

# Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



*Learn and Live*<sup>SM</sup>

## **Genotype-Phenotype Relationships for the Renin-Angiotensin-Aldosterone System in a Normal Population**

Françoise Paillard, Dominique Chansel, Eva Brand, Athanase Benetos, Frédérique Thomas, Stanislaw Czekalski, Raymond Ardaillou and Florent Soubrier

*Hypertension* 1999;34:423-429

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 1999 American Heart Association. All rights reserved. Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://hyper.ahajournals.org/cgi/content/full/34/3/423>

Subscriptions: Information about subscribing to Hypertension is online at  
<http://hyper.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:  
[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at  
<http://www.lww.com/reprints>

# Genotype-Phenotype Relationships for the Renin-Angiotensin-Aldosterone System in a Normal Population

Françoise Paillard, Dominique Chansel, Eva Brand, Athanase Benetos, Frédérique Thomas, Stanislaw Czekalski, Raymond Ardailou, Florent Soubrier

**Abstract**—The renin-angiotensin-aldosterone system plays an important role in blood pressure regulation by influencing salt-water homeostasis and vascular tone. The purpose of the present study was to search for associations of single nucleotide polymorphisms on 3 major candidate genes of this system with the plasma concentrations of the corresponding renin-angiotensin-aldosterone system components considered as quantitative phenotypes. Genotyping was performed in 114 normotensive subjects for different variants of the angiotensinogen (*AGT*) gene (C-532T, G-6A, M235T), the angiotensin I-converting enzyme (*ACE*) gene [4656(CT)<sub>2/3</sub>], the aldosterone synthase (*CYP11B2*), and the type 1 angiotensin II receptor (*AT1R*) gene (A1166C) by hybridization with allele-specific oligonucleotides (ASO) or enzymatic digestion of polymerase chain reaction products. Plasma levels of *AGT*, *ACE*, angiotensin II (Ang II), aldosterone, and immunoreactive active renin were measured according to standard techniques. Platelet binding sites for Ang II were analyzed by the binding of radioiodinated Ang II to purified platelets.  $B_{\max}$  and  $K_D$  values of the Ang II binding sites on platelets of each individual were calculated to examine a possible relationship between these parameters and the *AT1R* genotype. A highly significant association of the *ACE* 4656(CT)<sub>2/3</sub> variant with plasma *ACE* levels was observed ( $P < 0.0001$ ). ANOVA showed a significant effect of the *AGT* C-532T polymorphism on *AGT* plasma levels ( $P = 0.017$ ), but no significant effect was detectable with the other *AGT* polymorphisms tested, such as the G-6A or the M235T. A significant effect association was also found between the C-344T polymorphism of the *CYP11B2* gene and plasma aldosterone levels, with the T allele associated with higher levels ( $P = 0.02$ ). No genotype effect of the *AT1R* A1166C polymorphism was detected either on the  $B_{\max}$  or the  $K_D$  value of the Ang II receptors on platelets. (*Hypertension*. 1999;34:423-429.)

**Key Words:** angiotensinogen ■ angiotensin-converting enzyme ■ angiotensin II ■ aldosterone ■ polymorphism ■ blood pressure

Single nucleotide biallelic polymorphisms are powerful tools when used to search for a linkage disequilibrium between a marker genotype and a disease, mainly of multifactorial origin. With these markers, candidate genes of the renin-angiotensin-aldosterone system (RAAS), including those of angiotensinogen (*AGT*), the angiotensin I-converting enzyme (*ACE*), and type I angiotensin (Ang) II receptor (*AT1R*), have been investigated in association studies with cardiovascular diseases, such as hypertension or myocardial infarction. As examples of these associations, the *ACE* insertion/deletion (I/D) polymorphism was found associated with myocardial infarction, cardiac hypertrophy after physical exercise, and Alzheimer disease,<sup>1,2</sup> and the *AGT* or *AT1R* genes were found to be associated with hypertension.<sup>3,4</sup>

Therefore, it is of interest to establish relationships between these marker genotypes and intermediate phenotypes

related to the disease and to the gene tested to understand the physiopathology of these associations, thus helping to clarify inconsistent results issued from some association studies. Among these observations, the strong effect of the *ACE* I/D genotype on plasma *ACE* levels has been reproduced in several studies. Significant associations have also been found between *AGT* markers and plasma *AGT* in 2 independent studies.<sup>3,5</sup>

In this study, we analyzed the relationships between several marker genotypes of the *ACE*, *AGT*, aldosterone synthase (*CYP11B2*), and *AT1R* genes and biochemical parameters related to these genes in a group of normal individuals. We confirmed previously reported effects of *ACE* and *AGT* genes on their gene products in plasma and identified a polymorphism of the 5' region of *AGT* as a candidate functional variant, because it showed the most pronounced

Received April 6, 1999; first decision April 12, 1999; revision accepted April 21, 1999.

From INSERM U489, Hôpital Tenon (F.P., D.C., S.C., R.A.); INSERM U525, Hôpital Saint-Louis (E.B., F.S.); INSERM U337 (A.B.); and Investigation Préventive et Clinique (F.T.), Paris, France.

