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Research paper

The AGT and the GNB3 polymorphisms and insulin resistance in prehypertension

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ABSTRACT

OBJECTIVE: This study surveyed the frequencies of single nucleotide polymorphisms (SNPs) M235T AGT and C825T GNB3, and their association with insulin resistance, other biochemical markers and qualitative variables in subjects with high normal blood pressure and/or prehypertension in the Greek population. DESIGN: 330 men and women of Greek origin were divided into 3 groups: a) hypertensive, b) prehypertensive and c) control group. These groups were genetically tested for these polymorphisms and insulin resistance with the HOMA index. RESULTS: No statistically significant differences were found among the polymorphisms of the compared groups. However, the T allele carriers (CT/TT vs. CC) of the C825T polymorphism were associated with an increased BMI in all 3 groups (p=0.004). The HOMA index was higher in the hypertensive (p=0.006) and prehypertensive (p=0.016) versus the control group, and similar results were found for insulin (hypertensive vs. control p=0.012, prehypertensive vs. control p=0.001) without statistical significance between the first 2 groups (p=0.522). Additionally, there was a statistically significant difference between the control group and the hypertensive and prehypertensive groups regarding cholesterol (control vs. hypertensive p=0.001, control vs. prehypertension p=0.018) and triglycerides (control vs. hypertensive p=0.0001, control vs. prehypertension p=0.007). Differences were also noted between the control and the hypertensive group regarding the value of HDL (p=0.005) and LDL (p=0.013). CONCLUSION: This study failed to demonstrate a correlation between specific SNPs, blood pressure and insulin resistance in the 3 groups. However, T allele carriers of the polymorphism C825T were found to have an increased BMI. Similarly, increased insulin resistance and lipidemia were more common in the hypertensive and prehypertensive populations.

Key words: Genes, HOMA-IR, Insulin resistance, Prehypertension, SNPs

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INTRODUCTION

The association between increased blood pressure (BP) and coronary artery disease may be caused by concurrence of atherogenic biochemical abnormalities in hypertensive patients to form the metabolic cardiovascular syndrome. The JNC7[1] in 2003 defined a new category in the classification of BP, namely, prehypertension, including patients with BP 120-139/80-89 mmHg. Historically, Robinson et al[2] (1939) were the first to designate the values of BP = 120-139/80-89 mmHg as prehypertension when they noticed that most hypertensive patients started off from the prehypertensive zone. These individuals had twice the mortality of the population with BP<120/80 mmHg. According to Egan et al,[3] prehypertension is subdivided into first stage prehypertension (BP=120-129/80-89 mmHg) and second phase prehypertension (BP=130-139/85-89 mmHg). The incidence of prehypertension of the first stage is 31-37%, while that of the second stage is 12-14%.[4,5] In the U.S.A., 43 million people (22% of the adult population) are characterized as prehypertensive.[6] Prehypertension accounts for 3.4% of hospital admissions, 6.2% of house care and 8.5% of deaths.[6] Stage 2 prehypertensives[3] with more concomitant risk factors (increased body weight, hyperinsulinemia, lipidemia, insulin resistance, a large number of small dense LDL particles, high fibrinogen, PAI-1, inflammatory cytokines, endothelial dysfunction) and decreased coronary reservoir[7-9] or disease markers are three times as likely to develop hypertension or twice as likely to develop clinical cardiovascular disease. First stage prehypertensive patients also are at increased risk, but it is approximately half of that of stage 2.

The relationship between insulin resistance and hypertension is a particularly controversial issue. Hypertension and insulin resistance have been shown to be independently associated with confounding factors such as obesity,[10] but the relationship seems inconsistent.[11-13] Several mechanisms have been proposed in an effort to explain this relationship, but it is unclear which (if any) are important. Furthermore, some reports have demonstrated that prehypertension is associated with lower insulin sensitivity,[14] but its pathologic mechanisms have not as yet been clearly defined.

