

## Endothelin-1 but not Endothelial Nitric Oxide Synthase Gene Polymorphism is Associated with Sickle Cell Disease in Africa

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**ABSTRACT:** Sickle cell disease shows marked variability in severity and pathophysiology among individuals, probably linked to differential expression of various adhesion molecules. In this study, we investigated the differential distribution, genomic diversity and haplotype frequency of endothelial nitric oxide synthase (eNOS) and endothelin-1 (ET-1) polymorphisms, recently implicated as important in modification of disease severity. One hundred and forty five sickle cell disease patients (HbSS) and 244 adult and pediatric controls, without sickle cell disease (HbAA), were recruited from Mali. Genotypic analysis of the functionally significant eNOS variants (T786C, G894T and intron 4) and endothelin-1 (G5665T) was carried out with a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. Our results show that the wild type alleles are the most frequent for all eNOS variants between cases and controls. Allelic and genotypic frequencies of eNOS polymorphic groups are not significantly different between cases and controls ( $P > 0.05$ ). In addition, there is no association between eNOS variants and sickle cell disease, contrary to published reports. On the other hand, we report that endothelin-1 (G5665T) mutant variant had the lowest allelic frequency, and is significantly associated with sickle cell disease in Africa ( $P < 0.05$ ). Similarly, haplotype frequencies were the same between cases and controls, except for the haplotype combining all mutant variants (T, C, 4a;  $P = 0.01$ ). eNOS polymorphic variants are less frequent, with no significance with sickle cell disease in Africa. On the other hand, endothelin-1 is associated with sickle cell disease, and has the capacity to redefine pathophysiology and possibly serve as modulator of disease phenotype.

**KEYWORDS:** endothelial nitric oxide synthase, endothelin-1, sickle cell disease, polymorphisms, pathophysiology

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### Introduction

Sickle cell disease (SCD) is an inherited red cell disorder with multisystem complications and is characterized by chronic hemolytic anemia, vaso-occlusive crises and marked variability in disease severity between individuals. It is present in patients homozygous for the hemoglobin S (HbSS) gene, produced by a defective beta-globin gene on chromosome 11. It remains a major health problem in many parts of the world, with variants of the HbSS and other thalassemias

presenting similarly,<sup>1</sup> and four major sickle haplotypes associated with specific regions found in Africa.<sup>2</sup> The burden of sickle cell disease is presently at an alarming phase and was recently described as an emerging health burden,<sup>3–5</sup> whose greatest effects are probably exerted on a few countries.<sup>1</sup> In the United States, SCD remains a challenge in the African American and immigrant community, despite better comprehensive care and advanced genotype testing. The emotional toll from constant emergencies and multiple



transfusions arising from crisis and other complications remain a great concern.

Nitric oxide (NO) plays a significant role in the regulation of cardiovascular homeostasis by attenuating leukocyte adhesion to endothelium and is produced in endothelial cells by endothelial nitric oxide synthase (eNOS).<sup>6</sup> Published studies have suggested roles for several single nucleotide polymorphisms, and various risks for stroke, leg ulceration, pulmonary hypertension, priapism and osteonecrosis in sickle cell disease patients, including the endothelial nitric oxide synthase and endothelin-1 genes. These include a polymorphism in the promoter region (T786C; rs2070744), another in exon 7 (G894T; rs1799983), and the variable number of tandem repeats (VNTR) in intron 4<sup>7–11</sup> of the eNOS gene. The eNOS VNTR has been associated with the mean plasma nitric oxide level, while C-786 variant has been found to reduce eNOS gene promoter activity and is a genetic risk factor for acute chest syndrome in adult female sickle cell anemia patients.<sup>11,12</sup> A recently published report found a higher prevalence of mutant alleles and genotypes of all three eNOS single nucleotide polymorphisms in sickle cell disease individuals, implying an association between eNOS gene polymorphisms and sickle cell disease in India.<sup>13</sup> The finding of higher incidence of mutant alleles and genotypes among the SCD severe group in contrast to higher incidence of wild alleles and genotypes among the SCD mild group of patients indicate the eNOS gene probably acting as a genetic modifier of phenotypic variation among Indian patients.<sup>13</sup> We, and others have shown that there is a substantial interethnic diversity in the distribution of eNOS variants,<sup>14–18</sup> and this difference could potentially clarify interethnic differences in nitric oxide bioavailability, and potentially sickle cell pathophysiology.

