Prognostic Value of the Insertion/Deletion Polymorphism of the ACE Gene in Type 2 Diabetic Subjects

Results from the Non-Insulin-Dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR), Diabete de type 2, Nephropathie et Genetique (DIAB2NEPHROGENE), and Survie, Diabete de type 2 et Genetique (SURDIAGENE) studies

OBJECTIVE — We tested whether determination of the ACE insertion/deletion polymorphism is useful for renal and cardiovascular prognoses of type 2 diabetic subjects.

RESEARCH DESIGN AND METHODS — The French participants (3,126 of 4,912) in the Non-Insulin-Dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR) trial were studied for their prognosis over 4 years according to their ACE insertion/deletion polymorphism. We used two cohorts of French type 2 diabetic patients for replication: a 3-year follow-up study (n = 917; Survie, Diabete de type 2 et Genetique [SURDIAGENE] study) and a case-control study on diabetic nephropathy (n = 1,277; Diabete de type 2, Nephropathie et Genetique [DIAB2NEPHROGENE] study). We investigated the effect of the insertion/deletion polymorphism on the primary outcome in the DIABHYCAR trial (defined as the first of the following events to occur: cardiovascular death, nonfatal myocardial infarction, stroke, heart failure leading to hospital admission, or end-stage renal failure) and its components.

RESULTS — In DIABHYCAR, the primary outcome and most of its components were not affected by the ACE insertion/deletion genotype. Only renal outcome was favored by the I allele (P = 0.03). The risk of myocardial infarction was not affected by ACE genotype, but the probability of fatal outcome increased with the number of D alleles (P < 0.03). In SURDIAGENE, the association between the ACE I allele and renal outcome was not replicated. In DIAB2NEPHROGENE, no association was found with nephropathy.

CONCLUSIONS — We were not able to demonstrate the manifest usefulness of the ACE insertion/deletion polymorphism for the prognosis of type 2 diabetic subjects.
The ACE gene is an excellent candidate for determining prognosis for cardiovascular and renal risks: a single insertion/deletion polymorphism in intron 16 (rs1799752) of a 287-bp Alu sequence accounts for half of the interindividual variance of the circulating and cellular activities of this enzyme. ACE activity is highest in subjects homozygous for the D allele (DD genotype), lowest in those homozygous for the I allele (II genotype), and intermediate in heterozygotes (ID genotype). Although its prognostic value for myocardial infarction is controversial in the general population (11,12), its impact for renal prognosis is well established in type 1 diabetes (13–16). Clinical trials in type 1 diabetes have suggested that patients with the II genotype display a better renal response to ACE inhibitors than other patients (17).

We therefore wondered whether genotyping ACE for its insertion/deletion polymorphism would markedly contribute to individualization of renal and cardiovascular prognoses of type 2 diabetic subjects with raised urinary albumin concentrations in a substudy of the Non-Insulin-Dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR) trial (9), a clinical trial comparing a low dose of ramipril with placebo. We assessed the impact of the ACE insertion/deletion genotype on the principal outcome, a composite of cardiovascular death, nonfatal myocardial infarction, stroke, heart failure leading to hospitalization, and ESRF, and on each of its components. To replicate our initial findings, we tested the same hypothesis on two independent cohorts of French type 2 diabetic patients: a single-center follow-up study on cardiovascular and renal outcomes (the Survie, Diabete de type 2 et Genetique [SURDIAGENE] study), and a multicenter case-control study on diabetic nephropathy (the Diabete de type 2, Nephropathie et Genetique [DIAB2NEPHROGENE] study).

RESEARCH DESIGN AND METHODS

Primary cohort
The DIABHYCAR study design and results have been reported elsewhere (9,18). Participants were selected on the basis of type 2 diabetes, treatment with oral antidiabetic agents on enrollment, high urinary albumin concentration (76% microalbuminuric and 24% macroalbuminuric patients), age ≥50 years, and serum creatinine concentration ≤150 μmol/l. French patients were selected by their general practitioners, and ≥98% were Caucasians. The tested drug was low-dose ramipril (1.25 mg/day). The primary end point was the combined incidence of cardiovascular death, nonfatal acute myocardial infarction, stroke, heart failure leading to hospitalization, and ESRF (defined as a requirement for dialysis or kidney transplant). These outcomes were also analyzed separately. All events were adjudicated by an independent clinical event committee (18). Ramipril proved to be ineffective (9).

