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Influence of the I/D Polymorphism of the Angiotensin-Converting Enzyme Gene on the Outcome of Microalbuminuria in Essential Hypertension

Josep Redon, Felipe Javier Chaves, Youlian Liao, Jose Maria Pascual, Eduardo Rovira, Maria Eugenia Armengod, Richard S. Cooper

Abstract—The objective of the present study was to analyze the influence of the I/D polymorphism of the ACE gene on the outcome of microalbuminuria in essential hypertensive patients who were receiving antihypertensive treatment. One hundred thirty-six essential hypertensive patients who were <50 years old and had never previously received treatment with antihypertensive drugs were included in the study. During a 3-year period, patients received nonpharmacological treatment consisting of moderate salt restriction and a low-calorie diet they were obese, with or without a regimen of antihypertensive drugs based on β -blockers or ACE inhibitors. Hydrochlorothiazide was added when necessary to maintain the blood pressure goal of <135/85 mm Hg. At the beginning of the study and at yearly intervals, systolic and diastolic blood pressures (SBP and DBP, respectively), 24-hour urinary albumin excretion (UAE), renal function, and biochemical profile measurements were made. The insertion/deletion (I/D) polymorphism of the ACE gene was determined through the use of polymerase chain reaction. The variables used in the statistical analysis were the measurements at the start of the study and the increase or decrease detected during the follow-up, estimated as individual specific regression line slope values. At baseline, no differences in blood pressure or UAE values were observed among genotypes. Likewise, the genotype or allele frequency was not significantly different between normoalbuminurics and microalbuminurics. After the 3 treatment years, significant reductions in SBP, DBP, and UAE were found (SBP 151.6 ± 17.3 reduced to 137.2 ± 14.3 mm Hg, $P < 0.001$; DBP 96.6 ± 8.9 reduced to 84.5 ± 9.8 mm Hg, $P < 0.001$; UAE 36.7 ± 71.5 reduced to 28.3 ± 78.6 mg/24 h, $P < 0.05$). The slopes of these parameters over time did not differ significantly among genotypes. The slope of SBP was the main factor related to the slope of logUAE ($P < 0.003$). A significant positive correlation coefficient between the SBP and logUAE slopes was observed for the DD patients ($r = 0.57$, $P < 0.0001$) but was absent in patients carrying the I allele (II $r = -0.03$, $P = \text{NS}$; I/D $r = 0.01$, $P = \text{NS}$). Follow-up studies should be used to achieve a better understanding of the impact of candidate gene polymorphisms on the development of hypertension-induced organ damage. Assessment of the I/D polymorphism of the ACE gene may identify subjects who require a greatly lowered blood pressure to prevent organ damage and to reduce hypertension-associated complications and death. (*Hypertension*. 2000;35[part 2]:490-495.)

Key Words: hypertension, essential ■ albuminuria ■ genetics ■ angiotensin-converting enzyme gene

Microalbuminuria has attracted attention as an early marker of organ damage in hypertension, and preliminary data that support microalbuminuria as a prognostic factor for cardiovascular or renal risk in hypertension have recently been published.¹ Previous studies by our group and by others have demonstrated that in essential hypertension, microalbuminuria is related to blood pressure (BP) values^{2,3} and to the presence of hyperinsulinemia,^{4,5} as an expression of insulin resistance.⁵ Blood pressure and hyperinsulinemia, however, account for only part of the variation in microalbuminuria among hypertensives.⁴ Some studies that support the contribution of genetic background to the development of

microalbuminuria in hypertension have recently been published.⁶⁻⁸

The D allele of the polymorphism insertion/deletion (I/D) of the ACE gene, involving an intronic deletion (D) of a 287-bp sequence, has been linked to a higher prevalence of microalbuminuria^{6,8} and to nephroangiosclerosis⁸ among hypertensives. In other diseases that affect the kidney, such as diabetes or IgA nephropathy, subjects carrying the D allele also have the highest risk of the development of renal failure.^{9,10} Furthermore, the D allele has been linked to a failure of the renoprotective action of ACE inhibitors (ACEIs) to retard the development of end-stage renal disease.^{11,12}

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The influence of the I/D polymorphism on the outcome of microalbuminuria has not been explored in essential hypertension, and the objective of the present study was to analyze this relationship in patients receiving antihypertensive treatment during a 3-year follow-up period. The primary purpose of this study was to obtain information on the impact of the I/D polymorphism on the development of microalbuminuria in essential hypertension.

