Journal of Neonatal Surgery
ISSN(Online): 2226-0439
Vol. 14, Issue 32s (2025)
https://www.jneonatalsurg.com

OPEN ACCESS

Association of eNOS and ACE Gene Variants with Increased Susceptibility to Congenital Heart Defects

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Cite this paper as: Nandini Krishnamurthy, Jebaraj Rathinasamy, Ashok Kumar, Shuba S, Devi Krishna R, Sanjana Murali, Andrea Mary Francis, (2025) Association of eNOS and ACE Gene Variants with Increased Susceptibility to Congenital Heart Defects. *Journal of Neonatal Surgery*, 14 (32s), 4670-4678.

ABSTRACT

Background: Genetic polymorphisms in the endothelial nitric oxide synthase (*eNOS*) and angiotensin-converting enzyme (*ACE*) genes have been linked to the pathogenesis of congenital heart defects (CHDs). However, their precise role in CHD susceptibility remains to be fully elucidated. **Aim:** This study aimed to evaluate the association of *eNOS* (rs1799983) and *ACE* (rs4646994) gene polymorphisms with the risk of CHDs in an Indian population. **Methods:** A case-control study was conducted involving 112 children with CHDs and 112 age-matched healthy controls. Genomic DNA was extracted from peripheral blood, and genotyping was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Results were confirmed by Sanger sequencing.

Results: For *eNOS* (rs1799983), both the GT genotype P < 0.0001; OR = 4.7435 (2.5849 to 8.7050) and GG genotype P = 0.0051; OR = 3.3281 (1.4350 to 7.7189) were significantly associated with increased CHD risk. The dominant model (GT + GG) also showed a strong association P < 0.0001; OR = 4.3293 (2.4679 to 7.5945) For *ACE* (rs4646994), the ID genotype resulted in P= 0.0307; OR= 2.3824 (1.0841 to 5.2352) DD genotype was significantly more frequent among CHD cases P < 0.0001; OR = 5.2941(2.8342 to 9.8892) while the dominant model (ID/DD) demonstrated a strong association with CHD risk P < 0.0001; OR = 4.1294 (2.3629 to 7.2166). Allele frequency analysis revealed a significantly higher frequency of the G allele in *eNOS* (rs1799983) and D allele *ACE* (rs4646994) among cases compared to controls (both P < 0.0001), suggesting their roles as potential genetic risk factors for CHD.

Conclusion: Polymorphisms in *eNOS* (rs1799983) and *ACE* (rs4646994) genes are significantly associated with increased susceptibility to congenital heart defects in the studied Indian cohort. These findings provide valuable insight into the genetic basis of CHDs and support the potential for using these markers in genetic screening and risk assessment.

Keywords: *Congenital heart defects, eNOS polymorphism, ACE gene variant, PCR-RFLP, Genetic susceptibility, Case-control study.*

1. INTRODUCTION

Congenital heart disease (CHD) is one of the most common major birth defects although very little is known about its causes. A combination of environmental and genetic factors are involved. Though the exact mechanisms causing CHD are still unknown, few models of human and animal-induced pluripotent stem cells have demonstrated how these factors impair heart development [1]. With almost 1% of births affected annually, congenital heart defects (CHDs) are the most prevalent kind of birth defect. According to reports, there are 8–12 cases of congenital heart disease for every 1,000 live births. Over 200,000 children are thought to be born with congenital heart disease (CHD) in India each year. Approximately one-fifth of these are

probably going to have severe defects that need to be fixed within the first year of life [2,3]. Over the years, congenital heart defects (CHD) have become more prevalent worldwide. A systematic review and meta-analysis found that the birth prevalence of congenital heart disease (CHD) increased steadily between 1970 and 2017, peaking at 9.41 per 1,000 live births between 2010 and 2017. Improved detection of mild CHD lesions like patent ductus arteriosus, atrial septal defects, and ventricular septal defects is primarily responsible for this increase.

