ORIGINAL INVESTIGATIONS

Renal Function in Relation to Three Candidate Genes

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● We recently found that femoral intima media thickness, as well as the incidence of hypertension, is influenced by genes encoding the angiotensin-converting enzyme (*ACE*; insertion/deletion [*I/D*]) polymorphism, α -adducin (*Gly460Trp*), and aldosterone synthase ($-344C/T$). By interfering with blood pressure or sodium homeostasis, these genetic polymorphisms also may change renal function. We therefore investigated serum creatinine level, calculated and measured creatinine clearances, and 24-hour urinary protein excretion in subjects previously genotyped for these three polymorphisms. The 1,454 participants drawn at random from the population (64.3% of those invited) were aged 43.4 years and included 744 women (51.2%). Blood pressure, measured by study nurses at subjects' homes, averaged 123/76 mm Hg. Mean values were 90 $\mu\text{mol/L}$ for serum creatinine; 84 and 88 mL/min/1.73 m² for calculated and measured ($n = 855$) creatinine clearances, respectively; and 90 mg/d of protein for proteinuria ($n = 556$). The prevalence of mild renal dysfunction (creatinine clearance ≤ 60 mL/min/1.73 m²) was nearly 11%. In single-gene analyses with adjustment for significant covariables, the risk for mild renal dysfunction was positively associated with the *ACE D* allele. However, multiple-gene analyses showed that these associations were restricted to carriers of the mutated α -adducin *Trp* allele (40.1% of all subjects). Findings remained similar after hypertensive patients and women on hormonal therapy were excluded. In this phenotypically more homogeneous subgroup, serum creatinine level was 3.6 $\mu\text{mol/L}$ ($P = 0.02$) and relative risks for mild renal dysfunction and proteinuria were 1.7-fold ($P < 0.001$) and 26% ($P = 0.02$) greater in *ACE D* subjects than *ACE II* homozygotes, respectively. The aldosterone synthase *T* allele did not strengthen genetic associations with the *ACE D* allele considered alone or in combination with the α -adducin *Trp* allele. Thus, in the present cross-sectional analysis, renal function was slightly but consistently impaired when both the *ACE D* and α -adducin *Trp* alleles were present. These findings, together with experimental studies and our previous reports on femoral intima media thickness and the incidence of hypertension, constitute a growing body of evidence delineating a clinical entity genetically determined by the risk-carrying *ACE D* and α -adducin *Trp* alleles.

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INDEX WORDS: α -Adducin gene; angiotensin-converting enzyme (*ACE*); aldosterone synthase; chronic renal disease; genetic mechanisms; renal function.

IN A CROSS-SECTIONAL analysis of a white population, we recently found that systolic blood pressure and the prevalence of hypertension were significantly elevated among patients who carried both the α -adducin *Trp* allele (*Gly460Trp* polymorphism) and the aldosterone

synthase *T* allele ($-344C/T$ polymorphism).^{1,2} Intima media thickness of the femoral artery in a cross-sectional analysis of the same population³ and the incidence of hypertension over a median follow-up of 9 years^{1,2} were greater among carriers of the deletion (*D*) allele of the angiotensin-

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Received February 26, 2001; accepted in revised form July 6, 2001.

Supported in part by research grants no. G.0174.97 and G.0291.98 from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen, Brussels, Belgium; Bilaterale Wetenschappelijke en Technische Samenwerking Vlaanderen-China (project BIL98/15); special research grant Onderzoek-

stoelage OT99/28 from the Katholieke Universiteit Leuven; and grants no. Br 1589/1-1 (E.B.) and He 2852/1-1 (S.-M.H.) from the Deutsche Forschungsgemeinschaft. Additional families were recruited in the framework of the European Project on Genes in Hypertension, supported in part by the European Union (contract no. IC15-CT98-0329-EPOGH and QLG1-CT-2000-01137-EURNETGEN).

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*© 2001 by the National Kidney Foundation, Inc.
0272-6386/01/3806-0002\$35.00/0
doi:10.1053/ajkd.2001.29205*

converting enzyme (*ACE* insertion [*I/D*] gene, but only in the presence of the α -adducin *Trp* allele.

The hypothesis underlying our previous observations was that epistatic interactions between the three candidate genes might increase blood pressure through stimulation of sodium reabsorption in the kidney and chronic expansion of circulating fluid volume.^{1,2} These mechanisms may not only influence intima media thickness of large muscular arteries,³ but also directly or indirectly lead to changes in renal glomerular or tubular structure and function. Proteins up to a molecular weight of 70,000 d and creatinine, after glomerular filtration, are reabsorbed at the tubular level. We therefore investigated cross-sectional associations between serum creatinine level, measured and calculated creatinine clearances, and proteinuria and the previously mentioned three candidate genes in our population sample.^{1,2}

METHODS

Study Population

The Flemish Study on Environment, Genes and Health Outcomes started in 1985. The protocol was approved by the Ethics Committee of the University of Leuven, Belgium. From August 1985 to November 1990, we recruited a random sample of households living in a geographically defined area of northern Belgium,⁴ which has a stable population with little migration. From June 1996 to January 1999, we enlarged the cohort with additional family members, including children aged at least 10 years. Participants or their parents gave informed consent. The participation rate among all subjects contacted was 64.3%.^{1,2} We included 1,454 subjects who underwent at least one measurement of serum creatinine, creatinine clearance, or 24-hour protein excretion in the present cross-sectional analysis.

