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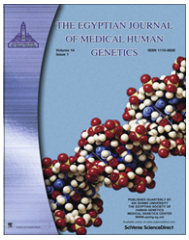

ORIGINAL ARTICLE

A study of the M235T variant of the angiotensinogen gene and hypertension in a sample population of Calabar and Uyo, Nigeria

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Abstract A common molecular variant of the angiotensinogen gene had been reported to predispose some ethnic groups to hypertension. This case–control study was designed to determine the frequency and association of the angiotensinogen M235T allele with hypertension in residents of Calabar and Uyo cities, south–south Nigeria.

The study involved 1308 subjects, 612 patients and 696 controls. The M235T variant was investigated using an allele specific polymerase chain reaction and enzymatic digestion to determine allele frequencies. Hypertensinogenic factors such as dietary habits, physical activity, smoking and drinking habits were assessed using questionnaires. Descriptive statistics, chi-square and multiple regression analysis were used to analyze the data obtained.

The M235T allele frequency was high (0.94 for hypertensives and 0.96 for controls) though it was not associated with hypertension status. The odds ratio for hypertension was 0.64 (95% confidence interval: 0.39–1.06) there were no significant differences between the genotype frequency of hypertensives and controls. By multiple regression, Hypertension was observed to be associated with age and was a predictor for systolic blood pressure in both patient $r^2 = 0.359$; $p < 0.05$ and control groups $r^2 = 0.26$.

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Age and body mass index were predictors for diastolic blood pressure in the control group, $r^2 = 0.28$.

Although the frequency of the M235T variant was high, it was not a significant risk factor for hypertension in the study population.

1. Introduction

Hypertension is a multifactorial disorder because of the interaction of many risk genes such as molecular variants of the angiotensinogen gene, angiotensin converting enzyme gene, angiotensin II receptor I gene and the corin gene [1–4], and environmental factors such as obesity, body mass index (BMI), dietary salt intake, alcohol consumption, stress and high-density lipid (HDL)–cholesterol levels. Genes determine approximately 20–60% of the variability in blood pressure in different populations [4,1].

Most studies so far have focused on the genes of the renin–angiotensin–aldosterone system such as the *M235T* variant in the angiotensinogen gene, which has been associated with increased circulating angiotensinogen levels and blood pressure in many distinct populations [5–7] and a common variant in the angiotensin-converting enzyme (ACE) gene that has been associated in some studies with blood pressure variation in men [8,9]. However, these variants seem to only modestly affect blood pressure, and other candidate genes have not shown consistent and reproducible associations with blood pressure or hypertension in larger populations [10] thus, demonstration of common genetic causes of hypertension in the general population remains elusive [6,11,12].

The renin–angiotensin (R–A) system is a powerful pressure system which influences salt and water homeostasis. Angiotensinogen (AGT) is a key component of this system, it is cleaved by renin to yield angiotensinogen 1 (AGT 1), which is cleaved by angiotensinogen converting enzyme (ACE) to yield angiotensinogen II (AGT II), responsible for carrying out a range of functions that include prompting the constriction of blood vessels causing a rise in blood pressure, ensuring the release of aldosterone which induces an increase in the re-absorption of sodium by the Kidneys [13].

Many research groups have reported that among the two important molecular variants of AGT characterized so far, the M235T allele is the predominant allele in Blacks accounting for a frequency between 80% and 93% in the population. Most of the studies carried out so far in Nigeria have been concentrated in individuals and population in the South Western part of the country. Little is found in literature on hypertension in other geographical and ethnic areas of Nigeria, and its genetic epidemiology, especially since variations in the dietary intake, culture (way of life) etc. exist in these places. In Blacks, differences have been observed in the expression of risk factors such as level of circulating angiotensinogen, urbanization, dietary factors and metabolic disorder for hypertension depending on the environment [14–16].

The aim of this study was to determine the frequency of the M235T allele of the angiotensinogen gene and its association with hypertension status in the study population.