Regional differences in the prevalence of CHD are substantial. At 2.315 per 1,000 live births, Africa has the lowest prevalence, whereas Asia has the highest, at 9.342 per 1,000 live births. Variations in healthcare access, diagnostic technology, and environmental or genetic factors can all be attributed for this variation [4]. Previous studies indicate that polymorphisms in both *eNOS* (endothelial nitric oxide synthase) and *ACE* (angiotensin-converting enzyme) are linked to CHD. A study carried out on an Iranian cohort revealed that specific polymorphisms in the *eNOS* and *ACE* genes were significantly correlated with a heightened risk of CHD. In particular, the *eNOS* T894G and *ACE* A2350G polymorphisms were identified as risk factors for CHD. These genetic variations could influence the production and regulation of nitric oxide and angiotensin II, which play vital roles in cardiovascular development and functionality. The research concluded that the AA and GA genotypes of the MTHFD1 G1958A, along with the TT and GT genotypes of *eNOS* T894G, and the AA and GA genotypes of *ACE* A2350G are conducive factors for CHD and may elevate the risk of developing these anomalies [5]. Studies have indicated that the *eNOS* rs1799983 and *ACE* I/D (rs4646994) polymorphisms are linked to congenital heart defects (CHD). The *eNOS* (rs1799983) polymorphism, also referred to as T894G, is associated with several cardiovascular abnormalities, including CHD, as it impacts nitric oxide production, which is vital for cardiovascular health and development [6]. Likewise, the *ACE* I/D polymorphism (rs4646994) involves the insertion or deletion of a 287-base pair Alu repeat sequence in the *ACE* gene and has been connected to a higher risk of CHD, especially in individuals with additional risk factors like type 2 diabetes mellitus. These genetic variations may affect the likelihood of developing CHD by influencing the regulation of nitric oxide and angiotensin II, both of which are crucial for cardiovascular development and function [7]. Therefore, we selected two functional single nucleotide polymorphisms (SNPs), *eNOS* (rs1799983) and *ACE* I/D (rs4646994)**,** and analyzed their gene expression in CHD cases and controls from the general population.

2. MATERIALS AND METHODS

Study Population and Bioethics:

This study adhered to all ethical guidelines and regulatory requirements, ensuring compliance with governmental policies. Blood samples were legally obtained from Sri Ramachandra Institute of Higher Education and Research (SRIHER) and were used exclusively for research purposes. Since the participants were children, written informed consent was obtained from their parents or legal guardians. Additionally, assent forms and patient questionnaires were collected. The present study was approved by the Institutional Ethics Committee of SRIHER, Chennai, India and the IEC reference number is IEC-NI/23/AUG/88/50.

A total of 112 children diagnosed with CHD (n=112) and 112 unrelated healthy individuals were included in this case-control study. CHD cases were confirmed through cardiac MRI and echocardiography, and only those who subsequently underwent surgical intervention were included. All participants initially completed a screening questionnaire to assess their willingness to participate, followed by a face to face questionnaire to collect demographic details. Subsequently, 5 mL of venous blood was collected from each participant.

To ensure the study's validity, individuals with additional congenital disorders or known chromosomal abnormalities were excluded. Furthermore, control subjects were selected from non-CHD outpatients within the same geographic region, matched for age and gender with the CHD cases. Only individuals without congenital anomalies were included in the control group. Maternal factors known to increase the risk of congenital anomalies, such as pre-existing diabetes, phenylketonuria, exposure to teratogens, or certain therapeutic drugs during pregnancy, were carefully considered, and participants with such histories were excluded. All subjects were genetically unrelated and belonged to Indian population.

Genotyping for *eNOS* rs(1799983):

Genomic DNA was extracted from peripheral blood leukocytes using the DNA Mini Kit (QIAGEN) following the manufacturer's protocol, involving cell lysis, protein digestion, and purification steps to obtain high-quality DNA suitable for downstream applications. The isolated DNA was quantified using a NanoDrop spectrophotometer to assess purity and concentration, with samples having an A260/A280 ratio between 1.8 and 2.0 considered optimal and subsequently stored at -20°C for further analysis. Polymerase chain reaction (PCR) amplification was carried out in a 30 µL reaction volume containing 10X PCR buffer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each primer, 1 U of Taq DNA polymerase, and 50 ng of genomic DNA. For (rs1799983), the primers used for PCR amplification were Forward: 5'-GTCACGGAGACCCAGCCAATG-3' and Reverse: 5'-GCCCTTCTTGAGAGGCTCAGGGAT-3'. To ensure the reliability of genotyping, approximately 10% of the samples were randomly selected for reanalysis, and the results were found to be 100% concordant, confirming the accuracy of the genotyping process.

