**RESEARCH**
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# THE RELATIONSHIP BETWEEN GENETIC VARIATIONS IN MTHFR C677T AND MTRR A66G GENES IN METABOLIC SYNDROME PATIENTS IN THE DEMOGRAPHIC OF NORTH INDIA

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**Abstract**
**Introduction:** Metabolic Syndrome (MetS) presents a complex interplay of metabolic factors contributing to cardiovascular diseases and type 2 diabetes, with genetic variations being implicated in its etiology.
**Methods:** This study explores the relationship between genetic variations in MTHFR C677T and MTRR A66G genes and MetS in a North Indian demographic. A case-control study involving 400 individuals was conducted, with clinical, demographic, and molecular parameters assessed.
**Results:** Genotyping analysis revealed no significant correlation between MTHFR C677T variation and MetS, as evidenced by adherence to Hardy-Weinberg equilibrium and nonsignificant chi-square values. Similarly, MTRR A66G variation showed no substantial association with MetS.
**Conclusion:** These findings suggest that these genetic variations may not confer susceptibility to MetS in this population. Further research is warranted to elucidate the role of these genes in other metabolic disorders and populations.
Keyword: Metabolic Syndrome, Systolic Blood Pressure, Diastolic Blood Pressure, Genotype, Allelic Frequency

## INTRODUCTION

Metabolic syndrome (MetS) is distinguished by the aggregation of metabolic factors such as abdominal obesity (1), dyslipidemia, elevated blood pressure, and cardiovascular disease (CVD) with an escalated susceptibility to type 2 diabetes (T2DM)(2), and sedentary lifestyles that are pervasive on a global scale contribute significantly to the emergence of MetS (3,4), a condition that is impacted by a multitude of genetic, environmental, and lifestyle factors (5-7). Various research endeavors have delved into the correlation between MetS and hyperhomocysteinemia (HHcy), which denotes an elevated level of homocysteine (Hcy), an amino acid that stems from methionine metabolism (8-13).

HHcy has been associated with DNA methylation which leads to endothelial dysfunction, cardiovascular disease, and insulin resistance (IR), all of which play pivotal roles in the development of MetS (11, 14, 16). The two most important genetic determinants such as methionine synthase reductase (MTRR) A66G polymorphisms and another methylenetetrahydrofolate reductase (MTHFR) C677T are crucial in modulating plasma Hcy levels. The responsibility of the MTHFR gene lies in the conversion process of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF), which is a crucial stage within the Homocysteine (Hcy) metabolic pathway (5) and its C677T polymorphism leads to a reduction in enzyme activity, consequently causing an increase in Hcy levels and a decrease in DNA methylation (15). Similarly, the MTRR gene aids in Hcy remethylation to methionine, and its A66G polymorphism detrimentally impacts enzyme activity, thereby contributing to HHcy and DNA hypomethylation.

Several studies propose a potential impact of the MTRR A66G polymorphism and MTHFR C677T level of plasma Hcy (17). These genetic variances can predispose individuals to dyslipidemia, hypertension, diabetes, and obesity, all of which are integral components in the diagnosis of MetS. Current research findings suggest that MTHFR C677T may exert a more pronounced influence on the components of MetS in comparison to MTRR A66G.

The researchers have recommended the role of MTHFR C677T plays a more significant role in components of MetS compared to MTRR A66G gene polymorphism
Hypothesizing a potential relationship between MTRR A66G polymorphisms and another MTHFR C677T with Metabolic Syndrome in the Indian population, a case-control study was performed to explore this hypothesis among individuals predominantly situated in North India.

## MATERIALS AND METHODS

**Participants**:
A 30-week randomized case-controlled study was implemented at King George's Medical University situated in Lucknow, Uttar Pradesh, to scrutinize the effects of a specific intervention on a selected set of participants. During the period between January 2019 to March 2023, 400 individuals between the age group 20 and 70 yrs were selected as per specific criteria of the study and they were requested to join the research initiative. Upon their agreement to take part in the conducted study, their voluntary informed consent forms were duly verified and obtained. Furthermore, the individuals provided written consent for venipuncture procedures and were informed about the intended purpose of the collected samples. The recruitment process involved 400 individuals from King George's Medical University in Lucknow, who were chosen through a multistage sampling. Among the total number of participants, 200 individuals met the criteria for being categorized as cases, while the remaining 200 individuals were classified as controls.