Many studies have sought to correlate SNPs with essential hypertension. Angiotensinogen (AGT) is a liver protein that interacts with renin to produce angiotensin I, the pro-hormone of angiotensin II. Angiotensin II is the major effector molecule of the renin-angiotensin-aldosterone system (RAAS) and plays a key role in the regulation of blood pressure (BP) by increasing vascular tone and promoting sodium retention. Genetic variants in the angiotensinogen gene modify the plasma concentration of angiotensinogen, which has been directly related to arterial blood pressure.[15] The molecular variant (M235T) of the AGT gene, encoding a threonine instead of a methionine at residue 235 of the mature protein, has been associated with a higher plasma AGT level and higher BP in patients homozygous for the T allele and occurs among various ethnic populations.[15-17] In a meta-analysis, the TT genotype was associated with a 32% increase in the risk of hypertension in whites but not in non-whites, when compared with the MM genotype.[18]

G proteins are signal transducers that communicate signals from many hormones, neurotransmitters, chemokines and autocrine and paracrine factors.[19] A C825T polymorphism of the gene encoding the G-protein b3 subunit (GNB3) is associated with the occurrence of alternative splicing, which causes the loss of 41 amino acids. This polymorphism is associated with increased intracellular signal transduction.[20] The 825T allele possibly increases the risk for phenotypes of metabolic syndromes and hypertension. There are only a few studies that examine these 2 SNPs in association with insulin resistance,[21,22] and even fewer associate them with prehypertension.[23]

In the present study, we investigated the association of the M235T polymorphism of the AGT gene and the C825T polymorphism of the GNB3 with insulin resistance in prehypertensive individuals.

MATERIALS AND METHODS

Study design and population

This is a cross-sectional observational study in which unrelated individuals were recruited from the Hypertension Unit of the 1st Department of Propaedeutic and Internal Medicine, "Laikon" General Hospital, Athens University Medical School, while the DNA extraction and PCR took place at the Laboratory

of the 1st Department of Cardiology, "Hippokration" General Hospital, Athens University Medical School. Individuals were divided into 3 groups: hypertensive (HTN), prehypertensive (PREHTN) and control group (NORMTN) under the JNC7 categories. All the patients had at least three office blood pressure (OBP) and one ambulatory blood pressure (ABP) measurements, except for the control group which had only the OBP. Body mass index (BMI) was calculated as weight (kg)/height(m).[2] All subjects included in the study were ≥18 years old and of Caucasian White Mediterranean European (Greek) origin. Informed consent was obtained from all the participants. Pregnant women and subjects with established coronary artery, cerebrovascular or peripheral arterial disease, congestive heart failure, valvular heart disease, chronic alcohol or drug abuse, morbid obesity and diabetes were excluded. At baseline, all study participants were measured under daily routine conditions and were subjected to a thorough screening program that included assessment of a detailed personal and family history, physical examination, determination of anthropometric parameters and measurement of various biochemical parameters, along with several other more specialized procedures. PREHTN and NORMTN subjects were not under treatment with antihypertensive or other medication.

Office and 24-hour Ambulatory Blood Pressure Measurement

Office BP measurement took place according to the ESC 2007 Guidelines for the management of arterial hypertension.[24] Patients sat for several minutes in a quiet room before beginning BP measurements. BP was measured at least twice, spaced by 1–2 minutes. A standard bladder (12–13 cm long and 35 cm wide) was used but a larger and a smaller bladder were also available for large and small arms, respectively. The cuff was at the heart level and BP was measured in both arms at the first visit to detect possible differences due to peripheral vascular disease. In this instance, the higher value was considered as the reference value. For those measurements OMRON 705 IT (Hoofddorp, The Netherlands device) was used. The 24-hour ambulatory blood pressure measurement was performed with the SpaceLabs Incorporation model 90207 monitoring system (Washington, USA).

Subjects were classified as hypertensive if they were receiving antihypertensive medication or if their mean daytime blood pressure was SBP>140 mmHg and/or DBP ≥90 mm Hg. Subjects classified in the prehypertensive group presented with SBP ≥120-139 mmHg and/or DBP=80-89 mmHg.

Laboratory Analyses

Venous blood was collected from subjects after an overnight fast. Total serum cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) and uric acid were determined with commercially available kits. Insulin resistance was determined with the homeostasis model (HOMA), first described by Matthews in 1985.[25] HOMA is a formula that assesses the function of beta cells and the insulin sensitivity using the values of fasting glucose and insulin. This model has been widely used since it was first published in its original form. The original model HOMA consists of a simple mathematical function: HOMA-IR = FRI (pU/ml)* FGR (mg/dl) / 405, where FRI is the fasting insulin concentration and FGR is the fasting plasma glucose, and 405 is a specific mathematical constant. Special attention was given to sampling, since insulin secretion is pulsatile. It is recommended that the mean of three samples taken be used at 5-minute intervals, which is theoretically better than just measuring one sample.[24] The 3 sample model was used in this study.