Endothelin-1 (rs5370) gene, on the other hand, plays a very unique role in maintaining vascular wall tone, is synthesized by many cell types<sup>19</sup> and has been associated with vasospasm, vascular damage and inflammation.<sup>20</sup> Recent evidence have shown that sickle cell disease affects blood vessel functions causing abnormal vascular tone and activated endothelium, and these abnormalities may promote dysfunction and destruction through active ET-1 production and signals.<sup>20–22</sup> High ET-1 urinary output observed in SCD subjects at steady state is thought to reflect increased tubular activation of ET-1 production; since the kidneys are implicated during vaso-occlusive ischemic attacks, high levels of ET-1 is a potent contributor to adverse outcomes. Published report suggests that endothelin-1 may be involved in the progression of chronic glomerulosclerosis affecting a number of SCD patients.<sup>23</sup>

Acute chest syndrome (ACS) and painful vaso-occlusive crises are common causes of hospitalization in sickle cell anemia, and published reports have suggested the involvement of endothelin-1 in the pathogenesis of these complications. Endothelin-1 genomic variants, G5665T and T8002C have been associated with abnormal vascular reactivity, with

the ET-1 C8002 allele appearing to increase the risk of acute chest syndrome.<sup>11</sup> This observation provided a basis for extensive investigations into the role of ET-1 gene polymorphisms in sickle cell pathophysiology. In a recent report, administering a sustained nitric oxide-releasing compound reverses dysregulated NO signal transduction in priapism, a common feature in sickle cell disease,<sup>24</sup> implying a need to understand how eNOS and ET-1 polymorphisms contribute to nitric oxide bioavailability. Additionally, there is a paucity of information on how this observation plays within and between sickle cell groups that are genetically homogeneous, since previous studies were carried out in genetically heterogeneous populations. To this end, clarifying the role of eNOS and ET-1 polymorphisms on genetic variability of disease among Africans (genetically homogeneous population) with sickle cell disease is highly imperative. We examined the genomic diversity and haplotype frequency of functionally significant endothelin-1 and endothelial nitric oxide synthase gene polymorphisms in individuals with sickle cell disease recruited from Mali. To our knowledge, this is the first report to examine these polymorphisms in an African sickle cell disease population.

## Materials and Methods

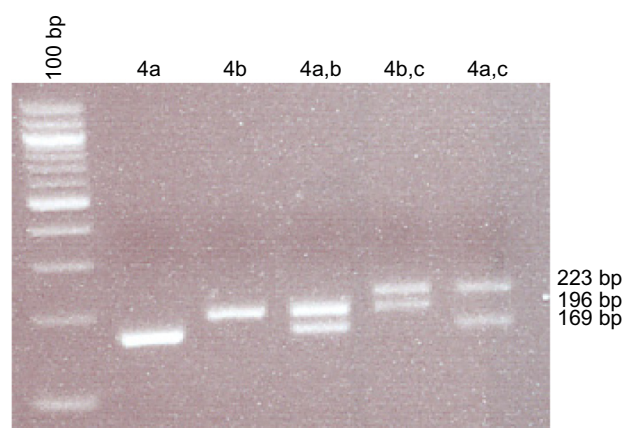
**Subjects (Patients and Controls).** This study was conducted at the Centre de Recherche et de Lutte contre la Drepanocytose (CRLD), a referral facility for managing sickle cell disease and its complications in Bamako, Mali. Approval for study was received after evaluation from the national ethical review board, and a written consent obtained before study initiation and patient recruitment. Inclusion criteria include diagnosis with sickle cell disease and presentation at the hospital during crisis or during regular follow-up. Volunteer subjects, predominantly family members, or recruited by word of mouth and able to provide informed, written consent for participation, and without a diagnosis of sickle cell disease (confirmed to be normal hemoglobin, HbAA) were recruited as controls. On presentation, demographic profiles and clinical symptoms were recorded, blood samples collected and electrophoretic analysis using High Performance Liquid Chromatography (HPLC) was performed to confirm sickle cell diagnosis.

