2 [incipient] to 4 [advanced nephropathy]), the relative risk for nephropathy due to one given genetic trait, and the prevalence of the genetic trait (48).

Results

Patients' description. A total of 551 patients were recruited in 1 yr; 57 patients were excluded because the characteristics of their diabetes or the severity of their diabetic retinopathy did not fulfill the inclusion criteria. Of the 494 remaining patients, 484 were Caucasians, 9 were Blacks, and 1 Asian. 157 patients had no nephropathy (32%), 104 had incipient nephropathy (21%), 126 established nephropathy (25%), and 107 advanced nephropathy (22%) (of them, 46 [9%] were on renal replacement therapy). The daily insulin doses were 0.63 ± 0.21 IU/kg/d (not different according to the stage of nephropathy; ANOVA, $P = 0.2953$), and the proportions of patients on one, two, three, or four daily insulin injections or portable insulin pumps were 1, 36, 30, 15, and 18%, respectively (not different according to the stage of nephropathy; χ^2 test = 11.8, $P = 0.4612$). Patient characteristics are given in Table I; those who had no nephropathy were older, had had diabetes for longer, and their time to retinopathy onset was longer and glycated hemoglobin lower than the others. Systolic and diastolic BP, plasma creatinine, urinary albumin, the number of antihypertensive treatments, and the proportion of patients on ACEI increased with the stage of renal involvement. The prevalence of cardiovascular complications increased with the stage renal involvement regarding cerebral stroke (3 patients [1.9%] without nephropathy, one [1.0%] with incipient, 6 [4.8%] with established, and 9 [8.6%] with advanced nephropathy [χ^2 for trend = 8.18; $P = 0.004$]) and lower limb arteriopathy (20 patients [12.7%] without nephropathy, 15 [14.4%] with incipient, 21 [16.7%] with established, and 30 [28.0%] with advanced nephropathy [χ^2 for trend = 9.2; $P = 0.002$]), but not regarding myocardial infarction (12 patients [7.6%] without nephropathy, 4 [3.9%] with incipient, 9 [7.1%] with established, and 12 [11.2%] with advanced nephropathy [χ^2 for trend = 1.24; $P = 0.25$]).

Fig. 2 illustrates the cumulated frequency of subjects ac-

Table I. Patient Characteristics

Stage of nephropathy	No nephropathy (n = 157)	Incipient nephropathy (n = 104)	Established nephropathy (n = 126)	Advanced nephropathy (n = 107)	All patients (n = 494)	P value
Age (yr)	46±13	43±13	42±12	44±11	44±12	0.0051*
Sex (M/F)	84/73	62/42	66/60	65/42	277/217	0.458‡
Age at diabetes onset (yr)	16±9	16±10	15±8	15±8	15±9	0.6976*
Diabetes duration (yr)	31±10	27±9	27±9	28±9	29±10	0.0011*
Time to retinopathy onset (yr)	26±9	23±8	23±8	23±8	24±8	0.0088*
Body mass index (kg/m ²)	23.8±2.9	23.7±2.9	23.6±3.3	23.3±3.4	23.6±3.1	0.7012*
Glycated hemoglobin (%)	8.4±1.6	8.6±1.9	9.1±2.1	8.5±1.6	8.6±1.8	0.0149§
Systolic/diastolic BP (mmHg)	129±14/74±9	137±18/79±12	141±18/82±11	148±20/85±11	138±19/79±11	0.0001/0.0001*
Plasma creatinine (μM)	80 (35–134)	84 (53–144)	103 (35–148)	193 (79–1047)	95 (35–1047)	0.0001§
Urinary albumin concentration (mg/liter)	6 (1–60)	32 (1–204)	585 (2–7032)	501 (1–7883)	36 (1–7883)	0.0001§
Percentage of patients treated with 0/1/2/3/4/5 antihypertensive agents	76/18/5/1/0/0	47/35/12/6/0/0	22/32/33/11/2/0	15/22/30/21/10/2	43/26/19/9/2/1	0.0001‡
Percentage of patients on ACEI	22	51	72	69	51	0.0001‡

Data are means±SD, or medians (ranges). Glycated hemoglobin was obtained from only 462 participants for technical reasons. Urinary albumin and plasma creatinine concentrations are those measured in patients on treatment at the time of study. *ANOVA; ‡χ² test; §Kruskall-Wallis test.