Methods

Selection of Study Participants and Design

Patients included in the study were selected from an outpatient clinic over the 5-year period of January 1990 through December 1994. All patients who fulfilled the inclusion criteria were invited to participate, and written consent was requested. The inclusion criteria included (1) diastolic BP (DBP) in the range of high normal to moderate essential hypertension, defined as being between 90 and 114 mm Hg (Korotkoff phase V, sitting position) for 3 visits at 1-month intervals; (2) age of 25 to 50 years; (3) the absence of cardiovascular events; (4) a glomerular filtration rate of $>60 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$; and (5) no previous treatment for hypertension. Patients with nephropathy, diabetes mellitus, urinary tract infection, fasting glucose in serum of $>120 \text{ mg/dL}$, or a positive dipstick for albumin or glucose were excluded. A total of 146 patients were included in the study. The study was approved by the Ethical Committee of the Hospital of Sagunto, and all patients granted their consent in writing.

After enrollment, and according to clinical criteria, which did not consider microalbuminuria, the patients were placed on a nonpharmacological treatment that consisted of moderate salt restriction and a low-calorie diet, if overweight, with or without a regimen of antihypertensive drugs based on β -blockers or ACEIs. Hydrochlorothiazide was added when necessary to maintain the BP goal of $<135/85 \text{ mm Hg}$. Of the initial 146 patients, 136 completed the 3-year study. Twenty-three received only the nonpharmacological treatment, 29 received β -blockers, 59 received ACEIs, and the remaining 25 had their treatment shifted over time due to a variety of reasons. During the 3 years of follow-up, urinary albumin excretion (UAE), renal function, and other parameters were assessed on a yearly basis.

Clinical and Analytical Procedures

All patients underwent a complete clinical work-up to rule out secondary hypertension. Blood pressure was measured in a quiet environment with the use of a mercury sphygmomanometer with the patient in a sitting position after 5 minutes of rest, according to the recommendations of the British Hypertension Society. Systolic BP (SBP) and DBP were measured according to Korotkoff phases I and V, respectively.

Blood samples were obtained in the mornings after that participants had fasted for a minimum of 8 hours. Serum biochemical profiles and lipids were measured with an autoanalyzer SMAC. The glomerular filtration rate was estimated on the basis of the clearance of endogenous creatinine as expressed in reference to body surface ($\text{mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$).

UAE for 2 separate 24-hour urine collections was measured with the use of an immunonephelometric assay (Behring Institute). Aliquots of urine were taken during a 24-hour period, stored in glass tubes at 4°C , and analyzed 1 to 7 days after collection. For each patient, the UAE was considered the mean of values obtained for the 2 separate 24-hour urine collections. If a difference in values obtained for the 2 samples was $>25\%$ of the higher value or of a creatinine excretion value during a 24-hour period was lower than expected for body size and gender, a third sample was requested. On the basis of UAE, hypertensive patients were classified as normoalbuminuric (UAE $<30 \text{ mg/24 h}$) or microalbuminuric (UAE 30 to 300 mg/24 h).

