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Five Polymorphisms in Gene Candidates for Cardiovascular Disease in Afro-Brazilian Individuals

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Associations of polymorphisms in the angiotensin I-converting enzyme (*ACE*), apolipoprotein B (*APOB*) and apolipoprotein E (*APOE*) genes with hypertension and variations in lipid serum levels were evaluated in 184 Afro-Brazilians with a familial history of coronary artery disease (CAD). *ACE* (*Ins/Del*) and *APOB* (*Ins/Del*, *XbaI*, and *EcoRI*) and *APOE* (*HhaI*) polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses on agarose, and polyacrylamide gel electrophoresis. Serum lipids were measured by means of routine enzymatic assays. The results showed a high frequency of hypertension (44%) in Afro-Brazilians that was increased in subjects >40 years old and those with a blood mass index (BMI) higher than 25 kg/m^2 ($P<0.001$). The *ACE Del* allele was associated with hypertension in men >40 years old ($P<0.05$). *APOE* (*HhaI*) and *APOB* (*XbaI* and *Ins/Del*) polymorphisms were not associated with hypertension or variations in serum concentrations of lipids, while subjects with the *APOB* E− allele had higher low-density lipoprotein cholesterol (LDL-C) levels than E+ carriers ($P<0.05$). These results suggest that *ACE Ins/Del* polymorphism is associated with hypertension, and *APOB EcoRI* polymorphism is associated with LDL-C variation in Afro-Brazilians. J. Clin. Lab. Anal. 18: 309–316, 2004. © 2004 Wiley-Liss, Inc.

Key words: angiotensin I-converting enzyme; apolipoprotein B; apolipoprotein E; polymorphism; hypertension; coronary artery disease

INTRODUCTION

Coronary artery disease (CAD) is a chronic disease caused by multiple genetic and environmental risk factors, such as cigarette smoking, stress, and a sedentary lifestyle (1–3). Specific genetic causes for CAD have been identified in members of families affected by monogenetic forms of hypercholesterolemia, such as familial hypercholesterolemia (4). However, in the general population, the multifactorial etiology of CAD is not fully understood (5). The cumulative effects of both genetic and environmental factors have been the focus of recent attention (6). However, the major challenge confronting researchers is to identify the predominant genetic factor, and determine how the environmental factor modulates the expression of candidate genes associated with risk for atherosclerosis and CAD. Several studies have shown an association between CAD and polymorphisms in genes related to the control of blood pressure and lipid metabolism; however, these results have not been consistent (6).

Angiotensin I-converting enzyme (ACE) plays an important role in blood pressure regulation and electrolyte balance by hydrolyzing angiotensin I into angiotensin II (a potent vasopressor), stimulating aldosterone secretion, and inactivating bradykinin (a potent vasodilator) (7).

A common insertion/deletion (*Ins*/*Del*) polymorphism in intron 16 of the ACE encoding gene has been associated with variations in serum enzyme activity (7,8). In previous studies, *ACE Ins*/*Del* polymorphism was associated with an increased risk for cardiovascular disease (9,10). The *Del* allele was recognized as being associated with high ACE activity in plasma, increased blood pressure, and myocardial infarction risk (11). However, other studies have failed to detect associations between *ACE* polymorphism and CAD or myocardial infarction in Caucasian, Asian, and African populations

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(12–17). These differences may be related to the ethnic origin of the population, as previously suggested (18).

Polymorphisms at the genes encoding the apolipoproteins B and E, among others, have been indicated as markers of predisposition for moderate hypercholesterolemia and consequently for CAD (3,5).

Apolipoprotein E (apo E) is an important constituent of triglyceride-rich lipoproteins that plays a fundamental role in liver uptake and catabolism of chylomicrons, chylomicron remnants, very-low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) subspecies (19). The apo E encoding gene (*APOE*) has a common *HhaI* polymorphism located in exon 4 that generates three alleles: ϵ2, ϵ3, and ϵ4 (19). The frequency of these alleles varies with each population, demonstrating a geographic cline. The common allele in several populations is ϵ3. It varies in frequency from 66.2% in Nigerians to 85.9% in American Indians and Mexican Americans (20). Higher frequencies of the ϵ4 allele are found in African Americans and African Nigerians (20% and 31%, respectively), and low frequencies of the ϵ2 allele are found in American Indians (1.7%), Japanese (3.7%), and Mexican Americans and Finns (3.9%) (20).

