GMR
The Original

MOLECULAR EVALUATION OF ENOS RS1799983 T<C POLYMORPHISM AND ITS ASSOCIATION WITH SICKLE CELL DISEASE IN SAUDI ARABIA.

Reef I. Hamdi[1,2], Reem I. Qarmush[1,2], Reem S. Alaida[1,2], Thikra A. Alwadai[2,3], Rawan Kaabi[4], Joud Hamadi[5], Rashid Mir [1,2], Abdullah Hamadi* [1,2],

[1]Faculty of Applied Medical Sciences, Department of Medical Laboratory Technology, University of Tabuk, 47512 Tabuk, Saudi Arabia
[2] Prince Fahad Bin Sultan Chair for Biomedical Research, University of Tabuk, 47512 Tabuk, Saudi Arabia
[3] Faculty of Medicine, Department of Internal medicine and Surgery, University of Tabuk, , 47512 Tabuk Saudi Arabia
[4] Sterilization Department, King Fahad Specialist Hospital, Tabuk Health Cluster, 47717 Tabuk, Saudia Arabia
[5] Tabuk International School, General Administration of Education in Tabuk, 47713 Tabuk, Saudia Arabia
*Corresponding author -Dr. Abdullah Aldhafri, a.aldhafri@ut.edu.sa

ABSTRACT

Background: Under the genetic factors, namely eNOS rs1799983 T<C have become an important tool to study the mechanism that underlies the pathogenesis of this disease. Therefore, we investigated the association of eNOS rs1799983 T<C gene variations with susceptibility of Sickle cell disease.

Methods: This study was conducted on 100 Sickle cell disease patients and 117 matched healthy individuals. Genotyping of the eNOS rs1799983 T<C gene variation was performed by using amplification refractory mutation system PCR method (ARMS-PCR).

Results: The distribution of eNOS rs1799983 T<C genotypes observed between patients and controls was significantly different (P=0.048). Moreover, the frequency of G allele (fG) was found to be significantly higher among patients than in controls (0.36 vs. 0.25). Our findings showed that the Hsa-miR-146a-5p C>G variant was associated with an increased risk of CAD in codominant inheritance model CC vs. CG genotype (OR = 1.84, 95 % CI, 1.02-3.31; p=0.040) and (OR = 3.18, 95 % CI, 1.02-9.9; p=0.045) for CC vs. GG genotype in dominant inheritance model. Whereas the G allele significantly increased the risk of coronary artery disease (OR =1,81, 95 % CI, 1.18-2.78; p=0.006) compared to C allele.

Conclusion: Our findings indicated that eNOS rs1799983 T<C (E298D) genotype and G allele are associated with an increased susceptibility to Sickle cell disease. A larger sample size can be the key to progress in establishing the genetic co-relation of miRNA gene polymorphisms and Sickle cell disease.

KEYWORDS: Sickle cell disease, eNOS rs1799983 T<C, Amplification refractory mutation system (ARMS-PCR)