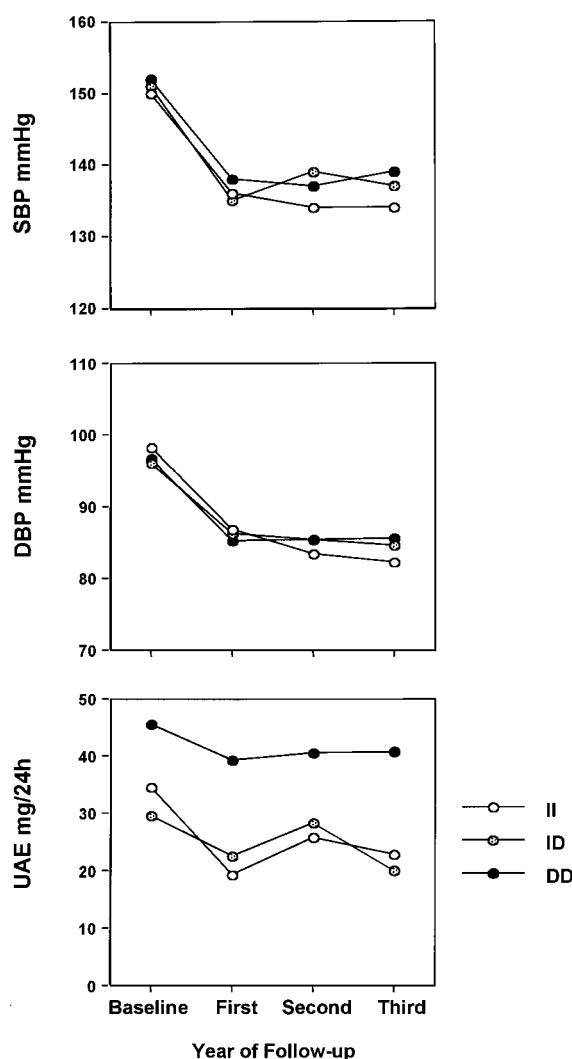


Figure 1. Values of SBP, DBP, and UAE at baseline and yearly during follow-up in 3 genotypes of I/D polymorphism of ACE gene (II $n=22$, I/D $n=59$, and DD $n=55$).

I/D Polymorphism of ACE Gene

Genomic DNA was extracted from blood according to the procedure of Tilzer et al.¹³ Genotyping of ACE I/D was assayed with the use of DNA amplification with the oligonucleotides described by Rigat et al.¹⁴ Reactions were performed in a final volume of $15 \mu\text{L}$ containing $0.75 \mu\text{mol/L}$ concentration of each primer, $2 \text{ ng}/\mu\text{L}$ DNA, $75 \mu\text{mol/L}$ concentration of each dNTP, 1.5 mmol/L MgCl_2 , 75 mmol/L Tris \cdot HCl (pH 9.0), 5 mmol/L KCl, 20 mmol/L $(\text{NH}_4)_2\text{SO}_4$, and $0.02 \text{ U}/\mu\text{L}$ Netzyme ADN polymerase (Need, SL). The DNA was amplified for 40 cycles with denaturation at 94°C for 30 seconds and extension at 72°C for 90 seconds (PTC-100 thermal cycler; M.I. Research). DNA sequencing confirmed that no mistyping of I/C heterozygotes occurred under these conditions. The polymerase chain reaction products were separated through 2% agarose gel electrophoresis, and DNA was visualized with ethidium bromide staining (Figure 1).

Statistical Analysis

For each variable, the values were expressed as $\text{mean} \pm \text{SD}$ values. Data on UAE were analyzed in 2 ways. First, the UAE data were categorized into normoalbuminuric and microalbuminuric groups, as previously defined, and second, a continuous variable with logarithmic transformation ($\log\text{UAE}$) was used. The variables used in the statistical analysis were the values of BP, UAE, and biochemical

TABLE 1. General Characteristics, Blood Pressure Values, Urinary Albumin Excretion, and the Slope of the Regression Lines Over Time, Grouped by Genotype of the I/D Polymorphism of the ACE Gene

	II (n=22)	I/D (n=59)	DD (n=55)
Age, y	39.7±7.6	38.6±8.1	41.2±7.3
Gender, M/F	16/6	32/27	23/32
Body mass index, kg/m ²	28.2±4.2	27.9±4.0	28.9±4.7
UAE>30 mg/24 h, n (%)	9 (41)	17 (29)	15 (27)
Baseline			
SBP, mm Hg	150.1±12.8	151.3±20.6	152.4±15.0
DBP, mm Hg	98.3±8.7	96.0±9.3	96.6±8.7
UAE, mg/24 h	34.4±47.1	29.4±51.5	45.4±94.6
Follow-up			
Slope SBP, mm Hg	-5.76±5.22	-3.99±5.61	-4.11±6.12
Slope DBP, mm Hg	-5.61±4.34	-3.55±3.31	-3.54±3.92
Slope UAE, mg/24 h	-2.87±10.8	-2.58±11.7	-1.29±15.1
Antihypertensive treatment			
Nonpharmacological	1	13	9
β-Blockers	4	14	11
ACEI	11	23	25
Others	6	8	11

Nonpharmacological indicates restriction of salt, calories, or both on the basis of dietary advice; β-blockers, atenolol or bisoprolol only; ACEIs, enalapril or lisinopril only; others, shifted treatments.