Apo E isoforms interact differently with lipoprotein receptors, altering their metabolism and consequently the plasma level of the circulating lipids (mainly cholesterol levels) (21). The correlation of *APOE* polymorphism with serum cholesterol levels in numerous populations of diverse racial origins has been well documented (19). The ϵ4 allele has been associated with hypercholesterolemia, increased risk for CAD, diabetes, and Alzheimer syndrome (20–24). In contrast, the ϵ2 allele tends to be associated with increased concentrations of apo E and triacylglycerols (TG), and decreased levels of apo B and cholesterol (19).

Apolipoprotein B (apo B) is essential for the synthesis and secretion of triglyceride-rich lipoproteins in both the intestine and the liver. Apo B is also involved in uptake of cholesterol-rich particles (such as LDL) from plasma, by the LDL receptor-mediated endocytosis present on the cell surface (25). The apo B gene (*APOB*) has several polymorphisms that are associated with variations in plasma lipid concentrations, and CAD or myocardial infarction in different populations (26–28).

In previous studies we found an association between the *APOB XbaI* polymorphism and CAD or increased total cholesterol (TC) and LDL-C in Brazilian Caucasian women (23,29). The association between *APOB Ins/Del* (signal peptide) and *3′HVR* polymorphisms was related to hypercholesterolemia in white individuals (30). In addition, the *X+Del* haplotype (*APOB Ins/Del* and *XhaI* polymorphisms) was associated with higher TC, LDL-C, and TG serum levels in white individuals with increased risk for CAD (31). However, *APOB* (*Ins/Del*, *XbaI*, and *EcoRI*) polymorphisms were not associated with myocardial infarction or variation in lipid plasma concentrations in other studies (32–34).

The aim of this study was verify whether the *ACE* (*Ins/Del*), *APOB* (*Ins/Del, XbaI*, and *EcoRI*), and *APOE* (*HhaI*) polymorphisms are associated with hypertension and lipid serum concentrations in Afro-Brazilian individuals with a familial history of CAD.

MATERIALS AND METHODS

Study Subjects

For this study, 184 nonrelated Afro-Brazilian subjects (103 females and 81 males, 20–83 years old) were selected from four communities in the countryside of Mato Grosso do Sul state. Each of these individuals had a familial history of CAD. Individuals with thyroid, renal, or liver diseases, and who were receiving antihypertensive medication were not included in the study. The subjects underwent clinical and laboratory examinations for evaluation based on these criteria. The study protocol was approved by the local ethics committee, and informed consent was given by all of the selected subjects.

Lipid and Lipoprotein Analysis

After the subjects were fasted overnight, serum lipids were determined by standard procedures and the use of commercially available kits (Boehringer Mannheim and Roche Diagnostic, São Paulo, Brazil). High-density lipoprotein cholesterol (HDL-C) was determined by means of dextran sulfate/magnesium chloride precipitation of apo B lipoproteins in a fully automated system (BM HITACHI, model 902; Roche Diagnostic, São Paulo, Brazil). LDL-C levels were calculated based on the Friedwald formula (35).

Arterial Blood Pressure

We measured arterial blood pressure twice in each patient at 2-min intervals using calibrated equipment from Tyco (Tokyo, Japan). All patients with hypertension were submitted to a second evaluation 10 days later. Hypertension was defined as systolic pressure $\geq$140 mmHg or diastolic pressure $\geq$90 mmHg, according to the Third Brazilian Consensus on Hypertension (36).

Analysis of APOB, APOE, and ACE Polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes by means of a salting-out procedure (37). The

ACE Ins/Del polymorphic region was amplified by polymerase chain reaction (PCR) and analyzed by 1.5% agarose gel electrophoresis according to previously described procedures (8). *APOB* (*Ins/Del*, *XbaI*, and *EcoRI*) and *APOE* (*HhaI*) polymorphic regions were analyzed by PCR restriction fragment length polymorphism (RFLP) as previously described (23). To monitor the accuracy of the genotyping, we used positive and negative controls for restriction sites in all sets of reactions, and reanalyzed 10% of randomly chosen samples.