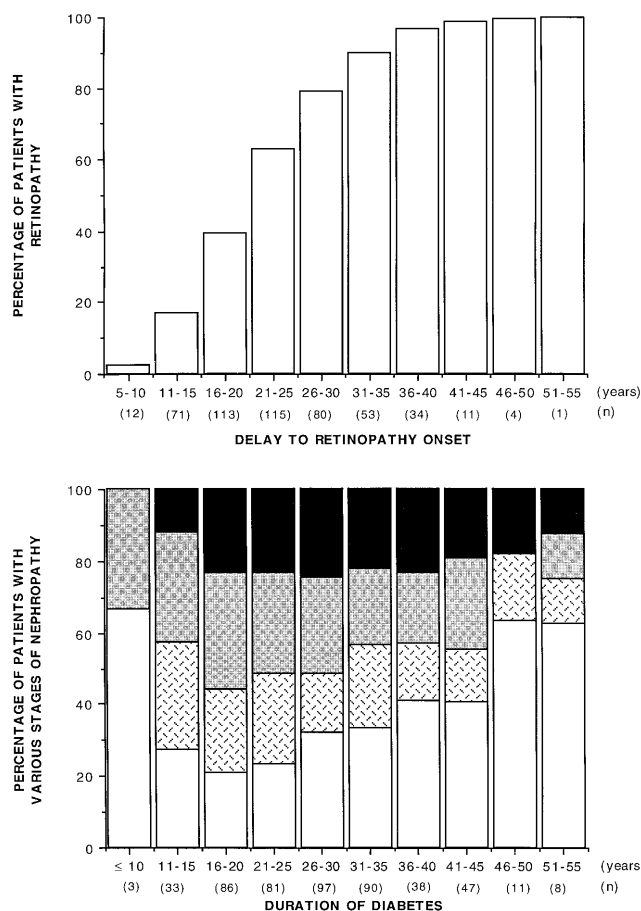


Figure 2. Cumulative frequency of patients in study according to the time to retinopathy onset (see Methods) (top) and proportion of dia-

abetic nephropathy stages according to diabetes duration (bottom): □ no nephropathy, ▨ incipient, ▩ established, and ■ advanced nephropathy. Numbers between parentheses indicate number of patients.

betically according to the time to retinopathy onset (*top*) and the relationship between renal involvement and diabetes duration (*bottom*). The median time to retinopathy onset was 23 yr; 75% of the patients had developed proliferative retinopathy before they had had diabetes for 29 yr. Conversely, the proportion of patients without nephropathy reached a nadir (21%) in patients who have had diabetes for 16–20 yr; then this proportion increased to 63% for patients suffering from diabetes for over 45 yr.

The amount of glycated hemoglobin was negatively correlated with the diabetes duration (Spearman's rank coefficient = -0.162; *P* = 0.0005) and with the time to retinopathy onset (Spearman's rank coefficient = -0.124; *P* = 0.0079).

The 346 control subjects were 180 men and 166 women, aged 44±9 yr, with 24.4±4.2 kg/m² body mass index, and 130±16/79±10 mmHg systolic/diastolic BP.

Relationships between renal involvement and angiotensin I converting enzyme. The distribution of ACE I/D polymorphism in patients was in Hardy-Weinberg equilibrium and not different from that of age- and sex-matched controls: DD genotype 167 (34%) vs. 117 (34%), ID genotype 237 (48%) vs. 154 (44%), II genotype 90 (18%) vs. 75 (22%), (χ² = 1.25, *P* > 0.50). There was a significant association between ACE I/D polymorphism and the stage of renal involvement in the diabetic patients (χ² for trend = 5.135; *P* = 0.023; Table II). The D allele frequency increased with nephropathy stages: 1, 0.5255; 2, 0.5865; 3, 0.5992; 4, 0.6215 (χ² for trend = 5.22; *P* = 0.022). This association was due to a reduced proportion of pa-

betically according to diabetes duration (*bottom*): □ no nephropathy, ▨ incipient, ▩ established, and ■ advanced nephropathy. Numbers between parentheses indicate number of patients.

