Polymorphisms of endothelial nitric oxide synthase gene in systolic heart failure: An haplotype analysis

Nidiane C. Martinelli, Kátia G. Santos [1], Andréia Biolo, Vanessa L. La Porta, Carolina R. Cohen, Daiane Silvello, Michael E. Andrades, Nadine Clausell, Luis E. Rohde *

Cardiovascular Experimental and Molecular Laboratory, The Heart Failure and Cardiac Transplant Unit, Cardiology Division at Hospital de Clínicas de Porto Alegre, and the Post-Graduate Program in Cardiology and Cardiovascular Science, Medical School of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil

ARTICLE INFO

ABSTRACT

Background: Endothelial nitric oxide synthase (eNOS) gene polymorphisms have been associated with the pathogenesis of cardiovascular diseases, but few studies have evaluated the role of eNOS haplotypes on the risk and prognosis of heart failure (HF). This prospective study was designed to analyze the impact of three eNOS polymorphisms (T-786C, VNTR4a/b and Glu298Asp) and their haplotypes on the susceptibility and clinical outcomes in HF outpatients with systolic dysfunction.
Methods and results: We conducted a case-control and a cohort study in which 316 HF patients and 360 healthy controls were recruited from a tertiary care university hospital. DNA was extracted from peripheral blood and eNOS polymorphisms were detected by PCR or PCR-RFLP. Patients were predominantly men, had a mean left ventricular ejection fraction of 31% and were followed-up for a median of 41 months; there were 96 deaths, including 58 HF-related deaths. Genotype distribution of the eNOS T-786C, VNTR 4a/b and Glu298Asp was similar between HF patients and controls. Haplotype frequencies differed between HF patients and controls only in African–Brazilians ($p = 0.043$). African–Brazilian patients that carried the haplotype -786C/4b/Asp298 had a better prognosis than patients that carried other haplotypes (log rank p value = 0.016 for all-cause mortality). In a Cox proportional hazard model adjusted for clinical variables of risk, the -786C/4b/Asp298 haplotype remained as an independent genetic predictor of survival (adjusted HR = 0.11; 95% CI = 0.01–0.83; $p = 0.03$).
Conclusions: The -786C/4b/Asp298 eNOS haplotype had a significant impact on HF susceptibility and prognosis, particularly in African–Brazilian patients.

Introduction

Basic and clinical evidence suggests that defects in nitric oxide (NO) generation and action are involved in the pathogenesis of several cardiovascular syndromes [1–3]. Physiological levels of NO appear to play an important protective role in heart failure (HF), but higher levels have also been associated to detrimental negative inotropic effects on the heart [4]. Endothelial NO synthase (eNOS) is the predominant source of vascular NO and its synthesis is constitutively mediated by a reaction that involves the conversion of ʟ-arginine to ʟ-citruline [5]. Variation in NO availability, an alleged risk for cardiovascular disease, could be explained in part by eNOS genetic polymorphisms that modify protein expression and/or activity [6–7].

The eNOS protein is encoded by the NOS3 gene, located on the chromosome 7q35-36 and is comprised of 26 exons [8]. A single nucleotide variation occurs on the promoter region of the eNOS gene (a T-to-C transition [T-786C], rs2070744). The -786C allele was associated with reduced eNOS activity in human platelets [9] and reduced mRNA levels in human cardiomyocytes [10]. The -786C variant was also linked to the risk of coronary artery disease and hypertension [1,3]. Another common eNOS polymorphism involves an amino acid substitution in exon 7 of the mature protein (a G-to-T transversion [G894T], rs1799983), leading to a glutamate-aspartate substitution at codon 298 (Glu298Asp). The Asp298 variant was reported to be a main risk factor for ischemic heart disease [11] and was associated with worse event-free survival in non-ischemic HF patients [2]. Finally, a 27-bp repeat in intron 4 (VNTR intron 4a/b) has been associated to physiologic variations of the plasmatic concentrations of NO metabolites [12]. This variant has also been linked to the severity of coronary stenosis [13] and the risk of myocardial infarction in the Tunisian population [14].

* Corresponding author. Address: Heart Failure and Transplant Unit, Cardiology Division, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos 2350, Sala 2061, Porto Alegre, RS 90035-003, Brazil. Fax: +55 51 33598344.
[1] Associate Professor at the Research Center in Medical Sciences of the Lutheran University of Brazil.