**Genomic DNA extraction.** This project was reviewed and approved by the Institutional Review Board, Rochester Institute of Technology. Blood samples analyzed in this study were all obtained at the Centre de Recherche et de Lutte contre la Drepanocytose. Discarded EDTA-anticoagulated blood samples, from a total of 389 subjects, comprising of 145 sickle cell disease patients and 244 normal, asymptomatic controls were spotted onto Whatmann filter papers (GE Healthcare Sciences), and shipped to the United States, per the shipping policy of the Centers for Disease Control and Prevention. Well-characterized genomic DNA samples were extracted from the dried, spotted samples with the Qiagen

Blood Mini Kit (Qiagen Inc., Valencia, CA), with minor adjustment to the manufacturer's instruction. Briefly, dried blood spots were clipped from filter papers and transferred to clean 1.5 ml centrifuge tubes for further processing. Rather than the three filter paper clips, per the manufacturer's guidelines, we used four filter paper clips to enhance DNA concentration, and suitability for downstream applications. Final elution volume was 100  $\mu$ l and DNA samples were stored at  $-20^{\circ}\text{C}$  until further analysis.

**Genotyping of the Variable Number of Tandem Repeats (VNTR) polymorphism in intron 4.** Amplification of DNA samples for polymorphic analysis of variable number of tandem repeats (VNTR) in intron 4 (27 bp TR) was performed with previously published<sup>17</sup> and modified protocols<sup>18</sup> using the following primer set.<sup>16</sup> Primers 5'-CTATGGTAGTGCCTTGGCTGGAGG-3' (forward) and 5'-ACCGCCAGGGAACTCCGCT-3' (reverse) and 1  $\mu$ l of genomic DNA template were used for PCR amplification, with conditions altered to 25  $\mu$ l final volume and amplified using the Lucigen EconoTaq Plus Green 2X Master Mix PCR system (Lucigen Corporation, Middleton WI). PCR cycling parameters followed published protocol<sup>17</sup> and 5  $\mu$ l of the amplified PCR product was evaluated on 2% ethidium bromide stained agarose gels, visualized and photographed. Estimation of product size was carried out with 5  $\mu$ l TriDye 100 bp DNA ladder (New England Biolabs, Boston MA) and product size analysis with the Doc-It LS Image Analysis Software (UVP Life Sciences, Upland CA). Fragment of 169, 196, 223 and 142 bp (Fig. 1) corresponding to eNOS alleles 4a, 4b, 4c and 4d, defined the presence of four, five, six and three 27 bp repeats respectively.<sup>18</sup>

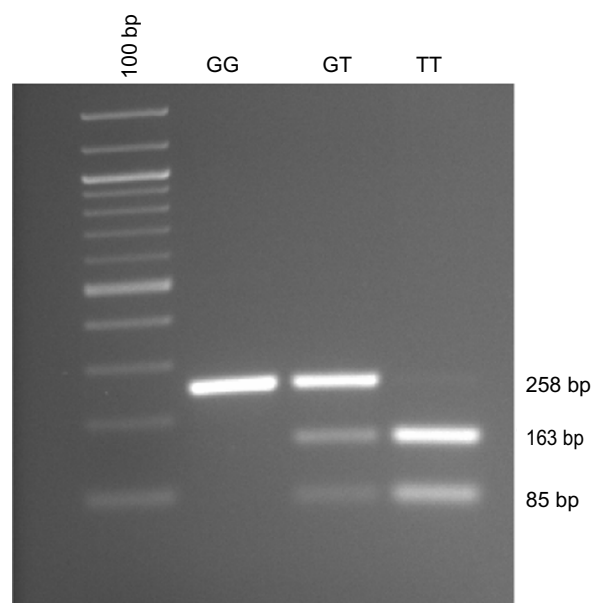
**Genotyping of G894T polymorphism in exon 7 and T786C polymorphism in the 5'-flanking region.** The G-to-T amino acid transversion (G894T; rs1799983), leading to a glutamate-aspartate substitution at codon 298 (Glu298Asp), and the T786C (rs2070744) polymorphism on the promoter region of the eNOS gene (a T-to-C transition) were genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, with the following primer pairs: 5'-AAGGCAGGAGACAGTGGATGGA-3' and 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' for G894T forward and reverse reactions and 5'-TGGAGAGTGCTGGTG TACCCCA-3' and 5'-GCCTCCACCCCA-CCCTGTC-3' for T786C forward and reverse reactions respectively.<sup>15</sup> PCR setup and reaction conditions are according to a previously published protocol.<sup>18</sup> For the G894T (rs1799983) polymorphism, amplified PCR products of 258 base pair band size were digested with 2U *BanII* (New England Biolabs, Boston MA) restriction endonuclease for 1 hour at  $37^{\circ}\text{C}$ . This enzyme cleaves the PCR product (Fig. 2) into fragments of 163 and 85 bp fragments (wild type) or no digestion (variant allele). In the case of T786C (rs2070744) polymorphism in the 5'-flanking region, PCR products of the appropriate band size were digested for 1 hour with 1  $\mu$ l *MspI*