Field Work

All subjects were visited repeatedly in their homes. Blood pressure was measured five times consecutively during each of two home visits after a 5-minute rest in the sitting position. Hypertension was diagnosed if the average of the 10 blood pressure readings was at least 140 mm Hg systolic or 90 mm Hg diastolic or subjects were administered antihypertensive medication. In the interval between home visits, usually 2 to 3 weeks, subjects collected a 24-hour urine sample in a wide-neck plastic container for the measurement of creatinine and protein excretion. Field workers explained to participants how to collect the 24-hour urine sample and gave them a leaflet with a summary of the procedure in writing. If urinary volume or creatinine excretion was outside published limits,⁵ results based on the urine sample were discarded. Subjects also completed a questionnaire

inquiring about their medical history, smoking habits, alcohol intake, and use of medications. Study nurses verified the questionnaire at the second home visit. Venous blood was sampled for serum creatinine measurement and genotyping.⁶⁻⁹

We used published formulas to compute body surface area¹⁰ and lean body mass.¹¹ Creatinine clearance was measured in 855 subjects. In addition, creatinine clearance was calculated in all 1,454 participants using the formula of Cockcroft and Gault.¹² Measured and calculated creatinine clearances were standardized to 1.73 m² of body surface area.¹⁰ In keeping with recent studies,¹³ mild renal dysfunction was defined as a calculated or measured creatinine clearance of 60 mL/min/1.73 m² or less.

Determination of Genotypes

Genomic DNA was extracted from peripheral blood. The *ACE I/D* polymorphism was detected as described by Lindpaintner et al.⁶ All samples initially genotyped as *DD* underwent a second polymerase chain reaction (PCR) with insertion-specific primers.^{6,8} Allelic discrimination of the *Gly460Trp* α -adducin polymorphism was performed using a 5' nuclease assay⁹ on an ABI Prism 7700 apparatus (Perkin Elmer, Norwalk, CT). Forward and reverse primers and *460Gly* and *460Trp* probes used in the TaqMan assay (Applied Biosystems, Foster City, CA) were 5'-CGTCCACACCTTAGTCTTCGACTT, 5'-GGAGAA-GACAAGATGGCTGAAGTC, 5'-FAM-TTCCATTCTGC-CCTTCCTCGGA-TAMRA, and 5'-TET-TTCCATTCTGC-CATTCTCGGAA-TAMRA, respectively. Per 25 microliters, PCR fluid contained 50 ng of DNA, 300 nmol of primers, 100 nmol of FAM-probe, and 50 nmol of TET-probe. Amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 minutes, and 62°C for 1 minute for 40 cycles. For determination of $-344C/T$ aldosterone synthase genotypes, PCR and subsequent genotyping were performed as described by Brand et al.⁷

Statistical Methods

We used SAS version 8.1 (SAS Institute, Cary, NC) for database management and statistical analysis. Measurements with a skewed distribution were normalized by logarithmic transformation. Comparisons of means and proportions were performed by means of the standard normal *z* test and Fisher's exact test, respectively. Significant covariables were traced by stepwise linear or logistic regression. We set *P* at 0.05 for independent variables to enter and stay in the model. In addition, potentially important covariables were forced into the models regardless of statistical significance. We used analysis of covariance to compare continuous measurements between genotypes, with adjustments for covariables. The presence of mild renal dysfunction in relation to genotype was studied by multiple logistic regression.

Genotypes were represented by dummy variables using the deviation from mean coding approach,¹⁴ which does not imply a genetic hypothesis. In single-gene analyses in which we tested independent hypotheses, we adjusted α levels and confidence intervals (CIs) for multiple testing using Bonferroni's method.¹⁵ Because family members are more likely to share identical alleles than randomly selected subjects, we

repeated the analyses using generalized estimating equations to account for the possible nonindependence of renal function measurements within families.^{16,17} In the PROC GENMOD procedure, we used normal and logit link functions for continuous and dichotomous variables, respectively. We defined intrafamilial correlation matrices from renal phenotypes in our own data set, with adjustments applied for covariables and confounders. Pedigrees were made for each family. However, because the association analysis, as well as the generalized estimating equation approach, does not imply a hypothesis about the mode of inheritance of the studied phenotypes, we did not account for possible consanguinity or nonpaternity.

RESULTS

Characteristics of Participants

The 1,454 participants included 710 men (48.8%). Age ranged from 10 to 84 years (Table 1). Mean arterial pressure (diastolic pressure plus one third of pulse pressure) was computed from the 10 systolic and diastolic blood pressure readings performed by study nurses in subjects' homes (Table 1). Mean pressure averaged 91.4 ± 11.4 (SD) mm Hg; 338 (23.3%) subjects were hypertensive. Two hundred twenty-two men (31.3%) were current smokers and 234 men (33.0%) reported intake of alcohol. In women, these numbers were 205 (27.6%) and 84 (11.3%),

respectively. One hundred forty-three women (19.2%) used oral contraceptives and 13 women (1.8%) were on hormonal replacement therapy.

Measurements of creatinine clearance and urinary protein excretion were available in 855 (58.8%) and 556 participants (38.2%), respectively. Characteristics of these subgroups were similar to those of the entire study population described in Table 1. Overt proteinuria (protein > 300 mg/d) was present in three men and three women.