Phenotype-genotype association studies, based on individual single nucleotide polymorphisms (SNPs), as those mentioned above, have been criticized because results are rarely reproducible across populations with different genetic backgrounds. In this scenario, it is well recognized that the expression and/or activity of proteins frequently vary according to haplotype clusters. Haplotype analysis might overcome some of the inconsistencies observed in studies that evaluated individual SNPs, providing enhanced predictive power and reproducibility in association studies [15]. Recently, several reports have pointed out that eNOS haplotypes might substantially interfere with NO formation, as well as with levels of plasma and whole blood nitrite [12–16]. In the present study we evaluated the role of the T-786C, VNTR 4a/b and Glu298Asp eNOS gene polymorphisms, individually and by haplotypes, as markers of disease susceptibility and prognosis in a South-Brazilian cohort of HF patients.

Methods

Subjects

Heart failure patients were recruited from a tertiary care university hospital in Porto Alegre, Brazil. Consecutive eligible patients who agreed to participate were enrolled at the Heart Failure and Transplant Outpatient Clinic between October, 2003 and October, 2007. A total of 316 patients with systolic HF (222 Caucasians and 94 African–Brazilians) were enrolled in the study. Inclusion criteria were age >18 years and left ventricular ejection fraction (LVEF) < 45%. Patients with other serious illnesses or with reduced life expectancies were excluded. The ethnical classification of all participants was self-reported. Demographic, clinical, and routine laboratory data from all patients were collected using a structured data form. We also evaluated 360 healthy blood donors from the blood bank center of the same hospital. Before blood donation, volunteers who chose to participate of the study underwent a systematic interview. Subjects who reported a positive family history of premature sudden death or cardiovascular diseases (in first degree relatives, irrespective of age), the presence of atherosclerotic risk factors, or any overt clinical disease were excluded from the protocol. However, no additional laboratory data was collected from blood donors. The study protocol was approved by the local institutional review board and by the National Agency of Ethics in Research and all subjects provided written informed consent.

Genotyping

Genomic DNA was extracted from samples of peripheral blood using a commercially available kit (Puregene; Gentra Systems, Minneapolis, USA). Gene fragments containing the T-786C and the G894T (Glu298Asp) variant sites in eNOS gene were amplified by polymerase chain reaction (PCR), using experimental conditions as previously described [17]. Amplification products were digested with the appropriate restriction enzymes under the conditions recommended by the manufacturer (MBI Fermentas, St. Leon-Rot., Germany). The digested fragments were then separated by electrophoresis on 6% polyacrylamide gels, followed by ethidium bromide staining, and directly visualized under ultraviolet light. The VNTR polymorphism in intron 4 was detected by PCR using primers and conditions as previously described [18] and the alleles were identified according to the length of bands after separation of PCR fragments on ethidium bromide-stained 2% agarose gels. The presence of four, five or six 27-bp repeats in intron 4 results in amplicons of 393 bp (a allele), 420 bp (b allele) or 447 bp (c allele), respectively.

Outcome evaluation

Enrolled patients were followed-up at the Heart Failure and Transplant Outpatient Clinic at our institution between October/2003 through October/2007. At the HF clinic, patients were scheduled to have regular visits, with pre-defined intervals from 1 to 4 months. Follow-up data was directly derived from reviewing all electronic clinical data from the institution's records (most patients had several follow-up visits). For patients that were not regularly visiting the HF clinic (or had lost the follow-up), a telephone contact was attempted to check for relevant clinical events, based on a structured telephone interview performed by trained nurses. For those who we were unable to be contacted by phone (approximately 20 patients), we checked their vital status through the State Death Certificate Database (that contains data about the main cause and date of all death in our state). Analyses were stratified by the presumptive cause of death, classified as (1) all-cause mortality and (2) HF-related, defined as sudden, unexpected death (within 1 h of initiation of symptoms) or caused by advanced refractory disease (pump failure).