Table II. ACE I/D Polymorphism and the Stages of Renal Involvement

ACE I/D polymorphism	No nephropathy (n = 157)	Incipient nephropathy (n = 104)	Established nephropathy (n = 126)	Advanced nephropathy (n = 107)
DD	48	36	44	39
n (%)	(31%)	(35%)	(35%)	(37%)
ID	69	50	63	55
n (%)	(44%)	(48%)	(50%)	(51%)
II	40	18	19	13
n (%)	(25%)	(17%)	(15%)	(12%)

Relationship between ACE I/D polymorphism and the stages of renal involvement: χ^2 for trend = 5.135; $P = 0.023$. Comparison of patients with the II genotype to the others: χ^2 for trend = 8.364; $P = 0.0038$. Comparison of patients with the DD genotype to the others: χ^2 for trend = 1.021; $P = 0.3123$. Comparison between patients with the DD or ID genotypes; χ^2 for trend = 0.001; $P = 0.9702$.

tients with the II genotype according to the stage of renal involvement (comparison to patients with the ID or DD genotypes [χ^2 for trend = 8.364; $P = 0.038$]), and not to an increased proportion of patients with the DD genotype (comparison to patients with the ID or II genotypes [χ^2 for trend = 1.021; $P = 0.3123$]). There was no difference for the stage of renal involvement between patients with the DD or ID genotypes (χ^2 for trend = 0.001; $P = 0.9702$; Table II).

Patient characteristics according to ACE I/D genotypes are shown in Table III. The BP, plasma creatinine, and the number of antihypertensive agents used by patients were associated to the ACE I/D genotype. Patients with II genotypes had lower systolic ($P = 0.0258$; Student's t test) and diastolic ($P = 0.0135$; Student's t test) BP and lower plasma creatinine ($P = 0.0056$; Mann-Whitney U test) than those with ID or DD genotypes and they were given fewer antihypertensive agents ($P =$

0.0321; Mann-Whitney U test) than those with ID or DD genotypes. There was no difference between patients with ID and those with the DD genotype regarding BP, plasma creatinine, and antihypertensive agents.

The possible influence of ACE I/D polymorphism on the risk of a diabetic nephropathy constitution was assessed by polytomous logistic regression analysis. Gender, age at diabetes onset, time to retinopathy onset, and diabetes duration (the last three variables were divided into tertiles of equal magnitude) were used as covariates. When the I and the D alleles of ACE were introduced into this model, the D allele was an independent contributor to diabetic nephropathy: adjusted odds ratio 1.889 (95% CI 1.209–2.952, $P = 0.0052$). The I allele was not contributive: odds ratio 1.046 (95% CI 0.731–1.496; Table IV). This suggests that the ACE D allele has a dominant effect on the risk of diabetic nephropathy. The concordance between the observed and predicted severity of diabetic nephropathy was 56.9%, using this model. In this population, the attributable risk for diabetic nephropathy due to the D allele was 42% (95% CI 15–61).

Among the 37 patients with myocardial infarction, 11 were homozygotes DD (6.6% of patients with DD genotype), 25 were ID (10.6% of those with ID genotype), and 1 was II (1.1% of those with II genotype) (χ^2 test = 8.68; $P = 0.013$). As myocardial infarction is more prevalent in insulin-dependent diabetic patients with nephropathy than in those without (2, 3), and as ACE polymorphism is associated with myocardial infarction (18), we repeated the logistic regression analysis described above after excluding the 37 patients with myocardial infarction. The D allele remained a significant contributor to diabetic nephropathy: adjusted odds ratio 1.755 (95% CI 1.117–2.756, $P = 0.0146$), population attributable risk 38% (95% CI 9–59).

There was no detectable relationship between ACE I/D polymorphism and cerebral stroke (6 patients with the DD genotype [3.6%], 11 with the ID genotype [4.6%], and 2 with the II genotype [2.2%] [χ^2 test = 1.08; $P = 0.588$]) or lower

Table III. Patient Characteristics According to ACE I/D Genotypes

ACE I/D genotype	DD (n = 167)	ID (n = 237)	II (n = 90)	P value
Age (yr)	43±13	44±12	44±13	0.7802*
Sex (M/F)	86/81	143/94	48/42	0.1787‡
Age at diabetes onset (yr)	16±9	15±9	15±8	0.8784*
Diabetes duration (yr)	28±10	29±10	29±10	0.4362*
Time to retinopathy onset (yr)	32±8	25±8	24±8	0.0637*
Body mass index (kg/m ²)	23.3±3.1	23.9±3.2	23.5±2.8	0.1608*
Glycated hemoglobin (%)	8.5±1.9	8.8±1.9	8.3±1.5	0.0913§
Systolic/diastolic BP (mmHg)	139±19/81±11	138±20/80±12	134±16/77±11	0.0804/0.0302*
Plasma creatinine (μM)	129 (35–1047)	130 (35–874)	113 (35–526)	0.0079§
Urinary albumin concentration (mg/liter)	507 (1–7032)	460 (1–7883)	409 (1–5254)	0.3474§
Percentage of patients treated with 0/1/2/3/4/5 antihypertensive agents	(46/25/18/9/1/1)	(37/27/22/9/4/1)	(52/26/13/9/0/0)	0.0221§
Percentage of patients on ACEI	38	45	34	0.1773‡