Analyses Involving Single Genes

Frequencies of the *ACE* I/D ($P = 0.07$), α -aducin *Gly460Trp* ($P = 0.98$), and aldosterone synthase $-344C/T$ ($P = 0.58$) genotypes did not deviate from Hardy-Weinberg equilibrium. In single-gene analyses, genotype and allele frequencies were not significantly different among subjects with normal renal function and those with mild renal dysfunction based on calculated creatinine clearance (Table 2). If measured instead of calculated ($n = 1,454$) creatinine clearance was used, mild renal dysfunction was present in 90 of 855 subjects (10.5%). In general, findings in this subgroup were not different from those listed in

Table 1. Characteristics of Participants

Characteristic	Men	Women	<i>P</i>
No. of participants	710*	744*	—
Age (y)	43.2 ± 16.1	43.5 ± 16.0	0.76
Lean body mass† (kg)	58.8 ± 4.6	38.5 ± 2.9	<0.001
Body mass index (kg/m ²)	25.5 ± 3.9	25.2 ± 5.2	0.26
Systolic pressure‡ (mm Hg)	125.3 ± 15.1	121.1 ± 17.1	<0.001
Diastolic pressure‡ (mm Hg)	76.9 ± 10.4	74.2 ± 10.4	<0.001
Taking antihypertensives	67 (9.4)	99 (13.3)	0.02
Patients with diabetes	6 (0.9)	15 (2.0)	0.06
Serum creatinine (μ mol/L)	98.1 ± 15.5	83.0 ± 16.3	<0.001
Urinary protein excretion (mg/d)	94.1 (88.6-100.0)	86.9 (82.1-92.0)	0.06
Creatinine clearance (mL/min/1.73 m ²)			
Calculated§			
Mean	85.2 ± 19.4	82.4 ± 20.3	0.007
≤ 60	68 (9.6)	100 (13.4)	0.02
Measured			
Mean	91.6 ± 22.2	85.1 ± 23.3	<0.001
≤ 60	36 (8.9)	54 (12.1)	0.13

NOTE. Values expressed as arithmetic mean $\pm$ SD, geometric mean (95% CI), or no. of subjects (percent).

*Urinary protein excretion and measured creatinine clearance were available in 260 and 407 men and 296 and 448 women, respectively.

†Calculated according to Kvist et al.¹¹

‡Mean of 10 blood pressure readings were performed by study nurses (5 readings at each of two separate home visits).

§Calculated using the formula of Cockcroft and Gault.¹²

Table 2. Genotype and Allele Frequencies in Subjects Without and With Mild Renal Dysfunction

Genes	Genotypes			P*	Alleles		P*
<i>ACE</i> gene	<i>II</i>	<i>ID</i>	<i>DD</i>		<i>I</i>	<i>D</i>	
Normal renal function	313 (24.3)	662 (51.5)	311 (24.2)		1,288 (50.1)	1,284 (49.9)	
Mild renal dysfunction†	28 (16.7)	99 (58.9)	41 (24.4)	0.20	155 (46.1)	181 (53.9)	0.45
α -Adducin gene	<i>GlyGly</i>	<i>GlyTrp</i>	<i>TrpTrp</i>		<i>Gly</i>	<i>Trp</i>	
Normal renal function	771 (60.0)	444 (34.5)	71 (5.5)		1,986 (77.2)	586 (22.8)	
Mild renal dysfunction	90 (53.6)	72 (42.9)	6 (3.6)	0.27	252 (75.0)	84 (25.0)	0.75
Aldosterone synthase gene	<i>CC</i>	<i>CT</i>	<i>TT</i>		<i>C</i>	<i>T</i>	
Normal renal function	262 (20.4)	646 (50.2)	378 (29.4)		1,170 (45.5)	1,402 (54.5)	
Mild renal dysfunction	26 (15.5)	84 (50.0)	58 (34.5)	0.51	136 (40.5)	200 (59.5)	0.25

*Fisher's exact test was used to compare frequencies between subjects with and without mild renal dysfunction.

†Mild renal dysfunction was defined as a calculated¹² creatinine clearance of 60 mL/min/1.73 m² or less.¹³

Table 2. However, among subjects with a measured creatinine clearance of 60 mL/min/1.73 m² or less, the *ACE II* genotype was slightly under-represented, whereas the opposite trend was observed for the *ID* genotype (mild renal dysfunction versus normal renal function, 12.2% (*II*) and 66.7% (*ID*) versus 23.5% (*II*) and 52.8% (*ID*); $P = 0.06$).

In additional single-gene analyses, we adjusted for sex, age, age squared, lean body mass,¹¹ body mass index, mean arterial pressure, smoking, alcohol intake, diabetes mellitus, and the use of antihypertensives and contraceptives, as appropriate.

With adjustment for these covariables, the relative risk for mild renal dysfunction using measured creatinine clearance was slightly but significantly ($P = 0.02$) greater in *ACE ID* heterozygotes than in the entire study population (Table 3). Statistical analyses that, in addition to previously mentioned covariables, accounted for nonindependence of renal phenotypes within families did not alter these results (Table 4).

Analyses Involving Multiple Genes

In an additional step of adjusted analyses, we observed that the association between serum

Table 3. Renal Function in Relation to Single Genes

Genes and Genotypes	Serum Creatinine* (μ mol/L)	Relative Risk for Mild Renal Dysfunction*		Urinary Protein Excretion* (mg/d)
		Estimated†	Measured†	
<i>ACE</i>				
<i>II</i>	89.4 $\pm$ 0.8	0.75 (0.52-1.08)	0.62 (0.35-1.08)	90.4 (82.9-101.9)
<i>ID</i>	90.7 $\pm$ 0.5	1.27 (0.97-1.68)	1.59 (1.06-2.37)‡	89.4 (83.7-95.6)
<i>DD</i>	90.8 $\pm$ 0.8	1.05 (0.75-1.45)	1.02 (0.62-1.67)	90.4 (81.2-100.6)
α -Adducin				
<i>GlyGly</i>	90.1 $\pm$ 0.5	0.98 (0.65-1.46)	1.01 (0.52-1.97)	89.7 (84.1-95.6)
<i>GlyTrp</i>	90.8 $\pm$ 0.7	1.33 (0.88-2.01)	1.39 (0.71-2.74)	91.0 (83.6-99.1)
<i>TrpTrp</i>	91.0 $\pm$ 1.7	0.77 (0.38-1.55)	0.71 (0.21-2.38)	91.3 (74.0-112.8)
Aldosterone synthase				
<i>CC</i>	89.3 $\pm$ 0.9	0.87 (0.60-1.25)	1.05 (0.65-1.69)	91.0 (81.2-102.1)
<i>CT</i>	90.4 $\pm$ 0.5	0.99 (0.75-1.31)	0.75 (0.51-1.10)	92.3 (86.0-99.0)
<i>TT</i>	91.2 $\pm$ 0.7	1.16 (0.86-1.57)	1.28 (0.86-1.91)	86.5 (79.1-94.5)