2. Subjects and methods

Venous blood (3 ml) was collected from each participant after they had given informed consent, into bottles containing anticoagulant EDTA. Plasma was obtained by centrifugation of blood samples. Blood and plasma were kept frozen in the freezer/cold room, then transported to the Department of Zoology University of Ibadan where samples were kept at −70 °C. Samples were then transported to freezers in the International Institute of Tropical Agriculture, Ibadan for subsequent lab investigations. DNA was extracted from blood for angiotensinogen (AGT) genotyping. For some participants who refused blood collection from the upper arm, blood was obtained from thumb pricks and blotted onto a filter (Whatman, No. 3) paper, allowed to dry at room temperature and preserved in plastic bags prior to DNA extraction.

Participants included in the study gave informed consent and ethical approval for the study was obtained from the joint UI/UCH Ethics Review Committee and each of the health establishment concerned – University Teaching Hospital Calabar, University of Uyo Teaching Hospital and the General Hospital, Calabar.

The wall in the collection centre was calibrated in meters. Individuals stood without foot or head wear facing the investigator, looking straight ahead and the investigator placed a ruler on top of the head of the individual and the reading in meters was recorded. Using the conventional weight scale, weight was measured in kilograms.

Blood pressure readings were taken using a sphygmomanometer in millimeters of mercury systolic and diastolic BP values were recorded. Before taking the measurement, the respondent was advised to sit quietly for 5 min, with the legs uncrossed and the right hand free from clothing. The right hand was placed on the table with the palm facing upward. The appropriate cuff size was selected and the cuff wrapped and fastened securely. The cuff was kept at the same level as the heart during measurement. The upper reading, the systolic blood pressure (SBP) and lower reading the diastolic blood pressure (DBP) were recorded, the first and second readings were taken twice and the average of the two used for the analysis.

2.1. DNA extraction

DNA was extracted according to the method developed by Dellaporta et al. [17] with a little modification. The DNA was re-suspended in 50 μl of Tris–EDTA buffer and stored in the freezer as stock solution.

An aliquot of the DNA stock solution was run on agarose gel electrophoresis to check the quantity of DNA. Each sample of genomic DNA (5 μl) was mixed with 2 μl of loading dye and transferred into the wells of the gel. A voltage of 110 V was applied for about 45 min. The samples were scored +, 2+,

3+ depending on the intensity of the bands to indicate the amount of DNA extracted.

DNA was extracted from filter paper according to Bereczky et al. [18]. Pieces (1–2) of the filter paper about 5 mm in diameter were cut using a sterile blade for each samples. These pieces were placed in an eppendorf tube, soaked in 65 µl of T.E. buffer.

The tube was incubated at 50 °C for 15 min in a water bath. The pieces were pressed gently at the bottom of the tube several times using a new pipette tip for each sample.

The eppendorf tubes were heated again for 15 min at 97 °C to elute the DNA. The liquid condensing on the lid and the walls of the tube was removed by a short centrifugation (2–3 s). The DNA extract that is the supernatant was kept at −20 °C before use.

2.2. Polymerase chain reaction

Stock DNA samples were diluted to 1/10, 1/50, 1/100 depending on the amount of DNA in the sample checked after extraction. A 1/10 dilution was done for all filter paper extracted DNA stock solutions.

Genomic DNA (2 µl) was amplified in a 15 µl reaction mix containing Go Taq® green master mix (Promega) 7.5 µl, upstream and downstream oligonucleotide primers 0.30 µl each and 4.9 µl of nuclease-free water.

Cycling conditions: Below is the primer sequence used for these experiments, an initial denaturation for 10 min at 95 °C was followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min 30 s at 72 °C and a final elongation of 10 min at 72 °C.

Primer sequence [19]

5^{1} CAGGGTGCTGTCCACACTGGACCCC 3^{1}
5^{1} CCGTTTGTGCAGGGCCTGGCTCTCT 3^{1}

2.3. Enzymatic digestion

The T235 variant of the *AGT* is induced by a single nucleotide change from thymine to cytosine at position 704 near the 3′ end of exon 2 of the angiotensinogen gene. The specific mismatch incorporated into the antisense primer creates a Tth 111l site if the T235 variant is present (the presence of a cytosine at position 704; GACN↓NNGTC) the enzyme cuts the PCR product into a 141 and a 24 bp fragment. If the 235 M variant is present, (the presence of thymine at position 704; GATNNNGTC) the enzyme does not cut, leaving a 165 bp PCR product with no restriction fragments [20]. A cocktail of 0.25 µl of the Tth111I enzyme, 1.5 µl of the 10× buffers and 3 µl of sterile water was added to 10 µl of the PCR product. The enzyme digestion was performed in a final volume of 14.75 µl at 65 °C for 2 h. The digested products were separated on 3% agarose gel stained with 1.2 µl of ethidium bromide for 30 min at 125 V.

