

Endothelial Nitric Oxide Synthase (–786T>C) and Endothelin-1 (5665G>T) Gene Polymorphisms as Vascular Dysfunction Risk Factors in Sickle Cell Anemia

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ABSTRACT: Sickle cell anemia (SCA) patients have vascular complications, and polymorphisms in endothelin-1 (*ET-1*) and endothelial nitric oxide synthase (*eNOS*) genes were associated with ET-1 and nitric oxide disturbance. We investigate the association of *ET-1* 5665G>T and *eNOS* –786T>C polymorphisms with soluble adhesion molecules (sVCAM-1 and sICAM-1), biochemical markers, and medical history. We studied 101 SCA patients; carriers of *eNOS* minor allele (C) had the highest levels of sVCAM-1, and carriers of *ET-1* minor allele had more occurrence of acute chest syndrome (ACS). The multivariate analysis suggested the influence of the *ET-1* gene on ACS outcome and an association of the *eNOS* gene with upper respiratory tract infection. We suggest that *eNOS* and *ET-1* gene polymorphisms can influence SCA pathophysiology and that *eNOS* variant in SCA patients might be important to nitric oxide activity and vascular alteration. We found an association of the *ET-1* minor allele in ACS, showing the importance of genetic screening in SCA.

KEYWORDS: sickle cell anemia, eNOS, endothelin-1, gene polymorphisms

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Introduction

A single amino acid substitution in the hemoglobin (Hb) molecule is the molecular basis for sickle cell anemia (SCA). However, the disease clinical evolution is heterogeneous and involves multiple factors. The SCA is a vascular disease and is already known that genetic differences associated with endothelial function contribute to its phenotypic diversity.¹

Endothelin-1 (ET-1) and nitric oxide (NO) are endothelium-derived mediators essential for maintaining vascular homeostasis. The correct balance between NO and ET-1 production seems to be essential in preventing vascular endothelial dysfunction.^{2,3}

The endothelin is an endothelium-derived molecule and an important vasoconstrictor. Among the three isoforms of endothelin, ET-1 is the only isoform produced by endothelial cells. Various stimuli, such as thrombin, inflammatory mediators, and hypoxia, increase ET-1 levels that play a pivotal role in vascular function regulation and act through

the smooth muscle producing vasoconstriction, cell growth, and cell adhesion.^{2,3} Because of the role of ET-1 in vascular pathophysiology, polymorphic gene coding ET-1 increases vascular reactivity in several vascular disorders. A single nucleotide polymorphism in the *ET-1* gene involving a G-to-T replacement at nucleotide 5665 in exon 5 was correlated with an increased susceptibility of acute chest syndrome (ACS) in SCA individuals.⁴

The NO is synthesized by a family of NO synthase (NOS), and the dominant NOS isoform in the vasculature is the endothelial NOS (eNOS), an enzyme that can metabolize L-arginine and generate NO.^{5,6} The NO is involved in the pathogenesis of several disease such as SCA and has vasodilator and antithrombotic properties that, if impaired, can contribute to the vasoconstriction that coupled with the adhesion of circulating cells may lead to the occlusion of microvessels.^{5,7} The *eNOS* polymorphic variant –786T>C is associated with a decreased NO production because of the reduction



in *eNOS* gene expression and consequently the molecule activity. This condition results in vasoconstriction, platelet aggregation, and thrombosis.^{3,8} The reduced or impaired NO production may result in endothelial cell activation and upregulation of adhesion molecules. Thus, shedding of soluble adhesion molecules into blood plasma can serve as markers either of endothelial dysfunction or of inflammation, with endothelial activation, a clinical situation present in SCA individuals.^{6,8,9} Recent studies have suggested the importance of several SNPs, including the *eNOS* and *ET-1* genes, as risk markers for stroke, leg ulceration, pulmonary hypertension, priapism, and osteonecrosis in sickle cell disease patients.¹⁰

The aim of this study was to investigate the *eNOS* -786T>C (rs2070744) and *ET-1* 5665G>T (rs5370) gene polymorphisms in SCA individuals and controls associating their presence with levels of soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1), biochemical markers, and medical history.