Data are means±SD, or medians (ranges). Glycated hemoglobin was obtained from only 462 participants for technical reasons. Urinary albumin and plasma creatinine concentrations are those measured in patients on treatment at the time of study. *ANOVA; ‡ χ^2 test; §Kruskall-Wallis test.

Table IV. Risk of Diabetic Nephropathy

Variable	Adjusted odds ratio	95% CI	P value
Males	1.169	0.838–1.630	0.3583
Age at diabetes onset*			
0–12 yr	—		
13–24 yr	0.985	0.689–1.410	0.9356
≥ 25 yr	0.792	0.501–1.272	0.3185
Diabetes duration*			
8–25 yr	—		
26–41 yr	0.744	0.514–1.078	0.1180
≥ 42 yr	0.475	0.254–0.889	0.0200
Time to retinopathy onset*			
5–15 yr	—		
16–31 yr	1.369	0.855–2.120	0.1586
≥ 32 yr	1.269	0.691–2.329	0.4424
ACE D allele	1.889	1.209–2.952	0.0052
ACE I allele	1.046	0.731–1.496	0.8074

Polytomous multivariate logistic regression analysis takes into account gender, age at diabetes onset, diabetes duration, time to retinopathy onset, and presence of ACE D or I alleles. The concordance between the observed and predicted severity of diabetic nephropathy was 56.9%. *Odds ratios express the risk of second vs. first, and third vs. first tertiles, respectively.

limb arteriopathy (28 patients with the DD genotype [16.8%], 45 with the ID genotype [19%] and 13 with the II genotype [14.4%] [χ^2 test = 1.01; $P = 0.604$]).

Fig. 3 shows the effect of the ACE I/D genotype and diabetes on the circulating ACE concentrations (log-transformed for ANOVA). Plasma ACE increased as expected from II to DD genotype ($F^2_{582} = 82$, $P < 0.0001$). Diabetic subjects had higher ACE levels than controls ($F^1_{582} = 75$, $P < 0.001$). But the effect of I/D polymorphism was the same in diabetic and nondiabetic subjects (interaction, $F^2_{582} = 0.44$, $P = 0.64$). The

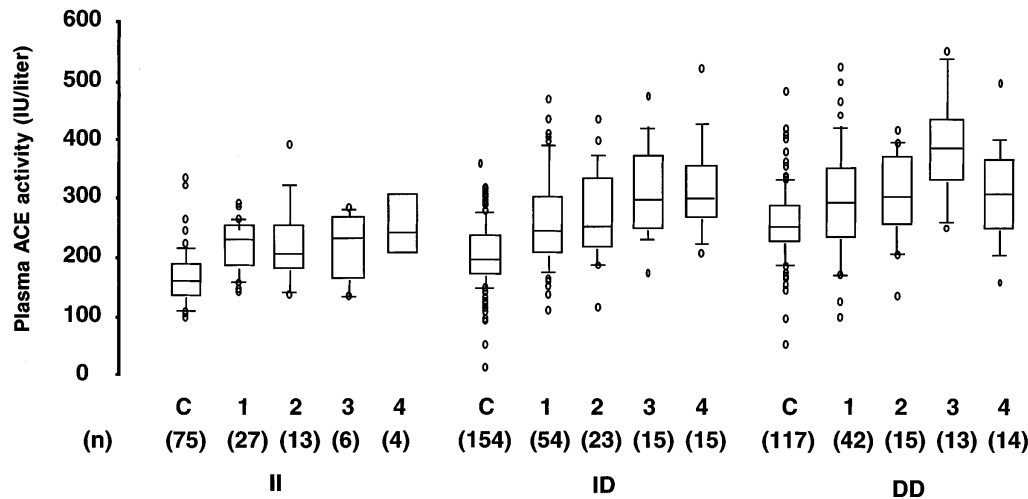


Figure 3. Plasma ACE activity according to ACE I/D polymorphism (II, ID, DD) in 346 control subjects (C), and in 242 insulin-dependent diabetic subjects according to their stages of diabetic nephropathy (1, no nephropathy; 2, incipient; 3, established; 4, advanced nephropathy). Results are presented as box-plots with outlier values. Numbers between parentheses indicate number of patients. Two-factor (ACE I/D polymorphism and diabetes) ANOVA on ACE levels (log-transformed): ACE I/D polymorphism effect $F^2_{582} = 82$, $P < 0.0001$; diabetes effect