Correspondence to Florent Soubrier, INSERM U 525, Hôpital Saint-Louis, 1, Av Claude Vellefaux, 75475 Paris cedex 10. E-mail soubrier@inserm.chu-stlouis.fr

© 1999 American Heart Association, Inc.

*Hypertension* is available at <http://www.hypertensionaha.org>

**TABLE 1. Primers Used for Amplification of the Regions Containing the Analyzed Polymorphisms**

Gene	Location	Primer	Product Size, bp	Annealing Temperature, %
AGT	5' region	5'-TTC CAG AAG GCA CTT TTC AC-3'	594	56
	Exon 1	5'-TAG TAC CCA GAA CAA CGG CA-3'		
AGT	Exon 2	5'-GAT GCG CAC AAG GTC CTG TC-3'	354	60
	Intron 2	5'-GCCAGC AGA GAG GTT TGC CT-3'		
ACE	3'UTR	5'-GAG TAC CTT GGA GGG CCT GCT-3'	177	67
	3'UTR	5'-GCT ACA CTC CAG CGT CTG AGG-3'		
AT1R	U6	5'-AGA AGC CTG CAC CAT GTT TTG AG-3'	404	60
	L6	5'-CCT GTT GCT CCT CTA ACG ATT TA-3'		

association with AGT plasma levels. Our results indicate that the AT1R sites on platelets are of limited density and that there is no effect of the genotype on receptor number or affinity. A significant increase of plasma aldosterone (pAldo) levels was associated with the T allele of the C-344T polymorphism of the *CYP11B2* gene.

## Methods

### Subjects

The study was approved by the review committee of Hôpital Saint-Antoine, and all subjects gave informed consent. One hundred fourteen white adults (87 men, 27 women) were selected from the Center for Preventive Medicine and the staff of the laboratory. Subjects with acute or chronic disease, a body mass index  $>28$  kg/m<sup>2</sup>, antihypertensive drug therapy, or an alcohol intake  $>50$  g/24 h were excluded. After 10 minutes of rest in the sitting position, subjects had their blood pressure measured with a sphygmomanometer.

### Biochemical Measurements

Blood was collected into tubes that contained heparin for determination of plasma concentrations of proteins, creatinine, sodium, and potassium. These determinations were performed immediately by the usual techniques adapted to a RAXT Technicon autoanalyzer. Untimed urine specimens were obtained by voiding for measurement of creatinine, sodium, and potassium concentrations with the same techniques as used for blood samples.

### Measurement of Plasma RAAS Parameters

#### Analytical Determinations

Blood was collected into cold tubes that contained potassium EDTA for determinations of the various parameters of the RAAS and were also supplemented with 2% ethanol, 2 mg/mL neomycin, and 25  $\mu$ mol/L 1,10-phenanthroline for Ang II determination. Plasma was stored at  $-20^{\circ}\text{C}$ . pAldo was measured by radioimmunoassay (RIA) according to a previously reported technique.<sup>6</sup> Ang II was measured according to the method of Nussberger et al<sup>7</sup> with an Ang II monoclonal antibody, which was obtained in the laboratory, that did not cross-react with Ang I.<sup>8</sup> Active renin was measured with a commercial kit (Sanofi Pasteur). This technique is an RIA adapted from a previously published report.<sup>9</sup> ACE was determined with a commercial kit (Sigma Diagnostics) on the basis of the hydrolysis of FAPGG, a synthetic substrate. Change in optical density was recorded at 340 nm according to the technique of Holmquist et al.<sup>10</sup> pAGT was measured by RIA of Ang I after the incubation of plasma in the presence of an excess of human recombinant renin (Roche). The assay of Ang I was performed according to the technique of Ménard and Catt.<sup>11</sup>

### Preparation of Platelets

Forty to 50 mL of blood was drawn from a forearm vein. The first milliliter of blood was discarded, the rest was transferred into a glass tube in ice, and sodium citrate was added as an anticoagulant. Platelets were separated according to Mann et al<sup>12</sup> with minor modifications. Platelet-rich plasma was obtained by centrifugation at 100g for 15 minutes at room temperature. It was then washed with 20 volumes of cold Tris-buffered saline (Tris-HCl 50 mmol/L, NaCl 154 mmol/L, Na<sub>2</sub>EDTA 5 mmol/L, pH 7.35) that contained 0.05% BSA and centrifuged at 1000g for 10 minutes at room temperature. Washings and centrifugations were repeated once more, and the platelets were then resuspended in medium 199 buffer (Gibco Life Technologies) that contained 5 mmol/L EDTA, 0.2% BSA, and 1 mg/mL bacitracin (pH 7.4 at 22°C) called an assay buffer. Platelet number in the final suspension was determined automatically in a Coulter counter.