Statistical Analysis

Allele frequencies and genotype distributions for each polymorphic site were calculated by gene counting. Chi-square analysis and Fisher's complementary test were used to test for Hardy-Weinberg equilibrium and to compare allele and genotype frequencies between the studied groups. Mean values were compared by Student's *t*-test and one-way analysis of variance (ANOVA). Logarithmic transformation was used when the data did not fit a normal distribution. A value of $P<0.05$ was considered statistically significant. A multivariate logistic regression was performed with hypertension as the dependent variable, and the following independent variables: *ACE*, *APOB*, and *APOE* genotypes; age; sex; body mass index (BMI); TC; LDL-C; and TG. Statistical tests were performed with the SAS system for Windows, v. 6.12 (SAS Institute Inc., Cary, NC).

RESULTS

The clinical profiles and lipid serum levels of 184 Afro-Brazilians from the countryside of Mato Grosso do Sul state are shown in Table 1. The frequency of hypertensive individuals (44%) was higher than that found in the general Brazilian population (12–31%). To evaluate the association between age and hypertension, we divided the subjects into two groups, using the mean age of the sample (40 years) as the cutoff age that is within the range of age (30–55 years) of onset of the risk for hypertension, according to the Third Brazilian Consensus on Hypertension (36). An increased frequency of hypertension was found in individuals >40 years old, and those with a BMI > 25 kg/m^2 ($P<0.001$). A multivariate logistic regression analysis showed that the risk for hypertension was 6.71 times higher (95% CI, 3.13–14.43) in individuals >40 years old, and 3.39 times higher (95% CI, 1.48–7.74) in those who were overweight. Conversely, the risk for hypertension was lower in women (OR = 1.06; CI, 0.59–1.91) and smokers (OR = 0.86; CI, 0.58–1.29).

TABLE 1. Clinical profile and serum levels of lipids in normotensive and hypertensive Afro-Brazilian individuals*

	Normotensive	Hypertensive[a]	
Patients	(103)	(81)	
Gender			
Men	57% (46)	43% (35)	$\chi^2=0.04$
Women	58% (57)	42% (46)	(*P = 0.844*)
Age (years)			
≤40	71% (73)	25% (20)	$\chi^2=38.69$
>40	29% (30)	75% (61)	(*P < 0.001*)
BMI (kg/m^2)			
<25	73% (75)	32% (26)	$\chi^2=28.74$
≥25	27% (28)	68% (55)	(*P < 0.001*)
Serum parameters (mg/dL)[b]			
Total cholesterol	172 ± 36	190 ± 41	(*P = 0.002*)
LDL-C	100 ± 30	113 ± 37	(*P = 0.012*)
HDL-C	46 ± 11	45 ± 12	(*P = 0.622*)
Triacylglycerols	113 ± 81	153 ± 101	(*P = 0.001*)

*Number of individuals in parenthesis.
[a]Systolic pressure ≥140 mmHg and diastolic pressure ≥90 mm Hg, according to the III Brazilian Consensus on Hypertension (36).
[b]Values are mean ± SD compared by *t*-test.
BMI, Body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

As shown in Table 1, serum levels of TC, LDL-C, and TG were higher in hypertensive individuals than in normotensive subjects ($P<0.02$). Moreover, serum glucose was also increased in hypertensive subjects (91 ± 29 mg/dL) compared to normotensive individuals (80 ± 15 mg/dL) ($P<0.001$).

Hypertensive individuals had a higher frequency of CAD (60.6%) compared to normotensive subjects (45.4%); however, this difference was not statistically significant ($P=0.091$). In addition, stroke occurred more frequently (46.5%) in the hypertensive group than in the normotensive group (29.2%) ($P=0.047$).