$F^1_{582} = 75$, $P < 0.001$; interaction $F^2_{582} = 0.44$, $P = 0.64$. Two-factor (nephropathy stage and ACE I/D polymorphism) ANOVA on ACE levels (log-transformed) in diabetic subjects: nephropathy effect $F^3_{230} = 2.6$, $P = 0.053$; ACE I/D polymorphism effect $F^2_{230} = 19.8$, $P < 0.001$; and interaction $F^6_{230} = 1.512$, $P = 0.17$.

ACE concentrations in diabetic subjects were determined by nephropathy ($F^3_{230} = 2.6$, $P = 0.053$) and ACE I/D polymorphism ($F^2_{230} = 19.8$, $P < 0.001$), and there was no interaction between the two variables ($F^6_{230} = 1.512$, $P = 0.17$).

Relationships between renal involvement and polymorphism of the angiotensinogen or angiotensin II subtype I receptor. The distributions of AGT M235T and T174M, and of AT1R T573C and A1166C polymorphism are given in Table V according to the stage of nephropathy. No significant association was detected. A logistic regression analysis using a model identical to the one used to test ACE I/D polymorphism was used to test each of these polymorphisms for their relationships with renal involvement. No significant association was found: the adjusted odds ratio for M235T 1.137 (95% CI 0.905–1.429, $P = 0.2699$), for T174M 1.001 (95% CI 0.997–1.004, $P = 0.6649$), T573C 0.999 (95% CI 0.997–1.001, $P = 0.3234$), and A1166C 0.9291 (95% CI 0.712–1.213, $P = 0.5895$).

Any interaction between the AGT or AT1R polymorphism and the ACE I/D polymorphism in determining renal disease was tested by introducing each of them separately into the same logistic polytomous regression model with the ACE I/D alleles. There was a significant interaction between the ACE D allele and AGT M235T polymorphism: adjusted odds ratio 2.101 (95% CI 1.144–3.858; $P = 0.0166$; Table VI). The concordance between the observed and predicted severity of diabetic nephropathy was 58.9% using this model. The meaning of this interaction is shown in Fig. 4. The proportion of patients with II genotypes having established and advanced nephropathy decreased nonsignificantly from MM to TT genotypes (χ^2 test = 5.95, $P = 0.43$), whereas it increased among patients with the ID and DD genotypes (χ^2 test = 14.13, $P = 0.0282$). The plasma creatinine in the 403 patients with ACE ID or DD genotypes was linked to AGT M235T genotypes: MM (44–874) 92 μ M, MT (35–1,047) 131 μ M, TT (53–735) 105 μ M (Kruskall-Wallis test, $P = 0.0058$). However, the systolic/diastolic BP were not different: MM 139±20/80±11 mmHg, MT 138±19/80±11 mmHg, TT 139±19/80±12 mmHg (ANOVA, $P = 0.9065/0.9929$).

Table V. Distributions of Substitution Polymorphisms M235T and T174M in Angiotensinogen, and T573C and A1166C in Angiotensin II Subtype 1 Receptor Gene According to Patient Stage of Nephropathy

Stage of nephropathy	No nephropathy	Incipient nephropathy	Established nephropathy	Advanced nephropathy	All patients	P value
M235T						
MM	49	38	46	25	158	0.2441
MT	78	49	49	56	232	
TT	29	17	31	26	103	
T174M						
TT	117	85	94	86	382	0.6741
TM	35	19	26	20	100	
MM	4	0	5	1	10	
T573C						
TT	34	19	27	18	98	0.9846
TC	67	51	54	53	225	
CC	53	34	44	36	167	
A1166C						
AA	75	59	64	54	252	0.6776
AC	64	39	57	45	205	
CC	14	6	5	8	33	

M235T genotypes were determined in only 493 patients, T174M in 492 patients, T573C in 490 patients, and A1166C in 490 patients. *P* values were obtained from χ^2 tests for trend.