**Ethical considerations**
This investigation received consent from the Institutional Ethics Committee (IEC) of KGMU, Lucknow, with reference code 99th ECM II Ph D/P2, indicating compliance with ethical standards in research involving human subjects. Furthermore, the study was meticulously documented and registered under the Clinical Trials Registry (CTRI), specifically under the registration number as CTRI/2020/07/026318, demonstrating transparency and adherence to regulatory protocols. The thorough review and endorsement by the ethics committee underscore the commitment to upholding the welfare and rights of participants, ensuring that the research was conducted ethically and with due consideration for ethical guidelines. By adhering to these established procedures and regulations, the study reinforces the importance of ethical integrity and accountability in scientific inquiry.

**Inclusion criteria**:
1. Metabolic syndrome can be stated when patients meet atleast three out of five specified criteria:
- Abdominal obesity, is characterized by a waist circumference of ≥ 90 cm (35.4 in) men and in women ≥ 85 cm (33.4 inches), or body mass index ≥ 22.9 kg/m2.
- HDL cholesterol level in male ≤40 mg/dl and in female ≤ 50 mg/dl
- Fasting blood glucose (FBG) ≥ 100 mg/dl
- Elevated serum triglyceride levels ≥ 150 mg/dl.
- Systolic blood pressure (SBP) ≥ 130 (mmHg) or/also diastolic blood pressure (DBP) ≥ 85 (mmHg).

2. Additionally, individuals who expressed a willingness to participate were enrolled in the study.

**Exclusion criteria**:
The study excluded individuals falling within the subsequent categories:
(a) Individuals following a particular regulated dietary regimen for any given cause within the preceding two months.
(b) Individuals who were not capable or unwilling to engage in the study. (c) Individuals who were currently consuming flaxseed, flaxseed oil, omega-3, insulin, or hypoglycemic medications.
(d) Expectant and nursing mothers.

**Clinical and laboratory evaluation**
**Data acquisition and detection**
Data about various demographic factors such as age, gender, medical history, and familial medical background were meticulously gathered and documented for analysis. To ensure standardized conditions, all participants refrained from consuming any food for a period exceeding 8 hours. After the overnight fasting period, measurements of blood pressure, BMI, height, waist circumference weight, and hip circumference were meticulously conducted. Additionally, the lipid profile and fasting plasma glucose (FPG) of collected samples were accurately determined through laboratory analysis.

**Laboratory evaluation**
5 milliliters of blood were from the vein of the front part of the humerus in the early hours following 12 hours of abstaining from food and refraining from taking any medication, and this process was completed within half an hour of the collection. The blood samples, measuring 5 mL, were obtained and placed in sterile tubes that contained EDTA (Vacutainer®). All the necessary material used was promptly ice placed, then the serum was isolated through centrifugation at around 1600 times gravity for about 10 minutes at' $4^0$C. Biochemical tests conducted on the serum included assessments of fasting glucose levels (FGS), HDL cholesterol, and Triglycerides at the beginning of the study and again after 12 weeks at end of the study. The various constituents of the serum lipid profile were analyzed using established enzymatic colorimetric methods. After separation, the serum was divided into smaller portions and stored at a freezing temperature of -80 degrees Celsius until it was required for further analysis and experimentation.

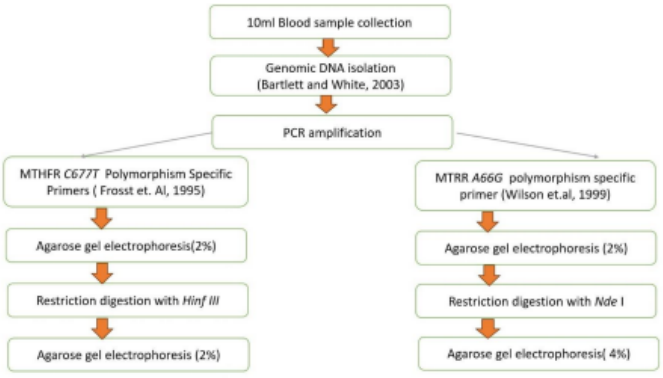


**Fig: 01 Study Design of MTHFR and MTRR gene polymorphism**

**1) Statistical analysis**
The allelic and genotypic frequencies in both the intervention and control groups were determined through the direct-counting method. To assess nonconformity from Hardy Weinberg equilibrium (HWE) and matched allelic frequencies between

two groups, $\chi 2$ analysis was conducted. The odds ratio (OR) 95% confidence interval was computed to determine the relative risk associated with various alleles and genotypes. The p-value < 0.05 was considered a significant level. All calculations were completed using the software Graph pad Prism.