2.4. Data management

The Statistical Package for Social Sciences – SPSS for Windows® Version 11.0 was used to statistically analyze the data obtained. Descriptive statistics was used to analyze all variables studied which include marital status, occupation, snack use, smoking practices, alcohol consumption and *AGT* genotype of the subjects. Genotype frequencies in control and hypertensive groups were compared by chi-square analysis. Continuous variables were compared between hypertensives and controls by independent *t* test.

Effect of *AGT* genotype on BP was analyzed using a general linear model. Influence of *AGT* genotype on continuous variable was analyzed using one way ANOVA. Multiple regression analysis was also carried out using SBP or DBP as dependent variable, then sex, age, body mass index (BMI) and other variables were used as independent variables. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Gel results

After digestion of the 165 bp PCR product with the Tth111I enzyme, the normal individual M235M gives an undigested 165 bp, a mutant individual M235T gives two fragments of 141 bp and 24 bp. A recessive individual T235T gives a 141 bp fragment. However agarose gel allows the visualization of a 165 bp fragment for M235M, a 141 bp fragment for T235T, a 165 bp and 141 bp for the M235T individuals respectively [19] Fig. 1.

3.2. Demographic data

There were a total of 1308 subjects recruited into the study, consisting of 612 hypertensives – 225 males and 387 females and 696 normotensives – 273 males and 423 females. The Efiks and the Ibibios (34.2; 32.4% respectively, $n = 612$) were the main ethnic groups among the patients. The Ibibios (41.5%, $n = 696$) were the predominant ethnic group among the controls Fig. 2. Most individuals had the T235T variant of the AGT gene in the ethnic groups of both the patient and the control population Figs. 3 and 4. The T235T allele was the predominant allele observed with a higher frequency among females than males, Fig. 5.

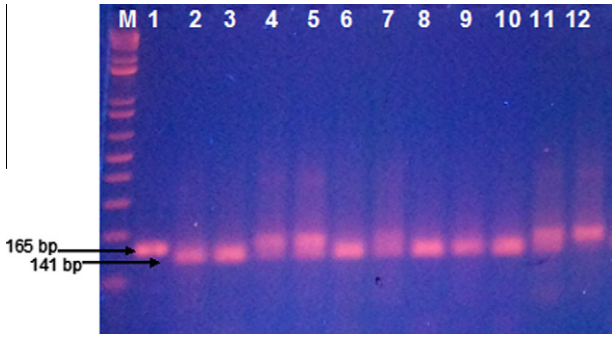

Figure 1 Agarose gel electrophoresis showing the amplification of the 165 bp fragment after enzymatic digestion with the Tth 1111 restriction endonuclease enzyme. Lane M is a 1 kb plus DNA ladder; lane 1 is a 165 bp PCR product; lane 2 is a 141 bp digested fragment showing a recessive homozygous individual; lanes 4 and 5 are 165 and 141 bp fragments showing a heterozygous individual; lane 12 is a 165 bp undigested fragment showing a normal wild typa individual.

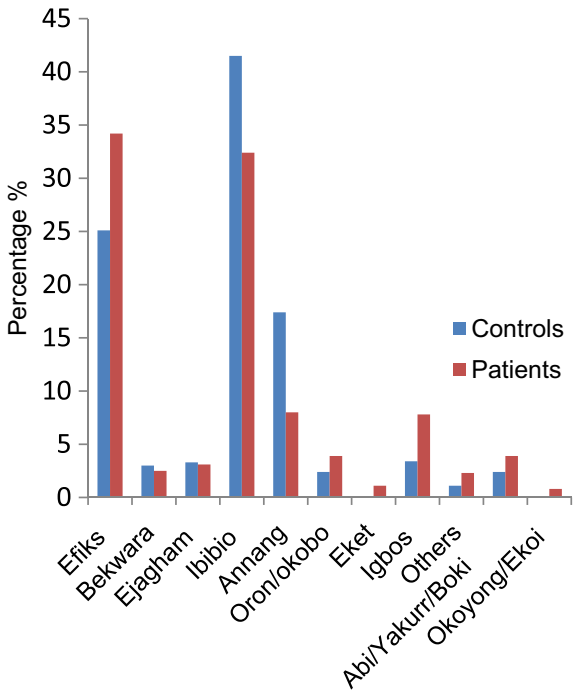

Figure 2 Ethnic distribution among patient and control groups.