INTRODUCTION

Sickle cell anemia (SCA) is a hereditary blood condition resulting from the presence of two copies of a mutation in the beta-globin gene on chromosome 11. SCA was the pioneering example of a molecular disease, in which advancements in technology and chemical analysis were employed to assess gene abnormalities [1] . According to the World Health Organization (WHO), the prevalence of world population is 5.2% affected by SCD [2], and this percentage varies in Saudi Arabia population according to provinces and areas . The Saudi population is considered at high risk of suffering from SCA due to traditional, cultural and social factors [3]. Significantly, there are other factors that contribute to the variation in clinical and hematologic characteristics observed in patients with sickle cell anemia (SCA). These factors include a single mutation in the beta-globin gene, environmental influences, and additional genetic modifiers. Using contemporary molecular techniques and sophisticated genotype testing, there is a lack of appropriate data regarding the analysis and frequency of nucleotide polymorphisms in SCA patients in Saudi Arabia. Endothelial nitric oxide synthase (eNOS) is one of the isoforms of NOS play a vital role in vasculogenesis and angiogenesis [4]. Elevated expression of the endothelial nitric oxide synthase (eNOS) gene was correlated with an augmented susceptibility to a range of illnesses. The G894T polymorphism is a frequently observed variation of the eNOS gene in many populations. This single nucleotide polymorphism (SNP), known as rs1799983, results in the substitution of glutamic acid with aspartic acid (Glu298Asp) at codon 298 in the seventh exon of the NOS3 gene [5]. The Glu298Asp (G894T, rs1799983) polymorphism of the NOS-3 gene, found on chromosome 7q35-36, is a significant genetic variation that occurs when a G nucleotide is replaced by a T nucleotide at position 894. This variation is known to decrease the generation of nitric oxide (NO) and has been implicated in the development of several diseases [6]. Plasma levels of endothelin-1, cytokines, and prostaglandin E2 were measured in individuals with sickle cell disease and acute vasoocclusive sickle crisis. The synthesis of nitric oxide by endothelial cells was examined in cases of acute chest syndrome. The relationship between polymorphisms in endothelial nitric oxide synthase and the development of atherogenesis was also investigated. Additionally, the role of a specific gene (3-gene) was explored. Endothelial nitric oxide synthase (eNOS) is a crucial factor in the development and progression of sickle cell anemia (SCA) and has a significant impact on its severity and prognosis [7]. Several studies showed that higher frequencies of mutant alleles C-786 has been found of sickle cell anemia patients in India [8], and Egypt [9] . Moreover, there was a strong association between this mutant and develop complications in SCD in Ghanaian patients [10]. Sharan and his colleagues recently conducted a study that revealed a noteworthy rise in eNOS gene variants

among African-American patients with sickle cell disease (SCD) [11]. Currently, there is no research that has demonstrated the allelic frequencies of eNOS rs1799983 G to T polymorphisms in sickle cell disease patients from Saudi Arabia. The objective of our work was to develop a fast and accurate molecular assay for identifying the eNOS rs1799983 G to T gene variant in SCD patients from the Saudi Arabian population. The study also assessed the genetic variety of eNOS rs1799983 G to T polymorphisms in sickle cell patients.

MATERIAL AND METHODS

Selection criteria of patients:

The subjects of consecutive patients with clinically confirmed newly diagnosed, pathologically and HPLC confirmed cases of Sickle cell disease patients (105). We have excluded any patients with any previous history of any chronic disease from this study.

Sample collection

All patient specimens were timed around routine blood drawn that was the part of routine workout, and hence will not require additional phlebotomy. In some cases, 3ml of peripheral blood sample was collected by venipuncture in EDTA tubes from SCD patient as well as from healthy controls (HC). The specimens obtained were newly diagnosed, pathologically and HPLC confirmed cases of SCD patients.

Healthy Controls

The study included of 100 healthy controls ranging from 20 to 50 years of age, visiting Hospital for routine checkup. The controls were enrolled from the general population of the same geographical region. Routine medical check-up was conducted (CBC, KFT, LFT etc) and history of illness if detected was recorded by a health practitioner. Those who appeared apparently healthy without any history of any significant disease or other chronic diseases were considered normal. A standard questionnaire was used to document the socio-demographical characteristics such as age, sex, and lifestyle.

Genomic DNA extraction

DNA was extracted using DNeasy Blood Kit (Cat No. 69506) Qiagen (Germany) as per the manufactures instructions and then the DNA was dissolved in nuclease-free water and stored at 4°C until use. The extracted DNA was dissolved in nuclease-free water and stored at 4 °C until use. The quality of the extracted DNA was checked by running the sample in 1% agarose gel. The quantity of the extracted DNA is determined by absorbance at 260 nm and 280nm using a spectrophotometer or NanoDrop™ (Thermo Scientific, USA).

Genotyping of endothelial nitric oxide gene polymorphism **(e*NOS-3 rs1799983 (Glu298Asp or 894 G>T) in SCD***

Amplification-refractory mutation system PCR was optimized using tetra-primers. It was performed on the genomic DNA using a tetra-primer ARMS PCR approach. The eNOS rs1799983 G to T (Glu298Asp) genotyping was detected by using amplification-refractory mutation system PCR. The ARMS primers were designed by using Primer3 software as depicted in Table 1.