Statistical analysis

Continuous data are expressed as mean ± standard deviation or median (interquartile ranges) and categorical variables are expressed as absolute numbers and percentages. Comparisons between groups were tested by the chi-square (χ^2), Student's t test, analysis of variance, or nonparametric statistics, as appropriate. Allele frequencies were determined by gene counting and deviations from the Hardy–Weinberg equilibrium were verified using the χ^2 test. The χ^2 test was also used to evaluate the allele and genotype distributions among groups of subjects and the residual analysis was used to identify the categories responsible for statistical differences. The linkage disequilibrium between all pair of loci was calculated and expressed in terms of r^2 and D' [19]. Haplotype frequencies were estimated by a Bayesian method using the software PHASE version 2.1 [20–21]. We also used PHASE program to compare the distribution of different eNOS haplotypes between HF patients and controls through permutation analyses of 1000 random replicates. Kaplan–Meier survival curves were constructed from the date of entry at the outpatient clinic until the last registry of follow-up or death, and compared by the log–rank statistics. Survival analysis for all patients (Fig. 1) was adjusted for self-reported race. Cox proportional hazard models were created and adjusted for age, gender, LVEF, serum sodium levels, duration of QRS, left ventricular diastolic diameter (LVDD), acute myocardial infarction, functional class (NYHA) and use of beta-blocker. A two-tailed p value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 18 for Windows.

Results

Clinical characteristics

The clinical and demographic characteristics of HF patients are presented according to self-reported ethnicity (Table 1). Both groups, Caucasians and African–Brazilians, were mainly males (67% and 73%), with mean age of 60 ± 13 and 59 ± 12 years, respectively. African–Brazilian HF patients had a higher prevalence of hypertensive etiology (34%) while Caucasians were predominantly of ischemic etiology (42%). Most HF patients in both groups had moderate to severe left ventricular dysfunction and were in New York Heart Association (NYHA) class I or II. Patients were taking standard background therapy for HF including angiotensin-converting enzyme (ACE) inhibitors and beta-blockers. Healthy
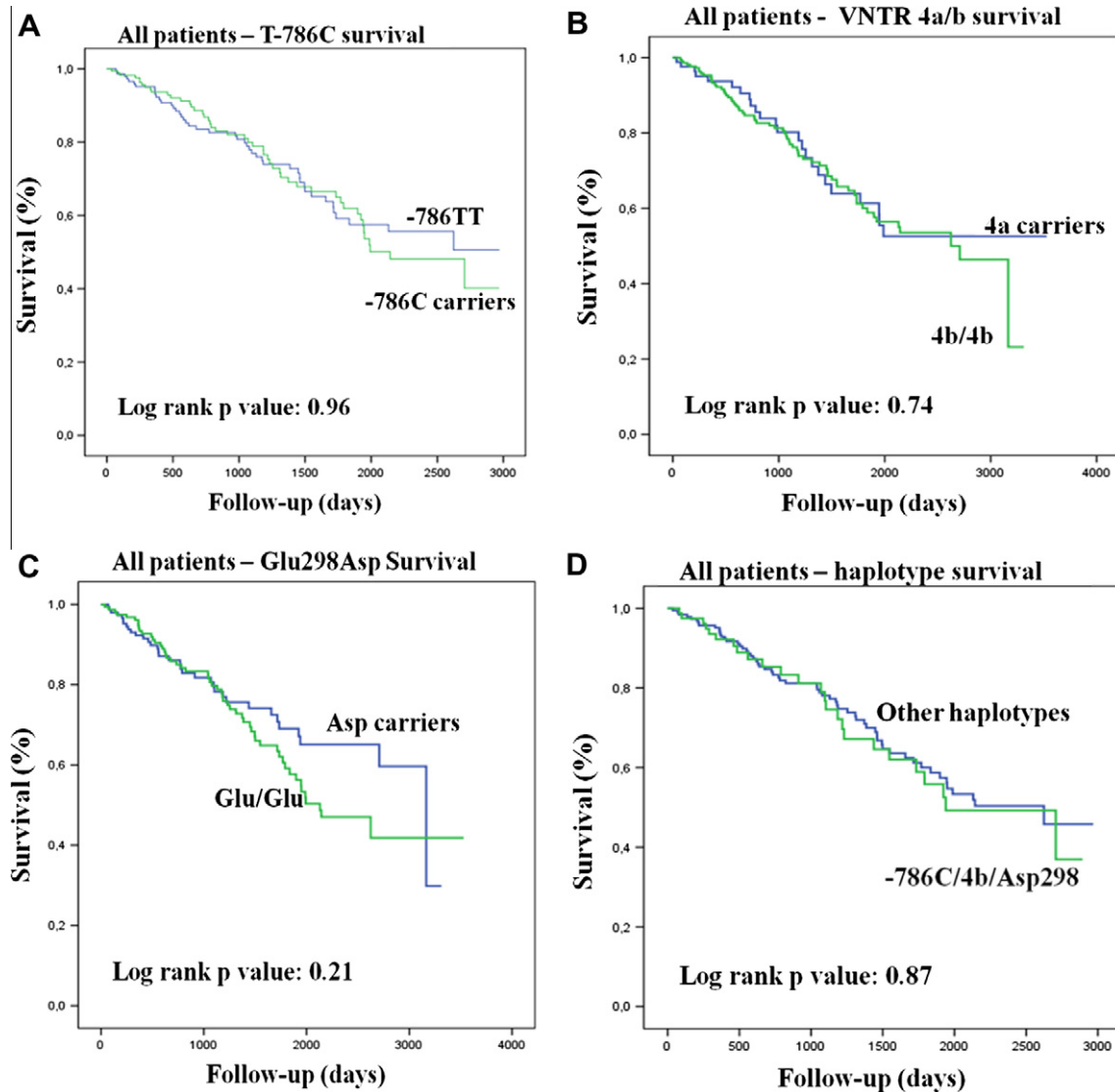
Fig. 1. Kaplan–Meier survival curves comparing all HF patients between eNOS polymorphisms and haplotypes (A – T-786C; B – Glu298Asp; C – VNTR 4a/b; D – eNOS haplotype).