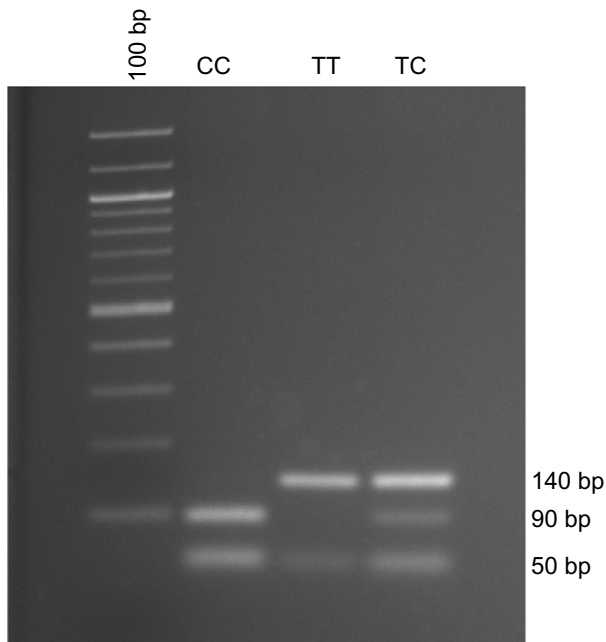
**Figure 1.** Agarose gel electrophoresis showing variants of the intron 4 (27-bp TR) polymorphism of the endothelial nitric oxide synthase gene. Ladder: 100 bp ladder, where the 500 bp band stains most intensely, was used as a molecular weight marker for all gels.

restriction enzyme at  $37^{\circ}\text{C}$ , to produce fragments (Fig. 3) of 140 and 40 base pairs for the wild-type (TT) allele, or 90, 50, and 40 base pairs for the variant (CC). Size of amplified PCR products and digested fragments were estimated with a TriDye 100 bp DNA ladder (New England Biolabs, Boston MA) and a Doc-It LS Image Analysis Software (UVP Life Sciences, Upland CA).

**Endothelin-1 (G5665T) polymorphism.** To genotype the G5665T (rs5370) polymorphism on the endothelin-1 (ET-1) gene, a revised version of a previously published PCR protocol<sup>35</sup> and a PCR-RFLP assay was employed. Primer pairs



**Figure 2.** Agarose gel electrophoresis showing variants of the G894T (rs1799983) polymorphism of the endothelial nitric oxide synthase gene. PCR products were digested in 2U of *BanII* restriction endonuclease. bp base pairs; Ladder: 100 bp ladder, where the 500 bp band stains most intensely, was used as a molecular weight marker.