Table VI. Risk of Diabetic Nephropathy

Variable	Adjusted odds ratio	95% CI	P value
Males	1.127	0.806–1.575	0.4859
Age at diabetes onset*			
0–12 yr	—		
13–24 yr	0.967	0.675–1.385	0.8557
≥ 25 yr	0.819	0.516–1.299	0.3956
Diabetes duration*			
8–25 yr	—		
26–41 yr	0.733	0.506–1.061	0.0999
≥ 42 yr	0.496	0.264–0.932	0.0293
Time to retinopathy onset*			
5–15 yr	—		
16–31 yr	1.346	0.869–2.086	0.1833
≥ 32 yr	1.230	0.668–2.263	0.5058
ACE D allele	0.465	0.137–1.574	0.2183
ACE I allele	1.025	0.715–1.468	0.8939
AGT M235T	0.625	0.359–1.089	0.0974
Interaction between ACE I/D alleles and AGT polymorphism	2.101	1.144–3.858	0.0166

Polytomous multivariate logistic regression analysis taking into account gender, age at diabetes onset, diabetes duration, time to retinopathy onset, ACE I/D alleles, and AGT M235T polymorphism. Due to interaction between ACE I/D alleles and AGT polymorphism, no independent effect was observed of ACE I/D alleles or AGT polymorphism alone on diabetic nephropathy severity, as illustrated by Fig. 4. The concordance between the observed and predicted severity of diabetic nephropathy was 58.9%. *Odds ratios express the risk of second vs. first, and third vs. first tertile, respectively.

No interaction was found in the model including ACE I/D polymorphism and AGT T174M (adjusted odds ratio 0.558 [95% CI 0.239–1.304, *P* = 0.1778]), AT1R T573 C (adjusted odds ratio 0.994 [95% CI 0.953–1.038, *P* = 0.7874]), or A1166C (adjusted odds ratio 0.687 [95% CI 0.386–1.223, *P* = 0.2019]) polymorphisms.

Discussion

This study indicates that genetic polymorphisms of ACE and AGT potentially affecting the levels of angiotensins and kinins in the kidney can affect the development of renal disease in insulin-dependent diabetic subjects whose diabetes is inadequately controlled. Brenner proposed that elevated hydraulic pressure within the glomerular capillaries can cause glomerular damage (49), especially in diabetes (10). As uncontrolled diabetes causes overall capillary vasodilation (50), a relative postglomerular vasoconstriction (as produced by angiotensin II) can lead to elevated intraglomerular capillary pressure (10). However, not all insulin-dependent diabetic subjects develop diabetic glomerulopathy (1, 2). Thus, the physiological factors that affect glomerular capillary pressure are potential risk factors for susceptibility to glomerulopathy due to diabetes. The present data are consistent with the hypothesis that constitutive levels of angiotensin II can affect the development of diabetic glomerulosclerosis. Subjects with the ACE II genotype have the lowest plasma and cellular ACE levels (14, 17). Because the conversion of angiotensin I is low in the kidney (51), it has been proposed that the plasma ACE circulating through the kidney is an important contributor but yet a limiting factor in angiotensin II production within the renal circulation (52). Upstream angiotensin II production, angiotensin I production can be limited by the amount of substrate for renin, which explains the interaction with the M235T polymorphism, because circulating AGT is partly determined by the AGT

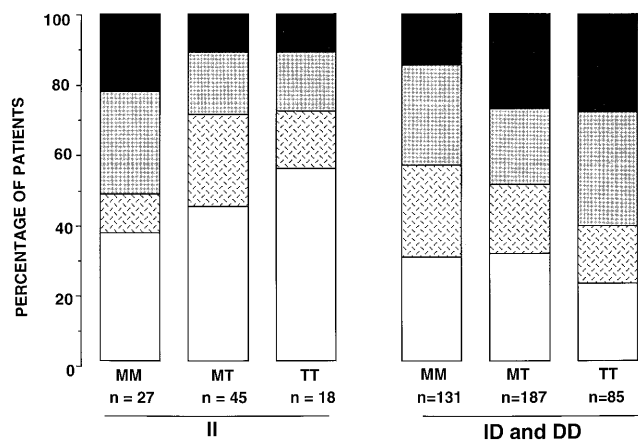


Figure 4. Proportions of patients with various stages of diabetic nephropathy according to their ACE II (left) or ID and DD (right) genotypes, and according to AGT M235T polymorphism (MM, MT, TT): □ no nephropathy, ▨ incipient, ▩ established, and ■ advanced nephropathy. Adjusted odds ratio of risk for diabetic nephropathy due to interaction between ACE D/I alleles and AGT M235T genotypes: 2.101 (95% CI 1.144–3.858, $P = 0.0166$). Association between AGT M235T genotypes and stages of diabetic nephropathy in 90 subjects with ACE II genotype $\chi^2 = 5.95$, $P = 0.43$ and in 403 subjects with ACE ID and DD genotypes: $\chi^2 = 14.13$, $P = 0.0282$.