NOTE. Values expressed as mean $\pm$ SE or relative risk (95% CI).

*The number of subjects included in the analysis is 1,454 for serum creatinine and renal dysfunction based on estimated¹² creatinine clearance, 855 for renal dysfunction based on measured creatinine clearance, and 556 for proteinuria. Significant covariables of each outcome variable were identified by stepwise regression (see Results). Analyses were adjusted for sex, age, age squared, lean body mass,¹¹ body mass index, mean arterial pressure, smoking, alcohol intake, diabetes mellitus, and the use of antihypertensive drugs and oral contraceptives, as appropriate.

†Estimated or measured creatinine clearance of 60 mL/min/1.73 m² or less.¹³

‡ $P = 0.02$ versus the overall risk in the entire study population.

Table 4. Renal Function in Relation to Single Genes in Adjusted Models Using Generalized Estimating Equations

Genes and Genotypes	Serum Creatinine* ($\mu\text{mol/L}$)	Relative Risk for Mild Renal Dysfunction*		Urinary Protein Excretion* (mg/d)
		Estimated†	Measured†	
<i>ACE</i>				
<i>II</i>	89.4 $\pm$ 0.8	0.78 (0.54-1.12)	0.63 (0.36-1.10)	89.7 (80.1-100.5)
<i>ID</i>	90.3 $\pm$ 0.6	1.22 (0.93-1.61)	1.57 (1.06-2.33)‡	89.2 (83.2-95.7)
<i>DD</i>	90.4 $\pm$ 0.8	1.05 (0.75-1.47)	1.01 (0.62-1.65)	92.1 (84.6-100.3)
α -Adducin				
<i>GlyGly</i>	90.0 $\pm$ 0.6	1.00 (0.66-1.52)	1.01 (0.51-2.00)	89.1 (83.3-95.3)
<i>GlyTrp</i>	90.3 $\pm$ 0.7	1.35 (0.86-2.13)	1.39 (0.70-2.78)	91.1 (83.8-98.2)
<i>TrpTrp</i>	90.0 $\pm$ 1.4	0.74 (0.34-1.60)	0.71 (0.20-2.49)	93.2 (76.2-113.8)
Aldosterone synthase				
<i>CC</i>	89.2 $\pm$ 0.8	0.87 (0.60-1.26)	1.03 (0.65-1.64)	89.4 (80.7-99.1)
<i>CT</i>	90.2 $\pm$ 0.6	1.02 (0.78-1.35)	0.76 (0.51-1.12)	92.6 (85.9-100.0)
<i>TT</i>	90.6 $\pm$ 0.8	1.13 (0.83-1.53)	1.28 (0.85-1.92)	86.4 (79.4-94.0)

NOTE. Values expressed as mean $\pm$ SE or relative risk (95% CI).

*Analyses were based on generalized estimation equations. For the number of subjects included in the analysis and the covariables considered, see Table 3.

†Estimated or measured creatinine clearance of 60 mL/min/1.73 m² or less.¹³

‡ $P = 0.02$ versus the overall risk in the entire study population.

creatinine level and *ACE* genotype was confined to carriers of the mutated α -adducin *Trp* allele (Fig 1; Table 5). Against this genetic background, serum creatinine levels were 4.0 $\mu\text{mol/L}$ (95% CI, 1.1 to 7.0; $P = 0.005$) and 4.9 $\mu\text{mol/L}$ (95% CI, 1.5 to 8.3; $P = 0.008$) lower in *ACE II* subjects than *ID* heterozygotes and *DD* subjects, respectively (Fig 1).

In keeping with our findings on serum creati-

nine level, the increased risk for mild renal dysfunction associated with the *ACE D* allele also was restricted to carriers of the α -adducin *Trp* allele (Fig 2; Table 5). Compared with the average risk in the entire population, the adjusted relative risk for renal dysfunction in this subgroup was 0.41 (95% CI, 0.21 to 0.80; $P = 0.009$) in *II* homozygotes and 1.89 (95% CI, 1.37 to 2.62; $P < 0.001$) in *ID* heterozygotes (Fig 2).

Fig 1. Serum creatinine levels by *ACE* genotype in carriers of the α -adducin *Trp* allele, aldosterone synthase *T* allele, or both (*Trp* and *T*). Values expressed as mean $\pm$ SE adjusted for sex, age, lean body mass, mean arterial pressure, diabetes mellitus, and antihypertensive drug treatment. The number of subjects is given for each genotype.

