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Title:	**Genetic Association of AGT rs699 with Hypertension in a Population from Rawalpindi, Pakistan**
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Genetic Association of AGT rs699 with Hypertension in a Population from Rawalpindi, Pakistan

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ABSTRACT

Background. Hypertension is a widespread chronic illness, and ranks as one of the leading causes of death. Some complications of having chronic hypertension include cardiovascular diseases (CVDs), strokes, and chronic renal diseases. Evidence from epidemiological studies represent that hereditary factors and genes are involved in the etiology of hypertension. Therefore, the current study aimed to discuss the association of rs699 point mutation of AGT gene, which results in the M268T substitution (Methionine>Threonine). The objective of this study was to investigate the association of hypertension with the SNP rs699 of AGT gene.

Methods. A total of 80 participants were included in this case-control study, with 50 hypertension patients and 30 controls. The blood samples were collected from multiple hospitals in Rawalpindi district. The genomic DNA was isolated. Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method was used to find allele frequencies.

Results. Genotypic analysis indicated a strong association of T allele of rs699 with hypertension. For T allele, both heterozygotes and homozygotes were found to have an elevated risk of hypertension. The study found that the odds ratio of TT genotype was 3.5 times higher for patients than those who had the CC genotype (OR=3.5), 95% Confidence Interval (CI) indicating a higher risk of hypertension. Whereas, the odds of disease in individuals with the "CT" genotype were lower than those with the "CC" genotype (OR=0.5), 95% CI."

Conclusion. These findings demonstrated that rs699 polymorphism of AGT gene is significantly associated with the susceptibility to hypertension.

Keywords: AGT gene, ARMS PCR, hypertension, rs699

Highlights

- The study focused on the polymorphism of the AGT gene, rs699 (M268T), in relation to hypertension in the Pakistani people of Rawalpindi area.
- A case-control study involving 80 participants was carried out using ARMS-PCR for genotyping analysis.
- The results indicated that the carriers of the T allele in rs699 are significantly at a

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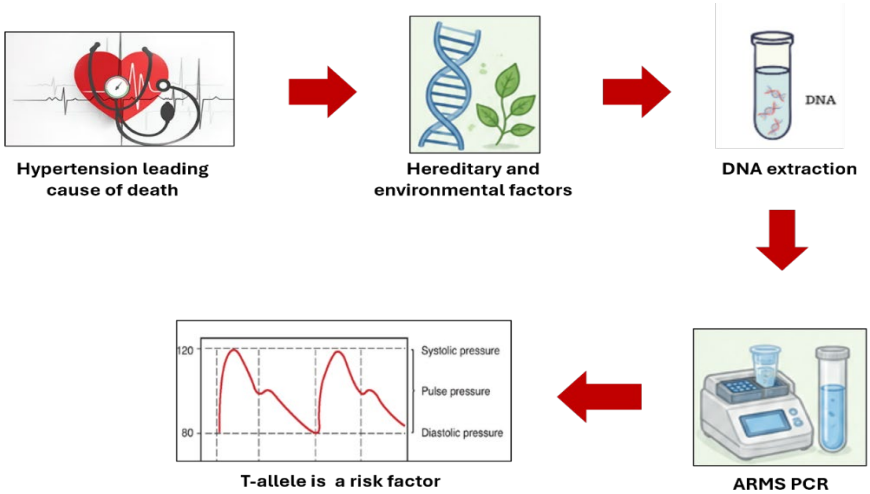
higher risk of hypertension in both heterozygous and homozygous models.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Hypertension is quite an important yet preventable risk factor towards renal impairment and cardiac diseases leading to fetal cases [1]. More than 1.3 billion adults worldwide are estimated to have high blood pressure. In 2010, 31.1% of the adult population (1.39 billion individuals) worldwide suffered from hypertension, defined as systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg [2]. World Health Organization (WHO) has ranked hypertension as the 10th leading cause of death in the world's mortality ranking. The global estimates attribute around 17 million deaths to cardiovascular diseases (CVDs) each year, out of which over 7 million are due to hypertension [3]. Moreover, another study described the prevalence of hypertension in Asian countries to range from 15–35% in urban adults, while rural population showed two to three times lower their prevalence [4]. In Pakistan, the overall prevalence of hypertension in people above 15 years of age is about 18%, with 16.2% in rural areas and 21.6% in urban areas. The proportion of patients with adequately controlled hypertension remains disquietingly low. About 70% of hypertensive individuals in Pakistan remain unaware of their health situation, including about 5.5 million men and 5.3 million women. Further, less than 3% of the patients achieve effective blood pressure control even with medication [5–7].