M235T polymorphism (15). Also, the vasodilator kinins, which are produced in the renal circulation and metabolized by ACE (53), may participate in the control of glomerular hemodynamics in diabetes and their inactivation can mediate the deleterious effect of ACE on kidney function (54).

This study was done on insulin-dependent diabetic subjects who had already expressed their risk of renal complications due to diabetes, because they had proliferative diabetic retinopathy. Patients with proliferative diabetic retinopathy are 20 and 24%, respectively, of all patients with insulin-dependent diabetes attending a French (35) or a Danish (55) diabetic clinic, and 37% of those with insulin-dependent diabetes for 30 yr or more attending diabetic clinics in Europe (56). The age at onset of diabetes, the diabetes duration, and the time to proliferative diabetic retinopathy in the studied patients all fit with previous descriptions (2, 5, 35, 55). The degree of renal involvement in these patients was graded using a classification derived from the one proposed by Mogensen et al. (1). The proportion of patients without nephropathy, or with incipient, established, or advanced nephropathy is consistent with previous cross-sectional studies (1, 35, 55). The relationship between the severity of clinical renal involvement and diabetes duration (Fig. 1) is in keeping with previous follow-up studies, that indicated a peak incidence of diabetic nephropathy (identified by proteinuria) after 10–30 yr of insulin-dependent diabetes (2, 57). Lastly, the blood glucose control of the studied patients (as assessed by glycated hemoglobin at time of data collection) was linked to the time required to develop diabetic retinal disease, and to diabetes duration. These data are consistent with previous studies showing that the degree of blood glucose control affects the incidence of diabetic retinal and renal complications and the vital prognosis of insulin-dependent diabetic patients (5, 6).

The possible role of genetic factors in the development of diabetic nephropathy could be tested using the present patient

cohort, because there was no uncertainty in these patients on the role of insulin-dependent diabetes in diabetic nephropathy constitution. Genetic determinants of the renin-angiotensin system were the first candidates to be tested. Taking the adjusted odds ratio as an estimate of relative risk (value 1.889 [95% CI 1.209–2.952]), the population risk for diabetic nephropathy attributable to ACE D allele was 42% (95% CI 15–61), because the ACE D allele is frequent in the population. In studies examining diabetic nephropathy in families with multiple siblings with insulin-dependent diabetes, the relative risk for siblings to develop diabetic nephropathy was reported to be 2.2, 3.3, and 4.8, respectively (7, 58, 59), if the proband had developed diabetic nephropathy. The familial component increased by 50% the absolute risk for diabetic nephropathy in the most recent study (59). Thus, the contribution of ACE gene polymorphism to the heritable part of risk for diabetic nephropathy seems important, and further suggests that most of the familial component of the risk for diabetic nephropathy is due to genetic factors, as supported by familial studies performed in patients with insulin-dependent diabetes living in New England (59) and in Pima Indians with noninsulin-dependent diabetes (8). However, we cannot reject from this study that nonglycemic, nongenetic variables can also affect the development of diabetic nephropathy, like smoking habits (60), although we found in the present study no relationship between past or current tobacco use and renal disease (data not shown), or the nongenetic components of BP.

The ACE D allele had a dominant effect, while the risk of nephropathy was relatively reduced in the insulin-dependent diabetic subjects with the II genotype. These data from the present multicenter study confirm those we reported in a previous monocenter case-control study (26) and those obtained in other studies (27, 28), but differ from two other published studies (30, 31). However, the controls used in these latter studies were not matched with cases for diabetic retinopathy severity and/or diabetes control (30, 31). As controls in these studies were less exposed to hyperglycemia than cases, then an effect of candidate genes able to modulate renal risk may have been masked.

