The molecular variants M235T and T174M of the angiotensinogen gene have been linked to essential hypertension in some populations, but there are discrepancies about this association in other studies. We studied 75 patients with essential hypertension (BP > 160/100 mm Hg) from our outpatient clinic, aged 55 ± 1 years, 30 men, systolic BP 182 ± 2.5, diastolic BP 109 ± 1 mm Hg (mean ± SEM), and a family history of the disease. Target organ damage was evaluated by measuring urinary albumin excretion rate, left ventricular hypertrophy, and fundoscopy. As a control group, 75 healthy subjects with BP < 130/85 mm Hg and with no family history of cardiovascular disease were selected. M235T and T174M angiotensinogen genotypes were determined by PCR and subsequent digestion of the products with SfaNI and NcoI, respectively. The frequency (q) of genotypes of the variant M235T in the patients with essential hypertension was MM 0.31, MT 0.41, and TT 0.28, not significantly different ($P = .93$) from that of the controls (MM 0.28, MT 0.44, and TT 0.28). For the variant T174M, the genotype frequencies in hypertensives were TT 0.83, TM 0.15, and MM 0.02, which was not significantly different ($P = .89$) from that of the controls (TT 0.86, TM 0.12, and MM 0.02). Similarly, there was no evidence for association between angiotensinogen genotypes and hypertension in subjects aged ≤ 40 years old (n = 24) or with severe (stage III) hypertension (n = 31). Within the group of patients with essential hypertension, there were no differences in genotype distribution between patients with and without retinopathy (n = 31), left ventricular hypertrophy (n = 37), or microalbuminuria (n = 14). This study shows that M235T and T174M variants are not associated either with essential hypertension or with target organ damage in a Spanish sample. Am J Hypertens 1998;11:439–444 © 1998 American Journal of Hypertension, Ltd.

KEY WORDS: Angiotensinogen, genes, hypertension, polymorphism.
role in salt and water homeostasis and vascular tone regulation. Jeunemaitre et al demonstrated linkage between the angiotensinogen (AGT) gene and EH in two populations. Moreover, several molecular variants were identified and a significant association with BP was demonstrated for two of them, M235T (Met → Thr substitution at codon 235) and T174M (Thr → Met substitution at codon 174). Although these variants have not been demonstrated to alter the kinetics of the RAS, M235T was associated with higher plasma levels of AGT. The pathophysiological relevance of these findings relies on the fact that a correlation between plasma AGT and BP has been demonstrated.

Genetic linkage of the AGT locus and EH has been demonstrated in several populations. In addition, since the initial report of Jeunemaitre et al, a number of association studies of the M235T polymorphism and EH have been conducted and have yielded conflicting results. In this article, we investigated the association between the M235T and T174M polymorphisms and EH in an unselected group of patients and its relation to end-organ target damage and severity of the disease. A review of the M235T and T174M association studies performed to date in white populations was evaluated by electrocardiography using the Cornell Index (Rv5 + Sv3 > 28 mm in men and > 20 mm in women) and the Minnesota classification (Rv5-6 > 26 mm, RII, III, aVF > 20 mm).

Fundoscopic evaluation was performed by the same observer in a blinded fashion and the Keith-Wagener classification was used. Left ventricular hypertrophy was measured by the single-shot clearance technique using the Cornelli Index (Rv1 + Sv3 > 28 mm in men and > 20 mm in women) and the Minnesota classification (Rv5-6 > 26 mm, RII, III, aVF > 20 mm). Detection of M235T and T174M Genotypes of Angiotensinogen Angiotensinogen M235T and T174M genotypes were determined by PCR amplification of genomic DNA followed by restriction enzyme digestion. Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. PCR was conducted in 25 µL volume containing 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.3, 200 µmol/L of each dNTP, 1U Taq polymerase, and 100 ng of genomic DNA, and 1 µmol/L each primer: 5' TGG ATG CGC ACA AGG TCC TGT C 3' and 5' CAG GGT GCT GTC CAC ACT GGC TCG C 3'. After an initial denaturation at 96°C for 5 min, thermocycling consisted of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 1 min for 35 cycles followed by a final extension for 5 min. The second primer bears a mismatched nucleotide, which creates a SfaNI restriction site during amplification. Digestion of PCR products with SfaNI (New England Biolabs, Beverly, MA) at 37°C yields a 266-bp band if codon 235 is ATG (M235), in contrast to the undigested 303-bp band (T235). The T174M genotype was determined by a separate digestion at 37°C of the same 303-bp amplified product with a native NcoI (New England Biolabs) restriction site. If the codon 174 is ATG (174M), digestion yields two fragments, 210 and 95 bp long. After digestion, the prod-
products were size-fractionated on ethidium bromide-stained 0.5% agarose/2.5% Nu.Sieve (Amersham, Arlington Heights, IL) and visualized by ultraviolet transillumination.