The genetic substudy of DIABHYCAR was conducted in the French participants only for logistic reasons. All gave written consent on a form separate from the trial consent form. As preliminary data had suggested that an ACE insertion/deletion polymorphism might be associated with the risk of myocardial infarction (11) and diabetic nephropathy (19), this analysis was planned prospectively (20). By assuming a D allele frequency of 60% and a composite event rate of 20% over the study period (20), the sample size of the French subgroup (18) gave an a priori power of 94% for detecting a 25% difference in the risk of morbidity among patients with the DD genotype and those with the ID or II genotype. The study design was approved by the Angers University Ethics Committee.

Replication cohorts
SURDIAGENE was a prospective single-center follow-up study of type 2 diabetic patients regularly being seen at the Diabete Department at Pottiers University Hospital, in 2001–2007, designed to identify the genetic determinants of microvascular and macrovascular diabetic complications in type 2 diabetes. The main exclusion criteria were residence outside the Pottiers area and/or evidence of nondiabetic kidney disease. Clinical events corresponding to the primary end point of the DIABHYCAR study were recorded prospectively from patients’ hospital records and interviews with general practitioners.

DIAB2NEPHROGENE was a multicenter case-control study (15 diabetes and 5 nephrology centers in France between 2001 and 2004 [additional information can be found in an online appendix at http://dx.doi.org/10.2337/dc07-2079]) designed to assess the genetic determinants of diabetic nephropathy in type 2 diabetes. Case patients were type 2 diabetic patients with high urinary albumin concentrations (≥20 mg/l or 30 mg/24 h on two of three sterile urine collections) and retinopathy. Control subjects were type 2 diabetic patients of the same geographic origin with urinary albumin concentrations <20 mg/l or 30 mg/24 h on two of three sterile urine collections who were not receiving ACE inhibitors and/or angiotensin receptor blockers and who had retinopathy and/or known diabetes duration ≥20 years.

The DIAB2NEPHROGENE and SURDIAGENE studies were approved by the Pottiers University Ethics Committee. All participants gave written informed consent.

Biological determinations
DNA extraction and genotyping methods have been described elsewhere (13). A1C was determined centrally using a high-performance liquid chromatography method: a DIAMAT analyzer (normal values 4.0–5.6%; Bio-Rad, Richmond, CA) in the DIABHYCAR study and ADAMS HA-8160 analyzer (normal values 4.0–6.0%; Menarini, Florence, Italy), in the DIAB2NEPHROGENE and SURDIAGENE studies. Urinary albumin was measured by nephelometry. Serum creatinine, lipid (total and HDL cholesterol, triglycerides, and calculated LDL cholesterol), and highly sensitive C-reactive protein concentrations were determined centrally in the fasting state using a colorimetric method, running on an automated analyzer (Kone Optima; Thermo Clinical Labsystems, Vantaa, Finland), and a nephelometric method (N High Sensitivity CRP; Dade Behring, Marburg, Germany), respectively.

Statistical analysis
In the DIABHYCAR and SURDIAGENE studies, we considered the time to occurrence of a predefined combined primary end point and of each of its components during the study. For the combined end point, we considered only the event that occurred first. A Kaplan-Meier survival curve was constructed to assess the effect of ACE genotype on the occurrence of end points with time to the event as the outcome variable and censoring at the end of the study. A multivariate Cox proportional hazards model was used to analyze the effect of ACE genotype and other covariates on study outcomes. We also used χ² and χ²* for trend tests, ANOVA after log-transformation if required, and mul-
tivariate regression analysis. All statistical analyses were performed with Statview 5 (SAS Institute, Cary, NC).

### RESULTS

#### DIABHYCAR trial

Baseline characteristics are presented according to the ACE insertion/deletion polymorphism in Table 1. The ACE insertion/deletion polymorphism was associated with a personal history of myocardial infarction ($\chi^2=6.53; P=0.038$), with the I allele being more frequent among participants with previous myocardial infarction than among others (49.4% vs. 40.6%, $P=0.01$). This association persisted ($P=0.0076$) in multiple logistic regression analysis after sex, systolic blood pressure, serum creatinine concentration, urinary albumin concentration, and HDL cholesterol concentration were considered and also after forcing age, A1C, and smoking into the model. During follow-up (median 4 years; range 0–6 years), a primary outcome occurred in 495 participants (Table 2). The occurrence of the primary combined outcome did not differ among ACE genotypes (supplemental Fig. 1A, available in an online appendix), with no interaction with treatment group (ramipril or placebo). No association was found regarding the inci-