Methods

Subjects. We studied 101 SCA patients (mean age 15.6 ± 12.11 years) from Northeast Brazil attending the outpatient clinic of the Fundação de Hematologia e Hemoterapia da Bahia. All SCA patients were in the steady state of the disease that was characterized as a time of three months without any acute clinical events and without using blood therapy 4 months prior to blood sampling. The exclusion criteria were the presence of infectious diseases, Hb profiles other than SCA, and inflammatory episodes during the blood collection. Determination of *eNOS* polymorphism was possible in 60 of these patients due to sample availability. One hundred eight healthy Brazilian subjects with normal Hb profiles were included as a control group for *ET-1* polymorphism, and 81 subjects with normal Hb profiles were included as a control group for *eNOS* polymorphism.

This study was approved by the Centro de Pesquisas Gonçalo Moniz da Fundação Oswaldo Cruz's Research Board, and all patients and their guardians provided written informed consent, in accordance with the Declaration of Helsinki of 1975 and its revisions. Clinical information was collected from the patients' records.

Polymorphisms genotyping. The *ET-1* 5665G>T (rs5370) and *eNOS* -786T>C (rs2070744) gene polymorphisms were investigated by the polymerase chain reaction and restriction fragment length polymorphism techniques as previously described.^{8,11}

Soluble adhesion molecule measurements. Soluble adhesion molecules, sICAM-1 and sVCAM-1, were estimated using the ELISA Kits (R&D Systems), according to the manufacturer's recommendations.

Biochemical and hematological analyses. Serum concentrations of bilirubin, lactate dehydrogenase (LDH), aspartate aminotransferase, alanine aminotransferase (ALT), total cholesterol and fractions, triglyceride levels,

and C-reactive protein (CRP) were determined using the commercially available biochemical kits (LABTEST). Electronic cell counter (Coulter Corporation) was used to quantify hematological parameters. Hb pattern and its concentration were estimated by high performance liquid chromatography (Bio-Rad).

Statistical analysis. Baseline characteristics were summarized as proportions and mean of selected variables. The Kolmogorov–Smirnov test determines the distribution of quantitative variables. Spearman's rank correlation coefficient measures the strength of a linear relationship between paired data. Nonparametric tests of Mann–Whitney and Kruskal–Wallis compare two or more groups of *ET-1* and *eNOS* alleles and sVCAM-1 and sICAM-1 levels measured as quantitative variables. The chi-square statistic test compares the tallies of categorical variables between two independent groups. Multivariate analyses were performed to show a possible interaction of *ET-1* 5665G>T gene polymorphisms, sVCAM-1, and LDH levels as risk factors on ACS outcome and of *eNOS* -786T>C gene polymorphisms, white blood cell, LDH, and CRP on infection outcome. Test analyses were significant if *P* values were less than 0.05. Data analyses were conducted using the software programs STATA 10 (StataCorp) and GraphPad Prism 5 (GraphPad Software).

Results

Polymorphisms frequencies. The *ET-1* 5665G>T polymorphism was analyzed in 101 SCA patients and 108 healthy individuals, while the *eNOS* -786T>C was investigated in 60 SCA patients and 81 healthy controls. Our results showed frequencies of 66.3% (67/101) for wild-type genotype (GG) and 33.6% (34/101) of heterozygous (GT) and 2.9% (3/101) of homozygous for the variant allele (TT) of *ET-1* 5665G>T gene polymorphism in SCA patients (Table 1). The *eNOS* -786T>C gene polymorphism analysis showed 56.7% (34/60) for wild-type genotype (TT) and 36.7% (22/60) of heterozygous (TC) and 6.5% (4/60) of homozygous for the variant allele (CC) in SCA patients (Table 1). Both polymorphisms were in Hardy–Weinberg equilibrium. In the control group, the frequency of *ET-1* 5665G>T gene polymorphism

Table 1. Frequencies of *ET-1* 5665G>T and *eNOS* -786T>C gene polymorphisms among healthy individuals and SCA patients.