The *ACE Ins/Del* genotype distribution in Afro-Brazilian individuals was 38.6%, 45.6%, and 15.8% for *Del/Del*, *Ins/Del*, and *Ins/Ins*, respectively. Hence, the overall frequency of the *ACE Del* allele (61.4%) was higher than that of the *Ins* allele (38.6%). As shown in Table 2, the relative frequency of the *ACE Del* allele was slightly higher in hypertensive individuals (0.654) compared to normotensive subjects (0.583), although the difference did not reach statistical significance level ($P=0.195$).

When Afro-Brazilian individuals were grouped according to gender, the relative frequency of the *ACE Del* allele was slightly higher (0.757) in hypertensive men than in normotensive men (0.641), but this difference was not statistically significant ($P=0.159$) (Table 2). Interestingly, the men bearing the *ACE Del* allele (*Del/Del* genotype) showed higher diastolic and systolic arterial pressure than the *ACE Ins* allele (*Ins/Ins* and

Ins/*Del* genotypes) carriers (Fig. 1). No differences in the *ACE Del* allele relative frequency (Table 2, $P = 0.525$) or arterial pressure were found in women.

When we evaluated individuals >40 years old (Table 3), we found that the relative frequency of the *ACE Del* allele was higher in hypertensive individuals (0.672) than in normotensive subjects (0.500) ($P = 0.037$). This difference was found in older men ($P = 0.032$), but not in older women ($P = 0.399$) or subjects <40 years old (0.838).

In the Afro-Brazilian population, the *APOB Ins*/*Del* genotype distributions were 55.2% (*Ins*/*Ins*), 33.9% (*Ins*/*Del*), and 10.9% (*Del*/*Del*). The *APOB Ins* allele frequency was higher (72.1%) than the APOB *Del* allele frequency (27.9%). An analysis of *APOB EcoRI* polymorphism showed that the genotype distributions for E+E+, E+E–, and E–E– were 89.6%, 9.8%, and 0.6%, respectively. The frequencies of the *APOB* E+ and E– alleles were 94.5% and 5.5%, respectively, in these individuals. Moreover, the genotype distributions for *APOB XbaI* polymorphism were 10.4% (X+X+), 39.3% (X+X–), and 50.3% (X–X–), and the allele frequencies were 30.1% (X+) and 69.9% (X–). The overall distributions of *APOE* genotypes E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4 in Afro-Brazilians were 5.5%, 19.8%, 51.6%, 21.4%, and 1.6%, respectively. The frequencies of the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles were 12.6%, 65.1%, and 22.3%, respectively.

The genotype distributions for *APOB Ins*/*Del*, *EcoRI*, and *XbaI* polymorphisms in hypertensive subjects were

TABLE 2. Relative allele frequencies of *ACE Ins*/*Del* polymorphism in normotensive and hypertensive Afro-Brazilian individuals, according to gender

Groups	Alleles	Normotensive	Hypertensive	
Total		($n = 103$)	($n = 81$)	
	Ins	0.417	0.346	$\chi^2 = 1.680$
	Del	0.583	0.654	($P = 0.195$)
Men		($n = 46$)	($n = 35$)	
	Ins	0.359	0.243	$\chi^2 = 1.986$
	Del	0.641	0.757	($P = 0.159$)
Women		($n = 57$)	($n = 46$)	
	Ins	0.456	0.402	$\chi^2 = 0.404$
	Del	0.544	0.598	($P = 0.525$)

n, number of individuals; *ACE*, angiotensin I converting enzyme.

TABLE 3. Relative allele frequencies of *ACE Ins*/*Del* polymorphism between normotensive and hypertensive Afro-Brazilian individuals, according to age

Groups	Alleles	Normotensive ($n = 103$)	Hypertensive ($n = 81$)	
≤40 years		($n = 73$)	($n = 20$)	
	Ins	0.384	0.350	$\chi^2 = 0.042$
	Del	0.616	0.650	($P = 0.838$)
>40 years		($n = 30$)	($n = 61$)	
	Ins	0.500	0.328	$\chi^2 = 4.334$
	Del	0.500	0.672	($P = 0.037$)
>40 years (men)		($n = 20$)	($n = 27$)	
	Ins	0.475 (19)	0.241	$\chi^2 = 4.621$
	Del	0.525 (21)	0.759	($P = 0.032$)
>40 years (women)		($n = 10$)	($n = 34$)	
	Ins	0.550 (11)	0.397	$\chi^2 = 0.916$
	Del	0.450 (9)	0.603	($P = 0.339$)

n, number of individuals; *ACE*, angiotensin I converting enzyme gene.