**RESULTS:**

In a case-control study of 400 candidates, the case was divided ass 200 patients, and 200 as control were collected from King George's Medical University, Lucknow.

**(a) Clinical and Demographic Parameters**

Table 1 shows clinical and demographic features of both intervention and control group. It is evident from the data that there was a notable similarity in the sex distribution and age among individuals in both groups. Nevertheless, a closer examination reveals substantial variations between the two cohorts in terms of key parameters such as anthropometric parameters, clinical parameters, and lipid profile parameters. These variations underscore the significance of considering various factors to help in diagnosing MetS.

| Characteristics | Intervention Group n= 200 | | Control Group n=200 | | |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | P value |
| Mean Age | 53.00 ± 5.6 | 47.00 ± 8.1 | 51.80 ± 2.3 | 50.44 ± 5.9 | 0.4759 |
| Sex (%) | 61% | 39% | 51% | 49% | --- |
| Weight (kg) | 79 ± 4.3 | 67 ± 6.85 | 75 ± 2.5 | 60 ± 3.6 | 0.0031* |
| Waist circumference (cm) | 90.7 ± 5.8 | 84 ± 3.7 | 88.61 ± 4.4 | 83.49 ± 7.2 | 0.0205* |
| Exercise duration | 30–40 mins | 20–30 mins | 20–30 mins | 10–20 mins | --- |
| Smoker % | 47% | 12% | 55% | 8% | 0.5481 |
| Nonsmoker % | 53% | 89% | 45% | 92% | --- |
| Residence (Urban) | 77% | 69% | 81% | 88% | --- |
| Residence (Rural) | 23% | 31% | 19% | 12% | --- |
| BMI (kg/m$^2$) | 30.79 ± 3.12 | 28.61 ± 9.41 | 23 ± 7.51 | 22.44 ± 9.17 | 0.0321* |
| SBP (mmHg) | 133.92 ± 18.50 | | 110.61 ± 1.59 | | <0.0001 |
| DBP (mmHg) | 85.27 ± 2.17 | | 75.05 ± 1.85 | | <0.0001 |
| TG (mmol/L) | 285.55 ± 3.80 | | 139.37 ± 2.37 | | <0.0001 |
| HDL-C(mmol/L) | 56.03 ± 2.74 | | 64.7 ± 1.05 | | <0.0001 |
| LDL-C(mmol/L) | 152.95 ± 7.81 | | 107.97 ± 6.24 | | 0.0021 |

Table 1: Data representing parameters of male and female participants in both intervention and control groups. Comparison is made between the intervention group and the control group. Data is presented as mean ± SD (N=400). P value was calculated using one-way ANOVA with Holm–Sidak method; the (*) marks indicate statistical significance (P <0.05). SBP; systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

**Molecular Parameters under Consideration in Intervention Group and Control Group Individuals**

**MTHFR C677T mutation analysis**

MOLECULAR ANALYSIS OF MTHFR GENE.

Though the study includes 200 cases and 200 controls, this number varies for every.

marker based on the result obtained after DNA analysis

MTHFR gene polymorphisms

MTHFR C677T genotyping analysis was performed using specific primers which gave a PCR product of 198 bp band.

The sequence of forward and reverse primers was as follows:

Figure 01: DNA band showing MTHFR gene of 198bp

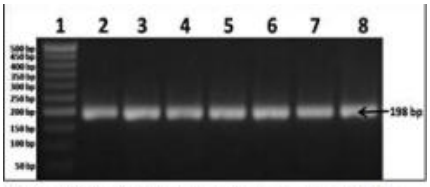


Figure 01: Dna Gel electrphoresis showing the MTHFR gene of 198 Bp With Ladder sequence.