subjects (control group) were predominantly men (68%) and Caucasian (77%), and their mean age was 45 ± 12 years ($p = 0.001$ vs. HF patients).

Genotype distribution in HF patients and controls

The genotype frequencies were in agreement with those predicted by the Hardy–Weinberg equilibrium for all eNOS polymorphisms in HF patients and controls. Supplementary Figures 1–3 illustrate the distribution of eNOS genotypes in HF patients and controls, stratified by self-reported race. Genotype frequencies for the three analyzed polymorphisms were similar between HF patients and controls in both Caucasians and African–Brazilians (Figure S1–S3). Regarding allele frequencies, we observed a small difference for the eNOS T-786C that was restricted to African–Brazilians (the C allele was more frequent in controls than in HF patients; 0.31 vs. 0.21, respectively, $p = 0.048$). There were no differences in allele frequencies for Glu298Asp and the VNTR intron 4a/b variants between African–Brazilian or Caucasians HF patients and controls (data not shown).

Linkage disequilibrium and haplotype analysis

Table S1 describes the linkage disequilibrium (LD) analysis among eNOS polymorphisms, stratified by cases and controls, and self-reported race. Most polymorphisms were not in LD, as indicated by r^2, but the T-786C polymorphism was weakly linked with the VNTR intron 4 variant and the Glu298Asp polymorphism in Caucasian HF patients. Among African–Brazilian HF patients, the Glu298Asp and T-786C polymorphisms were also in weak LD.

We next analyzed if haplotype clusters would be associated with HF using a Bayesian method to estimate the haplotype frequencies composed by the three studied eNOS polymorphisms. As the c allele of VNTR polymorphism in intron 4 was infrequent, carriers of the c allele were excluded from haplotype analysis and the VNTR intron 4 a/b polymorphism was considered a bi-allelic marker. The most frequent haplotype in patients and controls was -786T/4b/Glu298, both in Caucasians and African–Brazilians. The haplotypes -786C/4a/Asp298 and -786T/4a/Asp298 were not observed in African–Brazilians patients or in controls. In the Caucasian group, we also did not find the -786T/4a/Asp298 haplotype in HF cases and controls.

Table 1
Clinical characteristics of HF patients according to ethnicity.