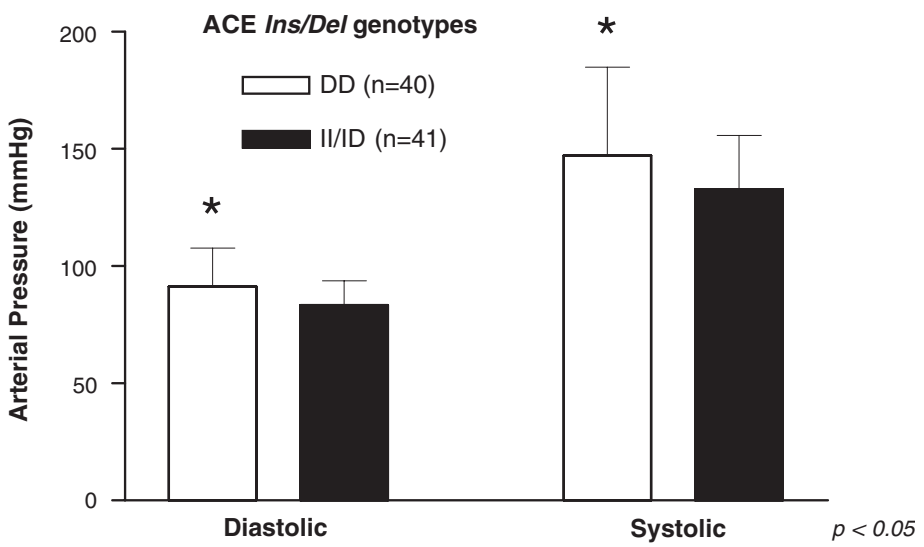

Fig. 1. Arterial blood pressure values in Afro-Brazilian men carrying the *ACE Del* allele (DD genotype) and *Ins* allele (II/ID genotypes). Values are represented as mean ± SD compared by Student's *t*-test.

similar to those found in normotensive individuals. Moreover, there were no differences in relative allele frequencies of these polymorphisms between hyper- and normotensive individuals ($P > 0.05$, Table 4). The relative frequencies of *APOE* alleles in hypertensives were similar to those in the normotensive group ($P > 0.05$, Table 4). These results indicate that *APOB* and *APOE* polymorphisms are not associated with hypertension in Afro-Brazilian population. Similar results were found when individuals were grouped according to gender or age (data not shown).

TABLE 4. Relative allele frequencies of *APOB EcoRI*, *XbaI* and *Ins/Del* polymorphisms and *APOE HhaI* polymorphism in normotensive and hypertensive Afro-Brazilian individuals

		Groups		
Genes	Alleles	Normotensive ($n = 102$)	Hypertensive (n = 81)	
APOB	Ins	0.7400.698$\chi^2 = 0.62$		
	Del	0.260	0.302	($P = 0.431$)
	E−	0.064	0.043	$\chi^2 = 0.39$
	E+	0.936	0.957	($P = 0.531$)
	X−	0.686	0.716	$\chi^2 = 0.25$
	X+	0.314	0.284	($P = 0.615$)
APOE	ϵ2	0.104	0.154	$\chi^2 = 2.12$
	ϵ3	0.663	0.636	($P = 0.347$)
	ϵ4	0.233	0.210	

n, number of individuals. *APOB*, apolipoprotein B gene; *APOE*, apolipoprotein E gene.

A logistic regression analysis showed that individuals with different *ACE* and *APOB* genotypes have similar risks for hypertension (data not shown). Conversely, subjects with the *APOE* E3E4 genotype had a 2.8 lower risk (OR = 0.36; 95% CI, 0.13–0.97) for hypertension than *APOE* E3E3 carriers.