### Binding Studies

Incubations were performed in a total volume of 100  $\mu$ L that included 80  $\mu$ L of platelet suspension. Saturation equilibrium binding studies were performed in the presence of increasing concentrations of <sup>125</sup>I-labeled Ang II (0.037 to 0.450 nmol/L). Nonspecific binding was ascertained in the presence of 1  $\mu$ mol/L of unlabeled Ang II. Incubation was performed for 120 minutes at 37°C in a shaking water bath. At the end of the incubation period, 2 mL of ice-cold assay buffer was added to each tube. The tubes were centrifuged at 2000g for 5 minutes at 4°C. The supernatants were discarded, and the pellets were resuspended again in 2 mL of ice-cold assay buffer. This procedure was repeated twice. Radioactivity of the final pellet was measured in a LKB-Wallac gamma counter with 60% efficiency. The results of the saturation binding experiments were analyzed by Scatchard transformation of the data with the "Ligand" program<sup>13</sup> to calculate the apparent  $K_D$  and the number of sites ( $B_{max}$ ).

### DNA Extraction

Genomic DNAs from subjects were extracted by standard techniques.<sup>14</sup>

### Genotyping for ACE, AGT, AT1R, and CYP11B2 Polymorphisms

For the A1166C polymorphism of *AT1R*,<sup>4</sup> polymerase chain reaction (PCR) products were digested overnight by the addition of 5 U of *DdeI* restriction enzyme. In the presence of the 1166C allele, the PCR product (404 bp) was cut into 2 fragments of 118 bp and 286 bp in length and visualized on ethidium bromide-stained 1.5% agarose gels.

Genotyping of the different variants of *AGT* (C-532T, G-6A, M235T)<sup>3</sup> and *ACE* [4656(CT)<sub>2,3</sub>],<sup>15</sup> was performed by hybridization with an allele-specific oligonucleotide.<sup>16</sup> DNA fragments that contained the polymorphisms were amplified by PCR with forward and reverse primers as indicated in Table 1. The PCR products were

**TABLE 2. Oligonucleotides Used for Detection of Polymorphisms by Specific Oligonucleotide Hybridization**

Gene	Location	Primer 1, Wild Type (Tm)	Primer 2, Mutation (Tm)
AGT	-532	5'-TGT GTT TTC CCC AGT GT-3' (50°C)	5'-ACA CTG GGA AAA ACA CA-3' (48°C)
AGT	-6	5'-ACC CGG CCA GGG GAA GA-3' (58°C)	5'-TCT TCC CCC GGC CGG GT-3' (60°C)
AGT	+704 (M235T)	5'-GCT CCC TGA TGG GAG CC-3' (58°C)	5'-GCT CCC TGA CGG GAG CC-3' (60°C)
ACE	+4656 (CT) <sub>2/3</sub>	5'-CAT GAC TCT GCT CG-3' (44°C)	5'-ATG ACT CTC TGC TC-3' (42°C)

denatured in 150  $\mu$ L of 0.5 mol/L NaOH and 1.5 mol/L NaCl and spotted onto nylon membranes (N+, ICN). Membranes were neutralized in 2 $\times$  SSC and cross-linked with UV light (DNA Transilluminator, Fotodyne). Each membrane was hybridized in 7% polyethylene glycol/10% sodium dodecyl sulfate at specific temperatures (Tm-5°C) for 4 hours, with 100 pmol of either of the 2 oligonucleotides (Table 2) that were end-labeled with [ $\gamma$ -<sup>32</sup>P]dATP with T4 polynucleotide kinase. The membranes were washed twice at room temperature in 1 $\times$  SSC for 5 minutes and then incubated for 5 minutes in 0.5 $\times$  SSC at specific temperatures (Tm-3°C) before autoradiography. Genotype determination of the C-344T polymorphism of *CYP11B2* was performed by allele-specific oligonucleotide hybridization as described previously.<sup>17</sup>

### Statistical Analysis

Results are expressed as mean $\pm$ SD.

#### Analysis of Variance

The association of the different polymorphisms with their corresponding gene product plasma levels or blood pressure levels was tested by 1-way ANOVA. Blood pressure values were adjusted for age and gender. Effects of the combination of the G-6A and C-532T polymorphisms of *AGT* were tested by 2-way ANOVA. Values are considered significantly different at  $P < 0.05$ . Biochemical values were log-transformed before analysis.

#### Multiple Regression Analysis

Multiple regression analyses were performed with the aim of evaluating the effects of the different polymorphisms on pACE, pAGT, pAldo, and on the  $K_D$  and  $B_{max}$  of platelet AT1R sites after adjustment for covariates [gender, systolic blood pressure (SBP), urinary Na<sup>+</sup> excretion].

## Results

### Anthropometric Variables and Biochemical Measurements

The main anthropometric characteristics and biochemical measurements are shown in Table 3. The mean age of the subjects was 49.5 $\pm$ 5.4 years. The number of subjects available for each measurement is given because in some cases, measurements failed for technical reasons. Significant positive correlations were found between plasma immunoreactive active renin (pIr-AR) and plasma Ang II (pAng II;  $r=0.69$ ,  $P < 0.0001$ ), pIr-AR and pAldo ( $r=0.31$ ,  $P < 0.0008$ ), and pAng II and pAldo ( $r=0.23$ ,  $P < 0.02$ ).