profile at the beginning of the study. The changes of parameters during the follow-up were estimated as the individual-specific regression line slope values. The type of treatment was included as nonpharmacological treatment (0), β-blockers (1), and ACEIs (2). One-way ANOVA and Kruskal-Wallis and Fisher tests were used to compare the parameters between patients with and without microalbuminuria and among patients with different genotypes. Multiple regression analysis was performed to assess the factors related to the changes in UAE during the follow-up and the influence of genotypes. Logistic regression analyses were performed to assess the influence of genotype on the presence of microalbuminuria. Values of $P<0.05$ were considered statistically significant.

Results

General Characteristics of the Study Population

The general characteristics, BP values, renal function, and UAE of the patients in each genotype of the I/D polymorphism who completed the study are shown in Table 1. Genotypes of the I/D polymorphism of the ACE gene were in Hardy-Weinberg equilibrium. No significant differences were observed in terms of age, body mass index, UAE, or SBP and DBP. Gender was the only characteristic that differed among the genotypes, with a predominance of women carrying the D allele. At the beginning of the study, 95 (70%) of the patients were normoalbuminuric and 41 (30%) were microalbuminuric.

I/D Polymorphism Genotypes and Microalbuminuria

Genotype distribution and allele frequencies of both polymorphisms in the initially normoalbuminurics and microalbuminurics are given in Table 2. No differences in the presence of microalbuminuria were observed among the genotypes of the I/D polymorphism. The allele frequencies also were not significantly different between the normoalbuminurics and microalbuminurics. Genotypes did not influence the presence of microalbuminuria when age, gender, and BP were taken into account in a logistic regression model (data not shown).

From the 41 initially microalbuminurics, 26 became normoalbuminurics during the 3 years of antihypertensive treatment. There were no significant differences in the normalization rates of microalbuminuria across the genotypes. From the 95 initially normoalbuminurics, 7 became microalbuminurics despite the antihypertensive treatment. Although with this small number it was not possible to observe whether the genotype influenced the occurrence rates, all subjects who developed microalbuminuria had the D allele: 5 homozygotes (DD genotype) and 2 heterozygotes (I/D genotype). The prevalence of the D allele increases from 0.57 at the beginning until 0.68 after the 3-year follow-up in the subjects who were microalbuminurics.

TABLE 2. Genotype and Allele Frequencies of I/D Polymorphism of the ACE Gene: Normoalbuminuric Versus Microalbuminuric Patients

UAE Status	Genotype			Allele	
	II	I/D	DD	I	D
Baseline					
Normoalbuminurics	13	42	40	0.36	0.64
Microalbuminurics	9	17	15	0.43	0.57
Pearson's χ^2 (P value)	$\chi^2=1.48$ (0.48)			$\chi^2=1.03$ (0.31)	
After 3 y					
Normoalbuminurics	20	50	45	0.39	0.61
Microalbuminurics	2	9	10	0.32	0.68
Pearson's χ^2 (P value)	$\chi^2=0.69$ (0.71)			$\chi^2=1.07$ (0.30)	
Became normoalbuminurics	7	10	9	0.44	0.56
New microalbuminurics	0	2	5	0.14	0.86

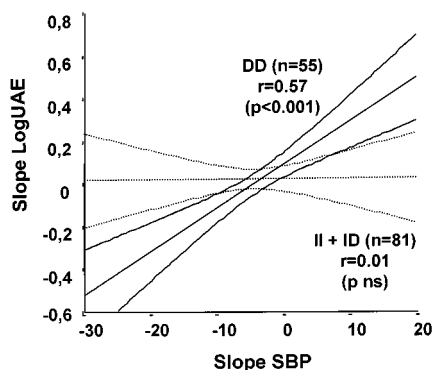


Figure 2. Regression lines and their corresponding 95% confidence interval between SBP and logUAE slope values in subjects who are homozygotes for D allele (DD genotype) and in those carrying I allele (II plus I/D genotypes).

I/D Polymorphism Genotypes, Blood Pressure, and UAE

The values of BP and UAE at the beginning and the changes throughout the study in patients with the different genotypes of I/D polymorphism are shown in Figure 1. At baseline, no differences in BP values were observed among the genotypes, and although UAE has the highest value in DD subjects, differences did not achieve statistical significance.