**Figure 3.** Agarose gel electrophoresis showing variants of the T786C (rs2070744) polymorphism of the endothelial nitric oxide synthase gene. PCR products were digested in 2U of *MspI* restriction endonuclease. bp base pairs; Ladder: 100 bp ladder, where the 500 bp band stains most intensely, was used as a molecular weight marker.

rs5370F: 5'-TCTTGCTTTATTAGGTCGGAGACC-3' and rs5370R: 5'-TTTGAACGAGGACGCTGGTC-3' was used to amplify a part of the gene, with PCR setup and amplification conditions as follows: 95 °C for 10 min, and 35 cycles of 95 °C for 1 min (denaturation), 61 °C for 1 min (annealing), 72 °C for 1 min and 30 s (extension) and 72 °C for 10 min (final extension). PCR products of 262 bp were digested with 2U *Cac8I* (New England Biolabs, Boston MA) restriction endonuclease (2 hour incubation) at 37 °C on an Eppendorf gradient Mastercycler. Digested products were examined on a 2% ethidium bromide stained agarose gels and size analysis carried out as described.<sup>47</sup> All PCR genotyping and restriction digestion were conducted anonymously, with 50% of samples subjected to repeat PCR and genotyping for quality control purposes with 100% concordance.

**Statistical analysis.** Of the four alleles in intron 4, the 4c and 4d alleles are rare; for analysis purposes, they were pooled with the 4a variant as reported elsewhere.<sup>18</sup> Thus, each of the four variants had two alleles. A simple PERL script (<http://www.perl.org>) was written to facilitate analysis of genotypic and allelic frequencies of each variant, and to convert original data files to an EH program format. Differences in genotype and allele frequencies between disease and controls were assessed by odds ratio. Haplotype frequencies were estimated and tested for disease differences with the EH program (<http://lab.rockefeller.edu/ott/programs>).<sup>48</sup> Individuals missing one or more genotypes were disregarded for analysis purposes (15 sickle cell disease patients and 13 controls).

## Results

We examined the genotypic and allelic diversities, as well as the haplotype frequencies of endothelial nitric oxide synthase (eNOS) gene and endothelin-1 (ET-1) gene polymorphisms between sickle cell disease patients and controls in Mali. The sickle cell disease group consists of 51.5% males and 48.4% females, (average: 21 years; range 1–51 years), and mostly of the Bambaran tribe. The polymorphisms analyzed for the eNOS gene are the variable number of tandem repeats in intron 4, eNOS 4a/b (27 bp TR); one in the promoter region, eNOS -786T > C (rs2070744), another in exon 7, eNOS 894G > T (rs1799983), and the ET-1 5665G > T (rs5370) polymorphism on the endothelin-1 gene. The genotypic and allelic frequencies for eNOS wild type, heterozygotes and mutants are shown on Tables 1 and 2, while the results for the endothelin-1 gene polymorphisms is summarized in Table 3. The homozygous wild types occur at a higher frequency for all polymorphisms in both sickle cell disease and control groups (71.6% versus 71.4% for T/T (rs2070744), 84.6% versus 80.5% for GG (rs1799983) and 46.2% versus 42.0% for 4b/4b (27 bp TR) of eNOS -786T > C, eNOS 894G > T and eNOS 4a/b respectively). The lowest frequencies for both case and controls were found in the homozygous mutants (2.3% versus 2.2% for C/C (rs2070744), 0.8% versus 0% for T/T (rs1799983) and 17.7% versus 13% for 4a/4a of eNOS -786T > C, 894G > T and eNOS 4a/b respectively). However, there was no significant difference in the genotypic frequencies of all eNOS variants (eNOS -786T > C, eNOS 894G > T and eNOS 4a/b) between sickle cell disease and controls ( $P = 0.995$ ,  $0.187$  and  $0.222$  for eNOS -786T > C, eNOS 894G > T and eNOS 4a/b respectively). Similar observation was made for allelic frequencies of eNOS polymorphisms, with no difference between sickle cell and control groups ( $P = 0.995$ ,  $0.452$  and  $0.928$  for eNOS -786T > C, eNOS 894G > T and eNOS 4a/b respectively).

In the case of G5665T (rs5370) polymorphism on the ET-1 gene, the highest (79.2% versus 68.3%;  $P = 0.027$ ) and lowest (0% versus 2.1%;  $P = \text{ND}$ ) genotypic frequencies (Table 3) were found for homozygous wild type (ET-1 5665G > G) and homozygous mutant (ET-1 5665T > T) respectively. A similar finding was recorded for allelic frequencies as well (highest 89.6% versus 83%;  $P = 0.015$  and lowest 10.4% versus 17%;  $P = 0.015$ ; Table 3). Unlike the observation recorded with eNOS gene polymorphisms, there was a significant difference between sickle cell disease and control groups. In addition, further analysis reveals an association between the rs5370 polymorphism and sickle cell disease ( $P = 0.015$  and  $0.014$  for genotypes and alleles respectively).