Genetic Analysis

Venous blood was collected in 5-mL tubes. Genomic DNA was isolated with the QIAamp DNA Blood Mini kit (cat.no 51106, Qiagen Corp. Germany) and stored at -18°C. Genetic analysis of the M235T polymorphism was investigated by polymerase chain reaction of genomic DNA followed by restriction endonuclease digestion.[26] Genomic DNA was used in a final volume of 50 uL containing 2.5 mmol/L MgC12, 50 mmol/L KC1, 10 mmol/L Tris hydrochloride (pH 9.0), 50 pmol of each primer (F 5'-GATGCGCA-CAAGGTCCTG-3' and R 5'-CAGGGTGCTGTC-CACACTGGCTCGC-3' Invitrogen Corp, Carlsbad, California USA), 250 μητοI/L each of the four dNTPs, Invitrogen Corp, Carlsbad, California USA) and 1 U Taq polymerase Invitrogen Corp, Carlsbad, California USA). A PCR device (Eppendorf Mastercycler, USA) was used for the procedure. There was an initial denaturation at 95°C for 10 minutes, followed by 30

one-minute cycles at 95˚C, 1 minute at 62˚C and 1.5 minutes at 72˚C. The final cycle was 10 minutes long at 72˚C and the final product was stored at 4˚C. The oligonucleotide is mismatched with genomic DNA, which created a SfaN1 (New England Biolabs, USA) restriction site during amplification. After enzymatic amplification of genomic DNA, each product was digested with SfaN1. TheM235 codon digestion with SfaNl yielded a 266-bp product as compared to the undigested 303-bp T235 product. The genotypes were analyzed by electrophoresis in 2% agarose gel (Applichem, Germany) and visualized by ethidium bromide staining (Fluka-Sigma, Aldrich, USA).

The same procedure was used for polymorphism C825T. Genomic DNA (250 ng) was amplified by polymerase chain reaction with 5-TGA CCC ACT TGC CAC CCG TGC-3 (forward primer) and 5-GCA GCA GCC AGG GCT GGC-3 (reverse primer) was used in a final volume of 50 uL containing 2.5 mmol/L MgCl-2, 50 mmol/L KC1, 10 mmol/L Tris hydrochloride (pH 9.0), 250 pmol of each primer and 1 U Taq Polymerase. There was an initial denaturation at 95˚C for 10 minutes, followed by 35 one-minute cycles at 94˚C, 1 minute at 65˚ and 1.5 minutes at 72˚C. The whole polymerase chain reaction mixture was subsequently digested overnight (16 hours) at 60˚ with 1 U of the restriction enzyme BsaJI (New England Biosystems), followed by denaturation at 80˚C for 20 minutes. BsaJI is an isochizomer of the restriction enzyme BseDI, which was used in the original protocol.[20] Fragments were separated on 2% agarose gels and visualized using ethidium bromide. The C allele was cut into 2 fragments of 116 and 152 bp, whereas the T allele remained uncut at 268 bp.

STATISTICAL ANALYSIS

The variables of the 3 groups were tested for normal distribution via the Kolmogorov-Smirnov test for normality and analyzed by the statistical method of one-way ANOVA and x^2 according to whether they were quantitative or categorical values. The least significant difference method was used for the post-hoc analysis between groups. In all cases, the statistical significance of the results was $p<0.05$. All measurements were registered and processed with statistical package SPSS ver. 17.0 (Chicago, Illinois, USA).