Around one-third of people aged more than 45 years in Pakistan are said to be hypertensive. The risk factors causing this include lifestyle, stress, obesity, high sodium and low potassium diets, aging, and/or diabetes [8,9].

As many as 28% of the population in South Asia and 46% in Africa have hypertension [10]. The prevalence of hypertensive disorders has been increasing steadily across the world due to the growing proportion of the elderly people being exposed to lifestyle risk factors. These include unhealthy diets characterized by excessive salt intake and/or potassium intake, as well as saturated fats, cholesterol, and/or caloric intake, and inactivity [2]. However, there

are variations in the prevalence of hypertensive disorders. In the previous twenty years, there has been a slight decrease in the prevalence of hypertensive disorders in countries with developing economies and high income. On the other hand, a considerable upsurge has been witnessed in the prevalence of hypertensive disorders in countries with low or medium level economy [11]. Despite advances in the technology of blood pressure measurement and the availability of safe and effective antihypertensive medications, the majority of patients still fail to receive a diagnosis and achieve BP with target values comparable to those of healthy individuals [12]. Hypertension is nearly twice as common among people suffering from diabetes as among people without diabetes, though the trend is even stronger among Mexican-Americans and African-Americans [13,14].

According to the guidelines from American College of Cardiology/American Heart Association (ACC/AHA) and European Society of Cardiology/European Society of Hypertension (ESC/ESH) in 2018, the criteria for blood pressure goals has been defined differently: <130/80 mmHg for ACC/AHA and <140/90 mmHg for ESC/ESH. Some other differences lie in the categorization of risk and management strategies for individuals with systolic blood pressure range, that is, 130-139 mmHg and diastolic pressures of 70-79 mmHg. This puts them under the hypertensive category of ACC/AHA but not ESC/ESH. The reduced cut-offs in the new guidelines led to an increased number of people being classified as hypertensive [15].

Hypertension is a multifactorial condition, involving both genes and environmental factors, and the number of cases in Pakistan is escalating day by day [16]. Some important factors relevant to hypertension include age of patients, which plays an important role. This is because as we get older, there is a greater chance of carrying this disease. Race and ethnicity are also among the important factors in causing hypertension, as black people face hypertension more than white people. Hispanics, Pacific Islanders, Asians, Alaska Natives, or American Indians, are mostly hypertensive. Similarly, it was found that hypertension levels vary between genders, and women have a higher tendency to develop hypertension issues with increasing age as compared to men [17]. Several genes are also associated with hypertension. These include caveolin-1 (CAV1), angiotensinogen (AGT), renin (REN), striatin (STRN), adducin-1 (ADD1), beta-2 adrenergic receptor (ADRB2), cytochrome P450 family 11 subfamily B member 2 (CYP11B2), cytochrome P450 family 17 subfamily A member 1 (CYP17A1), endothelin-1 (EDN1), estrogen receptor beta (ESR2), lysine-specific demethylase 1 (LSD1/KDM1A), serum- and glucocorticoid-inducible kinase 1 (SGK1), angiotensin-converting enzyme (ACE), bradykinin receptor B2 (BDKRB2), electrogenic sodium bicarbonate cotransporter 4 (SLC4A5), plasma membrane calcium-transporting ATPase 1 (ATP2B1), serine/threonine kinase 39 (STK39), Renin-Angiotensin system (RAS), WNK lysine deficient protein kinase 1 (WNK1), angiotensin receptor type 1 (AGTR1), fibroblast growth factor 5 (FGF5), and G protein subunit beta 3 (GNB3) gene [18].

An important gene among these is angiotensinogen (AGT). This is an essential player of

the renin-angiotensin-aldosterone system that regulates water and sodium homeostasis in body, thus affecting blood pressure [19]. The rs699 polymorphism (T>C substitution) in exon 2 of the AGT gene

results in the conversion of amino acid from Methionine (M) to another amino acid, Threonine (T), also known as M268T polymorphism [20,21]. Previous studies have indicated the association of this polymorphism in hypertension among various populations.