Genotyping by Restriction Fragment Length Polymorphism (RFLP) Analysis

Restriction Fragment Length Polymorphism (RFLP) analysis was performed using carefully selected restriction enzymes via Restriction Mapper 2.0 to ensure precise and efficient digestion of the amplified DNA fragments. PCR amplification produced a 325 bp fragment, which was digested using the restriction enzyme Mbo I in a 30 µL reaction volume containing 10X restriction buffer, 5 U of Mbo I enzyme, and 10 µL of PCR product, followed by incubation at 37°C for 4 hours to ensure complete digestion. The digested products were separated on a 2% agarose gel stained with ethidium bromide, and band patterns were visualized under a gel documentation system to determine the genotype. The digestion resulted in distinct banding patterns: homozygous wild-type (GG) produced 195 bp and 130 bp fragments, heterozygous mutant (GT) resulted in 325 bp, 195 bp, and 130 bp fragments, while homozygous mutant (TT) remained undigested at 325 bp. This method provided accurate genotype identification based on clear fragment separation.

Confirmation of Genotyping via Sanger Sequencing

Sanger sequencing was performed using the Thermo Fisher Sanger Sequencing Kit to validate genotyping results. PCR-amplified DNA fragments were purified using a PCR purification kit, and the purified products were subjected to cycle sequencing in a 10 µL reaction volume containing 2 µL of BigDye™ Terminator v3.1 Ready Reaction Mix, 1 µL of 5× sequencing buffer, 3 µL of deionized (RNase/DNase-free) water, 1 µL of M13 forward or reverse primer (3.2 µM), and 2 µL of purified PCR product. For batch processing in a 96-well plate, a master mix was prepared, consisting of 211 µL of BigDye™ Terminator v3.1 Ready Reaction Mix, 106 µL of 5× sequencing buffer, 317 µL of deionized water, and 106 µL of either M13 forward or reverse primer. The sequencing reactions were carried out under thermal cycling conditions of 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The sequencing products were purified via ethanol precipitation and analyzed using the ABI Genetic Analyzer (Applied Biosystems). The resulting chromatograms were examined using Chromas software to identify nucleotide variations, confirming the genotyping patterns, with most cases exhibiting the GT heterozygous mutant genotype than control groups, further validating the accuracy of the RFLP analysis.

Genotyping for *ACE* I/D rs (4646994):

The rs4646994 *ACE* I/D polymorphism, located in intron 16 of the *ACE* gene, involves insertion (I) or deletion (D) of a 287-base pair sequence, resulting in II, ID, or DD genotypes. It's associated with cardiovascular conditions like hypertension, coronary artery disease, CHD'S and heart failure. The D allele correlates with increased *ACE* activity and elevated angiotensin II levels, contributing to adverse cardiovascular outcomes. This polymorphism influences the renin-angiotensin-aldosterone system, affecting blood pressure regulation and fluid balance, thus impacting cardiovascular health and guiding personalized prevention and treatment strategies. The PCR protocol for distinguishing between D- and I- alleles involves amplifying genomic DNA using specific primers: Forward 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and Reverse 5′-GATGTGGCCATCAC ATTCGTCAGAT-3′. A 25 µL reaction mixture is prepared, including 50–100 ng of template DNA, 1.0 µL of each primer (10 µM), 0.5 µL of dNTP mix (10 mM), 2.5 µL of 10X PCR buffer, 1.5 µL of $MgCl^2$, 0.5 µL of Taq DNA Polymerase (5 U/µL), and nuclease-free water to adjust the volume. The thermal cycling conditions begin with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 45 seconds, concluding with a final extension at 72°C for 5 minutes and a hold at 4°C. Post-PCR, the products are analyzed using 2% agarose gel electrophoresis with ethidium bromide or SYBR Safe staining, running at 100–120V for 30–40 minutes in 1X TAE buffer. Visualization under gel documentation revealed fragment sizes, where a single 490 bp band indicates the wild-type homozygous (I/I) genotype, the presence of both 490 bp and 190 bp bands confirms the heterozygous mutant (I/D) genotype, and a single 190 bp band represents the homozygous mutant (D/D) genotype.

3. RESULTS

Distribution of variables among cases and controls:

The demographic characteristics of the 112 children included in the analysis are summarized in Table 1. There were no statistically significant differences between CHD patients and controls in terms of age ($P = 0.2324$) or gender ($P = 0.8034$). Among the 112 children with CHD, 56 were diagnosed with ventricular septal defect (VSD), 34 had atrial septal defect (ASD), and 22 had tetralogy of Fallot (TOF). A two-sided t-test was performed to compare age, while a chi-square (χ^2) test was used to analyze gender distribution. The distribution of demographic characteristics and CHD subtypes among the study participants is represented in (Table 1).