After the 3 treatment years, significant reductions in SBP, DBP, and UAE were noted (SBP 151.6 ± 17.3 to 137.2 ± 14.3 mm Hg, $P < 0.001$; DBP 96.6 ± 8.9 to 84.5 ± 9.8 mm Hg, $P < 0.001$; UAE 36.7 ± 71.5 to 28.3 ± 78.6 mg/24 h, $P < 0.05$). Values of SBP, DBP, and UAE, as well as the slopes of these parameters over time, did not differ significantly among genotypes (Table 1).

We analyzed the factors related with the changes in UAE by using multiple regression analysis. The slope of SBP was the main factor related to the slope of logUAE ($P < 0.003$), accounting for 7% of the variance. Neither gender, DBP, weight, glucose slopes, type of treatment, nor genotype influenced the changes in logUAE. The relationship between the SBP and logUAE slopes, however, strongly differed among the genotypes (Figure 2). A significant positive correlation coefficient was observed for the DD patients ($r = 0.57$, $P < 0.0001$) that was not present for the II ($r = -0.03$, $P = \text{NS}$) and I/D ($r = 0.01$, $P = \text{NS}$) patients.

The same analysis was performed for the 59 subjects treated with ACEIs. The relationship between the SBP and logUAE slopes remained significant for the subjects with DD genotype ($r = 0.78$, $P < 0.001$) and not for those with the II ($r = 0.06$, $P = \text{NS}$) or I/D ($r = 0.21$, $P = \text{NS}$) genotype or for subjects carrying the I allele (both II and I/D) ($r = 0.08$, $P = \text{NS}$).

Discussion

Patients with essential hypertension are heterogeneous in their clinical characteristics and prognosis. Factors influencing the variable prevalence of end-organ damage and the prognosis in patients with hypertension of similar severity are not well understood. Genetic background seems to be one of the main determinants of the heterogeneity, and a search for candidate genes has been initiated in the past few years. In the

present study, homozygotes for the D allele of the I/D polymorphism of the ACE gene showed a strong positive relationship between changes in SBP and UAE, something that was not observed in patients carrying the I allele. This positive correlation was also observed for patients treated with ACEIs.

We studied the influence of I/D polymorphism in the UAE of essential hypertensives by observing its impact on the outcome of microalbuminuria during antihypertensive treatment. This approach allowed us to consider the impact of genetics on the development of microalbuminuria, offering information complementary to that of association studies.⁶⁻⁸ To reduce potential confounding factors, we included hypertensives with selected characteristics: adults < 50 years old who were never previously treated with antihypertensive drugs and in whom diabetes mellitus and nephropathy had been excluded. To reduce day-to-day variability, UAE was measured in 2 separate 24-hour samples.

Another potential confounding factor, the type of the antihypertensive treatment that was used, merits special comment. Several studies have demonstrated that a reduction in BP values with the use of antihypertensive drugs was accompanied by a decrease in microalbuminuria in proportion to the reduction in BP. ACEIs and antagonists of the angiotensin II type 1 receptor have additional beneficial effects on microalbuminuria, independent of BP reduction.¹⁵ In the present study, the outcome of microalbuminuria was related to the SBP achieved throughout the 3 years of antihypertensive treatment, which is in agreement with previous studies. The changes in UAE values, however, were lower than those previously reported during short periods of observation,^{16,17} when only the changes in BP values and the effect of the drug itself influenced it. A longer observation period, such as the present 3-year study, allows us to analyze not only the effect of the changes in BP but also the impact of other factors on the UAE.