Table 5. Renal Function by *ACE* Genotype in α -Adducin *GlyGly* Homozygotes

Genes and Genotypes	Serum Creatinine ($\mu\text{mol/L}$)*		Relative Risk for Mild Renal Dysfunction* (95% CI)				Urinary Protein Excretion* (mg/d)	
	No.	Mean $\pm$ SE	No.†	Estimated‡	No.†	Measured‡	No.	Mean (95% CI)
Adjusted for confounders								
<i>GlyGly/II</i>	202	90.8 $\pm$ 1.0	202 (21)	1.09 (0.71-1.69)	112 (9)	0.89 (0.45-1.73)	68	99.7 (88.8-112.0)
<i>GlyGly/ID</i>	462	90.1 $\pm$ 0.7	462 (46)	0.98 (0.71-1.36)	292 (29)	1.33 (0.84-2.13)	191	87.5 (81.6-93.8)
<i>GlyGly/DD</i>	197	89.5 $\pm$ 1.1	197 (23)	1.01 (0.66-1.54)	120 (11)	1.01 (0.54-1.88)	77	86.8 (77.8-96.8)
Adjusted models using generalized estimating equations								
<i>GlyGly/II</i>	202	91.0 $\pm$ 1.1	202 (21)	1.12 (0.74-1.69)	112 (9)	0.90 (0.46-1.76)	68	97.5 (86.1-110.3)
<i>GlyGly/ID</i>	462	89.8 $\pm$ 0.8	462 (46)	0.94 (0.68-1.31)	292 (29)	1.31 (0.83-2.06)	191	87.1 (81.0-93.7)
<i>GlyGly/DD</i>	197	89.5 $\pm$ 0.9	197 (23)	1.03 (0.68-1.56)	120 (11)	1.01 (0.54-1.87)	77	87.5 (79.5-96.3)

*Significant covariables of each outcome variable were identified by stepwise regression (see Results). Analyses were adjusted for sex, age, age squared, lean body mass,¹¹ body mass index, mean arterial pressure, smoking, alcohol intake, diabetes mellitus, and use of antihypertensive drugs and oral contraceptives, as appropriate. For adjusted models using generalized estimating equations, nonindependence of renal phenotypes within families also was accounted for.

†No. of subjects with mild renal dysfunction in parentheses.

‡Estimated or measured creatinine clearance of 60 mL/min/1.73 m² or less.¹³

Compared with the *II* genotype, the adjusted relative risk for mild renal dysfunction was 4.62 (95% CI, 2.20 to 9.72; $P < 0.001$) in *ID* heterozygotes and 2.90 (95% CI, 1.29 to 6.53; $P = 0.01$) in *DD* homozygotes. We reproduced these results by using measured instead of calculated creatinine clearance (Fig 3; Table 5).

In adjusted multiple-gene analyses, a significant association between 24-hour proteinuria and

ACE genotype also became apparent in carriers of the mutated α -adducin (Fig 4; Table 5). In this subgroup, proteinuria was 17% (95% CI, -0.5 to 37; $P = 0.06$) and 26% (95% CI, 5 to 52; $P = 0.02$) lower in *ACE II* subjects than *ID* heterozygotes and *DD* homozygotes, respectively (Fig 4).

In all available subjects, as well as carriers of the α -adducin *Trp* allele, the presence of the aldosterone synthase *T* allele did not reinforce

Fig 3. Risk for having a measured creatinine clearance of 60 mL/min/1.73 m² or less by ACE genotype in carriers of the α -adducin Trp allele, aldosterone synthase T allele, or both (Trp and T). For further explanation, see Fig 2.

previously mentioned associations with the ACE genotype (Figs 1 to 4).

Further analyses confirmed that associations between renal phenotypes and the ACE genotype were confined to carriers of the α -adducin Trp allele and not present in GlyGly homozygotes. First, we evaluated the possible effects of confounders and excluded hypertensive patients and women on hormonal therapy. As noticed in the

entire study group, in ID heterozygotes and DD homozygotes, serum creatinine levels were 3.4 μ mol/L (95% CI, 0.2 to 6.6; $P = 0.03$) and 4.1 μ mol/L (95% CI, 0.4 to 7.8; $P = 0.04$) greater than in ACE II subjects, relative risks for mild renal dysfunction were 235% (95% CI, 38 to 715; $P = 0.008$) and 51% (95% CI, -47 to 329; $P = 0.43$) greater than in ACE II subjects, and proteinuria was 20% (95% CI, -1 to 47; $P =$

Fig 4. Urinary protein excretion by ACE genotype in carriers of the α -adducin Trp allele, aldosterone synthase T allele, or both (Trp and T). Values are geometric means adjusted for sex, age, and diabetes mellitus. Vertical lines denote 95% CIs of geometric means. For further explanation, see Fig 1.

0.07) and 44% (95% CI, 11 to 87; $P = 0.007$) increased, respectively. Second, in the entire study group, in addition to previously mentioned covariables, we also allowed for nonindependence of renal phenotypes within families. As observed in the main analysis in *ID* heterozygotes and *DD* homozygotes, serum creatinine levels were 3.7 $\mu\text{mol/L}$ (95% CI, 1.2 to 6.2; $P = 0.004$) and 4.4 $\mu\text{mol/L}$ (95% CI, 1.0 to 7.8; $P = 0.01$) greater than in *ACE II* subjects, relative risks for mild renal dysfunction were 312% (95% CI, 88 to 803; $P < 0.001$) and 168% (95% CI, 17 to 516; $P = 0.02$) greater than in *ACE II* subjects, and proteinuria was 16% (95% CI, 0 to 35; $P = 0.05$) and 24% (95% CI, 6 to 45; $P = 0.008$) increased, respectively.