**Forward primer 5 –**
**'TGAAGGAGAAGGTGTCTGCGGGA-3'**
**Reverse primer 5- 'AGGACGGTGCGGTGAGAGTG-3'**

PCR reaction was performed in total 15 µl and 30 cycles of PCR were run. **PCR-RFLP DIGESTION**

**MTHFR C677T allelic variants were genotyped by PCR-RFLP methods of Frosst et al. (1995).**

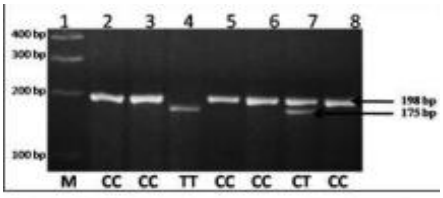


Figure 02: DNA gel showing the restriction digestion of MTHFR gene

Upon digestion of a 198bp DNA fragment using the Hinf I restriction enzyme, bands were observed at 198bp, 175bp, and 23bp in size. The uncut 198bp band was identified as representing the CC genotype, while the 175bp band was designated as the TT.

genotype. The presence of all three bands together indicated a heterozygous CT genotype.

MTHFR C677T mutation analysis

In the case of samples collected for MTHFR C677T, it was observed that the number of individuals with CC genotype was 150, individuals with CT genotype was 48, and individuals with TT genotype was 2. On the other hand, in the control sample, the count of individuals with the CC genotype was 168, individuals with the CT genotype was 32, and individuals with the TT genotype was 1. The analysis further revealed that in the intervention group the allelic frequency for the C allele was 0.92, while for the T allele, it was 0.08. Moreover, the frequency in the control group of the C allele alone was determined to be 0.87, whereas the frequency of the T allele was calculated to be 0.13.

**Table 02: Showing allelic frequency of MTHFR gene in a case-control study.**

| Subject | Genotype | | | No. of alleles | | Allelic frequency | | Genotypic frequency | | | X2 | P value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CC | CT | TT | C | T | C | T | CC | CT | TT | | |
| Case | 150 | 48 | 2 | 174 | 26 | 0.87 | 0.13 | 0.76 | 0.23 | 0.0169 | 0.1179 | 0.731 |
| Control | 168 | 32 | 1 | 184 | 17 | 0.92 | 0.08 | 0.85 | 0.15 | 0.0064 | 0.159 | 0.690 |

When conducting the chi-square test amid intervention and control groups, results indicated a chi-square value of 0.1179

and 0.159 for the intervention and control group respectively, with corresponding p-values of 0.731 and 0.690, respectively.

**Association of MTHFR C677T with MetS patients and Control**

| Variable | Case | Control | ODD RATIO | 95% CI | P- VALUE |
|---|---|---|---|---|---|
| Codominant | | | | | |
| CC | 150 | 167 | 1 | Ref | |
| CT | 42 | 32 | 1.47 | 0.877-2.433 | 0.072 |
| TT | 2 | 1 | 2.23 | 0.2-24.81 | 0.2575 |
| Dominant | | | | | |
| CC | 150 | 167 | 1 | Ref | |
| CT+TT | 50 | 33 | 1.69 | 1.03-2.76 | 0.0185 |
| Recessive | | | | | |
| CC+CT | 198 | 199 | 1 | Ref | |
| TT | 2 | 1 | 2.01 | 0.18-22.35 | 0.284 |
| Alleles | | | | | |
| C | 348 | 366 | 1 | Ref | |
| T | 100 | 66 | 0.435 | 0.297-0.638 | 0.127 |

Table 03: Representing the ODD RATIO and P value of MTHFR gene polymorphism.

**MTRR A66G mutation analysis**

MOLECULAR ANALYSIS:

Though the study includes 200 cases and 200 controls, this number varies for every marker based on the result obtained after DNA analysis

MTRR A66G gene polymorphisms

DNA ANALYSIS:

MTRR A66G genotyping analysis was performed using specific primers which gave a PCR product of 66 bp.