Similar to our previously published report,<sup>18</sup> four types of eNOS gene haplotypes were observed between sickle cell and control groups (Table 4). The most common, with the highest frequencies, combines the wild-type homozygotes for eNOS -786T > C and eNOS 4a/b with mutant eNOS 894G > T (44.9% versus 43.8% for sickle cell disease and control groups),

**Table 1.** Genotypic frequency of endothelial nitric oxide synthase (eNOS) gene polymorphisms in sickle cell disease and control groups.

POLYMORPHISM	GENOTYPE	SCD: N = 130 (%)	CONTROLS: N = 231 (%)	ODDS RATIO (95% CI)	P-VALUE
T786C (rs2070744)	T/T	93 (71.6)	165 (71.4)	1.01 (0.61–1.67)	1
	T/C	34 (26.1)	61 (26.4)	0.99 (0.58–1.65)	1
	C/C	3 (2.3)	5 (2.2)	1.07 (0.16–5.59)	1
G894T (rs1799983)	G/G	110 (84.6)	186 (80.5)	1.33 (0.73–2.51)	0.3925
	G/T	19 (14.6)	45 (19.5)	0.71 (0.37–1.31)	0.3149
	T/T	1 (0.8)	0 (0.0)	ND	ND
Intron 4 (27-bp TR)	4a/4a	23 (17.7)	30 (13.0)	1.43 (0.75–2.69)	0.2783
	4a/4b	47 (36.1)	103 (45.0)	0.70 (0.44–1.11)	0.1201
	4b/4b	60 (46.2)	97 (42.0)	1.17 (0.74–1.85)	0.5072

**Note:** Odds ratio was calculated by Fisher's two-tailed exact test.

**Abbreviations:** SCD, sickle cell disease; NS, not significant; CI, confidence interval; eNOS, endothelial nitric oxide synthase.

followed by wild type homozygotes for all three polymorphisms (31.6% versus 33.5%) for sickle cell disease and controls respectively. The haplotype combining G, T, 4a and G, C, 4a are completely absent in both groups, as well as the haplotype combining all mutant variants in sickle cell disease group (0%). There is no significant difference in haplotype frequencies between case and controls ( $P = 0.604$ ).

## Discussion

Sickle cell disease is still one of the most important single gene disorders, found in varying ethnic and regional groups globally. In the African continent, where access to comprehensive medical care is lacking, it is a significant contributor to childhood mortality.<sup>2,49</sup> Considering the diversity of clinical presentation and pathophysiology, it is imperative that the significance of polymorphisms with clinical relevance be delineated. Previous studies on variants of the 27 bp TR, rs2070744, rs1799983 and rs5370, and implications for their significance in sickle cell disease have been carried out in

other populations. To our understanding, this is the first report to investigate the role of endothelial nitric oxide synthase and endothelin-1 gene polymorphisms in an African population with sickle cell disease. Studies into endothelial nitric oxide synthase gene polymorphisms and their role in disease outcome or severity, or pharmacological observations have revealed the significance of this gene.<sup>25–29</sup> From our study, we report that there are no differences at all levels in the percentile distribution or frequencies of endothelial nitric oxide synthase polymorphisms between sickle cell disease and control groups. The genotypic and allelic frequencies are similar in distribution and the  $P$  value insignificant. All polymorphisms studied (the 27 bp TR, rs2070744 and rs1799983) were equally distributed, and followed the Hardy-Weinberg equilibrium (no deviations) in both sickle cell disease and control groups, showing this observation is not due to any selection pressure, attributable to disease.

Published records reveal a handful of reports examining the relationship between sickle cell disease and endothelial

**Table 2.** Allelic frequency of endothelial nitric oxide synthase (eNOS) gene polymorphisms in sickle cell disease and controls.