POLYMORPHISM	GENOTYPE	FREQUENCIES HEALTHY INDIVIDUALS (%)	FREQUENCIES SCA PATIENTS (%)
<i>ET-1</i> 5665G>T	GG	60.2 (65/108)	66.3 (67/101)
	GT	32.4 (35/108)	33.6 (34/101)
	TT	7.4 (8/108)	2.9 (3/101)
<i>eNOS</i> -786T>C	TT	54.3 (44/81)	56.7 (34/60)
	TC	42 (34/81)	36.7 (22/60)
	CC	3.7 (3/81)	6.5 (4/60)

Table 2. Association of soluble adhesion molecules levels (sVCAM-1 and sICAM-1) and *ET-1* 5665G>T and *eNOS* -786T>C gene polymorphisms.

	N	MEAN (\pm SD)	
		sVCAM-1 (ng/mL)	sICAM-1 (ng/mL)
ET-1	51		
Allele G	32	622.93 (\pm 393)	425.71 (\pm 165)
Allele T	19	574.09 (\pm 373)	407.06 (\pm 131)
*P value		0.815	0.693
eNOS	38		
Allele T	23	420.39 (\pm 161)	439.97 (\pm 193)
Allele C	15	584.09 (\pm 238)	411.61 (\pm 133)
*P value		0.028	0.906

Note: *Mann-Whitney test.

Abbreviations: sICAM-1, soluble intercellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1.

was 60.2% (65/108) for wild-type genotype and 32.4% (35/108) of heterozygous and 7.4% (8/108) of homozygous for variant allele (Table 1). The frequency of *eNOS* -786T>C was 54.3% (44/81) for wild-type genotype and 42% (34/81) of heterozygous and 3.7% (3/81) of homozygous for variant allele (Table 1).

Adhesion molecules and polymorphisms. We associated gene polymorphisms with the serum levels of soluble adhesion molecules (sVCAM-1 and sICAM-1) and found that patients' carriers of the minor allele of *eNOS* gene polymorphism had the highest levels of sVCAM-1. Table 2 shows the genotypes of *ET-1* and *eNOS* gene

polymorphisms and mean of serum levels of the studied soluble adhesion molecules.

Polymorphism and clinical data. Genotype frequencies were compared between SCA patients with and without clinical events. Table 3 summarizes the association of clinical data and the presence of polymorphisms in *ET-1* and *eNOS* genes. When the allele frequencies were evaluated, we found an association of the ACS in patients' carriers of the minor allele of the *ET-1* 5665G>T ($P < 0.001$) (Fig. 1).

Biochemical data. Biochemical data were assessed in SCA patients. Analyses of the 51 SCA show that patients in percentile 25% and 75% showed an association with the presence of the minor allele *ET-1* 5665G>T and levels of direct bilirubin and total cholesterol. Patients carrying the minor allele T had higher direct bilirubin (≥ 0.4 mg/dL) ($P = 0.021$, Fisher's exact test) as well as a higher concentration of total cholesterol (≥ 169.7 mg/dL) ($P = 0.03$, Fisher's exact test). Patients carrying the minor allele (T) had higher levels of direct bilirubin (≥ 0.4 mg/dL) ($P = 0.012$, unpaired *t* test) (Fig. 2). Other biochemical data did not show differences with gene polymorphisms, including the *eNOS* gene polymorphism. However, sVCAM-1 was negatively correlated with total cholesterol levels ($P = 0.027$, $r = -0.243$) and ALT levels ($P = 0.005$, $r = -0.307$) (Fig. 3).

Multivariate analysis. The multivariate analysis approach model investigates the interaction of the *ET-1* 5665G>T gene polymorphism, sVCAM-1, and LDH levels on ACS outcome (Table 4) and of the *eNOS* -786T>C gene polymorphism, white blood cell count, and LDH and CRP levels on upper respiratory tract infection (Table 5).

Table 3. *ET-1* 5665G>T and *eNOS* -786T>C gene polymorphisms association with clinical events among SCA patients.