### Platelet Binding Sites for Ang II

Competitive displacement experiments with unlabeled Ang II, [Sar<sup>1</sup>, Ala<sup>8</sup>]-Ang II, and nonpeptide AT<sub>1</sub> (losartan) and AT<sub>2</sub> (PD123177) receptor antagonists demonstrated that only AT<sub>1</sub> receptors were present at the platelet surface (data not shown). Saturation binding studies allowed the binding parameters to be characterized. Scatchard transformation was performed with the data from each saturation binding curve to calculate the number of sites ( $B_{max}$ ) and the apparent  $K_D$ . The

corresponding values (mean $\pm$ SEM) were 5.3 $\pm$ 0.54 fmol/ $8 \times 10^8$  platelets and 0.47 $\pm$ 0.04 nmol/L. This  $K_D$  value is similar to that observed for AT<sub>1</sub> receptors in another study on platelets.<sup>18</sup> The receptor number corresponds to 4 to 5 sites per platelet. This limited density is also in accordance with previously published studies.<sup>12,19</sup> A significant positive correlation was found between the  $K_D$  and the  $B_{max}$  values ( $r=0.35$ ;  $P=0.009$ ) and confirmed the general finding that the affinity of a ligand for its receptor is negatively correlated with the receptor density. No correlation was found to be significant with the other biochemical parameters.

### Association Between Gene Polymorphisms and Their Respective Intermediate Phenotypes

For all polymorphisms determined, there was no significant deviation from Hardy-Weinberg equilibrium in this group of subjects. Biochemical measurements of the RAAS (pIr-AR, pAGT, pACE, pAng II, pAldo) were analyzed according to the genotypes of the subjects by ANOVA and in multivariate analyses (Table 4).

### ACE Genotype

A marked difference in the pACE level was found between genotype groups of the ACE gene, with the 4656(CT)<sub>2/3</sub>

**TABLE 3. Clinical Parameters of the Population of the Tenon Study**

Variable	Subjects (M/F)	Mean	SD
Age, y	114 (87/27)	49.4	5.04
Weight, kg	114	74.8	13.6
Height, cm	114	171.6	8.6
SBP, mm Hg	114	127.4	14.8
DBP, mm Hg	114	79.7	11.3
U <sub>Na</sub> , mmol/L	112	119.4	47.6
U <sub>K</sub> , mmol/L	112	88.0	40.4
U <sub>creat</sub> , mmol/L	112	15.1	6.2
ACE, U/L	112	29.9	21.4
pAng II, pg/mL	95	7.6	6.5
pIr-AR, pg/mL	114	10.2	6.00
$B_{max}$ , fmol/ $8 \times 10^8$ platelets	86	5.5	6.4
$K_D$ , pmol/L	86	421.9	340.1
U <sub>Na</sub> /U <sub>creat</sub>	112	8.9	4.3
U <sub>K</sub> /U <sub>creat</sub>	112	6.5	3.5
pAldo, pg/mL	114	170.4	99.8
pAGT, ng $\cdot$ mL <sup>-1</sup> $\cdot$ h <sup>-1</sup>	114	1675.9	358.3

U<sub>Na</sub> indicates urinary sodium excretion; U<sub>K</sub>, urinary potassium excretion; and U<sub>creat</sub>, urinary creatinine secretion.

**TABLE 4. Genotype-Phenotype Relationships for the ACE and AGT Genotypes**

ACE 4656 (C/T) <sub>2/3</sub>	2/2	2/3	3/3	
N=84	52.3±23.2 (18)	29.6±15.0 (47)	11.8±12.7 (19)	pACE (U/L) $P<10^{-8}$
AGT C-532T	CC	CT+TT		
N=114	1635.7±335.5 (95)	1850.6±423.5 (20)		pAGT (ng · mL <sup>-1</sup> · h <sup>-1</sup> ) $P=.015$
	31.9±22.3 (91)	21.0±13.6 (21)		pACE (U/L) $P<0.04$
AGT G-6A	GG	GA	AA	
N=114	1627.3±345 (35)	1656.9±367.0 (61)	1822.8±352.0 (18)	pAGT (ng · mL <sup>-1</sup> · h <sup>-1</sup> ) $P=0.15$
AGT M235T	MM	MT	TT	
N=114	1626.7±353.0 (35)	1669.9±357.1 (62)	1799.3±366.0 (17)	pAGT (ng · mL <sup>-1</sup> · h <sup>-1</sup> ) $P=0.26$
CYP11B2 C-344T	CC	CT	TT	
N=114	130±62.5 (27)	194.5±108.0 (53)	164.5±100.1 (34)	pAldo (pg/mL) $P=0.02$

Values are mean±SD. Intergenotype comparisons performed by ANOVA. Biological parameters (units) are indicated in the right column with the  $P$  value of the tests.