3.3. Blood pressure

For patients the mean diastolic blood pressure was 93.25 ± 13.768, the mean systolic blood pressure was 161.14 ± 23.247. For the controls, the mean systolic blood pressure was 116.76 ± 9.19; the mean diastolic blood pressure was 72.181 ± 8.41. According to the seventh report of the Joint National Committee of Prevention, Detection, Evaluation and Treatment of High blood pressure (JNC VII) classification on hypertension, 281 patients had stage one hypertension and 331 patients had stage two hypertension, for the systolic BP measurement.

From the diastolic BP measurement, 381 patients were grouped into the stage 1 category and 231 patients had stage 2 hypertension. For the systolic BP measurement in controls, 395 were classified into the prehypertension group while 301 were classified as normal. For the diastolic BP measurement, 309 controls were classified into the prehypertension group and 387 controls as normal.

3.4. Age

The hypertensive subjects ranged from 24 to 90 years old with a mean age of 51.3 years ± 13.76. Among the patient group, 464 (75.8%) persons were more than 40 years of age and 148 (24.2%) patients were less than 40 years. Normotensives ranged from 20 to 73 years old with a mean age of 31.9 years ± 10.27, 569 (81.7%) controls were less than 40 years of age while 127 (18.3%) controls were above 40 years.

3.5. Body mass index

In the patient population, BMI below 24.9 kg/m^2 was observed in 234(38%) persons, BMI between 25 and 30 kg/m^2 was observed in 193(32%) persons and BMI above 30 kg/m^2 was found in 185(30%) persons. In the controls, BMI above 30 kg/m^2 was found in 89(13%) persons, BMI between 25 and 30 kg/m^2 was found in 140(20%) persons and a BMI below 24.9 kg/m^2 was observed in 467(67%) persons.

3.6. Genotype frequencies

The prevalence of *AGT* mutation was 88.4% for hypertensives and 92.2% for controls (homozygous mutation), 10.9% hypertensives and 7.5% control for the heterozygous mutation Table 1. The wild type allele was prevalent at 0.3% and 0.7% for patients and controls respectively. There were no significant differences between the genotype frequencies of hypertensive and the control groups by χ^2 analysis. When continuous variables were compared between hypertensive and control

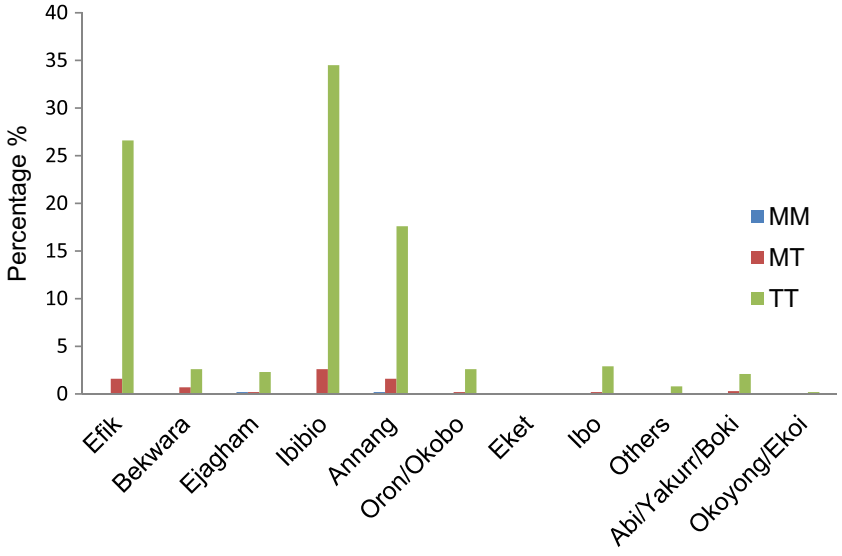

Figure 3 Distribution of the AGT M235T allele among ethnic groups in the control population.