Table 1: Comparison of selected variables between case and control groups.

Variables	Cases		Control		P value
	N	Percentage%	N	Percentage %	
Age Years [mean ± SD]	7 ± 4.20		6.4 ± 3.9		0.2324
Gender					
Male	67	59.82	64	57.14	0.8034
Female	45	40.18	48	42.86	
Types of CHD as classified					
Ventricular septal defect (VSD)	56	50			
Atrial Septal Defect (ASD)	34	30.36			
Tetralogy of Fallot (TOF)	22	19.64			

Association of *eNOS* (rs1799983) and *ACE* (rs 4646994) with CHD:

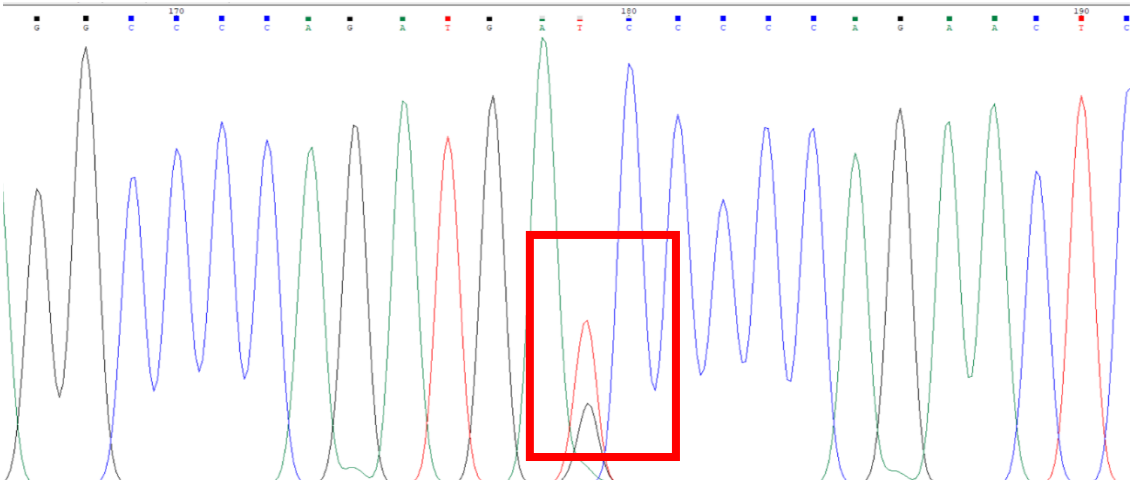
Our study examined the genotype and allele distributions in cases and controls to determine whether genetic polymorphisms (rs1799983) and (rs4646994) are linked to a person's vulnerability to CHDs. The detailed genotypic and allelic distributions, along with their statistical associations, are presented in (Table 2).

Genetic Variation of *eNOS* (rs1799983)

Genotypic distribution of *eNOS* (rs1799983) revealed a significant correlation between the presence of the G allele and elevated risk of congenital heart defects (CHDs). The TT genotype occurred more in the control group (71 individuals, 63%) than in cases (32 individuals, 28%), and was the reference genotype. The GT heterozygous genotype occurred much more often in cases (62, 55%) than in controls (29, 25%), with a highly significant P-value ($P < 0.0001$) and an odds ratio (OR) of 4.7435 (95% CI: 2.5849 to 8.7050), suggesting a strong association with CHD risk increase. The GG genotype was also more common among cases (18, 16%) than controls (12, 10%), with a statistically significant relationship ($P = 0.0051$) and an OR of 3.3281 (95% CI: 1.4350 to 7.7189), indicating a moderate increase in risk.

In the dominant model (GT + GG), those with at least one G allele (GT or GG) were highly significantly more common in the case group (80, 71%) than in the control group (41, 36%) with a highly significant P-value (< 0.0001) and an OR of 4.3293 (95% CI: 2.4679 to 7.5945)

As to allele frequencies, the T allele was more frequent in controls (171, 76%) than in cases (126, 57%) and was used as the reference allele. The G allele was, however, much more frequent in cases (98, 43%) than in controls (53, 24%), with a highly significant P-value (< 0.0001) and an OR of 2.5094 (95% CI: 1.6725 to 3.7652). These results strongly indicate that the G allele is implicated in increased susceptibility to CHDs. Sequencing data also ratified that the majority of samples exhibited the heterozygous GT genotype, supporting the data obtained by genotyping (Figure 1).