Characteristics	Caucasians N = 222	African–Brazilians N = 94	p
Age (years)	60 ± 13	59 ± 12	0.45
Males	149 (67)	69 (73)	0.33
HF etiology			
Ischemic	93 (42)	25 (27)	0.02
Hypertensive	43 (19)	32 (34)	0.01
Idiopathic	69 (31)	23 (24)	0.29
Chagas disease	4 (2)	5 (5)	0.17
Functional class			
I and II	174 (81)	68 (73)	0.19
III and IV	42 (19)	25 (27)	
Comorbidities			
Myocardial infarction	81 (36)	20 (21)	0.01
Hypertension	122 (55)	64 (68)	0.04
Cerebrovascular disease	16 (7)	10 (11)	0.42
Diabetes mellitus	74 (33)	21 (22)	0.07
Current smoking	21 (9)	17 (18)	0.05
Echocardiography			
LV ejection fraction (%)	31 ± 8	31 ± 8	0.84
LV diastolic diameter (cm)	6.6 ± 1.0	6.6 ± 0.8	0.66
LA dimension (cm)	4.7 ± 0.8	4.8 ± 0.9	0.86
PASP (mmHg)	49 ± 13	49 ± 14	0.94
Electrocardiogram			
Atrial fibrillation	40 (18)	17 (18)	>0.99
Left bundle branch block	65 (29)	27 (29)	>0.99
QRS duration (ms)	130 ± 34	128 ± 33	0.82
Laboratory variables			
Creatinine (mg/dL)	1.3 ± 0.5	1.3 ± 0.5	0.52
Urea (mg/dL)	58.5 ± 31.0	54.0 ± 27.5	0.27
Sodium (mEq/L)	140.6 ± 3.2	140.0 ± 3.4	0.20
Potassium (mEq/L)	4.5 ± 0.6	4.4 ± 0.5	0.46
Hemoglobin (g/dL)	13.1 ± 1.6	12.9 ± 1.9	0.32
Initial drugs			
Beta-blockers	197 (89)	80 (85)	0.47
ACEi	191 (86)	86 (91)	0.24
Spironolactone	91 (41)	30 (32)	0.17
Hidralazine	38 (17)	23 (24)	0.05
Isosorbide	50 (23)	25 (27)	0.09

Data are presented as means ± standard deviations or absolute numbers (percentages).
HF, heart failure; LV, left ventricle; LA, left atrium; PASP, pulmonary artery systolic pressure; ACEi, angiotensin-converting enzyme inhibitor.

The haplotype frequencies in Caucasian HF patients were remarkably similar to those of apparently healthy blood donors ($p > 0.05$; Table 2). However, for the African–Brazilians, haplotype frequencies were different between HF patients and controls; this difference was mainly due to the -786C/4b/Asp298 haplotype ($p = 0.043$; Table 2). We found a protective effect of this haplotype on HF susceptibility (OR = 0.41; 95% CI = 0.21–0.82; $p = 0.011$), but this protection was partially lost when adjusted for gender and age (adjusted OR = 0.46; 95% CI = 0.21–1.01; $p = 0.053$).

Event-free survival

Patients with HF were followed-up for a median of 41 months. During the follow-up there were 96 events, including 58 HF-related deaths. We have evaluated the impact of eNOS promoter, exon 7 and intron 4 polymorphisms and did not find any effect of these polymorphisms on survival for all patients (Fig. 1 A, B and C, respectively). In addition, there was no significant effect of all three eNOS polymorphisms on survival in the analysis restricted to Caucasian patients (data not shown). However, the presence of the Glu298 variant was associated with a worse survival when compared to Asp298 carriers in African–Brazilian patients ($p = 0.034$; Fig. 2A). In a Cox regression analysis adjusted for LVEF, age, gender, sodium levels and use of beta-blockers, the Glu298 variant did not remain as an independent predictor for all-cause mortality (adjusted HR = 2.77; 95% CI = 0.93–8.19, $p = 0.066$; Asp298 carriers as the reference group).

We further analyzed clinical outcomes in HF patients in relation to the presence of one copy of the -786C/4b/Asp298 haplotype. We did not find any association of this haplotype with all-cause mortality or HF-related death in analysis involving all patients (Fig. 1D), and neither for analysis restricted to the Caucasian group (data not shown). However, African–Brazilians carrying the -786C/4b/Asp298 haplotype had a better prognosis when compared to patients that carry other haplotypes ($p = 0.016$ Fig. 2B). After Cox regression analysis adjusted for several other clinical predictors of risk (Table 3), the 786C/4b/Asp298 haplotype persisted as independent genetic predictor of survival (adjusted HR = 0.11; 95% CI = 0.01–0.83; $p = 0.03$).