RESULTS

The population of the study consisted of three hundred and thirty subjects, of whom 149 were females and 181 males. Table 1 shows the clinical characteris-

Table 1. All continuous variables are expressed as mean ± standard deviation (SD). P-values Column refers to the ANOVA F test within groups

	HTN n1=114 (34%), 62 male 52 female Mean ± SD	PREHTN n2=106 (32%), 67 male 39 female Mean ± SD	NORMTN n3=110 (33%,) 52 male 58 female Mean ± SD	Total n=330 (100%), 181 male (55%), 149 female (45%) Mean ± SD	P value
Age	56.5 ± 11.6 *	48.4 ± 13.7 ***	46.7 ± 15.2	50.6 ± 14.2	**<0.001**
BMI	28.3 ± 4.2	28.5 ± 4.5	27.5 ± 5.3	28.1 ± 4.7	0.280
Glucose	98.1 ± 18.6	96.1 ± 11.3	93.2 ± 20.4	95.8 ± 17.5	0.130
Insulin	14.5 ± 9.3 *	15.5 ± 9.5 **	10.5 ± 10.8	13.3 ± 10.2	**0.002**
HOMA IR	3.1 ± 2.5 *	2.9 ± 2.6 **	2.2 ± 1.5	2.7 ± 2.2	**0.011**
Cholesterol	223.4 ± 46.8 *	217.6 ± 44.0 **	202.0 ± 46.3	214.2 ± 46.6	**0.002**
Triglycerid	136.7 ±78.5 *	129.3 ± 56.5 **	102.3 ± 50.0	122.4 ± 68.0	**0.001**
HDL	51.2 ± 13.0 *	48.1 ± 13.5	46.0 ± 13.2	48.5 ± 13.4	**0.020**
LDL	139.3 ± 45.5 *	129.9 ± 56.5	122.0 ± 45.0	130.1 ± 49.3	**0.040**
SBP 24h	138.3 ± 13.0	129.9 ± 6.8		133.6 ± 11.0	**<0.001**
DBP 24h	86.3 ± 12.4	80.3 ±7.5		83.1 ± 10.5	**<0.001**

HTN = hypertensive group, PREHTN = prehypertensive group, NORMTN = control group
* HTN vs NORMTN $p<0.05$, ** PREHTN vs NORMTN $p<0.05$, *** HTN vs PREHTN $p<0.05$.

tics of all groups. A statistical significance was found in insulin, HOMA-IR, cholesterol, triglycerid, HDL and LDL quantitative variables between the 3 groups. The post-hoc analysis revealed a statistically significant difference in the NORMTN against HTN and PREHTN values of cholesterol ($p=0.001$, $p=0.020$, respectively) and triglycerides ($p<0.001$, $p=0.007$). No statistical significance was observed between the hypertensive and prehypertensive groups for the previous variables. Similarly, there was a statistically significant difference in the NORMTN versus the HTN group regarding HDL ($p=0.005$) and LDL ($p=0.010$).

HOMA-IR was higher in the HTN and PREHTN groups as compared to the NORMTN ($p=0.006$ and $p=0.020$, respectively), while there was no statistically significant difference between the first 2 groups ($p=0.590$). Insulin yielded similar results (NORMTN vs. HTN $p=0.010$, NORMTN vs. PREHTN $p=0.001$) with no statistical significance between the first 2 groups ($p=0.520$).

There was no statistical difference in the frequency of polymorphisms of the genes studied among the 3 groups. The distribution is shown in Figures 2 and 3. There was also no significant difference between the alleles of the AGT M235T polymorphism and these

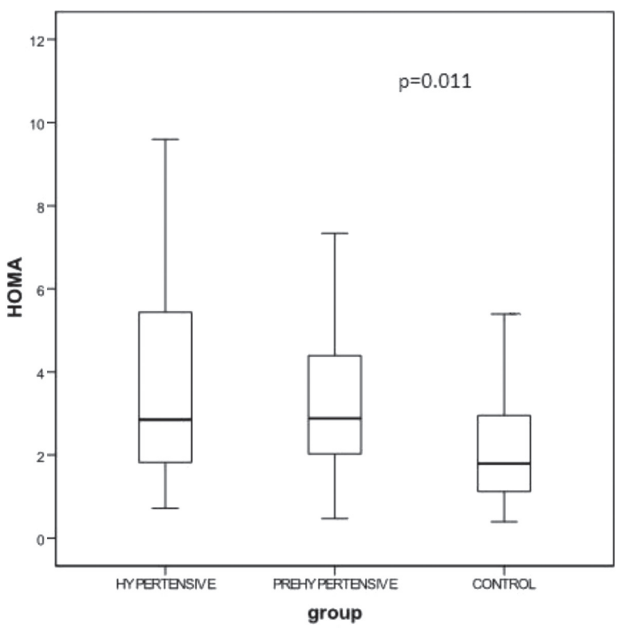

Figure 1. Means of HOMA-IR between groups.