Number of subjects in each genotype class in parentheses. N=total number of subjects genotyped.

repeat of the 3' region of the *ACE* gene. There was a nearly 5-fold lower level of mean plasma ACE in subjects homozygous for the 4656(CT)<sub>3/3</sub> versus subjects homozygous for the (CT)<sub>2/2</sub> genotype. No other relationships were found for this genotype, in particular with pAng II and pAldo.

#### AGT Genotype

Three different polymorphisms of the *AGT* gene were tested for their effect on pAGT levels. Higher levels of pAGT were found with the less frequent alleles of the M235T and of the G-6A polymorphisms (235T and -6A alleles, respectively), but none of these variations reached statistical significance. Only the C-532T polymorphism was associated with a significant difference in pAGT; higher levels of pAGT were associated with the less frequent allele (C-532T allele) of the polymorphism. By multiple regression analysis that included SBP, age, and all the polymorphisms tested, C-532T was the single polymorphism that significantly explained the AGT variance ( $P<0.02$ ;  $n=87$ ).

An analysis of the effect of the combination of the G-6A and C-532T polymorphisms on pAGT was also performed. These 2 polymorphisms are in complete linkage disequilibrium, as previously observed<sup>20</sup>; the T allele of the C-532T polymorphism is always associated with the A allele of the

G-6A polymorphism. As shown on Table 5, there was no significant difference in pAGT between the 3 genotype classes for the G-6A polymorphism among the CC subjects for the C-532T polymorphism. Inversely, pAGT was significantly increased in subjects with the CT or TT genotype versus those with the CC genotype of the C-532T polymorphism, in the 2 genotype classes of the G-6A polymorphism (GA or AA) in which these genotypes can be observed, as a consequence of the complete linkage disequilibrium.

There was also a weakly significant effect of the C-532T polymorphism on the pACE level, and lower plasma values were observed with the T allele (Table 4). There was no effect of the genotype on pAng II or pAldo levels.

#### A1166C Genotype of the AT1R Gene

No relationship was found between this polymorphism and any of the biochemical measurements performed. In particular, the AT1R binding sites ( $K_D$  and  $B_{max}$ ) were not affected by this genotype.

#### C-344T Polymorphism of the CYP11B2 Gene

pAldo levels were significantly different in the 3 genotype groups ( $P=0.02$ ). Heterozygous subjects exhibited the highest values, and TT homozygotes exhibited intermediate levels.

#### Association Between Gene Polymorphisms and Blood Pressure

No significant association between the polymorphisms of ACE, AGT, and CYP11B2 was found with either SBP or diastolic (DBP), age-, and gender-adjusted blood pressure. For the A1166C polymorphism of *AT1R*, a borderline association was found with DBP but not with SBP when subjects with the AC or CC genotype were compared with those with the AA genotype (79.8 versus 75.7 mm Hg;  $P=0.06$ ).

#### Discussion

The aim of this study was to assess the effect of polymorphisms of the RAAS genes on biochemical components

**TABLE 5. Plasma AGT Values According to the AGT C-532T and G-6A Genotype Combinations**

	CC-532	C-532T and -532TT
-6GG	1627.3 (345.6)	$n=0$
G-6A	1636.5 (352.3)	1732.3 (423.8)
-6AA	1656.9 (248.8)	2154.5 (295.1)

pAGT, mean (±SD) in ng · mL<sup>-1</sup> · h<sup>-1</sup>.

Significance of the effect of the G-6A polymorphism,  $P=0.14$ ; significance of the effect of the C-532T polymorphism,  $P=0.03$ .

Each line corresponds to a genotype for the G-6A polymorphism, and each column for the C-532T polymorphism (heterozygotes and homozygotes for the T allele are pooled).

directly related to these genes, such as pACE, pAldo, and pAGT, but also including other parameters that have not been studied previously in relation to these genotypes, such as platelet Ang II receptors and pAng II levels. This study was performed in a series of untreated subjects without primary selection for hypertension, in whom sodium excretion was assessed, and who did not receive any treatment that could alter the biochemical variables studied.

### Association of *AGT* Polymorphisms With Plasma AGT Levels

Different studies showed that the 235T allele of the M235T polymorphism was associated with higher pAGT levels versus noncarriers.<sup>3,5</sup> However, these results were found in a single series of hypertensive subjects and in children. Thus, our study is the first to be performed on untreated normotensive adults with 3 biallelic polymorphisms of the *AGT* gene, from the 5' region (C-532T and G-6A) and from exon 2 (M235T) of the gene. Although trends were observed for higher levels of pAGT in subjects with the 235T allele and with the -6A allele, these associations were not significant.

Only the C-532T polymorphism was significantly associated with pAGT concentration, both by 1-way ANOVA and by multivariate analysis. The M235T polymorphism is not considered to be functional, but the G-6A polymorphism was considered to be functional, at least in an *in vitro* assay with the *AGT* promoter fused to a reporter gene.<sup>21</sup> In another study of 130 normal nuclear families, the C-532T polymorphism was the one best supported by statistics (ANOVA and combined segregation-linkage analysis) as the functional variant among all tested polymorphisms of the *AGT* gene (E.B., N. Chatelain, F.P., L. Tiret, S. Visvikis, M. Lathrop, F.S., F. Demeuais, unpublished data, 1999).