The significance of microalbuminuria in essential hypertension is much broader than expected, and there are several main factors that determine the presence of microalbuminuria.¹⁵ Blood pressure level has been considered the most important factor related to microalbuminuria in essential hypertension, supported by the higher prevalence of microalbuminuria in patients with severe hypertension and by a positive relationship between UAE and BP values.^{3,18} Furthermore, elevated ambulatory BP values and their persistence during sleep have been associated with the presence of microalbuminuria in essential hypertension.¹⁹

Beside BP values, the overactivity of the renin-angiotensin system (RAS) and insulin resistance have been linked to the development of microalbuminuria. Among essential hypertensives, microalbuminurics have higher renal vascular resistance than normoalbuminurics despite similar BP levels, and a higher activated RAS was observed in young patients with essential hypertension who had albuminuria than was in those who did not.²⁰ Hyperinsulinemia, as an expression of insulin resistance, also has been related to microalbuminuria in hypertensive subjects.^{4,5} Higher peripheral resistance to the action of insulin, as estimated with as euglycemic clamp, has

been observed in microalbuminuric patients but not in normoalbuminurics in essential hypertension.^{5,21}

The impact of the I/D polymorphism on BP levels, RAS activation, and insulin resistance was reported recently. Although the I/D polymorphism seems to not influence BP values,²² the presence of the D allele has been linked to higher levels of renal damage among hypertensives. Our group observed a significant relationship between 24-hour ambulatory BP and UAE in essential hypertensives carrying the DD genotype, a relationship that was not observed for the II or I/D genotypes.²³ Higher ACE plasma levels, as well as higher local ACE activity, as described for subjects with the D allele, have been advocated to account for the highest rate of end-organ damage associated with this genotype.²⁴ The increased activity of ACE leads to higher local levels of angiotensin II, a peptide that produces vasoconstriction, aldosterone release, sodium and water retention, and cellular growth.²⁵

In contrast, in hypertensives carrying the I allele, higher rates of insulin resistance and hyperinsulinemia have been reported.^{26,27} Although no plausible explanation has been offered until now, recent evidence suggests a tight connection between insulin resistance and RAS. Angiotensin II infusion into normotensive volunteers has been shown to improve insulin resistance on the basis of hemodynamics through a redistribution of blood flow in skeletal muscle and on the basis of nonhemodynamic mechanisms.²⁸ It is conceivable that the slightly reduced ACE expression in ACE I allele carriers is associated with reduced insulin sensitivity. Whether the association between the I allele and insulin resistance is dependent on a lower RAS activation or is dependent on functional polymorphisms of another gene in linkage disequilibrium with the ACE gene is not fully understood. Among the genes located near the ACE locus in the 17q23 chromosome are members of the human growth hormone family that can influence glucose metabolism.

The most relevant observation in the present study was the relationship between changes in BP and changes in UAE, which was observed only in subjects who were homozygotes for the D allele. In accordance with this, the higher the SBP reduction, the higher was the UAE fall in the DD genotype, and consequently, better renal and vascular protection could be achieved. The relationship between BP values and microalbuminuria observed in our previous study²³ and in the present study in subjects who were homozygotes for the D allele might explain the association between genotypes of the I/D polymorphism and either RAS activation or insulin resistance. In subjects carrying the I allele, among whom the highest reduction in microalbuminuria was observed, UAE may be more dependent on the presence of insulin resistance or on other unknown factors and, consequently, less influenced by BP reduction than in subjects who are homozygotes for the D allele.

A reduction in BP values²⁹ and the administration of ACEIs³⁰ are 2 of the main ways to decrease or retard the progression of renal damage toward end-stage renal disease. According to previous studies, subjects with the DD genotype, however, seem to show the highest risk of developing renal damage and the poorest renal function outcome during

treatment.^{11,12} Whether further BP reduction is necessary to improve renal protection in subjects with DD genotype is an attractive hypothesis to be tested.

Finally, another aspect of the results deserve further comment. At the beginning, no differences in the prevalence of microalbuminuria were observed among patients with the genotypes of the I/D polymorphism. In contrast, the frequency of the D allele increases in the patients who were microalbuminuric after the treatment, limiting the occurrence of microalbuminuria to patients with the D allele. Moreover, subjects carrying II genotype achieved the highest reduction rate in microalbuminuria. This observation may help us understand the association between the D allele and microalbuminuria or renal damage previously reported in hypertensives.^{6,8} If patients who had previously received antihypertensive treatment were included in the study, a treatment-induced bias may be present, increasing the presence of the D allele among patients with the highest UAE or the poorest renal function.

In conclusion, follow-up studies may be useful to obtain a better understanding of the relationship between candidate gene polymorphisms and the development of hypertension-induced organ damage. An assessment of the I/D polymorphism of the ACE gene may identify subjects who require a large reduction in BP to protect organs from damage and to reduce hypertension-associated complications and death.

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