Statistical Analysis Quantitative variables are expressed as mean ± standard error of the mean (SEM). Genotype distribution and allele frequency were compared between groups using the $\chi^2$ test or the Fisher’s exact test. Differences between quantitative variables were analyzed by parametric test (analysis of variance). Only when the distribution was not normal (UAE rate) was a nonparametric test (Kruskal-Wallis) performed. A $P$ value < .05 was considered significant.

RESULTS The clinical characteristics of the hypertensive patients studied were the following: 30 men and 45 women, aged 55 ± 1 years, with SBP of 182 ± 2.5 and DBP of 109 ± 1.1 mm Hg. The mean creatinine level was 76 ± 2 $\mu$mol/L (0.9 ± 0.02 mg/dL) and the mean duration of hypertension was 10 ± 6.5 years. Microalbuminuria was detected in 14 patients (19%) and the mean urinary albumin excretion rate was 19.6 ± 12.5 $\mu$g/min. The renal hemodynamic study disclosed a GFR of 99 ± 3.5 mL/min/1.73m$^2$ and a ERPF of 438 ± 14 mL/min/1.73m$^2$, resulting in a FF of 0.22; this value was not significantly different from the reference values of the laboratory.

The genotype distribution of the AGT gene M235T polymorphism in hypertensive patients and in normotensive controls is depicted in Table 1A. There was no difference in genotype distribution between the two groups ($P = .93$) (Table 1A). Similarly, there was no difference in genotype distribution between groups for the T174M polymorphism ($P = .89$) (Table 1B).

In the studies reporting an association between these polymorphisms and hypertension, the relationship is especially strong in severe hypertensives or in patients with early-onset disease. Therefore, we evaluated the genotype distribution of M235T and T174M polymorphisms in hypertensives with early-onset of the disease, before the age of 40 years, and in severe hypertensives, stages III and IV, BP $>$ 180/110 mm Hg. As shown in Table 1, the AGT M235T and T174M polymorphism genotype distribution was not different between controls and hypertensives with early-onset disease or with severe hypertension in our study.

The renin-angiotensin system has been implicated in the development of target organ damage in EH, which in some patients occurs independently of the levels of BP, pointing to an individual predisposition. For target organ damage evaluation, we performed fundoscopic examination, ECG, and urinary albumin excretion rate measurements in all patients and analyzed the distribution of AGT polymorphism genotype in this subgroup.

Thirty-one patients (41%) displayed hypertensive changes on fundoscopy. The frequency of the 235T and 174M alleles was not different between patients with and without retinopathy: (0.47 vs 0.50; $P = .74$) and (0.05 vs 0.12; $P = .10$), respectively. Thirty-seven patients (49%) displayed left ventricular hypertrophy

| TABLE 1. GENOTYPES OF THE ANGIOTENSINOGEN M235T (A) AND T174M (B) VARIANTS AND FREQUENCIES (Q) OF 235T (A) AND 174M (B) ALLELES IN THE HYPERTENSIVE PATIENTS AND CONTROLS |
|-----------------------|-----------------------|-----------------------|-----------------------|
|                       | Controls* (n = 75)    | Hypertensives (n = 75) | Hypertensives, Onset ≤ 40 yr (n = 24) | Hypertensives, BP > 180/110 mm Hg (n = 31) |
| A) M235T variant       |                       |                       |                       |
| MM                    | 21 (0.28)             | 23 (0.31)             | 10 (0.42)             | 12 (0.39)             |
| MT                    | 33 (0.44)             | 31 (0.41)             | 10 (0.42)             | 12 (0.39)             |
| TT                    | 21 (0.28)             | 21 (0.28)             | 4 (0.16)              | 7 (0.22)              |
| $P$                   | .93                   | .36                   | .55                   | .55                   |
| 235T                  | (.50)                 | (.49)                 | (.37)                 | (.42)                 |
| $P$                   | .91                   | .85                   | .76                   | .76                   |
| B) T174M variant      |                       |                       |                       |
| TT                    | 64 (0.86)             | 62 (0.83)             | 22 (0.92)             | 26 (0.84)             |
| TM                    | 9 (0.12)              | 11 (0.15)             | 2 (0.08)              | 3 (0.10)              |
| MM                    | 2 (0.02)              | 2 (0.02)              | 0 (0)                 | 2 (0.06)              |
| $P$                   | .89                   | .62                   | .62                   | .62                   |
| 174M                  | (0.10)                | (0.10)                | (0.04)                | (0.11)                |
| $P$                   | .84                   | .53                   | .13                   | .13                   |

* Number of patients; parentheses indicate frequencies.
Genetic linkage between EH and a variable tandem repeat of the AGT gene has been replicated in four different populations by three different studies\(^\text{1,3,4}\) that used the affected-pedigree-member method. These studies support the important role of AGT in the control of BP and highlight a potential importance of the AGT locus in the pathophysiology of EH.