CLINICAL DATA	<i>ET-1</i>			<i>eNOS</i>		
	GENOTYPE GG	GENOTYPE GT AND TT	P VALUE	GENOTYPE TT	GENOTYPE TC AND CC	*P VALUE
Transfusion	14/21	7/21	0.580	8/15	7/15	0.689
Leg ulcers	7/9	2/9	0.302	1/5	4/5	0.188
Acute chest syndrome	3/8	5/8	0.114	1/3	2/3	0.329
Splenic sequestration	1/3	2/3	0.268	1/3	2/3	0.435
Avascular necrosis	3/4	1/4	0.569	0/1	1/1	0.376
Retinopathy	2/2	0/2	0.418	1/2	1/2	0.829
Splenectomy	1/3	2/3	0.268	1/3	2/3	0.435
Hepatomegaly	3/4	1/4	0.569	0/2	2/2	0.127
Stroke	1/1	0/1	0.654	–	–	–
Osteomyelitis	2/2	0/2	0.418	2/2	0/2	0.403
Hand foot syndrome	1/1	0/1	0.654	1/1	0/1	0.650
Infection	10/16	6/16	0.517	6/14	8/14	0.231
Pneumonia	4/8	4/8	0.255	4/8	4/8	0.920
Cholelithiasis	6/6	0/6	0.054	2/3	1/3	0.801
Aplastic crisis	0/1	1/1	0.346	1/1	0/1	0.650

Notes: *Chi-square statistic test. **Fisher exact test.

Abbreviations: *ET-1*, endothelin-1; *eNOS*, endothelial nitric oxide synthase.

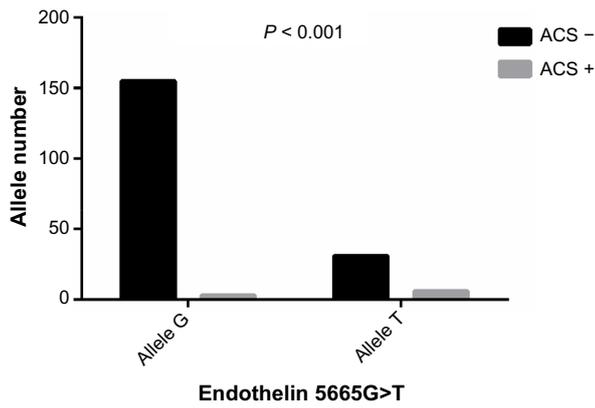


Figure 1. Presence of *ET-1* 5665G>T alleles and occurrence of acute chest syndrome among SCA patients.

Discussion

This study shows a new interesting result regarding the association of *eNOS* -786T>C gene polymorphism and sVCAM-1 levels. SCA patient's carrier of the minor allele (C) had higher sVCAM-1 levels, suggesting a contribution of this polymorphism on vascular inflammation. Based on the information that *eNOS* polymorphic variant is related to decreased NO production because of the reduction in gene promoter activity,^{12,13} decreasing NO production in the minor allele carriers (C) is supposed to upregulate vascular adhesion molecules (as sVCAM-1) and the antiinflammatory role of NO on vascular environment and, consequently, increase the endothelial damage. It is known that NO inhibits platelet activation and the expression of endothelial adhesion molecules, thus participating in healthy endothelial function and the maintenance of blood flow.^{14,15}

These results suggest a role of these molecules on SCD mechanism. In addition to endothelial dysfunction, SCA patients have a decrease in vasodilator responses to NO donors such as sodium nitroprusside and nitroglycerin,^{16,17} molecules that promote vascular smooth muscle relaxation.