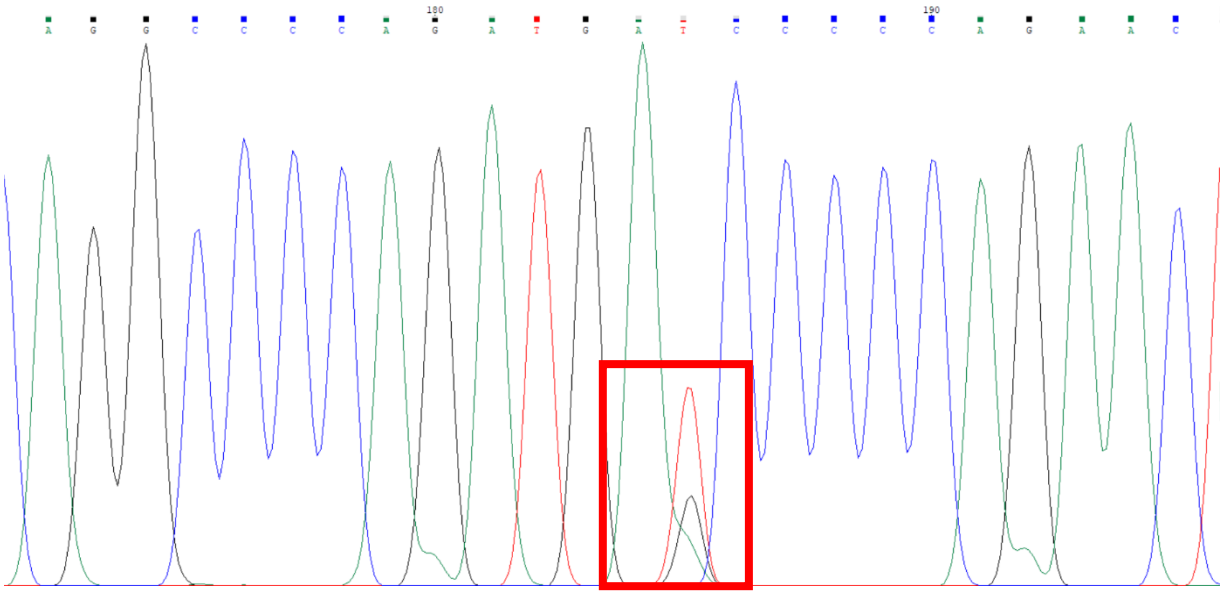
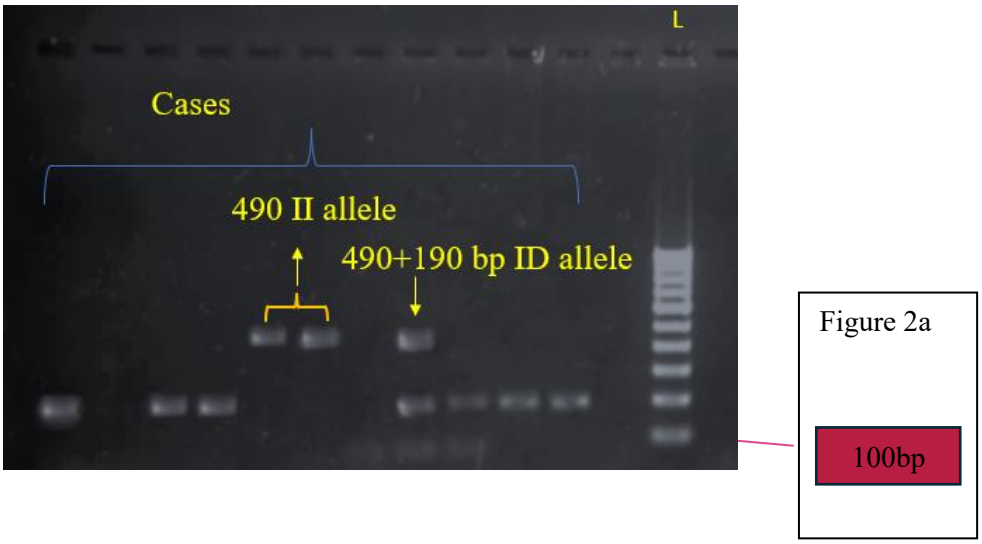


Figure 1: Sanger sequencing chromatograms of case samples confirming the GT heterozygous mutant genotype for rs1799983. The sequencing results show overlapping peaks in both samples, indicating the presence of both the wild-type (T) and mutant (G) alleles.