In Pakistan, rs699 has been studied in Mardan and Karachi. However, no association was found between hypertension and M268T in studied groups [22,23]. Nevertheless, in Rawalpindi, this has not been reported so far. Therefore, this study analyzed the rs699 polymorphism of AGT gene in healthy subjects and hypertensive patients, using the tetra-ARMS PCR.

2. MATERIALS AND METHODS

2.1. Sampling

To identify the rs699 polymorphism of AGT gene, the study performed Tetra-ARMS PCR on DNA samples obtained from controls and patients.

Blood samples of 50 hypertensive patients, ranging in age from 35-70 years, were collected from well-known hospitals, such as NESCOM and Holy Family Hospitals. The methodology used was convenience sampling. While, 30 control group samples were collected randomly from volunteers. Moreover, the informed consent of participants was obtained. The data is given in Table 1 and 2.

2.1.1. Inclusion Criteria. Cases: SBP ≥140 mmHg and/or DBP ≥90 mmHg on at least two separate occasions, or already on antihypertensive medication; Controls: SBP <130 mmHg and DBP <85 mmHg, no family history of hypertension in first-degree relatives.

2.1.2. Exclusion Criteria. Secondary hypertension, pregnancy, diabetes mellitus, chronic kidney disease, cardiovascular events in the past 6 months, refusal to consent. Blood pressure was measured using a standard mercury sphygmomanometer after around 10 minutes of rest. Some patients were already on antihypertensive medication, their blood pressure values represented their BP conditions after daily medicine. However, the status of hypertension was confirmed from medical records prior to initiation of therapy or as reported by them in case of missed dose of medicine.

2.2. Extraction of DNA

In this step, DNA was extracted from the blood samples through inorganic method. The extraction procedures had 3 steps which included cell lysis, separation of protein, and purification of DNA.

First step was to add 1 ml chilled T.E (Tris-acetate-EDTA) buffer in 200 µl blood, mixed, and centrifuged for 15min at 4000rpm. Afterwards, the supernatant was removed and 900 µl of chilled T.E buffer was added and mixed again by vigorous shaking. It was centrifuged again at 4000 rpm for 15 min. Afterwards, the same step was repeated and the supernatant was discarded, and 800 µl T.E buffer was added. It was mixed well and centrifuged for 15 min at 4000 rpm. After the centrifugation, the pellet was discarded and 200 µl TEN/Al buffer, 20 µl SDS, and 10 µl proteinase-K solution was added. The solution was incubated at 56°C overnight in water bath. The next day, tubes were placed on the ice tray and 50µl 6M NaCl was added and mixed vigorously. Then for settlement of proteins and salts, it was spun in centrifuge for 15min at 4000rpm. The supernatant was transferred in new Eppendorf, an equal amount of chilled isopropanol was added in it, and the tube was inverted slowly and gently so that the DNA could be visible. Afterwards, it was spun at 8000rpm for 1min. Supernatant was discarded and pellet was

washed with 75% ethanol twice. At last, the supernatant was discarded carefully and 60µl of low TE buffer was added in it, and the DNA was stored at -20°C [24,25].

2.3. ARMS PCR Procedure

The Amplification-Refractory Mutation System (ARMS) is a technique used for the detection of gene mutations. In this technique, sequence-specific PCR primers are used through which amplification could be attained only when target allele is present in the sample. Primers were designed using online software PRIMER1 (https://primer1.soton.ac.uk/primer1.html). The primer sequences for outer and inner primers are given in Table 3. Touchdown PCR protocol was employed to obtain the required amplicons. PCR reaction mixture was prepared in a total volume of 25 µl. In Tetra-ARMS PCR, instead of 2 primers, 4 primers were used, that is, that is PFo (outer), PFi (inner), PRo (outer), and PRi (inner). Reaction mix comprised of 9.8µl PCR-grade water, 1.3µl each primer at a final concentration of 0.5 µM, 2.5µl of 1× PCR buffer, 2.5 µl dNTPs, 2µl $MgCl_2$, and 1µl Taq DNA polymerase and 2 µl DNA.