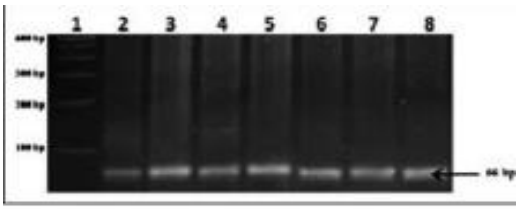


Figure 03: DNA gel Showing MTRR gene of 66bp

**Forward primer- 5-**
**'GCAAAGGCCATCGCAGAAGACAT-3'**
**Reverse primer – 5-**
**'GTGAAGATCTGCAGAAAATCCATGTA-3'**

PCR reaction was performed in total 15 µl and 30 cycles of PCR were run.

**PRCR- RFLP DIGESTION**

**MTRR A66G polymorphism identification was done by PCR-RFLP according to the methods of Wilson et al. (1999).**

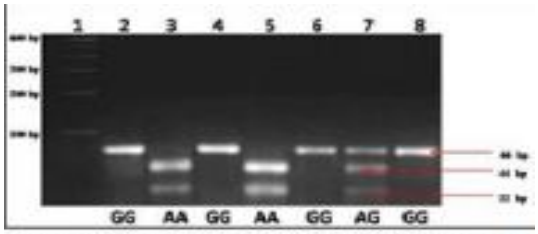


Figure 04: DNA band showing Restriction digestion of MTRR gene

Upon digestion of a 66 bp DNA fragment using the Ndel I restriction enzyme, bands were observed at 66 bp, 44 bp, and 22 bp in size. The uncut 66 bp band was identified as representing the GG genotype, while the 44 bp, 22 bp band was designated as the AG genotype.

**MTRR A66G mutation analysis**

In the investigation concerning samples for MTRR A66G, it was observed that the quantities of AA, AG, and GG genotypes were 19, 121, and 60 respectively. Specifically, the frequency of the A allele was determined to be 0.39, while the frequency of the G allele was calculated to be 0.60 within the case group.

On the other hand, in the control sample, the numerical values for AA, AG, and GG genotypes were reported as 11, 124, and 65 respectively. Notably, the allelic frequencies for the A and G alleles were ascertained to be 0.36 and 0.635 in the control group.

| Table 05: Representing the Genotype and allelic Frequency of MTRR gene polymorphism. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subjects | Genotype | | | No. of alleles | | Allelic frequency | | X2 | P value |
| | AA | AG | GG | A | G | A | G | | |
| Case | 19 | 121 | 60 | 159 | 241 | 0.39 | 0.60 | 13.84 | 0.0001 |
| Control | 11 | 124 | 65 | 146 | 254 | 0.365 | 0.635 | 22.78 | 0.0018 |

When conducting the chi-square test amid intervention and control groups, the results indicated a chi-square value of 13.84 and 22.78 for the intervention and control group respectively, with corresponding p-values of 0.0001 and 0.0018, respectively.

**Association of MTRR A66G with MetS patients and Control.**

| Table 06: Representing the ODD ratio and p-value of prevalence of MTRR gene polymorphism. | | | | | |
|---|---|---|---|---|---|
| **Variable** | **Case** | **Control** | **ODD RATIO** | **95% CI** | **P- VALUE** |
| **Codominant** | | | | | |
| AA | 19 | 11 | 1 | **Ref** | |
| AG | 121 | 124 | 0.56 | 0.258-1.237 | 0.07 |
| GG | 60 | 65 | 0.534 | 0.235-1.21 | 0.06 |
| **Dominant** | | | | | |
| AA | 19 | 11 | | **Ref** | |
| AG+GG | 181 | 189 | 0.554 | 0.257-1.198 | 0.06 |
| **Recessive** | | | | | |
| AA+AG | 140 | 135 | | **Ref** | |
| GG | 60 | 65 | 0.89 | 0.583-1.35 | 0.29 |
| **Alleles** | | | | | |
| A | 159 | 146 | | **Ref** | |
| G | 241 | 254 | 0.87 | 0.655-1.15 | 0.17 |
| | | | | | |

## DISCUSSION

### MTHFR C677T mutation analysis

Though the study includes 200 cases and 200 controls, this number varies for every marker based on the result obtained after DNA analysis