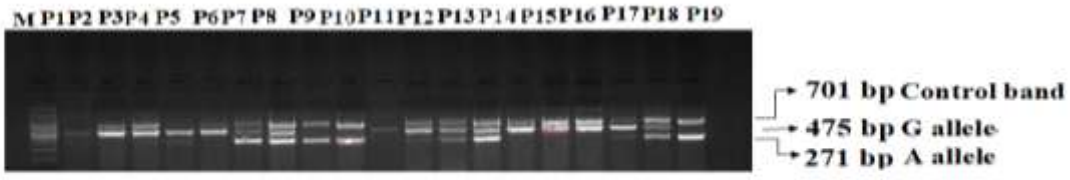
Preparation of PCR cocktail:

The ARMS-PCR was performed in a reaction volume of 25 µL containing template DNA (50 ng), FO -0.20 µL, RO -0.20 µL, FI-0.25 µL, RI -0.25 µL of 25 pmol of each primer and 10 µL from DreamTaq Green PCR Master Mix (2X) (K1081) (Thermo Scientific, USA). The final volume of 25 µL was adjusted by adding nuclease-free ddH_2O. Finally, 2 µL of DNA was added from each patient.

Thermocycling conditions:

PCR amplification (touchdown) was carried out at 95 ºC for 2 min, followed by denaturation at 95 ºC for 20 sec, first annealing at 68 ºC (10 cycles). In the remaining cycles (25 cycles), annealing was carried out at 69 ºC for 1 min and extension at 72 ºC for 50 sec, followed by a final extension for 5 min.

Gel electrophoresis: The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5 µg/mL ethidium bromide and visualized on a UV transilluminator. Primers FO and RO flank the exon 7 of the endothelial nitric oxide gene, resulting a band of 701 bp to act as a control for DNA quality and quantity. Primers Fwt and RO amplify a wild-type allele (G allele), generating a band of 475 bp, and primers FO and Rmt generate a band of 271 bp from the mutant allele (T allele) as depicted in figure 1.



M-100 bp DNA ladder
Heterozous-p1,p4, p6,p7,p11,p12,p13,p1,p18
Homozygous GG genotype -p2,p2,p5,p10,p14,p15,p16,
Homozygous AA genotype -p8,p9,p19

Figure 1: Genotyping endothelial nitric oxide gene polymorphism (eNOS-3 rs1799983 G>T) ARMS-PCR in SCD

Statistical analysis: Group differences were compared using Student's two-sample t-test or one-way analysis of variance (ANOVA) for continuous variables and Chi-squared for categorical variables. Deviations from Hardy-Weinberg disequilibrium (HWD) was calculated by Chi-square (χ^2) goodness-of-fit test. The differences in the miR-423 gene allele and genotype frequencies between groups were evaluated using Chi-square test. The associations between nitric oxide gene polymorphism (e*NOS-3 rs1799983 (Glu298Asp or 894 G>T)* genotypes and SCD were estimated by computing the odds ratios (ORs), risk ratios (RRs) and risk differences (RDs) with 95 % confidence intervals (CIs). Allele frequencies among cases as well as controls were evaluated by using the Chi–square Hardy-Weinberg equilibrium test. A p-value < 0.05 was considered significant. All statistical analyses were performed using Graph Pad Prism 8.4 and SPSS 20.

RESULTS

The Hardy-Weinberg Equilibrium Analysis: The genotype distributions and allele frequencies of the SNPs located in the miR-423 gene showed that no deviation was detected in HWE (> 0.05) (χ^2 = 0.73, *P*=0.39) in the patient group similarly the genotype distributions and allele frequencies of the nitric oxide gene polymorphism (e*NOS-3 rs1799983 (Glu298Asp or 894 G>T)* obeyed HWE (P= 0.83) (χ^2 = 0.043 *p*=0.83) in the control group. Thus, we chose 10% samples from normal control group randomly to review genotyping results, showing that the accuracy rate was more than 99%.