Associations between *ACE*, *APOE*, and *APOB* (*XbaI* and *Ins/Del*) polymorphisms and variations in lipid serum levels in Afro-Brazilians were not found. On the other hand, *APOB* E-allele carriers (E + E– genotype) had higher serum concentrations of LDL-C than noncarriers (E + E + genotype) ($P < 0.05$, Fig. 2).

DISCUSSION

The genetic risks implicated in CAD have been reported over the last decade. Although many of these genetic factors are well established, others are still controversial. The *ACE Ins/Del* polymorphism has been correlated with variations in ACE activity and blood pressure, and risks for myocardial infarction and other cardiovascular disorders (10,11,38–40). However, other studies have failed to demonstrate such associations in different populations (14–16,41). These discrepant results may be related to the different ethnic origins of the studied groups. It has been proposed that individuals of a specific ethnic origin are more susceptible to certain diseases than others (18).

In this study, Afro-Brazilian individuals showed a higher frequency of hypertension compared to the

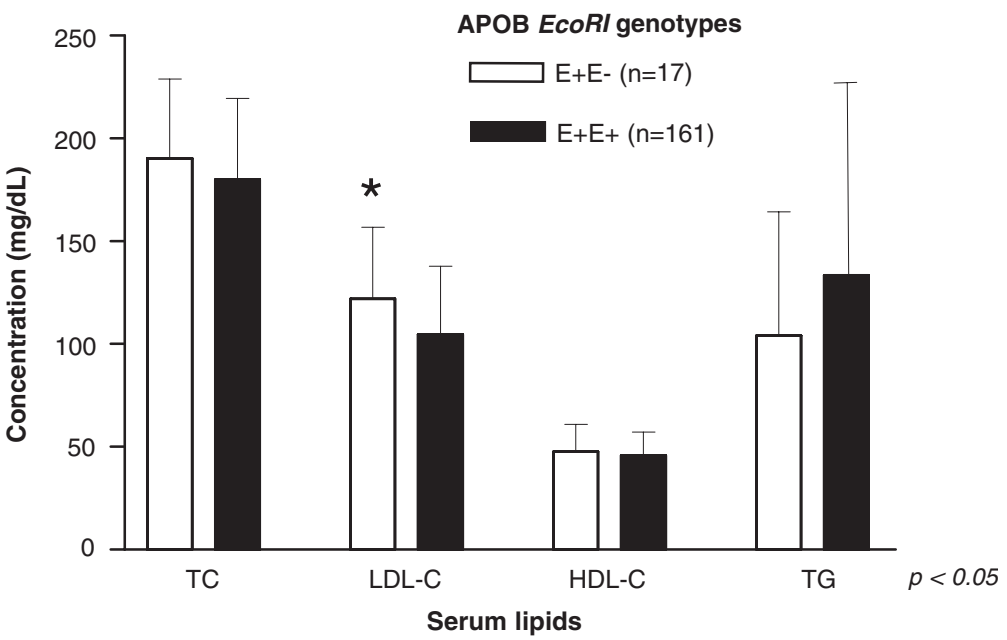

Fig. 2. Serum lipids concentrations in Afro-Brazilian carrying the *APOB EcoRI* genotypes. Values are represented as mean ± SD compared by Student's *t*-test.

Caucasian Brazilian population (42–44). The association between higher BMI and increased blood pressure indicates that overweight may play an additional role in the development of hypertension in Afro-Brazilians. When the effect of age on hypertension was evaluated, we found a significant relation between advanced age and frequency of hypertension in Afro-Brazilians. Therefore, the strong association between hypertension and advanced age and overweight may explain the high prevalence of CAD (54%) in these Afro-Brazilian communities, as shown previously in other populations (1).

The increased serum concentrations of TC, LDL-C, TG, and glucose observed in hypertensive individuals were similar to those found in patients with metabolic syndrome and type 2 diabetes (45,46). Consequently, these metabolic abnormalities, which are usually associated with insulin resistance and obesity, may represent additional risks for CAD in Afro-Brazilians.