Genetic Variation of *ACE* (rs4646994):

The genotypic distribution of the *ACE* I/D gene polymorphism (rs4646994) demonstrated a significant association with congenital heart defects (CHDs). The II genotype was considerably more frequent in the control group (72 individuals, 64%) compared to CHD cases (34 individuals, 30%), suggesting a protective effect. The ID genotype was observed in 18 cases (16%) and 16 controls (14%), showing a modest but statistically significant association with CHD risk ($P = 0.0307$; OR = 2.3824, 95% CI: 1.0841–5.2352). A stronger association was found with the DD genotype, which occurred more frequently in cases (60, 53%) than in controls (24, 21%), indicating a robust association with increased CHD risk ($P < 0.0001$; OR = 5.2941, 95% CI: 2.8342–9.8892). Under the dominant model (ID + DD), 78 cases (69%) carried at least one D allele, compared to 40 controls (35%), further supporting the association ($P < 0.0001$; OR = 4.1294, 95% CI: 2.3629–7.2166). In terms of allele frequencies, the I allele was more prevalent among controls (160, 71%) compared to cases (86, 38%), supporting its protective role. Conversely, the D allele was significantly more common in cases (138, 61%) than in controls (64, 28%) ($P < 0.0001$; OR = 4.1294, 95% CI: 2.3629–7.2166), suggesting its association with increased susceptibility to CHDs. The comparative genotypic and allelic distribution of *ACE* (rs4646994) among CHD cases and controls is illustrated in Figure 2a and 2b, highlighting the significant differences that support its involvement in disease pathogenesis.



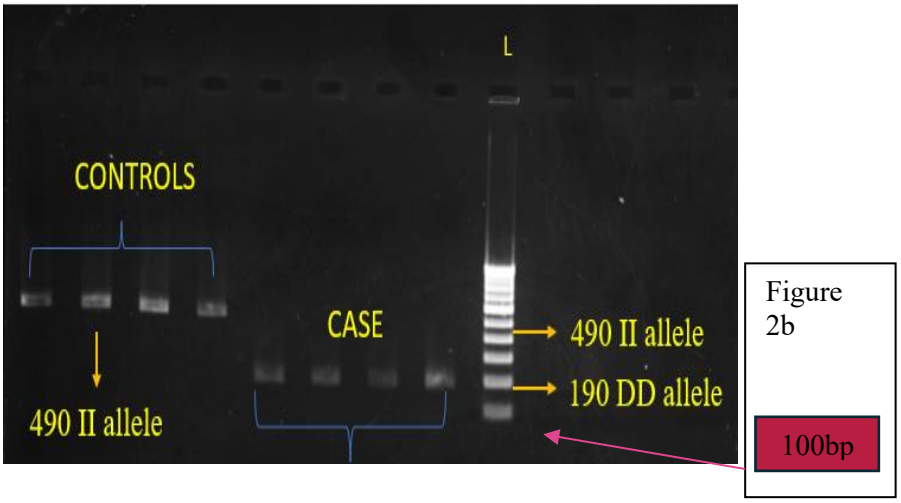


Figure 2: The agarose gel electrophoresis illustrates genotyping results for the *ACE* I/D polymorphism following PCR amplification from 50 to 100 ng of genomic DNA. Three distinct profiles are observed: a single band at 190 base pairs, indicating the homozygous mutant DD genotype; two bands at 490 bp and 190 bp, representing the ID heterozygous genotype; and a single band at 490 bp representing the II wild-type genotype. These band patterns align with the expected fragment sizes associated with each genotype. Figure 2A specifically shows lanes with the homozygous wild type with 490 bp band corresponding to the II allele, the heterozygous genotype represented by both 490 bp and 190 bp bands (ID), and the 190 bp band indicating the homozygous mutant DD genotype. Additionally, 2B includes representative cases with the homozygous mutant 190 bp (DD) genotype and a control sample showing the 490 bp (II) genotype.