Hardy–Weinberg equilibrium (HWE) test for endothelial nitric oxide (e*NOS-3 rs1799983 (Glu298Asp or 894 G>T)* genotypes.

We performed the $\chi 2$ goodness-of-fit test to test if the genotypes of the SNP eNOS rs1799983 GG, GT &TT genotypes of controls are obeying HWE (Table 2).

Genotype frequency and distribution of Nitric oxide gene polymorphism (e*NOS-3 rs1799983 (Glu298Asp or 894 G>T)* in Sickle cell disease

The genotypes frequency of e*NOS-3 rs1799983 (Glu298Asp or 894 G>T)* GG, GT and TT in Sickle cell disease patients was 33, 66 and 06 while it was 50, 41, 09 in healthy controls respectively (Table 3). This result indicated that genotype frequency of (rs4986893 G>A) polymorphism was significantly different between the Sickle cell disease patients and healthy controls with P value<0.007 (Table 3).

Risk of Sickle cell disease with eNOS-3 rs1799983 (Glu298Asp) in Sickle cell disease

A multivariate analysis based on logistic regression like odds ratio (OD) and risk ratio (RR) with 95% confidence intervals (CI) were calculated for each group to estimate the association between eNOS-3 G>T (Glu298Asp) genotypes and risk to coronary artery disease. Result showed that in the Codominant model the eNOS-3 G>T (Glu298Asp)-GT genotype (heterozygosity) was associated with Sickle cell disease with OR 2.43(1.35-4.38), RR 1.57(1.16-2.11) P=0.002 whereas non-significant association was reported with TT genotype OR1.01 (0.32-3.10) RR1.00 (0.64-1.57) P=0.98. Result showed that in the dominant model the eNOS-3 G>T (Glu298Asp)-(GT+TT) genotype was associated with Sickle cell disease with OR 2.18(1.23-3.85), RR 1.46(1.11-1.93) , P=0.007.In the recessive model results indicated that eNOS-3 G>T (Glu298Asp)-(GG+GT) genotype is not associated with T2D OR 0.61(0.20-1.78) RR 0.79(0.51-1.23) P=0.310.In allelic comparison, the G allele was compared with the T allele, there was a non significant association between G and T allele and Sickle cell disease .Result also showed that the T allele is not associated with T2D with OR= 1.41(0.93-2.13), RR=1.19(0.95-1.50), P-value>0.10 (Table 4). This result indicates a potential dominant effect of eNOS-3 heterozygosity on susceptibility to this disease. The increased susceptibility to Sickle cell disease in Saudi population was found to be associated with eNOS-3 GT genotype.

DISCUSSION

One of the variants of the eNOS gene, known as the rs1799983 TC variation, is a single nucleotide polymorphism (SNP) that alters an amino acid at position 298 from glutamic acid (Glu) to aspartic acid (Asp). This mutation is associated with a decrease in nitric oxide (NO) generation as well as a decrease in eNOS activity [12]. The molecule known as NO is a vasodilator, which means that it causes blood vessels to expand and improves blood flow. In addition, it can help prevent blood clots and reduce inflammation. It is possible for endothelial dysfunction to occur when the generation of NO decreases and a significant risk factor that can lead to a stroke [13]. Several research have been conducted to investigate the correlation between the eNOS rs1799983 TC polymorphism and the likelihood of experiencing a stroke within the population. Having the T allele, which is the most prevalent form of stroke, was found to increase the likelihood of having an ischemic stroke, according to a study of 22 studies that were conducted simultaneously. The odds ratio (OR) for the main model (TT+GT versus GG) was 1.249, which indicated that the strongest connection was observed in this model. However, it has also been demonstrated that the eNOS rs1799983 TC polymorphism is associated with a more severe kind of stroke as well as an increased likelihood of experiencing another stroke. The precise mechanism by which the eNOS rs1799983 TC polymorphism contributes to an increased risk of stroke is not completely understood [14]. The decrease in eNOS activity and NO output, on the other hand, is thought to have some connection to this phenomenon. Endothelial dysfunction, vascular inflammation, and thrombosis are all conditions that can be brought on by this, and they all contribute to an increased risk of having a stroke [15]. It is essential to keep in mind that the eNOS rs1799983 TC polymorphism is one of the many factors that can increase the likelihood of experiencing a stroke. Additionally, factors like as age, high blood pressure, diabetes, and excessive cholesterol all have a role in the development of this condition [16]. The frequency of allele C of rs2070744 polymorphisms was comparable in the Colombian population, however the white Colombian population displayed a greater frequency of the T allele of rs1799983 polymorphisms compared to the other ethnic groups with total of 890 participants . [17].