### Table 1—Baseline clinical and biological characteristics according to the ACE insertion/deletion polymorphism in the DIABHYCAR trial population

| Characteristic                          | ACE ID | ACE II | ACE DD | P value: ID vs. II | P value: ID vs. DD |
|----------------------------------------|--------|--------|--------|-------------------|-------------------|
| n                                      | 1,463  | 549    | 1,114  |                   |                   |
| Randomization group (placebo/ramipril) | 272/277(50) | 723/740(51) | 582/532(48) | 0.327             |                   |
| Age (years)                            | 65.3±8.3 | 65.7±8.4 | 65.7±8.2 | 0.621             |                   |
| Sex (male/female)                      | 395/154 (28) | 1,061/402 (27) | 828/286 (26) | 0.481             |                   |
| Known diabetes duration (years)        | 10.1±7.6 | 10.3±7.7 | 10.2±7.8 | 0.802             |                   |
| Systolic blood pressure (mmHg)         | 145±15 | 145±14 | 144±14 | 0.331             |                   |
| Diastolic blood pressure (mmHg)        | 82±8 | 82±8 | 82±9 | 0.601             |                   |
| BMI (kg/m²)                            | 29.6±4.6 | 29.3±4.6 | 29.3±5.0 | 0.460             |                   |
| Baseline urinary albumin (mg/l)        | 81 (42–227) | 75 (40–175) | 75 (39–183) | 0.281             |                   |
| Smokers (yes/no)†                      | 80/170 (83) | 201/1,054 (94) | 168/789 (82) | 0.481             |                   |
| Serum creatinine (umol/l)              | 88±19 | 90±21 | 89±20 | 0.224             |                   |
| A1C (%)                                | 8.0±1.8 | 7.9±1.7 | 7.8±1.8 | 0.016             |                   |
| Total cholesterol (mmol/l)             | 5.9±1.1 | 5.8±1.1 | 5.8±1.1 | 0.239             |                   |
| LDL cholesterol (mmol/l)               | 3.6±0.9 | 3.5±0.9 | 3.5±0.9 | 0.601             |                   |
| HDL cholesterol (mmol/l)               | 1.3±0.3 | 1.3±0.4 | 1.3±0.4 | 0.940             |                   |
| Total triglycerides (mmol/l)           | 1.9 (1.3–2.8) | 1.8 (1.3–2.7) | 1.8 (1.3–2.6) | 0.564             |                   |
| C-reactive protein (mg/l)              | 2.9 (1.4–6.6) | 3.1 (1.4–6.5) | 3.2 (1.5–7.0) | 0.438             |                   |
| Personal history of myocardial infarction (yes/no) | 39/7 (93) | 85/6/1,378 (94) | 47/4/1,067 (96) | 0.0108             |                   |
| Personal history of stroke (yes/no)    | 17/3/532 (97) | 59/4/1,404 (96) | 45/4/1,069 (96) | 0.4212             |                   |

Data are expressed as means ± SD, *medians (25th–75th percentile), or †n (%). †Missing data for 433 patients.

### Table 2—Incidence of the combined primary end point and of each of its various components, and all-cause death during the DIABHYCAR trial according to the ACE insertion/deletion polymorphism

| Study end point                          | All patients | ACE II | ACE ID | ACE DD | P value (log-rank): II vs. ID vs. DD |
|------------------------------------------|--------------|--------|--------|--------|-----------------------------------|
| n enrolled                               | 3,126        | 549    | 1,463  | 1,114  | 0.489                             |
| Primary combined end point               | 495/3.78 (3.45–4.10) | 97/4.20 (3.38–5.02) | 230/3.76 (3.28–4.23) | 168/3.60 (3.06–4.23) | 0.489                             |
| Cardiovascular death                     | 208/1.52 (1.31–1.72) | 48/1.99 (1.43–2.55) | 84/1.30 (1.02–1.58) | 76/1.56 (1.22–1.91) | 0.060                             |
| Myocardial infarction (fatal and nonfatal) | 95/0.69 (0.55–0.83) | 18/0.74 (0.40–1.09) | 49/0.76 (0.55–0.97) | 28/0.58 (0.36–0.79) | 0.493                             |
| Stroke (fatal and nonfatal)              | 157/1.17 (0.98–1.35) | 32/1.36 (0.89–1.82) | 73/1.15 (0.89–1.42) | 52/1.09 (0.79–1.38) | 0.615                             |
| Stroke (fatal and nonfatal)              | 136/1.00 (0.84–1.17) | 21/0.89 (0.51–1.27) | 67/1.06 (0.80–1.31) | 48/0.99 (0.71–1.26) | 0.769                             |
| Heart failure requiring hospitalization  | 18/0.13 (0.07–0.19) | 7/0.29 (0.07–0.51) | 8/0.12 (0.04–0.21) | 3/0.06 (0.01–0.13) | 0.034                             |
| ESRF                                     | 45/3.13 (3.01–3.61) | 88/3.68 (2.92–4.43) | 191/2.95 (2.53–3.36) | 176/3.61 (3.09–4.14) | 0.071                             |