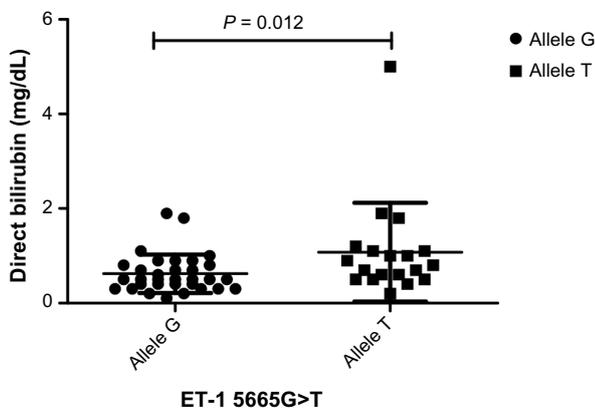


Figure 2. SCA patients carrying the minor allele (T) of the polymorphism *ET-1* 5665G>T had higher levels of direct bilirubin (≥ 0.4 mg/dL) ($P = 0.012$).

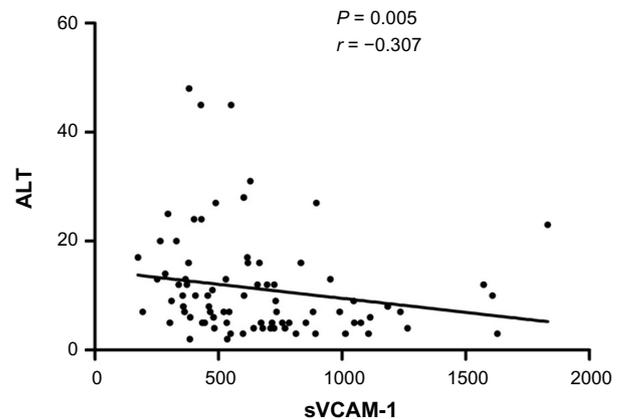
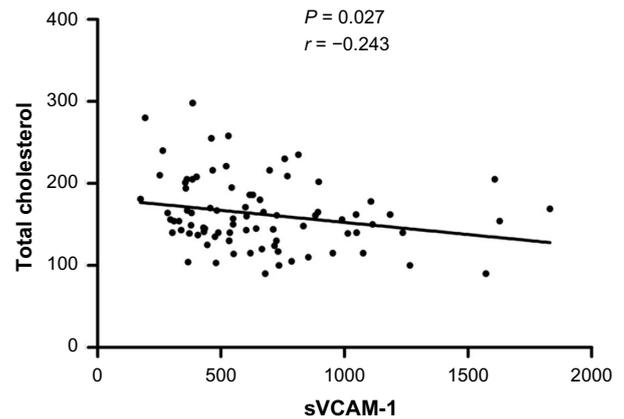


Figure 3. Correlation between sVCAM-1, total cholesterol, and ALT among 83 SCA patients.

This phenomenon is related to vascular cell dysfunction and NO resistance where a portion of exogenous NO is scavenged by reactive oxygen species or free serum heme before it can stimulate vascular smooth muscle.¹⁸ The *eNOS* -786T>C minor allele can be associated with an enhancement of the NO resistance state in the SCA individuals.^{3,8}

Also, in the current study, the *ET-1* 5665G>T minor allele was associated with the occurrence of ACS in SCA patients, confirming previous results.⁴ The ACS is a combination

Table 4. The multivariate model of the association of *ET-1* 5665G>T gene polymorphism, sVCAM-1, and lactate dehydrogenase (LDH) levels in acute chest syndrome.

VARIABLE	B	SE	T	P VALUE
Model 1				
<i>ET-1</i> 5665G>T	0.351	0.127	2.772	0.011
sVCAM-1 (ng/mL)	0.058	0.122	0.472	0.641
Model 2				
<i>ET-1</i> 5665G>T	0.358	0.126	2.843	0.009
sVCAM-1 (ng/mL)	0.057	0.121	0.467	0.645
LDH (U/L)	0.165	0.145	1.136	0.268

Abbreviations: B, coefficient; SE, standard error.

Table 5. The multivariable model of the association of *eNOS* -786T>C gene polymorphism, white blood cell (WBC) count, lactate dehydrogenase (LDH), and C-reactive protein (CRP) levels on upper respiratory tract infection.