Another research published in the journal Atherosclerosis found a significant association between the eNOS rs1799983 T^C polymorphism and a 1.8-fold increased risk of stroke in a population of over 50,000 individuals [18]. Additionally, the study revealed that the correlation was most pronounced in Western nations. In the United States, the likelihood of experiencing a stroke was 2.5 times greater, whereas in Europe, it was 2.1 times more. The mechanism by which the eNOS rs1799983 T<C polymorphism increases the risk of stroke is still not fully understood, leading to considerable uncertainty. Alternatively, it is possible that the polymorphism is responsible for a reduction in the production of nitric oxide (NO). Nitric oxide (NO) causes vasodilation, resulting in an enlargement of blood vessels. The blood pressure decreases and blood circulation improves. Vasoconstriction occurs when the synthesis of nitric oxide (NO) decreases, causing blood vessels to narrow. This can increase the likelihood of experiencing a stroke. The researchers determined that the G298T polymorphism has an impact on the production of endothelial nitric oxide. Percentage of the population that is white: 26.5%Frequency of the black population: 14.2%In a research involving 79 individuals with Greek Sickle Cell Disease (SCD) and 48 healthy individuals as controls, it was shown that the frequency of allele C at position 786 was greater than that of allele T. Specifically, the C allele accounted for 62.7% and 68.7% among SCD patients and healthy controls, respectively, whereas the T allele accounted for 37.3% and 31.3% among SCD patients and healthy controls, respectively [19].
Hundred and thirty-four Ghanaian patients suffering from SCD were recruited in a cross-sectional study, among which 46 HbSS patients were without complications and 88 with complications. The frequency of T allele in T786C (rs2070744) variants was 28.5% in the HbSS patients with complications and 24.4% in those with no complications; while the allele C of the same variant was 71.5% and 75.6% respectively [20]. In an investigation of the variant allele frequencies and the distribution of NOS3 gene polymorphism, a study of 173 Brazilian patients (100 controls and 73 SCD patients) observed no statistical difference between the control group and SCD patients as well as between those with severe clinical symptoms [21]. A study involving 150 Indian control group and 150 SCD matched group showed a significant increase in the frequency of eNOS gene polymorphisms among the severe group of SCD patients [8]. To conclude, Our study indicated that eNOS rs1799983 T<C (E298D) genotype and G allele are associated with an increased susceptibility to Sickle cell disease. However, a larger sample size can be the key to progress in establishing the genetic co-relation of miRNA gene polymorphisms and Sickle cell disease.

Conflicts of Interest: There exists no conflict of interest to declare.

Funding source: The authors extend their appreciation to the Deanship of Scientific Research, University of Tabuk, Saudi Arabia, for funding this research work through project number 0030-S-1441.

Acknowledgement: The authors acknowledge the research center Prince Fahad Bin Sultan Chair for Biomedical Research, University of Tabuk, for research facilities.

Data availability: We have included the data associated with the study in the manuscript. In case of specific queries corresponding, authors can be contacted.

Patient consent for publication: Not applicable

Conflicts of Interest: All the contributing authors declare that they do not have any conflict of interest.