Table 2: Comparative Genotypic and Allelic Distribution of *eNOS* (rs1799983) and *ACE* (rs4646994) Polymorphisms in CHD Cases and Controls

Genotypes	Cases (N=112)	Controls (N=112)	P value	OR (95% CI)
rs1799983				
TT	32 (28%)	71 (63%)		Ref
GT	62 (55%)	29 (25%)	P < 0.0001	4.7435 (2.5849 to 8.7050)
GG	18 (16%)	12 (10%)	P= 0.0051	3.3281 (1.4350 to 7.7189)
GT+GG	80 (71%)	41 (36%)	P < 0.0001	4.3293 (2.4679 to 7.5945)
T allele frequency	126 (57%)	171(76%)		Ref
G allele frequency	98 (43%)	53 (24%)	P < 0.0001	2.5094 (1.6725 to 3.7652)
rs 4646994				
II	34 (30%)	72 (64%)		Ref
ID	18 (16%)	16 (14%)	P=0.0307	2.3824 (1.0841 to 5.2352)
DD	60 (53%)	24 (21%)	P < 0.0001	5.2941(2.8342 to 9.8892)
ID/DD	78 (69%)	40 (35%)	P < 0.0001	4.1294 (2.3629 to 7.2166)
I allele frequency	86 (38%)	160 (71%)		Ref
D allele frequency	138 (61%)	64 (28%)	P < 0.0001	4.1294 (2.3629 to 7.2166)

4. DISCUSSION

Nitric oxide synthase (NOS) enzymes convert l-arginine to l-citrulline, which results in the production of nitric oxide (NO). Three isoforms of the NOS enzymes have been discovered thus far: neuronal (nNOS), inducible (iNOS), and endothelial

(*eNOS*) [8]. By inhibiting 5-methyltetrahydrofolatehomocysteine methyltransferase in the vascular system, the *eNOS* enzyme, which is the primary enzyme needed for NO synthesis, regulates homocysteine levels. Pl*ACE*ntal perfusion and the fetus's supply of oxygen and nutrients may be negatively impacted by changes in the endothelial cells' basal NO production [9]. The molecule NO is involved in many physiological processes, such as the control of blood vessel tone, inflammation, mitochondrial activity, and apoptosis [10]. The most significant isoform is *eNOS*, which has several other vasoprotective and antiatherosclerotic effects in addition to maintaining blood vessel dilatation and regulating blood pressure .The *eNOS* gene located on chromosome 7 encodes endothelium-derived NO synthase [11]. Numerous *eNOS* gene polymorphisms and their connections to different diseases have been investigated recently. Due to their functional significance to *eNOS* activity, three specific polymorphisms have drawn the most attention which include the 4b4a polymorphism (a VNTR) in intron 4 of the *eNOS*, a single nucleotide polymorphism in the promoter region (T-786C), and an 894T/G polymorphism that results in amino acid substitution at [12].The *eNOS* gene's exon 7 especially contains the common polymorphism rs1799983, which results in an amino acid substitution at position 298 from glutamine to aspartic acid (Glu-298Asp) [13]. Given the potent vasoactivity of substances like angiotensin II and bradykinin, genetic variations in the renin-angiotensin and RAS pathways may prove to be significant pathophysiological mechanisms affecting coronary heart disease, essential hypertension, and congenital heart defects (CHD) [14]. According to earlier experimental research, these compounds may also be involve in the regulation of cardiac protein synthesis and development [15]. A polymorphism in *ACE* gene has been linked in other studies to an increased risk of myocardial infarction, hypertension, cardiomyopathy, and sudden demise [16]. To fully understand the implications of *ACE* polymorphisms in patients with cardiovascular disorders, more research is necessary. Interactions between *ACE* and angiotensin II type-1 receptors have recently been discovered to have potential clinical implications for the diagnosis and prevention of cardiac defects. A study found that left ventricular hypertrophy was linked to *ACE* gene polymorphisms [17]. The 2350 G/A polymorphic locus at exon 17 had a significant impact on the plasma *ACE* levels revealed from the association analysis of 13 *ACE* gene polymorphic loci [18]. Also, a study concluded that CHD is a major global health concern, particularly in low- to middle-income countries like India. The genetic influence of the *ACE* insertion/deletion (I/D) polymorphism in 667 CHD cases and 104 controls were reported in this study where, the DD genotype was associated with increased CHD risk in females ($p = 0.036$), abnormal hemoglobin ($p = 0.049$), and primigravida cases ($p = 0.05$). Conversely, the II genotype elevated CHD risk in the offspring of tobacco-consuming fathers ($p = 0.029$). Cyanotic cases, especially Tetralogy of Fallot (TOF), exhibited a strong association with *ACE* I/D mutations ($p = 0.024$). The DD genotype was also linked to st*eNOS*is and pulmonary artery hypertension. Notably, maternal transmission of the D allele was observed in combined and acyanotic cases, while paternal transmission was noted in ventricular septal defects. These findings highlight the critical role of genetic factors in CHD susceptibility [19]. *ACE* activity is frequently increased in patients with essential hypertension, and it is essential in controlling blood pressure and vascular resistance. It is common in practice to prescribe *ACE* inhibitors to treat essential hypertension [20]. Our findings suggest a strong genetic association between *eNOS* (rs1799983) and *ACE* (rs4646994) polymorphisms and the risk of CHDs. The presence of the G allele in rs1799983 was significantly correlated with an increased susceptibility to CHDs. The TT genotype was more frequent in the control group, indicating a protective effect, while individuals carrying the GT or GG genotypes exhibited a higher risk. The dominant model (GT + TT) further reinforced this association, suggesting that individuals with at least one G allele have an elevated likelihood of developing CHDs. The allele frequency analysis also supported this observation, as the G allele was significantly more prevalent among cases than controls. These findings align with previous studies suggesting that *eNOS* polymorphisms may influence vascular function and nitric oxide bioavailability, potentially contributing to congenital cardiovascular anomalies. Similarly, the genotypic distribution of the *ACE* (rs4646994) polymorphism revealed a significant relationship with CHD risk. The II genotype was more frequent in the control group, indicating a protective effect, whereas the DD genotype showed a strong association with increased CHD susceptibility. The dominant model (ID + DD) confirmed this trend, further emphasizing the potential role of the D allele in CHD risk. The allele frequency analysis showed a significantly higher prevalence of the D allele in cases, reinforcing the hypothesis that it may contribute to CHD pathogenesis. The *ACE* gene is known to regulate the renin-angiotensin system, which plays a critical role in cardiovascular development, and alterations in its expression due to genetic variations could predispose individuals to CHDs.