VARIABLE	B	SE	T	P VALUE
Model 1				
<i>eNOS</i> -786T>C	0.420	0.187	2.249	0.037
WBC ($\times 10^9/L$)	0.373	0.190	1.959	0.066
Model 2				
<i>eNOS</i> -786T>C	0.268	0.176	1.517	0.149
WBC ($\times 10^9/L$)	0.502	0.177	2.838	0.012
CRP (mg/L)	0.332	0.182	1.823	0.087
LDH (U/L)	0.443	0.209	2.125	0.05

Abbreviations: B, coefficient; SE, standard error.

of radiographic evidence of new pulmonary infiltrates and respiratory symptoms and is a frequent cause of hospitalization in SCA patients.¹⁹ Pathophysiology of events leading to ACS progress in SCA was not determined but was considered similar to those observed in other organ systems. The ACS likely involves alterations of normal homeostatic functions of vascular endothelium in the lungs.²⁰ In addition to adherence, interaction of plasma factors and/or sickle red blood cells with endothelial cells may modify endothelial production of vasoactive mediators.² The plasma ET-1 levels were clearly elevated during the initial period of ACS and decreased by the third day of hospitalization.²¹ This suggests a contribution of ET-1 on ACS events probably by deregulating the mediators' balance because of the presence of *ET-1* 5665G>T minor allele, once this gene polymorphism is related to abnormal vascular reactivity and ET-1 plasma levels.^{12,22} In the present study, we found the association of the minor allele of *ET-1* 5665G>T with ACS but not the homozygous state of the minor allele, and we emphasize that further study including a higher number of SCA patients is necessary to confirm the association of these genotypes as a high-risk of ACS among these patients.

It was suggested that an imbalance between ET-1 and NO may contribute to changes in endothelial tone observed in the SCA,³ and consequently, the presence of those polymorphisms can break such balance by abnormal expression or activity of these mediators contributing to the vascular impairment.

In this study, we found negative significant correlation of sVCAM-1 and total cholesterol and ALT. Low total cholesterol levels were associated with the severity of hypertension and intracerebral hemorrhage, followed by the magnetic resonance imaging changes²³; also, a decrease in high-density lipoprotein cholesterol, which may have influence on the total cholesterol levels, has been related as an independent marker of endothelial activation, and also with an increase in inflammatory and oxidative stress molecules, such as sVCAM-1.²⁴ The negative correlation with ALT levels may suggest that

the increase in sVCAM-1 in this studied SCA group was not associated with hepatocellular damage.²⁵

Our results of multivariate analysis described a possible influence of the *ET-1* gene on ACS outcome and the association of *eNOS* gene with upper respiratory tract infection, showing a pivotal role of vascular mediators, like ET-1 and NO, in SCA pathophysiology, and also an interaction of the investigated gene with molecules and cells commonly involved in the hemolytic and inflammatory processes. Further studies will clarify the role of *ET-1* and *eNOS* gene polymorphisms and will advance our understanding of the altered endothelial state and clinical complications in SCA patients. It would be interesting to show whether -786C minor allele has a reduced promoter activity and eventually less eNOS transcription and whether endothelial cells with -786C minor allele have lower levels of eNOS and eventually NO production.

Conclusion

We suggest that *eNOS* -786T>C and *ET-1* 5665G>T gene polymorphisms may participate in the SCA pathophysiology. Our data show that *eNOS* variant in SCA patients might be important to NO activity and anti-inflammatory vascular process. Also, the ACS, a major clinical feature in SCA, which leads to patient morbidity and mortality, was associated with the *ET-1* minor allele showing the importance of the screening of genetic biomarkers and their mechanisms.

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Author Contributions

Carried out the polymorphism typing and statistical analysis, participated in the study design, and drafted the article: WV-B. Carried out the polymorphism typing and participated in the study design: CVBF. Helped to draft the article: TNP, RPS, SSS, CCG. Helped in the sample collection and contributed to the experimental work: MOS, AMDZ. Performed the biochemical and statistical analyses, participated in the study design, and drafted the article: BAVC. Participated in the design and coordination of the study: MSG. All authors reviewed and approved the final article.

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