Declaration: We clarify the all information in the manuscript are true and complete in the best of our knowledge.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki revised in 2013 and approved by the ethics committee of the University of Tabuk (protocol code UT-134-100-2021 and date of approval 24/04/2020.

REFERENCES

1. Cruz, L.J., et al., *PLGA-nanoparticles for intracellular delivery of the CRISPR-complex to elevate fetal globin expression in erythroid cells.* Biomaterials, 2021. **268**: p. 120580.
2. Abu-Shaheen, A., et al., *Sickle cell disease in Gulf Cooperation Council countries: A systematic review.* Expert Review of Hematology, 2022. **15**(10): p. 893-909.
3. Baothman, A., et al., *Prevalence of Red Blood Cell Alloimmunization Among Pediatric Patients with Sickle Cell Disease in Saudi Arabia.* European Journal of Health Sciences, 2023. **9**(2): p. 24-36.
4. Ungvari, Z., et al., *Endothelial dysfunction and angiogenesis impairment in the ageing vasculature.* Nature Reviews Cardiology, 2018. **15**(9): p. 555-565.
5. Zhu, Y., et al., *The genetic association between iNOS and eNOS polymorphisms and gastric cancer risk: a meta-analysis.* OncoTargets and therapy, 2018: p. 2497-2507.
6. Severino, P., et al., *Potential role of eNOS genetic variants in ischemic heart disease susceptibility and clinical presentation.* Journal of Cardiovascular Development and Disease, 2021. **8**(9): p. 116.
7. Silva, M. and P. Faustino, *From stress to sick (le) and back again–oxidative/antioxidant mechanisms, genetic modulation, and cerebrovascular disease in children with sickle cell anemia.* Antioxidants, 2023. **12**(11): p. 1977.
8. Nishank, S.S., et al., *Endothelial nitric oxide synthase gene polymorphism is associated with sickle cell disease patients in India.* Journal of human genetics, 2013. **58**(12): p. 775-779.

9. Yousry, S.M., H.N. Ellithy, and G.H. Shahin, *Endothelial nitric oxide synthase gene polymorphisms and the risk of vasculopathy in sickle cell disease.* Hematology, 2016. **21**(6): p. 359-367.
10. Ofori-Acquah, S.F., *Sickle cell disease as a vascular disorder.* Expert Review of Hematology, 2020. **13**(6): p. 645-653.
11. Germano, I., et al., *Genetic modulation of anemia severity, hemolysis level, and hospitalization rate in Angolan children with Sickle Cell Anemia.* Molecular Biology Reports, 2022. **49**(11): p. 10347-10356.
12. Aouf, S., et al., *Contribution of Nitric oxide synthase 3 genetic variants to nasopharyngeal carcinoma risk and progression in a Tunisian population.* European Archives of Oto-Rhino-Laryngology, 2019. **276**: p. 1231-1239.
13. Tousoulis, D., et al., *The role of nitric oxide on endothelial function.* Current vascular pharmacology, 2012. **10**(1): p. 4-18.
14. Luo, Z., et al., *Associations of the NOS3 rs1799983 polymorphism with circulating nitric oxide and lipid levels: a systematic review and meta-analysis.* Postgraduate Medical Journal, 2019. **95**(1125): p. 361-371.
15. Daiber, A., et al., *New therapeutic implications of endothelial nitric oxide synthase (eNOS) function/dysfunction in cardiovascular disease.* International journal of molecular sciences, 2019. **20**(1): p. 187.
16. Jankovic, M., et al., *Genetic and epigenomic modifiers of diabetic neuropathy.* International journal of molecular sciences, 2021. **22**(9): p. 4887.
17. Oliveira-Paula, G.H., R. Lacchini, and J.E. Tanus-Santos, *Clinical and pharmacogenetic impact of endothelial nitric oxide synthase polymorphisms on cardiovascular diseases.* Nitric Oxide, 2017. **63**: p. 39-51.
18. Butnariu, L.I., et al., *Etiologic puzzle of coronary artery disease: how important is genetic component?* Life, 2022. **12**(6): p. 865.
19. Armenis, I., et al., *Data on eNOS T786 and G894T polymorphisms and peripheral blood eNOS mRNA levels in Sickle Cell Disease.* Data in brief, 2017. **10**: p. 192-197.
20. Antwi-Boasiako, C., et al., *Association between eNOS gene polymorphism (T786C and VNTR) and sickle cell disease patients in Ghana.* Diseases, 2018. **6**(4): p. 90.
21. Chenou, F., et al., *Endothelial nitric oxide synthase (eNOS) gene polymorphisms and markers of hemolysis, inflammation and endothelial dysfunction in brazilian sickle cell anemia patients.* Biochemical genetics, 2020. **58**: p. 580-594.