DISCUSSION

To the best of our knowledge, our study provides the first evidence of a combined effect of two candidate genes on various indices of renal function in a white population. In the presence of the mutated α -adducin *Trp* allele, serum creatinine level, relative risk for mild renal dysfunction, and proteinuria were 4.3 $\mu\text{mol/L}$ ($P = 0.003$), threefold ($P < 0.001$), and 20% ($P = 0.02$) greater in *ACE D* carriers than *ACE II* homozygotes. The aldosterone synthase *T* allele did not strengthen the associations between renal function and the *ACE D* allele considered either alone or in combination with the α -adducin *Trp* allele.

Numerous studies¹⁸⁻²² investigated the possible relationship between the *ACE I/D* polymorphism and development and progression of nephropathy in patients with various diseases. Several reports showed that the *ACE D* allele was associated with a greater risk for diabetic nephropathy¹⁸⁻²² or hypertensive renal complications²³⁻²⁵ or faster progression of renal dysfunction, regardless of the underlying cause.^{26,27} Three quantitative overviews^{18,19,21} found a stronger association between diabetic nephropathy and the *ACE I/D* polymorphism in Asians than whites. In the former race, common odds ratios associated with the *D* allele^{18,19,21} were compatible with a nearly twofold increase in the risk for diabetic nephropathy. Conversely, the common odds ratio in whites with non-insulin-dependent diabetes mellitus was only 1.10 (95% CI, 0.83 to 1.45).²¹ For insulin-dependent white patients, the

pooled odds ratio was not computed because of significant heterogeneity among the individual studies.²¹

The most recent reports on possible effects of the *ACE I/D* polymorphism on the pathogenesis of renal disorders continue to produce conflicting results.²⁰⁻²² Several factors may account for these disparate findings,²² such as selection bias in case-control studies and differences in the genetic or racial background of subjects, diagnostic criteria for renal disease, or environmental factors. Few studies enrolled subjects based on a sample drawn from the general population. However, an alternative explanation for the divergence between the studies²⁰⁻²² is the failure to account for combined effects of several genes on renal function. In the present study, we selected three genetic polymorphisms that may influence renal function through their effects on sodium reabsorption or blood pressure. The presence of the *ACE D* allele leads to greater systemic ACE levels²⁸ and probably also stimulates the generation of angiotensin II,²⁹ which reduces the slope of the renal pressure–natriuresis relationship at low nonpressor doses.³⁰

Second, hypertensive patients carrying the 460*Trp* allele of the gene encoding the cytoskeleton protein α -adducin show enhanced proximal tubular renal reabsorption of sodium compared with those with the wild-type *Gly460* variant³¹ and experience greater blood pressure changes in response to sodium loading or diuretic treatment.^{8,32} These patients also have lower plasma renin activity, which may reflect a chronically expanded circulating blood volume or greater salt sensitivity.⁸

Third, the presence of the *T* allele at the $-344C/T$ locus in the promoter area of the aldosterone synthase gene stimulates aldosterone synthesis independent of regulation by angiotensin II and potassium.^{33,34}

With regard to proximal tubular sodium reabsorption, Barlassina et al⁸ described a synergistic action between the *ACE D* and α -adducin *Trp* alleles. However, further research should elucidate the mechanisms by which the *ACE* and α -adducin genes may interact in the overall determination of renal function. The intrarenal renin-angiotensin system is located not only in proximal tubules, but also in glomeruli and arterioles.³⁵ Increased angiotensin II generation may modu-

late glomerular hemodynamics through vascular functional (vasoconstriction) or structural (thickening of arterial wall) changes.³⁶ Infusion of low doses of angiotensin II directly into the rat kidney resulted in changes in the renal vasculature compatible with dose-related structural remodeling of the preglomerular resistance vessels around a narrowed lumen and a dose-related increase in arterial pressure, which was attenuated if the contralateral kidney was left intact.³⁷ In addition, the relation between blood pressure and glomerular filtration rate was shifted to the right.³⁷ Chronic volume expansion in the presence of the α -adducin *Trp* allele is accompanied by greater glomerular filtration rates and lower fractional sodium reabsorption before hypertension develops.³⁸ Glomerular hyperfiltration at an early stage in life in the long run may promote proteinuria and accelerate the decay of renal function with age.³¹ The presence of the *ACE D* allele through increased local or systemic generation of angiotensin II^{28,29} may mimic the effects recently observed in rat experiments,³⁷ blunt the homeostatic mechanisms activated in response to the mutated α -adducin, and ultimately lead to reduced glomerular filtration and accelerated increase in blood pressure, as observed in our prospective population study.^{1,2}

Our findings were consistent regardless of whether calculated¹² or measured creatinine clearance was used. In carriers of the mutated α -adducin, the relative risk for mild renal dysfunction was greater in *ID* subjects than *DD* homozygotes. These findings may be explained by random variation in creatinine clearance or the complex regulatory and counterregulatory mechanisms if two genes interact and jointly influence a remote phenotype, such as creatinine clearance. One more speculative possibility is that the combined influence on a complex phenotype may involve epistasis,^{31,39} which is the suppression or potentiation of a gene by other nonallelic genes.⁴⁰ Under these circumstances, the phenotype may not be accurately predicted by the summation of single-gene effects, and the genetic interaction may become nonlinear.

The present results must be interpreted within the context of their limitations. We used a single measurement of serum creatinine as an index of renal function. Serum creatinine levels may fluctuate over time and may be influenced by several

factors, such as fluid balance, diet, and concomitant diseases. Conversely, we included all available subjects, with correction for many potential confounders, including diabetes mellitus, use of antihypertensives, and hormonal treatment. This approach may increase the external validity of our findings and reduce selection bias. Our study was undertaken in a white population, and our findings remain to be confirmed in populations of Asian or African extraction.