When the effect of the C-532T on plasma AGT is analyzed in combination with the G-6A polymorphism, there is no detectable effect of the G-6A polymorphism on plasma AGT across the subjects according to this genotype in CC subjects. An effect on plasma AGT is detectable only according to the C-532T genotype, although the complete linkage disequilibrium between the 2 polymorphisms does not allow this effect to be fully analyzed. No functional study has yet been performed on the C-532T polymorphism. Because the C-532T polymorphism is located within a consensus sequence to the transcription factor AP-2, it might modulate *AGT* gene transcription.

We searched for an influence of the *AGT* genotypes on pAng II and pAldo levels through their effect on the AGT level, because we detected positive and significant correlations between pIr-AR and pAng II, pIr-AR and pAldo, as well as pAng II and pAldo. In this study, we were unable to detect an effect of the *AGT* genotype either on pAng II or on the pAldo concentration. This can be due to variations of these 2 parameters, with a variety of environmental factors that are difficult to standardize in a clinical study in humans. Alternatively, the differences in the pAGT levels may be too weak to produce a detectable effect or are counterbalanced by physiological retrocontrols. We observed a weakly significant ( $P=0.046$ ) association between the *AGT* C-532T polymorphism and pACE level, with an apparently codominant effect

and lower ACE values in subjects with the T allele of the C-532T polymorphism, which is associated with the highest levels of pAGT. If not spurious, this association might be due to a negative feedback of Ang II on ACE, as suggested in a previous study,<sup>22</sup> although no effect in pAng II could be detected in our study.

### *AT1R* Polymorphism

Because a polymorphism located in the 3' untranslated region of this gene (A1166C) was shown to be associated with hypertension or increased pulse wave velocity, we searched for an association of this polymorphism with different parameters of the RAAS.<sup>23</sup> The biological phenotypes that most directly related to the *AT1R* gene, and the most accessible, are the parameters of the Ang II binding sites that belong to the *AT1R* subtype and present on circulating cells. We confirmed that platelet Ang II binding sites are of the type 1 subclass by ligand binding specificity. The  $K_D$  value of Ang II binding in platelets was similar to that found in renal and vascular preparations,<sup>24</sup> whereas the  $B_{max}$  value corresponded to a much lower number of receptor sites, which was in agreement with previously published reports.<sup>12,18,19</sup> Our data are consistent with those already reported by Duggan et al.<sup>25</sup> We did not detect any significant association between the genotypes for the A1166C polymorphism and the parameters of *AT1R* binding sites. Because of their low expression, one can hypothesize that the *AT1R* in platelets are not or are weakly submitted to transcriptional regulatory mechanisms that act in the other target tissues of Ang II. Our results indicate that this measurement cannot be used as an intermediate phenotype for the *AT1R* gene. No other biological variable was significantly associated with the *AT1R* polymorphism.

The *AT1R* A1166C polymorphism is the single polymorphism for which we found a nonsignificant but borderline association with blood pressure values. The subjects bearing the C allele have a slightly higher DBP than those who do not bear this allele ( $P=0.06$ ). This result is consistent with the higher prevalence of this allele that we previously observed in hypertensive subjects.<sup>4</sup>

### Association of *ACE* Polymorphisms With Plasma ACE Levels

The 4656(CT)<sub>2/3</sub> polymorphism in the 3'-untranslated region of the *ACE* gene consists of a repetition of 2 or 3 CT dinucleotides (CT)<sub>2/3</sub> and is in complete linkage disequilibrium with the *ACE* I/D variant.<sup>15</sup> We observed a highly significant association of the *ACE* 4656(CT)<sub>2/3</sub> variant with pACE levels ( $P<0.0001$ ). No effect on other variables was detected; in particular, no effect on pAGT, pAng II, or pAldo.

### Association of *CYP11B2* Polymorphism With pAldo Levels

The association we found between *CYP11B2* polymorphisms and pAldo levels corroborate those of 2 independent studies that showed a similar relationship. In a series of 92 normal men, aged 30 to 55 years, Hautanen et al<sup>26</sup> found a higher rate of urinary Aldo secretion in TT carriers than in CC carriers

( $P=0.016$ ) of the C-344T polymorphism. Similarly, in a series of 486 normal subjects, Davies et al<sup>27</sup> found a higher excretion of tetrahydroaldosterone in T allele-bearing subjects than in those not bearing the T allele ( $P=0.024$ ). As in our study for pAldo, the urinary levels of tetrahydroaldosterone were higher in heterozygotes bearing the T allele. Contrasting results were reported by Pojoga et al,<sup>28</sup> who found a linear relationship between pAldo and the C-344T allele in a series of hypertensive subjects, but in this case, higher levels were found in C-bearing subjects.