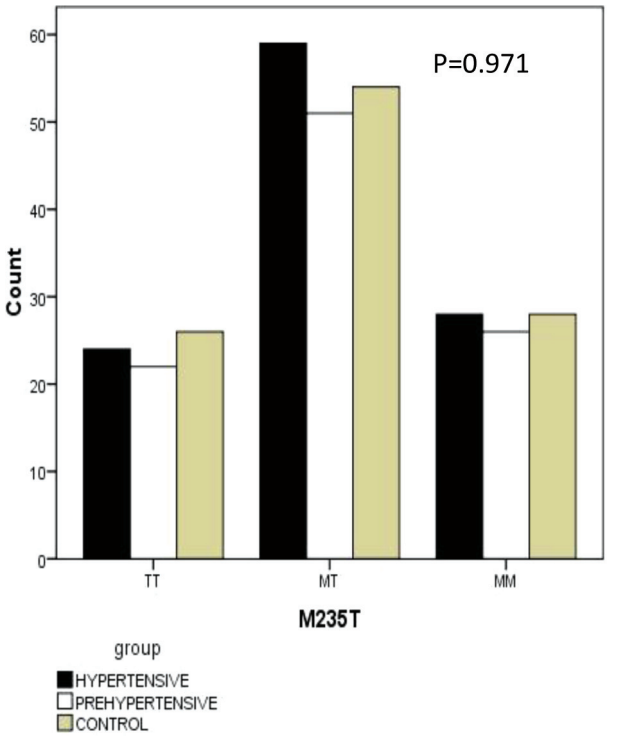

Figure 2. Distribution of M235T AGT gene.

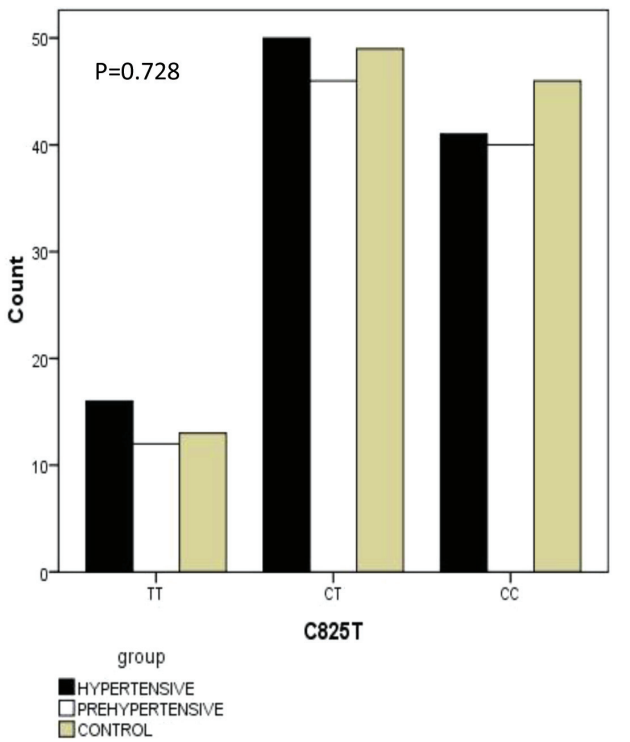

Figure 3. Distribution of C825T GNB3 gene.

variables, nor was there a statistically significant difference between the 3 groups with regard to qualitative variables (sex, insulin resistance, obesity). As for the GNB3 C825T polymorphism, the results indicated that the T allele (CT/TT vs. CC) was associated with an increased BMI (TT 29.5±4.4, CT 29.1±4.7, CC 27.6±3.9, p=0.004).

DISCUSSION

The aim of this study was to establish the distribution frequency of 2 polymorphisms, namely C825T and M235T, in the Greek population, as well as their correlation with hypertension and insulin resistance, especially in people with borderline blood pressure, i.e. prehypertensives.

Prehypertension was associated with insulin resistance in the large MESYAS study[27] and our results are in agreement with this observation (HTN vs. NORMTN p=0.006, PREHTN vs. NORMTN p=0.016). However, the correlation between the AGT and GNB3 genes and insulin resistance is unknown.