After size separation, the next process was nucleic acid fractionation, which was done by using agarose gel electrophoresis. This proceeded the further purification of molecules of interest. For DNA visualization, 1% gel was prepared. Moreover, to visualize the bands after ARMS PCR, 2.5% gel was made. To observe results, the study employed gel documentation system that uses UV light for gel visualization.

2.4. Statistical Analysis

Statistical Analysis was done using online software Social Science Statistics (SSS) (https://www.socscistatistics.com/) as done by [26]. Test applied included Chi-square. A p-value less than 0.05 was considered significant.

The outer primers (forward and reverse) together amplify a 305 bp control band. The inner forward with outer reverse gives 206 bp (C allele), and inner reverse with outer forward gives 144 bp (T allele).

Table 1. Data of Samples according to Age

Category	Total	35-45yr	46-55yr	56-65yr	65-70yr
Control	30	06	10	11	03
Hypertensive	50	08	21	16	05

Table 2. Data of Hypertensive Patients according to Age and BP Range

Category	Gender		Age Range	Blood Pressure Range
	Male	Female		
	03	05	35-45 yrs.	130/90
Hypertensive Patients	06	15	46-55 yrs.	140/90
50 samples	07	09	56-65 yrs.	130/90
	02	03	66-75 yrs.	150/100

Table 3. Details of Primers

Primer ID	Sequence 5'---3'	Length	Tm	Product

				size
rs699_Fwd_Inn_C	ATGGAA-GACTGGCTGCTCCCTTAC	24	66	206
rs699_Rev_Inn_T	TGCTGTCCACACTGGCTCACA	21	66	144
rs699_Fwd_Outer	TGGCTCTC-TATACCCCTGTGGTCC	24	66	305
rs699_Rev_Outer	TCAAGGGTGGTCAC-CAGGTATGTC	24	66	

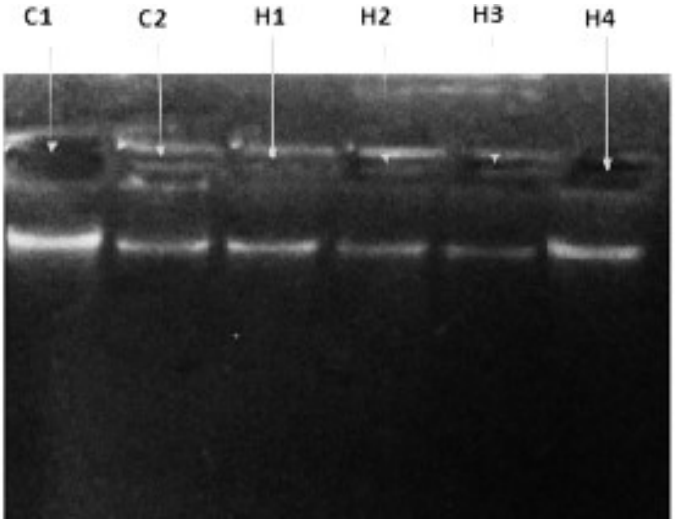


Figure 1. DNA Extraction from participants

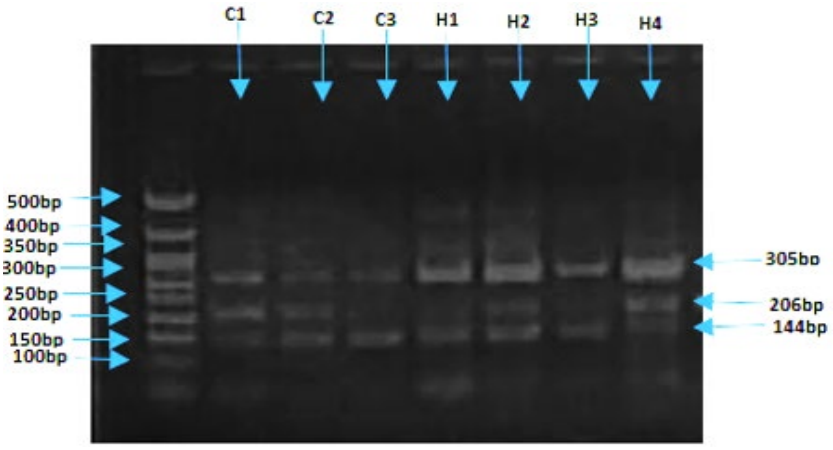


Figure 2. ARMS PCR indicating various alleles

3. RESULTS

After DNA extraction, ARMS PCR was carried out on 50 samples of the hypertensive group and 30 samples of the control