In conclusion, the present findings, together with experimental studies and our previous reports on the incidence of hypertension^{1,2} and femoral intima media thickness,³ constitute a growing body of evidence delineating a syndrome genetically determined by the risk-carrying *ACE D* and α -adducin *Trp* alleles. When confirmed, our findings may have important clinical implications. Renal dysfunction not only precedes the development of end-stage renal disease, but also predicts total and cardiovascular morbidity and mortality.^{13,41,42} Therefore, identification of the genetic mechanisms underlying the pathogenesis of renal dysfunction or hypertension may lead to a more accurate risk stratification and tailored strategies to prevent these disorders and their complications.

ACKNOWLEDGMENT

The Flemish Study on Environment, Genes, and Health Outcomes would not have been possible without the collaboration of family physicians of the participants. The municipality Hechtel-Eksel (Belgium) provided logistic support. The authors acknowledge the expert assistance of Leszek Bieniaszewski, MD, Rina Bollen, Lut De Pauw RN, Hilde Celis, MD, Primož Dolenc, MD, Paul Drent, RN, Dmitri Emelianov, MD, Jerzy Gasowski, MD, Heng Fan, Lieve Gijssbers, Alida Hermans, Tatyana Kuznetsova, MD, Tim Nawrot, BSc, Lutgarde Thijs, BSc, Yvette Toremans, Sylvia Van Hulle, RN, Inge Van Bael, Wouter Vinck, MD, and Renilde Wolfs (Study Coordinating Centre, Leuven, Belgium); Brigitte Egbers (Berlin, Germany) for determining aldosterone synthase genotypes; and Harry A.J. Struijker-Boudier and Elisabeth J. Balkestein (Universiteit Maastricht, The Netherlands) for helpful comments.

REFERENCES

1. Staessen JA, Wang JG, Brand E, Barlassina C, Bianchi G, Cusi D, Egbers B, Fagard R, Sharma AM, Soubrier F: Association between blood pressure, hypertension and polymorphisms of the aldosterone synthase and α -adducin genes in a Belgian population study. *J Mol Med* 78:B8, 2000 (abstr)
2. Staessen JA, Wang JG, Brand E, Barlassina C, Birkenhäger WH, Herrmann SM, Fagard R, Tizzoni L, Bianchi G:

Effects of three candidate genes on prevalence and incidence of hypertension in a Caucasian population. *J Hypertens* 19:1349-1358, 2001

3. Wang JG, Balkestein EJ, Staessen JA, Barlassina C, Bianchi G, Brand E, Den Hond E, Fagard R, Herrmann SM, Van Bortel LM, Struijker-Boudier HAJ: Singular and epistatic effects of three candidate genes on carotid and femoral intima-media thickness in a Caucasian population. *Am J Hypertens* 14:121A, 2001 (abstr)

4. Staessen JA, Roels HA, Emelianov D, Kuznetsova T, Thijs L, Vangronsveld J, Fagard R, for the Public Health and Environmental Exposure to Cadmium (PheeCad) Study Group: Environmental exposure to cadmium, forearm bone-density, and risk of fractures: Prospective population study. *Lancet* 353:1140-1144, 1999

5. Staessen J, Bulpitt CJ, Fagard R, Joossens JV, Lijnen P, Amery A: Salt intake and blood pressure in the general population: A controlled intervention trial in two towns. *J Hypertens* 6:965-973, 1988

6. Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F, LaMotte F, Buring J, Hennekens CH: A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease. *N Engl J Med* 332:706-711, 1995

7. Brand E, Chatelain N, Mulatero P, Féry I, Curnow K, Jeunemaitre X, Corvol P, Pascoe L, Soubrier F: Structural analysis and evaluation of the aldosterone synthase gene in hypertension. *Hypertension* 32:198-204, 1998

8. Barlassina C, Schork NJ, Manuta P, Citterio L, Sciarone MT, Lanella G, Bianchi G, Cusi D: Synergistic effect of α -adducin and ACE genes causes blood pressure changes with body sodium and volume expansion. *Kidney Int* 57:1083-1090, 2000

9. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K: Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* 4:357-362, 1995

10. Gehan EA, George SL: Estimation of human body surface area from height and weight. *Cancer Chemother Rep* 54:225-235, 1970

11. Kvist H, Chowdhury B, Grangård U, Tylén U, Sjöström L: Total and visceral adipose-tissue volumes derived from measurements with computed tomography in adult men and women: Predictive equations. *Am J Clin Nutr* 48:1351-1361, 1988

12. Cockcroft DW, Gault MH: Prediction of creatinine clearance from serum creatinine. *Nephron* 16:31-41, 1976

13. Ruilope L, Salvetti A, Jamerson K, Hansson L, Warnold I, Wedel H, Zanchetti A: Renal function and intensive lowering of the blood pressure in the hypertensive subjects of the Hypertension Optimal Treatment (HOT) Study. *J Am Soc Nephrol* 12:218-225, 2000

14. Hosmer DW Jr, Lemeshow S: *Applied Logistic Regression*. New York, NY, John Wiley and Sons, 1989, pp 38-81

15. Bland JM, Altman DG: Multiple significance tests: The Bonferroni method. *BMJ* 310:170, 1995

16. Zeger SL, Liang KY, Albert PS: Models for longitudinal data: A generalized estimating equation approach. *Biometrics* 44:1049-1060, 1988

17. SAS Institute Inc: The GENMOD procedure, in *SAS Online Doc version 7.1: SAS/STAT*. Cary, NC, SAS Institute Inc, 2000, pp 1311-1411

18. Staessen JA, Wang JG, Ginocchio G, Petrov V, Saavedra AP, Soubrier F, Vlietinck R, Fagard R: The deletion/insertion polymorphism of the converting-enzyme and cardiovascular-renal risk. *J Hypertens* 15:1579-1592, 1997