There was also an association between ACE I/D polymorphism and myocardial infarction, in agreement with previous reports (18–24), including on insulin-dependent diabetic subjects with nephropathy (61), and in noninsulin-dependent diabetic subjects (19, 24). Insulin-dependent diabetic subjects with nephropathy are especially prone to coronary heart disease (2, 3). The coexistence of myocardial infarction with diabetic nephropathy can therefore be a source of confusion for assessing the role of the ACE gene polymorphism in the constitution of diabetic nephropathy. This study shows that the association between the ACE D allele and nephropathy is still present after subjects with myocardial infarction are excluded, indicating an association between this polymorphism and diabetic nephropathy that is independent of any coexisting coronary heart disease.

There was an effect of the diabetes state on the plasma ACE that was independent of diabetic nephropathy and of I/D polymorphism. This confirms earlier reports (26, 62, 63). As a consequence, the relationship between plasma ACE levels and ACE I/D polymorphism was shifted upwards by the diabetes state. The effect of diabetes state on plasma ACE levels is currently not explained. It seems to be independent of the degree of diabetes control (26, 30). It may be due to alterations in

ACE metabolism, because ACE can be released from endothelial cells and trapped by the reticuloendothelial system, and because both endothelial cells and reticuloendothelial system can be altered by diabetes (50). If we accept that the risk of renal damage is linked to ACE levels, which are increased in diabetes, then the subjects with the lowest ACE levels, i.e., mostly those with the II genotype, would indeed appear to be at reduced risk of diabetic nephropathy.

As the plasma ACE levels were weakly associated to the severity of renal disease ($P = 0.053$), independently of I/D polymorphism, increments in plasma ACE levels can also be secondary to renal disease, although previous reports give conflicting results on the relationship between kidney function and plasma ACE levels (26, 30).

Thus, the ACE II genotype seems to reduce risk for renal disease in insulin-dependent diabetes, which is consistent with our first report (26) and with others about insulin-dependent (27, 28) and noninsulin-dependent diabetic patients (32–34). Pharmacological blockade of ACE protects kidney function of insulin-dependent diabetic patients with incipient or established diabetic nephropathy, and seems to do so independently of its blood pressure lowering effect (64–66). Thus, ACE may well be a risk factor for the progression of diabetic renal disease. Longitudinal studies of renal function and the response to ACEI should now be done on insulin-dependent diabetic patients according to their ACE I/D genotypes. Until then, it is still premature to formulate recommendations for the care of patients with insulin-dependent diabetes according to ACE I/D genotypes. Recommendations for the use of ACEI must still rely on identification of microalbuminuria or proteinuria in these patients.

The suggestion of a role for ACE and its inhibition in the progression of renal disease was first done for insulin-dependent diabetic subjects (26, 64, 66). Since then, similar observations have been reported in nondiabetic renal diseases, dealing with progression to renal failure according to ACE I/D polymorphism (67–69), and with retardation of renal failure by ACEI (70, 71). Constitutional ACE levels seem to be critical for the susceptibility of the glomerular circulation to external injury, whatever the nature of the injury (52).

The AGT polymorphism does not appear to be an independent risk factor for diabetic nephropathy, in agreement with Tarnow et al. (72) and Doria et al. (73), but in contrast with Fogarty et al. (74) who found a direct association between AGT M235T polymorphism and diabetic nephropathy. But there was in the present study a significant interaction between ACE I/D and AGT M235T polymorphisms. As the AGT M235T polymorphism can partly determine the AGT level (15), this interaction suggests that the level of AGT, the precursor of angiotensin I, can also affect the glomerular circulation of insulin-dependent diabetic subjects, and that this effect would only be apparent in subjects in whom angiotensin I conversion is not strongly limited by low ACE availability. This interaction between ACE and AGT polymorphisms now needs to be tested in nondiabetic renal diseases. The AGT genetic polymorphism has also been linked to familial hypertension (15, 16) which may share genetic determinants with diabetic nephropathy (75).

No relationship was found between AT1R polymorphism and diabetic nephropathy, directly or through interaction with the ACE I/D polymorphism, although an association between AT1R polymorphism and blood pressure has been reported

(25), and previous studies reported alterations in sensitivity to angiotensin II in diabetes (76, 77). The physiological significance of the AT1R polymorphism is unknown, and the present data do not rule out a role for the AT1R gene in susceptibility to diabetic nephropathy.

Finally, this study strongly suggests that genetic polymorphisms in the renin–angiotensin and kallikrein–kinin systems can affect the progression to renal failure of patients with inadequately controlled insulin-dependent diabetes. This possibility now needs to be confirmed by prospective studies. The effects of other candidate genes or acquired factors should also be tested in these patients (78).

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