5. CONCLUSION

In conclusion, our study highlights the significant impact of *eNOS* (rs1799983) and *ACE* (rs4646994) polymorphisms on CHD susceptibility. The presence of the G allele in *eNOS* and the D allele in *ACE* were strongly associated with increased CHD risk, while the TT and II genotypes appeared to have protective effects. These findings suggest that genetic screening for these polymorphisms could aid in early risk assessment and prevention strategies for CHDs. Future studies with larger sample sizes and functional analyses are warranted to further elucidate the molecular mechanisms underlying these associations.

Abbreviations:

CHD – Congenital Heart Defect; *eNOS* – Endothelial Nitric Oxide Synthase; *ACE* – Angiotensin Converting Enzyme; PCR – Polymerase Chain Reaction; RFLP – Restriction Fragment Length Polymorphism; SNP – Single Nucleotide Polymorphism; DNA – Deoxyribonucleic Acid; VSD – Ventricular Septal Defect; ASD – Atrial Septal Defect; TOF –

Tetralogy of Fallot; OR – Odds Ratio; SD – Standard Deviation; I/D – Insertion/Deletion; bp – Base Pairs; IEC – Institutional Ethics Committee; SRIHER – Sri Ramachandra Institute of Higher Education and Research.

Ethics approval and consent to participate

This present study was approved by the Institutional Ethics Committee of Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, Tamilnadu, India, with reference number **IEC-NI/23/AUG/88/50**. Written informed consent was obtained from the parents or legal guardians of all participating children prior to sample collection and inclusion in the study.

Consent for Publication:

Not Applicable.

Acknowledgements:

The Department of Human Genetics provided the facility, for which all authors are grateful, and the Udayar Founder Chancellor Fellowship from Sri Ramachandra Institute of Higher Education and Research provided stipend, which was essential in conducting this research. We would like to thank all of the participants for willingly participating in this study. We also value the samples that the Department of Pathology and the Department of Cardiology at SRIHER provided.

Conflict of Interest:

Authors does not have any conflict of interest.

Funding: This study received no external funding.

Author contributions

NK designed the study and analyzed data. DK, SM and collected the data from the journals. JR, AK, JSNM and AMF revised the manuscript. All authors contributed to the article.

Availability of Data and Material:

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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