POLYMORPHISM	ALLELE	SCD: N = 260 (%)	CONTROLS: N = 462 (%)	ODDS RATIO (95% CI)	P-VALUE
T786C (rs2070744)	T	220 (84.6)	391 (84.6)	0.9987 (0.6440–1.5652)	1
	C	40 (15.4)	71 (15.4)	1.001 (0.6389–1.5529)	1
G894T (rs1799983)	G	239 (91.9)	417 (90.3)	0.8145 (0.4494–1.4349)	0.503
	T	21 (8.1)	45 (9.7)	1.2278 (0.6969–2.2251)	0.503
Intron 4 (27-bp TR)	4a	93 (35.8)	163 (35.3)	0.9856 (0.7092–1.3727)	0.9356
	4b	167 (64.2)	297 (64.3)	1.0147 (0.7285–1.4101)	0.9356

**Note:** Odds ratio and P-value was calculated by two-tailed Fisher's exact test.

**Abbreviations:** SCD, sickle cell disease; NS, not significant; CI, confidence interval; eNOS, endothelial nitric oxide synthase.

**Table 3.** Genotypic and allelic frequency of endothelin-1 (G5665T) gene polymorphism in sickle cell disease and control groups.

POLYMORPHISM	GENOTYPE	SCD: N = 130 (%)	CONTROLS: N = 230 (%)	ODDS RATIO (95% CI)	P-VALUE
Endothelin-1 (rs5370)	G/G	103 (79.2)	157 (68.3)	0.5646 (0.3259–0.9583)	0.0277
	G/T	27 (20.8)	68 (29.6)	1.5992 (0.9386–2.7790)	0.0813
	T/T	0 (0.0)	5 (2.1)	ND	ND
	ALLELE	SCD: N = 260 (%)	CONTROLS: N = 460 (%)	ODDS RATIO (95% CI)	P-VALUE
Endothelin-1 (rs5370)	G	233 (89.6)	382 (83.0)	0.5699 (0.3513–0.8993)	0.01586
	T	27 (10.4)	78 (17.0)	1.7547 (1.1112–2.8467)	0.01586

**Note:** Odds ratio and *P*-value was calculated by two-tailed Fisher's exact test.

**Abbreviations:** SCD, sickle cell disease; ND, not determined; CI, confidence interval; ET-1, endothelin-1.

nitric oxide synthase gene polymorphisms. Among such is a Brazilian study, which reported no association between sickle cell disease and eNOS variants.<sup>30</sup> Further reports on sickle cell disease in children and adult females shows significant association of eNOS  $-786T > C$  (rs2070744) variant with disease symptoms<sup>11,12</sup> and are all contrary to a recent report from India showing a significant association between sickle cell disease and all three eNOS gene polymorphisms.<sup>13</sup> In fact, they showed that disease severity is significantly associated with the mutant homozygous eNOS variant. Our results are contrary to this observation, and potentially confirm previous reports of extensive interethnic diversity of eNOS gene polymorphisms. This diversity might be a contributory factor in our present observation.<sup>14,17,18</sup> In India, there is a huge divergence in sickle cell disease estimates across different regions plus sub-ethnic diversification, probably contributing to genomic heterogeneity. Our population (cases and controls) is a homogeneous African population from Mali

compared to the potential ethnic admixture in the Indian continent, and the possibility of skewing conclusions in this group.

On the other hand, our observation on endothelin-1 (G5665T) gene polymorphisms in relation to sickle cell disease is unique. We found a significant difference in the frequencies of endothelin-1 (G5665T) polymorphisms between sickle cell disease and control groups ( $P < 0.05$ ). This is the first time an association between endothelin-1 (G5665T) gene polymorphism and sickle cell disease has been established. Association between this gene and other disease syndromes is widely established with its homozygous variant contributing to disease outcome or severity.<sup>31–35</sup> The present observation demands extensive genome-wide analysis in different population groups, particularly other African and African American sickle cell groups, considering the phenotypic similarity and genotypic heterogeneity in the latter. The observation of geographic-structured alignment in