A search for molecular variants in the AGT gene with SSCP identified 15 polymorphisms, of which eight were in the coding sequence.\(^\text{1}\) An association of the AGT gene with EH was found for variants M235T and T174M, and this association was more striking by selecting patients with severe hypertension.\(^\text{1}\) This finding was confirmed by other studies in different populations.\(^\text{3}\) Importantly, some studies of the M235T variant were capable of demonstrating a significant association only when selecting patients for early-onset hypertension (<40 years)\(^\text{6}\) or strong family history of the disease.\(^\text{7}\) More recent studies in different populations demonstrate no association, even in severe hypertensives.\(^\text{8–10}\)

In the present study we found no evidence for an association between the M235T or T174M variant of the AGT gene and EH (Table 1). Although this finding contrasts with some authors,\(^\text{1,5}\) it is in agreement with the more recent studies performed also in white populations.\(^\text{3,6–11}\) In addition, when patients with early-onset hypertension or severe hypertension were analyzed, no significant association with the M235T or T174M variant was found (Table 1). Table 2 shows a summary of 10 studies performed in white populations, making evident the enormous discrepancy between studies. We purposely have not included studies in other populations, ie, black Americans or Japanese, because the different distribution of genotypes in the control populations of different ethnic origin makes comparison difficult.\(^\text{12}\) The genotype distribution in our control group is in agreement with that reported in studies of white populations.\(^\text{1,3,9}\)

Most of the studies examining the T174M polymorphism have resulted in no association with hypertension, whereas the M235T polymorphism results have been controversial. Although population-based differences could have accounted for the discrepancies, Table 2 shows that in three cases, different studies performed in the same country (thus assuming homogeneous ethnicity) were contradictory. It is possible that methodologic problems—mainly in the design of case-control association studies, which can result in bias and confounding—may account for the differences. In this regard, in some studies, like the present one, one has to take into account the possibility of a type II error. Nevertheless, it is remarkable that two careful population-based studies, one in the United States\(^\text{8}\) and the other in Finland,\(^\text{9}\) have demonstrated no association of the M235T polymorphism with EH. In addition, there is the possibility of misclassification of hypertensives and normotensives when BP is taken as a dichotomous variable, and most studies, like ours, have tried to overcome this problem by selecting severe hypertensives. However, in two recent studies, BP was taken as a continuous variable both in normotensive and hypertensive patients and no association between the M235T polymorphism and BP was found,\(^\text{9,11}\) despite adjustments for possible confounding variables like age, gender, body mass index, or alcohol consumption. Even treating BP as a continuous variable in a population-based sample, however, a negative result may be due to insufficient power, because a relative weak contribution to BP is expected for AGT.

Some of the positive studies have selected hypertensive patients by positive familial history or through specialized centers, and there may be a bias in the selection for patients with cardiovascular complications. In this sense, the RAS, and specifically, a deletion polymorphism in the angiotensin-converting enzyme gene, has been shown to be associated with increased risk for target organ damage in EH, including left ventricular hypertrophy, retinopathy, and microalbuminuria.\(^\text{13,14}\) To our knowledge, no other study has explored the association of AGT polymorphisms with target organ damage in EH. Despite the fact that our patients were moderate-severe hypertensives, no association between M235T or T174M and the presence of either left ventricular hypertrophy, retinopathy, or microalbuminuria was found.

Our study, in agreement with the most recent reports, suggests that the contribution of the M235T polymorphism to EH may be less than initially reported. This is in concordance with biochemical studies, which have demonstrated that neither M235T nor T174M polymorphisms affect the kinetics of AGT interaction with renin,\(^\text{15}\) which is the rate-limiting step in the RAS cascade.

However, it is not possible to exclude AGT as a BP-determining gene from negative association studies. The fact that linkage studies between the AGT locus and EH using the affected sibpair method have been consistently positive indicates that a relevant
mutation may be in close proximity to this locus, and that the M235T variant may act as a marker. In this sense, a common variant \( [A \ (-6)] \) has been identified in the promoter region of the AGT gene in close linkage with M235T and demonstrated to affect (increase) basal transcription AGT in vitro.\(^{15}\) However, the possibility exists that linkage without association may be present when there are many independent trait-causing genes in a population, thus making association with any particular allele weak.

In conclusion, we found no association between the M235T or T174M polymorphisms of the AGT gene and EH in a case control study, in agreement with recent reports performed in different white populations. Even by selecting more severe hypertensives or patients with early onset of the disease, we were unable to find an association. In contrast with other genes of the RAS, our results suggest that these variants of the AGT gene are not involved in the target organ damage in EH.

NOTE ADDED IN PROOF
A recent paper by Niu et al (J Clin Invest 1998;101: 188–194) has demonstrated absence of linkage in 310 Chinese hypertensive sibling pairs, highlighting that the disease relevance of the AGT gene may vary depending on ethnicity.

ACKNOWLEDGMENTS
We are indebted to Dr. Mazzara from the Blood Bank of the Hospital Clinic for his help in recruiting the control population. We also thank Dr. Fiera for performing the isotopic studies in the hypertensive patients and Dr. Dalfo for including some of his patients in the study.

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