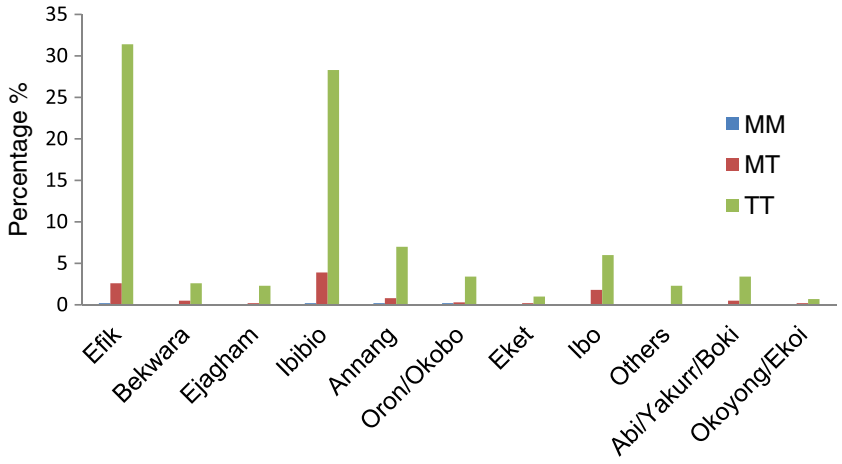

Figure 4 Distribution of the AGT M235T allele among ethnic groups in the patient population.

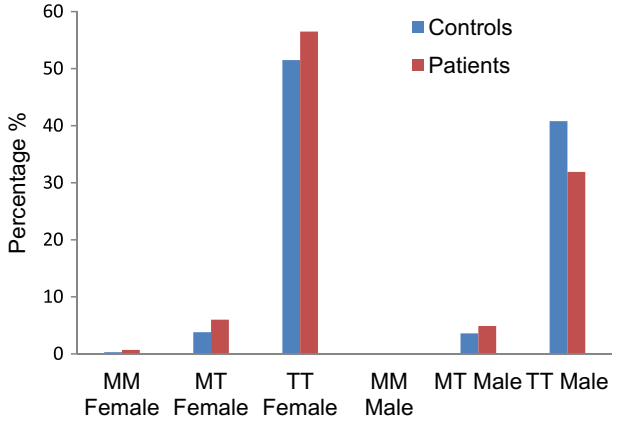

Figure 5 Distribution of the AGT M235T allele by gender in the control and patient groups.

Table 1 Genotype and allele frequency of the M235T polymorphism of the angiotensinogen gene in the control and patient populations.

Groups	*N*	Genotypes			Alleles	
		MM	MT	TT	M	T
Hypertensives	612	4	67	541	37	575
%		0.7	10.9	88	6	94
Normotensives	696	2	52	642	28	66.2
%		0.3	7.5	92	4	96

groups, significant differences existed between the age, BMI, systolic and diastolic blood pressure of controls and patients.

By multiple regression analysis, age was the predictor for SBP in the hypertensive group $r^2 = 0.359$; $p < 0.05$. Age and BMI were predictors for DBP in the control group, age was also the predictor for SBP in the control group. Gender, body mass index, and AGT genotype were not predictors for SBP and DBP in the hypertensive group. The influence of AGT genotype on continuous variable was compared using one way ANOVA. There were significant differences in the age and systolic in the control group and the systolic in the patient group but no significant differences between the continuous variation of other groups. When blood pressure and other variables using general linear model ANOVA were analyzed with no adjustments, age was significant for the MT and TT variables.

Using Hardy–Weinberg calculations for genotype and Allele frequency

Genotype frequencies of sample population

	MM	MT	TT
Patient	4	67	541
Control	2	52	642

Hardy–Weinberg calculations

M	T	MM	MT	TT	
0.05	0.95	0.004	0.09	0.9	observed frequency
		0.05	0.095	1.9	expected frequency according to Hardy–Weinberg

The observed are not equal to those frequencies predicted by Hardy–Weinberg which means there are evolutionary mechanisms influencing the loci under consideration. Thus the population does not meet the assumption of the Hardy–Weinberg theory.