In view of physiopathological hypotheses that proposed an inappropriate secretion of aldosterone in hypertension, the present results must be examined in the context of other results that associate the *CYP11B2* polymorphism with hypertension in other case-control studies. Indeed, the T allele of the -344 genotype that we found associated with higher pAldo levels was also found significantly more frequently ( $P=0.010$ ) in hypertensive patients (0.561) than in normotensive controls (0.488) by Brand et al.<sup>17</sup> A similar frequency of the T allele in white hypertensives (0.56) was also found in the study by Pojoga et al.<sup>28</sup> Davies et al also found a significant increase of the T allele in essential hypertensives from Scotland ( $P=0.009$ ).<sup>27</sup> Thus, it could be proposed that the T allele is more frequent in hypertensives and is associated with a higher activity of the *CYP11B2* gene, which leads to higher aldosterone secretion at a given level of sodium intake. This would necessitate higher blood pressure to allow for the sodium excretion rate required to maintain sodium homeostasis. This hypothesis needs to be supported by further studies specifically designed for this purpose.

The potential functional role of the C-344T polymorphism was analyzed in few studies. By gel shift assay, White and Slutsker<sup>29</sup> found that the -344C allele of the *CYP11B2* promoter binds the steroidogenic transcription factor 1 (SF-1) 4 times more than the -344T allele on a molar basis. In contrast, Clyne et al<sup>30</sup> reported that the -344 surrounding sequence binds SF-1 but without functional consequences. Two different elements at positions --71/-64 and --129/-114 that consist of SF-1 and COUP-1 binding sites, respectively, are required for both basal and Ang II- or  $K^+$ -stimulated *CYP11B2* transcription. Because of its reduced affinity to SF-1, the T allele might bind a smaller amount of the SF-1 factor, thus allowing more transcription factor to be bound on the functional site of the promoter.

## Conclusions

This study shows that the C-532T polymorphism of the 5' region of the *AGT* gene is most significantly associated with pAGT levels and that this effect can be detected in a relatively small sample of subjects ( $n=87$ ). This result suggests that this polymorphism should be used preferentially in association studies with the *AGT* gene. We also detected a significant increase of pAldo levels associated with the T allele of the C-344T polymorphism of *CYP11B2*.

Although these results, together with those from the literature, suggest that the polymorphisms studied might be implicated in the predisposition to hypertension, we were unable to detect any significant relationship between the

polymorphisms and blood pressure in our group of normal subjects.

We have been unable to detect relationships between the *AT1R* polymorphism and any biochemical parameters, including the number of Ang II binding sites of the  $AT_1$  type on platelets. It is clear that the tissue concentration of the different components and the kinetics of the system in the tissue microenvironment are not accessible in clinical studies. Therefore, pharmacological responses or physiological responses that reflect these kinetics should be also studied in relation with polymorphisms, in addition to biochemical parameters, to elucidate the associations found between marker genotypes and diseases.

## Acknowledgments

This work was supported by a clinical research contract from the Délégation la Recherche Clinique of the Assistance Publique-Hôpitaux de Paris. E.B. was supported by research grants from the Deutsche Forschungsgemeinschaft (DFG, Br 1589/1-1) and the Société Française d'Hypertension Artérielle. We thank Dr Laurence Tiret, Viviane Nicaud from INSERM U525, Jean-François Morcet from IPC for statistical analysis, and Dr Alain Kanfer (Hôpital Tenon) and the staff from Investigation Préventive et Clinique for their help in the collection of the subjects.

## References

- Villard E, Soubrier F. Molecular biology and genetics of the angiotensin I-converting enzyme: potential implications in cardiovascular diseases. *Cardiovasc Res*. 1996;32:999-1007.
- Kehoe PG, Russ C, McIlroy S, Williams H, Holmans P, Holmes C, Liolitsa D, Vahidassr D, Powell J, McGleenon B, Liddell M, Plomin R, Dynan K, Williams N, Neal J, Cairns NJ, Wilcock G, Passmore P, Lovestone S, Williams J, Owen MJ. Variation in DCP1, encoding ACE, is associated with susceptibility to Alzheimer disease. *Nat Genet*. 1999; 21:71-72.
- Jeunemaitre X, Soubrier F, Kotelevtsev Y, Lifton R, Williams CS, Charrou A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, Corvol P. Molecular basis of human hypertension: role of angiotensinogen. *Cell*. 1992;71: 169-180.
- Bonnardeaux A, Davies E, Jeunemaitre X, Fery I, Charrou A, Clauser E, Tiret L, Cambien F, Corvol P, Soubrier F. Angiotensin II type I receptor gene polymorphisms in human essential hypertension. *Hypertension*. 1994;24:63-69.
- Bloem LJ, Foroud TM, Ambrosius WT, Hanna MP, Tewksbury DA, Pratt JH. Association of the angiotensinogen gene to serum angiotensinogen in blacks and whites. *Hypertension*. 1997;29:1078-1082.
- Pham-Huu-Trung MT, Corvol P. A direct determination of plasma aldosterone. *Steroids*. 1974;24:587-608.
- Nussberger J, Brunner DB, Waeber B, Brunner HR. True versus immunoreactive angiotensin II in human plasma. *Hypertension*. 1985;7(suppl 1):I-1-I-7.
- Budisavljevic M, Geniteau-Legendre M, Baudouin B, Pontillon F, Verroust PJ, Ronco PM. Angiotensin II (AII)-related idiotypic network, II: heterogeneity and fine specificity of AII internal images analyzed with monoclonal antibodies. *J Immunol*. 1988;140:3059-3065.
- Simon D, Hartmann DJ, Badouaille G, Caillet G, Guyenne TT, Corvol P, Pau B, Marchand J. Two-site direct immunoassay specific for active renin. *Clin Chem*. 1992;38:1959-1962.
- Holmquist B, Bunning P, Riordan JF. A continuous spectrophotometric assay for angiotensin converting enzyme. *Anal Biochem*. 1979;95: 540-548.
- Ménard J, Catt KJ. Measurement of renin activity, concentration and substrate in rat plasma by radioimmunoassay of angiotensin II. *Endocrinology*. 1972;90:422-430.
- Mann JF, Sis J, Ritz E. <sup>125</sup>I-Angiotensin II binding to human blood cells. *J Hypertens*. 1985;3:131-137.
- Munson PJ, Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem*. 1980;107: 220-239.