19. Fujisawa T, Ikegami H, Kawaguchi Y, Hamada Y, Ueda H, Shintani M, Fukuda M, Ogihara T: Meta-analysis of association of insertion/deletion polymorphism of angiotensin I-converting enzyme gene with diabetic nephropathy and retinopathy. *Diabetologia* 41:47-53, 1998

20. Krolewski AS: Genetics of diabetic nephropathy: Evidence for major and minor gene effects. *Kidney Int* 55:1582-1596, 1999

21. Kunz R, Bork JP, Fritsche L, Ringel J, Sharma AM: Association between the angiotensin-converting enzyme insertion/deletion polymorphism and diabetic nephropathy: A methodologic appraisal and systematic review. *J Am Soc Nephrol* 9:1653-1663, 1998

22. Taal MW: Angiotensin-converting enzyme gene polymorphisms in renal disease: Clinically relevant? *Curr Opin Nephrol Hypertens* 9:651-657, 2000

23. Kario K, Kanai N, Nishiuma S, Fujii T, Saito K, Matsuo T, Matsuo M, Shimada K: Hypertensive nephropathy and the gene for angiotensin-converting enzyme. *Arterioscler Thromb Vasc Biol* 17:252-256, 1997

24. Pontremoli R, Ravera M, Viazzi F, Nicoletta C, Berutti V, Leoncini G, Giacomelli F, Bezante GP, Sacchi G, Ravazzolo R, Deferrari G: Genetic polymorphism of the renin-angiotensin system and organ damage in essential hypertension. *Kidney Int* 57:561-569, 2000

25. Redon J, Chaves FJ, Liao Y, Pascual JM, Rovira E, Armengod EM, Cooper RS: Influence of the I/D polymorphism of the angiotensin-converting enzyme gene on the outcome of microalbuminuria in essential hypertension. *Hypertension* 35:490-495, 2000

26. Harden PN, Geddes C, Rowe PA, McIlroy JH, Boulton-Jones M, Rodger RSC, Junor BJR, Briggs JD, Connell JMC, Jardine AG: Polymorphisms in angiotensin-converting-enzyme gene and progression of IgA nephropathy. *Lancet* 345:1540-1542, 1995

27. van Essen GG, Rensma PL, de Zeeuw D, Sluiter WJ, Scheffer H, Apperloo AJ, de Jong PE: Association between angiotensin-converting-enzyme gene polymorphism and failure of renoprotective therapy. *Lancet* 347:94-95, 1996

28. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F: An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 86:1343-1346, 1990

29. Müller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Ménard J, Luft FC: Vascular angiotensin-converting enzyme expression regulates local angiotensin II. *Hypertension* 29:98-104, 1997

30. Hall JH, Mizelle HL, Woods LL: The renin-angiotensin system and long-term regulation of blood pressure. *J Hypertens* 4:387-397, 1986

31. Bianchi G, Cusi D: Association and linkage analysis of α -adducin polymorphism: Is the glass half full or half empty? *Am J Hypertens* 13:739-743, 2000

32. Cusi D, Barlassina C, Azzani T, Casari G, Citterio L, Devoto M, Glorioso N, Lanzani C, Manunta P, Righetti M, Rivera R, Stella P, Troffa C, Zagato L, Bianchi G: Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension. *Lancet* 349:1353-1357, 1997
33. Curnow KM, Tusie-Luna MT, Pascoe L, Natarajan R, Gu JL, Nadler JL, White PC: The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. *Mol Endocrinol* 5:1513-1522, 1991
34. Clyne CD, Zhang Y, Slutsker L, Mathis JM, White PC, Rainey WE: Angiotensin II and potassium regulate human CYP11B2 transcription through common *cis* elements. *Mol Endocrinol* 11:638-649, 1997
35. Dzau VJ: Tissue renin-angiotensin system in myocardial hypertrophy and failure. *Arch Intern Med* 153:937-942, 1993
36. Teranishi M, Ono H, Ishimitsu T, Matsuoka H: Insertion/deletion angiotensin converting enzyme gene polymorphism affects the microvascular structure of the kidney in patients with nondiabetic renal disease. *J Hypertens* 17:351-356, 1999
37. Stevenson KM, Edgley AJ, Bergström G, Worthy K, Kett MM, Anderson WP: Angiotensin II infused intrarenally causes preglomerular vascular changes and hypertension. *Hypertension* 36:839-844, 2000
38. Manunta P, Cusi D, Barlassina C, Righetti M, Lanzani C, D'Amico M, Buzzi L, Citterio L, Stella P, Rivera R, Bianchi G: α -Adducin polymorphisms and renal sodium handling in essential hypertensive patients. *Kidney Int* 53:1471-1478, 1998
39. Hegele RA: The genetic basis of atherosclerosis. *Int J Clin Lab Res* 27:2-13, 1997
40. Frankel WN, Schork NJ: Who's afraid of epistasis? *Nat Genet* 14:371-373, 1996
41. Culleton BF, Larson MG, Wilson PWF, Evans JC, Parfrey PS, Levy D: Cardiovascular disease and mortality in a community-based cohort with mild renal insufficiency. *Kidney Int* 56:2214-2219, 1999
42. Wang JG, Staessen JA, Fagard RH, Birkenhäger WH, Gong L, Liu L, for the Systolic Hypertension in China (Syst-China) Trial Collaborative Group: Prognostic significance of serum creatinine and uric acid in older Chinese patients with isolated systolic hypertension. *Hypertension* 37:1069-1074, 2001