Upon digestion of a 198bp DNA fragment using the Hinf I restriction enzyme, bands were observed at 198bp, 175bp, and 23bp in size. The uncut 198bp band was identified as representing the CC genotype, while the 175bp band was designated as the TT genotype. The presence of all three bands together indicated a heterozygous CT genotype

The analysis of the MTHFR C677T mutation is a topic of discussion in this study. In the control group, the distribution of CC, CT, and TT genotypes was 84%, 16%, and 0.5%, respectively, while in the MetS group, the distribution was 75%, 24%, and 1%, respectively. It is noteworthy that the genotype distribution of this genetic variation followed the Hardy-Weinberg equilibrium in both the control group, as evidenced by a chi-square value ($X^2$) 0.1179, p-value of 0.731, and the intervention group shows a chi-square value 0.159 and a p-value 0.69.

When comparing allele frequencies, the odds ratio (OR) for the T allele was found to be 0.435 (95% - CI 0.297-0.638, p value 0.127) with C allele serving as the reference. These results suggest that the MTHFR C677T gene variation may not confer genetic susceptibility to MetS. Furthermore, the chi-square value in both the case and control groups falls below the degrees of freedom, while the p-value remains statistically insignificant, indicating a deviation from Hardy-Weinberg equilibrium principles.

In conclusion, based on the findings of our research project, it can be inferred that the presence of the MTHFR gene does not exhibit a statistically significant correlation with individuals

diagnosed with Metabolic Syndrome (MetS). This conclusion is drawn from the analysis of
data collected and analyzed through rigorous scientific methodologies, suggesting that other genetic or environmental factors may play a more prominent role in the development or manifestation of MetS among patients.

**MTRR A66G mutation analysis**
**MOLECULAR ANALYSIS:**
The chi-square test serves as a statistical tool utilized to assess the independence of observed and expected frequencies within a contingency table, commonly employed in research to examine the relationship between variables. Typically, this test is applied to investigate whether there exists a significant difference in the distribution of genotypes (AA, AG, GG) between the case group and the control group, shedding light on potential associations between genetic factors and certain conditions under scrutiny. When the p-value associated with the chi-square test is notably low, such as below 0.05, it signifies a statistically meaningful distinction between the anticipated and actual frequencies, thus implying that the genotypic distribution is not random but rather influenced by the group classification (case or control).
The elevated chi-square values observed in the control group relative to the case group point towards a more pronounced departure from the expected genotype frequencies among individuals in the control cohort, which in turn could signify a stronger correlation between MTRR A66G genotypes and the control set. These findings hint at a potential link between MTRR A66G genotypes and the targeted health condition, particularly evident within the control group where both the chi-square value and the p-value suggest a substantial deviation from the expected genotype distribution.
Based on the outcomes of our investigation, it is plausible to infer that no significant relationship exists between MTRR gene polymorphism and the subgroup of patients diagnosed with Metabolic Syndrome, implying a lack of association between this genetic variant and the specified health condition within the study population.

**CONCLUSION**
Based on the comprehensive analysis undertaken within the framework of this research endeavor, a definitive assertion can be made that there exists no substantial correlation between the genetic variation of the MTHFR C677T gene and individuals afflicted with Metabolic Syndrome (MetS). The observed genotype distribution within both the control group and the MetS cohort adhered to the principles of the Hardy-Weinberg equilibrium, thereby signifying
an absence of inherent genetic predisposition to MetS stemming from this specific gene variant. These findings underscore the intricate interplay between genetic factors and metabolic disorders, shedding light on the nuanced complexities that govern the pathogenesis of MetS.
Furthermore, the comparison of allele frequencies revealed an odds ratio (OR) for the T allele that did not show a statistically significant association with MetS.
Thus, further research is warranted to explore the potential role of the MTHFR gene variation in other metabolic disorders and patient populations to fully elucidate its impact. Our study found no significant association between MTRR gene polymorphism and Metabolic Syndrome among the study population. While the chi-square test suggested a potential correlation between MTRR A66G genotypes and the control group, further analysis revealed no meaningful relationship with the targeted health condition. Thus, these findings indicate a lack of association between this genetic variant and Metabolic Syndrome in our study cohort.

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**Conflicts of Interest:**
The authors declare no conflict of interest.

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