Data are expressed as n or number of events per 100 patient-years (95% CI). Primary combined end point: time to cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, nonfatal heart failure requiring hospitalization, or ESRF.
ACE genotype: cardiovascular and renal outcome

Table 3—Baseline clinical and biological characteristics according to the ACE insertion/deletion polymorphism in the SURDIAGENE population

| Characteristic                      | ACE II   | ACE ID   | ACE DD   | P value: II vs. ID vs. DD |
|------------------------------------|----------|----------|----------|--------------------------|
| **n**                              | 118      | 448      | 351      |                          |
| **Age (years)**                    | 66.1 ± 10.4 | 65.2 ± 10.6 | 64.9 ± 10.4 | 0.604                    |
| **Sex (male/female)**              | 68 (58/50 (42) | 258 (58/190 (42) | 184 (52)/167 (48) | 0.310                    |
| **Known diabetes duration (years)**| 15.2 ± 9.7 | 15.1 ± 10.1 | 15.4 ± 10.0 | 0.099                    |
| **Systolic blood pressure (mmHg)** | 131 ± 16  | 135 ± 18  | 135 ± 19  | 0.103                    |
| **Diastolic blood pressure (mmHg)**| 72 ± 10   | 73 ± 11   | 73 ± 11   | 0.709                    |
| **BMI (kg/m²)**                    | 31.0 ± 6.8 | 31.0 ± 5.8 | 31.1 ± 6.0 | 0.923                    |
| **Baseline urinary albumin (mg/l)**| 22 (8–95) | 23 (8–129) | 25 (8–135) | 0.841                    |
| **Smokers (yes/no)**               | 10 (8)/107 (92) | 52 (12)/389 (88) | 30 (8)/317 (92) | 0.288                    |
| **Serum creatinine (µmol/l)**      | 81.5 (72–100) | 84.5 (70–104) | 84 (70–102) | 0.488                    |
| **A1C (%)**                        | 7.8 ± 1.5 | 7.9 ± 1.5 | 7.9 ± 1.5 | 0.673                    |
| **Personal history of myocardial infarction (yes/no)** | 11 (9)/107 (91) | 62 (14)/386 (86) | 60 (17)/291 (83) | 0.099                    |
| **Personal history of stroke (yes/no)** | 4 (3)/114 (97) | 21 (5)/427 (95) | 19 (5)/332 (95) | 0.665                    |
| **ESRD†‡**                         | 2 (2)/116 (98) | 6 (1)/442 (99) | 7 (2)/344 (98) | 0.774                    |

Data are expressed as means ± SD, medians (25th–75th percentile), or n (%). *Missing data for 12 patients. †Percent calculated among nephropathic patients.

Of 28 patients with the DD genotype (χ² for trend P = 0.024). D allele frequency was 0.85 in those with fatal myocardial infarctions and 0.52 in those with nonfatal myocardial infarctions (P = 0.005). The deleterious effect of the DD genotype on postmyocardial infarction mortality risk persisted in multivariate regression analysis (adjusted relative risk vs. ID or II genotype 9.62 [95% CI 2.11–43.9]) (supplemental Table 2, available in the online appendix).

**SURDIAGENE cohort**

The baseline characteristics of the 917 participants according to the ACE insertion/deletion polymorphism are presented in Table 3. No association was found between the ACE insertion/deletion polymorphism and diabetic nephropathy or personal history of myocardial infarction.

Table 4—Incidence of the combined primary end point and of each of its various components, and all-cause death according to the ACE insertion/deletion polymorphism in the SURDIAGENE cohort