**Table 4.** Estimated eNOS gene haplotype frequencies between sickle cell disease patients and controls.

HAPLOTYPE			HAPLOTYPE FREQUENCIES			ODDS RATIO (95% CI)	P-VALUE
GLU298 ASP	T786C	INTRON 4	SCD	CONTROLS	SCD VERSUS CONTROL		
Glu	T	4b	0.315932	0.334733	0.329187	0.91 (0.65–1.28)	0.62
Glu	C	4b	0.087914	0.054878	0.06555	1.70 (0.90–3.19)	0.09
Asp	T	4b	0.449453	0.437508	0.442287	1.05 (0.77–1.45)	0.76
Asp	T	4a	0.080769	0.07408	0.074787	1.11 (0.60–2.01)	0.77
Asp	C	4b	0.065932	0.075479	0.071563	0.85 (0.43–1.60)	0.65
Asp	C	4a	0	0.023323	0.016626	--	0.009

**Notes:** Odds ratio and *P*-value was calculated by two-tailed Fisher's exact test. Haplotype analysis was carried out with the EH program (lab.rockefeller.edu/ott/programs).

**Abbreviations:** SCD, sickle cell disease; ND, not determined; CI, confidence interval; eNOS, endothelial nitric oxide synthase.



genetic markers among African Americans is consistent with underlying genetic differences in geographic variation for disease susceptibility<sup>36–41</sup> and how this relates to sickle cell pathogenesis makes for an interesting conclusion. Previous analyses of results from restriction fragment length polymorphisms assay of the beta-globin gene<sup>2,42–45</sup> have shown that there are possibly up to 5 independent and spontaneous clusters of sickle cell gene mutations, with the majority of these in Africa, giving rise to haplotypes such as ‘Senegal’ (Atlantic West Africa), ‘Benin’ (Central West Africa), ‘Bantu’ (Central African Republic) and ‘Cameroon’. The fifth haplotype, ‘Arab-Indian’, predominates in India and parts of the Middle East. How these haplotype diversities play in our current observation *vis-a-vis* the report from India, and possible contribution to genetic evolution in African American sickle cell population deserves further analysis. In addition, deciphering the unique contribution of environmental factors (compare West Africa to Central Africa to East Africa to Middle East-India) to genetic makeup of sickle cell patients, and how this attenuates or exacerbates clinical severity of disease demands painstaking deconvolution.

Several factors including nitric oxide bioavailability have been shown to be significant mechanisms contributing to endothelial dysfunction in sickle cell disease, which is further exacerbated by genetic polymorphisms including eNOS and ET-1 polymorphisms. Other reports have shown a role for an overlap of several unrelated processes, for which impaired NO bioavailability is central, and its consequence including endothelial cell activation and upregulation of endothelin-1, ultimately contributing to disease pathophysiology.<sup>46</sup> The significance of eNOS and ET-1 polymorphisms, classified along ethnic lines on disease outcomes, plus disease pressures due to endemic co-morbidities are currently unknown, and would significantly benefit from the present report.

Conclusively, endothelial nitric oxide synthase mutants are less frequent and lack any functional significance among patients with sickle cell disease in Africa, contrary to other reports.<sup>11–13</sup> In addition, we found a potential role for endothelin-1 polymorphisms in sickle cell disease that might impact clinical outcome in African cases. Expanding the sample size and extending this study to other sickle cell haplotypes within the African subcontinent would further assist in elucidating the clinical and therapeutic values of endothelial nitric oxide synthase and endothelin-1 gene polymorphisms in disease pathophysiology. Our conclusions would be further assisted by disease stratification (severe versus mild) as well as interaction analysis of ethnicity and clinical outcome, which is currently unknown, in this population.

### List of Abbreviations Used

eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; SCD: sickle cell disease; OR: odds ratio; CI: confidence interval; HbAA: hemoglobin AA; HbSS: hemoglobin SS;

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; VNTR: variable number of tandem repeats; ACS: acute chest syndrome.

### Author Contributions

BNT conceived and designed the experiment, and optimized protocols. AG and DAD carried out the clinical sample collection and sickle cell genotyping. BNT carried out DNA extraction. BNT, TJT and LRC carried out eNOS and ET-1 genotyping and restriction digestion. BNT and IGI drafted the manuscript. BNT and YL carried out the statistical analyses. All authors read and approved the final version of the manuscript.

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