The analysis of the *ACE Ins/Del* polymorphism showed that the overall frequency of the *Del* allele in Afro-Brazilians was similar to that found in Afro-Americans and Afro-Caribbeans (17,18,47), and black individuals from an urban population in Brazil (48), and was higher than in Caucasian populations of other countries (14,18,40).

The association of the *ACE Del* allele with high diastolic and systolic arterial pressures in men suggests that *ACE Ins/Del* polymorphism is related to alterations in blood pressure in the Afro-Brazilian population. A positive association between *Del* allele frequency and increasing blood pressure was found in black individuals from an Afro-Caribbean sample, but not in white people (17). Moreover, other studies have shown that blood pressure is not influenced by *ACE* genotypes in Caucasian populations (14,49). On the other hand, Israeli subjects bearing the *Del* allele have higher blood pressure levels than those with the *Ins/Ins* genotype (50). These findings show the importance of studying *ACE* polymorphism in genetically homogeneous populations, such as the Afro-Brazilian communities.

The higher frequency of the *ACE Del* allele found in hypertensive Afro-Brazilian men >40 years old suggests that the association between *ACE Ins/Del* polymorphism and hypertension may depend on gender, as previously found in the Framingham Heart Study (51). In that large population-based sample, the *ACE* locus was associated with diastolic blood pressure in men but not in women (51). The association between *ACE Ins/Del* polymorphism and hypertension found in older men in our study was also previously demonstrated in Japanese men (52). The results from these studies suggest that gender, age, and ethnic origin contribute to differences in the association of *ACE Ins/Del* polymorphism with hypertension and blood pressure variations.

The distribution of the *APOB Ins/Del*, *EcoRI*, and *XbaI* genotypes in Afro-Brazilians was similar to that found in white Brazilian populations, including those with a high risk for CAD (29–31). In addition, *XbaI* and *EcoRI* allele frequencies were similar to those found in a population in Senegal (53).

Polymorphisms of the *APOE* and *APOB* (*XbaI*, *EcoRI*, and *Ins/Del*) have been associated with hypercholesterolemia or increased risk for CAD in Caucasian-Brazilians (29–31,54–56) and other populations (57–61). In addition, the 3′ hypervariable region (HVR) of the *APOB* has been associated with essential hypertension in a United Arab Emirates population (62). In our study, *APOB* polymorphisms were not associated with either hypertension or variation in blood pressure in our sample population.

Despite the lack of association between *APOB* (*Ins/Del* and *XbaI*) polymorphisms and variation in serum lipids in Afro-Brazilians (data not shown), individuals bearing the *APOB* E− allele had higher LDL-C concentrations than *E+* allele carriers. A similar effect was also recently found in other populations. In Indians with CAD, the genotype EcoR1−/− was associated with increased TC and LDL levels (63). Moreover, in a Chinese population, the concentration of LDL-C was higher in patients with gallstone disease bearing the E+E− genotype than in E+E+ genotype carriers (64).

In Afro-Brazilians, the allele frequencies of the *APOE* (*HhaI*) polymorphism were similar to those found in African-Americans and African blacks (20,58). The frequency of the ϵ4 allele in our sample was similar to that found in other African populations, but it was higher than that observed in Caucasian populations (20,51,57–59). The similarity of relative allele frequencies of *APOE* polymorphism between normo- and hypertensive individuals suggests that *APOE* polymorphism is not associated with hypertension in Afro-Brazilians. It is noteworthy that the reduced risk for hypertension in ϵ4 allele (E3/E4 genotype) Afro-Brazilian carriers was also found in a large genetic epidemiological study carried out in Japan (65). In contrast to the reported effects of *APOE* polymorphism on serum cholesterol levels in European and African populations examined in other studies (19,57–59), *APOE* genotypes were not associated with variation in serum concentrations of lipids in Afro-Brazilians (data not shown).

In conclusion, Afro-Brazilian individuals have a high frequency of hypertension that is associated with increased age, higher BMI, and *ACE Ins/Del* polymorphism in men. Furthermore, *APOB EcoRI*

polymorphism is associated with variations in LDL-C concentrations in this Afro-Brazilian sample.

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