| Study end point                         | All patients | ACE II | ACE ID | ACE DD | P value (log-rank): II vs. ID vs. DD |
|----------------------------------------|--------------|--------|--------|--------|--------------------------------------|
| **n enrolled**                         | 917          | 118    | 448    | 351    |                                      |
| Primary combined end point             | 1877.28 (6.28–8.29) | 268.32 (5.26–11.39) | 90.17 (5.70–8.53) | 71.18 (5.57–8.78) | 0.769                                |
| Cardiovascular death                   | 73.2 (2.07–3.28) | 15/4.57 (2.31–6.83) | 29.2/14.1 (1.37–2.91) | 29.2/7.7 (1.77–3.77) | 0.057                                |
| Myocardial infarction (fatal and nonfatal) | 31.15 (0.75–1.55) | 5/1.53 (0.19–2.86) | 14/1.04 (0.50–1.59) | 12/1.16 (0.50–1.81) | 0.771                                |
| Stroke (fatal and nonfatal)            | 25/0.92 (0.56–1.28) | 3/0.91 (0.11–1.94) | 11/0.81 (0.33–1.30) | 11/0.6 (0.43–1.68) | 0.842                                |
| Heart failure requiring hospitalization | 70/2.63 (2.02–3.23) | 11/3.45 (1.44–5.45) | 29/2.18 (1.40–2.97) | 30/2.95 (1.91–3.99) | 0.358                                |
| ESRF                                   | 26/0.97 (0.60–1.34) | 2/0.61 (0.03–1.46) | 15/1.13 (0.56–1.70) | 9/0.87 (0.30–1.43) | 0.633                                |
| Death (all-cause)                      | 108/3.93 (3.20–4.65) | 20/6.04 (3.47–8.61) | 45/3.29 (2.34–4.23) | 43/4.08 (2.89–5.28) | 0.125                                |

Data are n and number of events per 100 patient-years (95% CI). Primary combined end point: time to cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, nonfatal heart failure requiring hospitalization, or ESRF.
among the ACE genotypes. No association was found for the incidence of myocardial infarction, stroke, heart failure leading to hospitalization, and ESRF. However, cardiovascular death occurred more frequently in patients with the II genotype compared with patients with the ID or DD genotypes ($P = 0.057$).

Eight of the 31 patients having a myocardial infarction during the follow-up period died: none of the 5 patients with the II genotype, 3 of the 14 patients with the ID genotype, and 5 of the 12 patients with the DD genotype ($\chi^2$ for trend $P = 0.0631$). D allele frequency was 0.81 in those having fatal myocardial infarctions and 0.54 in those having nonfatal myocardial infarctions ($P = 0.057$). The deleterious effect of the DD genotype on postmyocardial infarction mortality risk was not significant (relative risk vs. ID or II genotype 2.16 [95% CI 0.60–7.79]).

The ACE insertion/deletion polymorphism appeared ideal to introduce some genetic components into the calculation of cardiovascular and renal risks, particularly in type 2 diabetes. However, the present results do not support this possibility. Thus, the widespread use of this polymorphism for individualizing cardiovascular and renal prognoses in type 2 diabetes seems ruled out by our findings.

The association between the ACE I allele and previous myocardial infarction in DIABHYCAR participants went against our hypothesis, although it was not replicated in the other two cohorts. For myocardial infarction, conflicting data have been obtained in case-control studies (11,12). However, we must consider the possibility that a selection bias might have been operating in DIABHYCAR, in which the I allele was unexpectedly related to a history of myocardial infarction. This finding did not fit with the so-called mendelian randomization principle. In DIABHYCAR and SURDIAGENE, death after myocardial infarction was related to the D allele. Only small numbers of events were recorded, but ACE D allele frequency was higher in those dying after myocardial infarction (79 vs. 55%); this difference was significant ($P = 0.005$) when the two studies were pooled. This result is consistent with an autopsy study conducted in Belfast: individuals who died of coronary heart disease displayed the DD genotype more frequently than the ID or II genotypes (22). Thus, we can speculate that type 2 diabetic patients with the highest cardiovascular risk, favored by the DD genotype, were not included in the DIABHYCAR trial because of premature death. This may account for the statistically significant but not clinically valuable association between the ACE D allele and myocardial infarction reported in a meta-analysis of several cross-sectional studies (12). However, these are only secondary end points, and this analysis should be considered as exploratory only.

We and others have shown through follow-up and experimental studies (14–16) that the ACE II genotype protects against renal failure in type 1 diabetes. The association of the II genotype with ESRF in DIABHYCAR, contradicting this hypothesis, was not replicated in the SURDIAGENE cohort. Thus, the possibility of a chance finding is likely. As previously suggested for Caucasians (23), our results do not support the theory that renal involvement is related to the ACE insertion/deletion polymorphism in type 2 diabetes, contrary to type 1 diabetes.

In summary, we were not able to identify the ACE insertion/deletion polymorphism as a marker for the cardiovascular and renal prognosis of patients with type 2 diabetes, even if an effect of modest amplitude could have been missed owing to insufficient statistical power. Although of great interest for pathophysiological studies, this genetic variant does not seem ready for use in routine clinical practice.
ACE genotype: cardiovascular and renal outcome

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