group. The study found 28 patients out of 50 having homozygous genotype (TT), while 4 members of control group showed this genotype. On the other side, 21 members out of 30 control subjects exhibited heterozygous genotype, and 12 out of 50 diseased groups had a heterozygous genotype. The study found that there is a link of T allele in a homozygous situation with hypertension phenotype. The results of DNA electrophoresis are shown in Figure 1, while those of ARMS PCR are shown in Figure 2.

Data collected from ARMS PCR was subjected to statistical analysis to know about the statistical significance. The result of genotype frequencies was also analyzed in terms of percentage, as shown in Tables 4 and 5. Distribution of alleles among controls and patients is given in Table 6.

Table 4. Genotype Frequency of the Hypertensive and Control Groups

Category	CC	CT	TT
Control	5	21	4
Patients	10	12	28

Table 5. Genotype Frequencies in Terms of Percentage

	CC	CT	TT
Control	5/30 (16.6 %)	21/30(70%)	4/30 (20%)
Patients	10/50 (20%)	12/50 (24%)	28/50 (56%)

Note. p-value is .000108. The result is significant at $p < .05$.

Table 6. Allele Distribution among Controls and Patients

Group	C allele (*n*)	C allele (%)	T allele (*n*)	T allele (%)	Total alleles
Control	31	51.70%	29	48.30%	60
Patients	32	32.00%	68	68.00%	100

According to the observed genotypes (Table 4), the study assessed Hardy-Weinberg equilibrium (HWE) in the control group to find if the observed genotype frequencies show any deviation from expected frequencies under genetic equilibrium (Table 7).

Frequency of C allele (p): p = [2(CC) + CT] / 2N = [2(5) + 21] / 60 = 31/60 = 0.517

Frequency of T allele (q): q = [2(TT) + CT] / 2N = [2(4) + 21] / 60 = 29/60 = 0.483

Since p + q = 1, the calculated allele frequencies are valid.

Table 7. Expected Genotype Frequencies under HWE

Genotype	Observed	Expected	$(O-E)^2 / E$
	(O)	(E)	
CC	5	8.01	1.13
CT	21	14.97	2.43
TT	4	6.99	1.28

Chi-square (χ^2) = 1.13 + 2.43 + 1.28 = 4.84

Degrees of freedom (*df*) = 1

Critical χ^2 value at $p = 0.05$ is 3.84

This indicates that the genotype frequencies of the healthy individuals significantly deviate from Hardy-Weinberg equilibrium ($\chi^2 = 4.84$, $p < 0.05$). This means that the observed genotype distribution does not confirm to expected equilibrium proportions.

The values of Odds ratio (OR) with 95% confidence interval represent higher odds of TT genotype with clinical

hypertension as compared to other genotypes (Table 8).

Table 8. Odds Ratio among Observed Genotypes

Comparison	Odds Ratio (OR)	Interpretation
CT vs CC	0.29	Individuals with the CT genotype showed lower odds of disease as compared with the CC genotype, suggesting a possible protective effect.
TT vs CC	3.5	Individuals with the TT genotype had 3.5 times higher odds of disease as compared with individuals carrying the CC genotype.
(CT + TT) vs CC	0.8	Carrying at least one T allele did not show a strong increase in disease risk as compared with the CC genotype.
TT vs (CC + CT)	8.27	Individuals with the TT genotype had approximately 8.3 times higher odds of disease as compared with carriers of at least one C allele.
T allele vs C allele	2.27	The T allele was associated with approximately 2.3 times higher odds of disease as compared with the C allele.