14. Marcadet A, O'Connell P, Cohen D. Standardized Southern blot workshop technique. *Histocompatibility Testing*. 1986;1:553-1560.
15. Villard E, Tirt L, Visvikis S, Rakotovo R, Cambien F, Soubrier F. Identification of new polymorphisms of the angiotensin I-converting enzyme (ACE) gene, and study of their relationship to plasma ACE levels by two-QTL segregation-linkage analysis. *Am J Hum Genet*. 1996;58:1268-1278.
16. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified  $\beta$ -globin and HLA-DQ  $\alpha$  DNA with allele-specific oligonucleotide probes. *Nature*. 1986;324:163-166.
17. Brand E, Chatelain N, Mulatero P, Fery I, Curnow K, Jeunemaitre X, Corvol P, Pascoe L, Soubrier F. Structural analysis and evaluation of the aldosterone synthase gene in human hypertension. *Hypertension*. 1998;32:198-204.
18. Moore TJ, Taylor T, Williams GH. Human platelet angiotensin II receptors: regulation by the circulating angiotensin level. *J Clin Endocrinol Metab*. 1984;58:778-782.
19. Makariou M, Pawlak M, Campbell LV, Timmermans V, Duggan K, Charlesworth JA, Macdonald GJ. The platelet angiotensin II receptor in type I diabetes: studies in patients with and without nephropathy. *Eur J Clin Invest*. 1993;23:517-521.
20. Jeunemaitre X, Inoue I, Williams C, Charru A, Tichet J, Powers M, Sharma AM, Gimenez-Roqueplo AP, Hata A, Corvol P, Lalouel JM. Haplotypes of angiotensinogen in essential hypertension. *Am J Hum Genet*. 1997;60:1448-1460.
21. Inoue I, Nakajima T, Williams CS, Quackenbush J, Puryear R, Powers M, Cheng T, Ludwig EH, Sharma AM, Hata A, Jeunemaitre X, Lalouel JM. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J Clin Invest*. 1997;99:1786-1797.
22. Schunkert H, Ingelfinger JR, Hirsch AT, Pinto Y, Remme WJ, Jacob H, Dzau V. Feedback regulation of angiotensin converting enzyme activity and mRNA levels by angiotensin II. *Circ Res*. 1993;72:312-318.
23. Benetos A, Gautier S, Ricard S, Topouchian J, Asmar R, Poirier O, Larosa E, Guize L, Safar M, Soubrier F, Cambien F. Influence of angiotensin converting enzyme and angiotensin II type 1 receptor gene polymorphisms on aortic stiffness in normotensive and hypertensive patients. *Circulation*. 1996;94:698-703.
24. Ardaillou R. Angiotensin II receptors. *J Am Soc Nephrol*. 1999;10(suppl 11):S30-S39.
25. Duggan J, Kilfeather S, O'Brien E, O'Malley K, Nussberger J. Effects of aging and hypertension on plasma angiotensin II and platelet angiotensin II receptor density. *Am J Hypertens*. 1992;5:687-693.
26. Hautanen A, Lankinen L, Kupari M, Janne OA, Adlercreutz H, Nikkila H, White PC. Associations between aldosterone synthase gene polymorphism and the adrenocortical function in males. *J Intern Med*. 1998;244:11-18.
27. Davies E, Holloway CD, Ingram MC, Inglis GC, Friel EC, Morrison C, Anderson NH, Fraser R, Connell JM. Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2. *Hypertension*. 1999;33:703-707.
28. Pojoga L, Gautier S, Blanc H, Guyene TT, Poirier O, Cambien F, Benetos A. Genetic determination of plasma aldosterone levels in essential hypertension. *Am J Hypertens*. 1998;11:856-860.
29. White PC, Slutsker L. Haplotype analysis of CYP11B2. *Endocr Res*. 1995;21:437-442.
30. 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*. 1997;11:638-649.