4. Discussion

The renin–angiotensin–aldosterone system plays a major role in blood pressure regulation and AGT, a key substrate in this pathway has been an attractive candidate gene for the study of hypertension by many investigators [21–23]. Results have been conflicting; some studies have indicated that the M to T amino acid substitution at position 235 was associated with hypertension in several ethnic groups including Caucasian and Japanese populations [24–26]. Other research groups have reported no association of hypertension with the M235T variant.

The M235T allele is over 90% prevalent in the study population (0.94 for hypertensives and 0.96 for controls) which is very high, this is consistent with literature. Nigerians have been reported to have a high frequency of this molecular variant, between 80% and 93% [27,28]. The frequency of the M235T allele among hypertensive Malaysians was reported as 0.45 and 0.75 among the Japanese [29,30].

In this study, it was found that the M235T polymorphism of the AGT gene is not associated with hypertension implying that other polymorphisms are involved in the expression of the phenotype. The odds ratio for hypertension is 0.64 95% CI 0.39 to 1.06. Association studies in Africans and African-Americans also found a negative association [31,27] though the T allele was associated with increased plasma AGT [32].

Jeunemaitre et al. [5] were the first to report the linkage of the molecular variant M235T with hypertension in Whites/Caucasians. Subsequent studies among Whites/Caucasians supported an association [33] while others did not support any association [34,35].

Studies in the Japanese population found a positive association between hypertension and the M235T variant with an odds ratio of 2.67 [24,30]. Some other studies did not report any association [36,37]. A study among Malaysian subjects did not find an association between hypertension and the M235T allele [29]. Chinese and Taiwanese studies have also reported positive associations [11,38]. The mechanism by which the molecular variant M235T allele of the AGT gene is related to hypertension is poorly understood. Although the AGT 235T allele was found to be in complete linkage disequilibrium with a guanine to adenosine transition at −6 bp upstream of the initiation site of transcription [21]. In vitro test of promoter activity and DNA binding studies with nuclear proteins show that this nucleotide substitution affects the basal transcription rate of the gene in various cell lines thereby affecting the *AGT* T235 variant, increased plasma AGT levels that might lead to increased blood pressure [39].

The Efiks (384) and the Ibibios (487) made up the main ethnic groups in this study as is logical since the study was conducted in Calabar and Uyo, Nigeria. Most patients were married and advanced in age (above 40 years). Increase in age is thought to increase blood pressure because the arteries become hardened, less active, kidney function decreases and the body does not process salt as well as before [40]. It was observed that most young persons below the age of 40 years were normotensives.

In the control population, more individuals (467) had the normal body mass index which is $\geqslant$24.9 (67%), 140 controls (20%) were overweight and 89 controls (13%) were obese. In the patient population, 23(439%) patients had normal BMI of $\geqslant$24.9, more patients were overweight 193(38%) and obese 185(30%). Positive associations between body mass index and blood pressure have been reported in cross sectional studies in different populations [41–43].

Most females (both in controls and patients) were of the T235T genotype. A study has indicated that homozygosity for the T235 allele predicted risk for hypertension in women and not in men [44]. This variant was also reported to be associated with preeclampsia [45,19]. Markovic et al. [23] reported an association of nucleotide base substitutions at position-6, -29, -793, and -776 with hypertension in their study population. Other studies provided no evidence for association with essential hypertension while stratifying for sex [35,34]. Age was the predictor for SBP in the hypertensive group and was also the predictor for SBP in the control group. Age and body mass index were predictors for DBP in the control group, age, gender, body mass index, and AGT genotype were not predictors for SBP and DBP in the hypertensive group. All other hypertensinogenic factors considered did not contribute significantly to the disease when regression analysis was carried out.

The population under study does not conform to the Hardy–Weinberg equilibrium theory which means some evolutionary forces are acting on the locus under consideration.

The results obtained from this study will form a baseline information for these areas but more work still needs to be carried out to look for other gene polymorphisms that could be positive predictors for hypertension in this population.

5. Conclusion

The M235T variant was investigated for an association with hypertension in a sample population in Calabar and Uyo, Nigeria. The frequency of the M235T variant of the AGT gene is high particularly the AGT T235T homozygous genotype but the M235T mutation is not an independent risk factor for hypertension in individuals living within Calabar and Uyo.

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