4. DISCUSSION

In the current study, a notable difference in genotype distribution was observed between hypertensive patients and normotensive controls. The frequency of the TT genotype was considerably higher in the hypertensive group (56%) compared with the control group (20%). Whereas, the CT genotype was more prevalent among controls. The higher value of OR (3.5) with 95%CI for TT genotype represents a higher risk associated with this genotype in pathogenesis of hypertension. These findings indicate that the T allele of the AGT M235T polymorphism contributes to disease susceptibility. Previously, several studies have demonstrated a significant role of M235T polymorphism in the pathogenesis of hypertension thus reinforcing the validity of the present observations [27–29]. Studies in Uzbek males [30], Malaysian [28], South Indian [20], Tunisian [31], and Hong Kong Chinese cohorts [32] have indicated association of this polymorphism of AGT gene with hypertension.

Additionally, a study conducted in an Egyptian population revealed a significant correlation between the AGT M235T polymorphism and the risk of essential hypertension. This further supported the relevance of this genetic variant across different populations [21]. Moreover, [27] reported a significant association of two polymorphisms of AGT gene, M235T, and T174M with hypertension, indicating that multiple variants within the AGT gene may collectively contribute to disease risk.

The higher prevalence of the TT genotype in hypertensive individuals supports the hypothesis that the results of the current study are consistent with earlier studies reporting a significant association between the TT genotype and hypertension. Similar findings were documented by [33–35] as they also observed a higher frequency of the TT genotype among hypertensive subjects of Ethiopian, European, and Egyptian populations, respectively. In the Polish

population and in ethnicities of Gornaya Shoria mountains, [36,37] found similar results, indicating significance of TT genotype in clinical hypertension.

However, in some populations, C allele of rs699 was found to be associated with hypertension. Hu et al. [38] analyzed Han population of China, which had relevance of C allele with elevated risk of hypertension. Moreover, Nathaniel et al. [39] found the higher influence of C allele on hypertension pathophysiology in Pakistani women [39]. Repchuk et al. [35], Makuc et al. [40], and Mondorf et al. [41] could not find any association of either allele of rs699 with hypertension in European population. This kind of variations can be due to differences in lifestyle of studied populations, ethnic heterogeneity, or variation in sample size in different studies.

In addition to genetic factors, environmental influences, such as diet, lifestyle, and stress are known to play a substantial role in the development of hypertension. Vascular tone changes, endothelial function, and arterial wall thickness are mediated by cellular processes that involve magnesium as a regulator of vascular reactivity and cardiovascular function [29,42]. In diabetic patients, between 35% and 75% of cardiovascular events are attributed to hypertension, underpinning the requirement for prompt blood pressure control, in addition to metabolic control. Genetic predisposition, for instance, angiotensinogen genes (AGT or the renin-angiotensin system (RAS), as well as age, gender, and race, are known demographic factors in determining hypertension [13]. Other factors contributing to essential hypertension include age, body weight, gender, ethnicity, level of physical activity, dietary habits, tobacco smoking, stress levels, hormonal imbalances, and the presence of associated disorders, such as diabetes mellitus. Therefore, type of medications may vary among different patients for managing hypertension [43].

4.1. Conclusion

In conclusion, the current study supported the existence of a significant association between M235T polymorphism of AGT gene and hypertension, where T allele confers an increased risk in studied subjects. These findings highlight the importance of genetic factors in hypertension and underscore the need for larger, population-based studies to further elucidate gene-environment interactions and ethnic variations in disease susceptibility.

4.2. Limitations

This study is the first one to report the data of hypertensive patients in Rawalpindi/Islamabad area based on rs699 polymorphism of AGT gene. It was a pilot study, conducted to analyze the association of M235T polymorphism in available participants. However, the small number of subjects and single ethnicity demand a detailed study considering larger population size including more gene variants. Moreover, no formal adjustments were performed. However, the study attempted to include age- and gender-matched participants in each group of cases and controls, thus minimizing confounding effect. However, the fact that there can be some biasness on the basis of demographic data and biological variations, cannot be excluded. In conclusion, the study highlighted the significance of M235T variant for further investigations, which can be done using bioinformatic tools for the development of advanced therapeutic strategies.

Author Contribution

Fazila Mumtaz: data curation, writing original draft. **Yasir Ali:** data curation. **Akhtar Ali:** formal analysis. **Mubin Mustafa:** formal analysis

Ayesha Maqbool: Writing, conceptualization, formal analysis, supervision.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

Data supporting the findings of this study will be made available by the corresponding author upon request.

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Generative AI Disclosure Statement

The authors did not use any type of generative artificial intelligence software for this research.

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