Table 1: Genotyping ARMS-PCR primers for endothelial nitric oxide gene polymorphism (eNOS-3 rs1799983 (, Glu298Asp or 894 G>T) in SCD			
eNOS-3 Fo	5′-AGCCTCGGTGAGATAAAGGATG-3′	701 bp	**69^{0}C**
eNOS-3 Ro	5′-CCTGGACCTGCTCTGATTGTC-3′		
eNOS-3 FI G	5′-GCTGCTGCAGGCCCCAGATAAG-3′	475 bp	
eNOS-3 RI T	5′-GCAGAAGGAAGAGTTCTGGGAGA-3′	271 bp	

Table 2: Hardy-Weinberg equilibrium analysis for healthy population		
Genotypes	**Observed**	**Expected**
Homozygote reference	50	49.7
Heterozygote	41	41.6
Homozygote variant	9	8.7
Var allele freq	0.30	100
Chi-squared value	0.020	
Chi-squared test P value	0.88	

Table 3: Association of eNOS rs1799983 G to T gene variation in SCD cases and controls									
Subjects	**N=**	**GG**	**GT**	**TT**	**df**	**X2**	**G**	**T**	**P value**
Cases	105	33	66	06	2	9.81			0.007
Controls	100	50	41	09					

Table 4: Association of eNOS-3 rs1799983 (Glu298Asp or 894 G>T) gene variation in SCD cases and controls					
Genotypes	Healthy controls	CAD cases	OR (95% CI)	Risk Ratio(RR)	P-Val
	(N=105)	(N=100)			
Codominant					
e*NOS-3* –GG	50	33	1(ref.)	1(ref.)	
e*NOS-3* –GT	41	66	2.43(1.35-4.38)	1.57(1.16-2.11)	0.002
e*NOS-3* –TT	09	06	1.01(0.32-3.10)	1.00(0.64-1.57)	0.98
Dominant					
e*NOS-3* –GG	50	33	1(ref.)	1(ref.)	
e*NOS-3* –(GT+TT)	50	72	2.18(1.23-3.85)	1.46(1.11-1.93)	0.007

Recessive					
e*NOS-3* –(GG+GT)	91	99	1(ref.)	1(ref.)	
e*NOS-3* –TT	09	06	0.61(0.20-1.78)	0.79(0.51-1.23)	0.310
Allele					
e*NOS-3* —G	141	132	1(ref.)	1(ref.)	
e*NOS-3* —T	59	78	1.41(0.93-2.13)	1.19(0.95-1.50)	0.10

Table 5: A summary of T786C genotype frequency in different countries

Country	**N=**	**Genotypic frequencies**		
		C/C	T/C	T/T
Saudi Arabia	106 sicklers (62 male , 44 female and 100 controls	32.2%	62.9%	4.9%
Columbia	890 SCD and 917 controls	64.1%	31.585	4.32%
Greece	127(79 sicklers and49 controls)	46.8%	31.7%	21.5%
Ghana	134(88 with complications and 49 without)	51.2% and 62.2% respectively	40.7% and 26.7% respectively	8.1% (in those with complications); 11.1% in those without complications)
Brazil	173;100 controls and 73 ss patients (35 males and 38 females)	5%	25%	70%
India	300; 150 SCD patients and 150 matched control	16.6% SCD; 1.33% controls	52.6% SCD; 38.6% controls	30.6% SCD; 60.0% controls