In our study, the BMI was similar in all 3 groups. Statistical analysis showed that cholesterol and triglycerides in both the hypertensive and prehypertensive groups were different compared with the control group, with these biochemical markers being higher in the first two groups (Cholesterol: HTN vs. NORMTN p=0.001, PREHTN vs. NORMTN p=0.018, HTN vs. PREHTN p=0.377), (TG: HTN vs. NORMTN p=0.001, PREHTN vs. NORMTN p=0.007, HTN vs. PREHTN p=0.464). Furthermore, a statistically significant difference was observed for HDL and LDL, but this occurred only in the hypertensive group (HDL: HTN vs. NORMTN p=0.005, PREHTN vs. NORMTN p=0.285, HPN vs. PREHTN p=0.117), (LDL: HTN vs. NORMTN p=0.013, PREHTN vs. NORMTN p=0.307, HTN vs. PREHTN p=0.163). These results can be explained considering that both the hypertensive and the prehypertensive group had a higher HOMA index (insulin resistance) compared with the control group. Unlike what happens in normal subjects, in these groups insulin failed to suppress the production of VLDL apoB, although it suppressed the concentrations of NEFA[28] (Non-Esterified Fatty Acids). Insulin resistance seems to be a significant contributor to the mechanism underlying the increase in serum triglycerides in prehypertensive and hypertensive patients.[29] HDL in blood is reduced in insulin-resistant patients with high serum triglycerides. HDL particles are enriched with triglycerides, which make them a good substrate for hepatic lipase, which then removes them at an accelerated pace. Abnormal activity of lipoprotein lipase (LPL) may further reduce the levels of HDL by decreasing the conversion of HDL3 to HDL2.[30] Impaired insulin action in intravascular lipolysis, reflects the action of LPL in adipose tissue that hydrolyzes triglycerides in chylomicrons and VLDL, thus providing adipocytes with NEFA.[31] It is known that insulin strongly increases LPL activity in adipose tissue,[32,33] which seems to be associated with subjects with insulin-resistance.[34]

The polymorphisms examined have been extensively studied in previous studies. However, there are limited findings and conclusions in the prehypertensive group. Angiotensinogen has long been regarded simply as a “tank” which actives renin. Several studies strongly suggest that it is associated with essential hypertension and in some cases with gestational hypertension. It has been shown that a molecular variant of AGT (position 235T/-6A) is associated with an increased rate of gene transcription of AGT, which could, in turn, lead to small increases in AGT concentrations in plasma and tissues. The plasma and tissue concentration of AGT causes overstimulation of the renin, induces renal sodium reabsorption, vascular hypertrophy and/or increased activity in the sympathetic nervous system and predisposes to the development of common cardiovascular diseases. Controversial results exist in the literature and new studies are constantly emerging. Our study did not find any difference in the frequency or in the qualitative and quantitative variables of the gene polymorphisms of AGT M235T in the 3 groups studied, which agrees with the results found by by Cauifield et al,[25] Barley et al,[35] Fornage et al,[36] Hingorani et al,[37] Kiema et al[38] and the meta-analysis of Mondry et al.[39]

The G protein contains more than 1000 receptors, including enzymes and ion channels. G protein consists of α, β and γ subunits. The activity of the G protein is regulated by the binding of a substrate, leading to the hydrolysis of GTP. The C825T polymorphism is associated with an increased intracellular

signal transduction and carriers of this allele have an increased chemotactic response that has been associated with hypertension, obesity and possibly insulin resistance. As demonstrated by our results, the T allele carriers (CT/TT vs. CC) are more often overweight. This finding is in agreement with many previous studies.[22,40-43] There are ongoing studies using compounds for weight reduction (sibutramine) in individuals carrying the T allele polymorphism in the C825T GNB3.[44,45] Our results did not demonstrate a significant correlation between insulin resistance and hypertension.

CONCLUSION

This study failed to demonstrate a correlation between specific SNPs and blood pressure or insulin resistance in the 3 groups. However, T allele carriers of the C825T polymorphism were found to have an increased BMI. Similarly, increased insulin resistance and lipidemia were more commonly observed in the hypertensive and prehypertensive populations.

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