Discussion

Haplotype analysis has arisen as a more informative approach to appraise the genetic influence on diseases rather than testing isolated genetic markers. In the present study, haplotype analysis of eNOS polymorphisms had a significant impact on HF prognosis in self-reported African–Brazilian patients. However, we failed to demonstrate an association between isolated eNOS polymorphisms and HF susceptibility or clinical outcomes. This finding

Table 2
Haplotype frequencies of eNOS polymorphisms in African–Brazilian and Caucasian patients and controls.

Haplotype[*]	African–Brazilians		Caucasians	
	Controls, n (%)	HF cases, n (%)	Controls, n (%)	HF cases, n (%)
Number of chromosomes	160	180	560	440
-786T/4b/Glu298	86 (53.5)	111 (61.4)[†]	241 (43.2)	207 (47.1)[‡]
-786T/4b/Asp298	12 (7.5)	13 (7.5)	67 (11.9)	53 (12)
-786T/4a/Glu298	10 (6.5)	15 (8.4)	22 (3.9)	15 (3.4)
-786C/4b/Glu298	13 (7.8)	6 (3.4)	32 (5.8)	25 (5.6)
-786C/4b/Asp298	32 (20.2)	21 (11.6)[#]	124 (22.1)	92 (21.0)
-786C/4a/Glu298	7 (4.5)	14 (7.7)	72 (12.8)	44 (10.1)
-786C/4a/Asp298	–	–	2 (0.3)	4 (0.8)

PHASE program version 2.1 was used for estimating haplotype frequencies and for comparing cases and controls.
[*] As the c allele (intron 4 VNTR) was very rare, carriers of this allele were excluded from haplotype analysis and the VNTR intron 4 a/b polymorphism was considered as a bi-allelic marker.
[‡] P = 0.51 for Caucasian patients vs. controls.
[†] P = 0.043 for African–Brazilian patients vs. controls.
[#] Represents haplotype which contributes significantly to χ^2 test, as indicated by residual analysis.

strengthens the concept that eNOS variability is significantly related to racial phenotype, a finding that has been suggested by other investigators [2,17].

The effect of eNOS polymorphisms on susceptibility to cardiovascular disease has been previously evaluated in several scenarios. Despite the fact that eNOS polymorphisms are linked to eNOS activity [6,7] and thus can be expected to influence blood pressure control, no association was found between these polymorphisms and the presence of hypertension in both Caucasian and African–Brazilian patients [22]. A meta-analysis evaluating the association between three eNOS polymorphisms and ischemic heart diseases showed that there is a slightly increased risk associated with the presence of Asp298 and intron 4a alleles (OR = 1.17 and OR = 1.34, respectively), whereas no association was found between -786C allele and ischemic heart disease. It is noteworthy that ethnic background was not a source of inter-study heterogeneity in this meta-analysis [11]. Velloso et al. have demonstrated an increased prevalence of the Glu298Glu genotype and Glu298 allele in patients with chronic HF [23]. On the other hand, a Canadian study that has evaluated several polymorphisms in patients maximally treated for HF, including T-786C and Glu298Asp, has failed to demonstrate any association of these polymorphisms with the disease [24]. Similar to these reports, we failed to demonstrate any major association between three eNOS polymorphisms and HF susceptibility. Studies in hypertensive patients, however, have suggested that ethnic diversity and haplotype analysis could contribute significantly to hypertension susceptibility. Sandrim et al. have demonstrated that even in the absence of association between isolated eNOS polymorphisms and hypertension, one haplotype (-786C/4b/Asp298) was more frequent in hypertensive patients [22]. These findings were partially reproduced by our data, as we observed a significant effect of the -786C/4b/Asp298 haplotype on HF susceptibility in African–Brazilians.

Few studies have previously evaluated the role of eNOS gene variants on HF prognosis. For instance, McNamara et al. have demonstrated that Asp298 allele was associated with a poorer survival in predominantly white (approximately 90%) non-ischemic HF patients [2]. We failed to reproduce the detrimental effect of the Asp298 variant in our mixed sample (Fig. 1A); on the contrary,

we observed that African–Brazilian HF patients carrying the Asp298 allele had lower all-cause mortality (Fig. 2A) in univariate analysis. This association was partially lost after adjustment for other predictors of risk. Also, eNOS genotype influences blood pressure and left ventricular remodeling but the polymorphisms T-786C, Asp298Glu, and VNTR intron 4 had no impact on event-free survival in African–American HF patients [25]. Thus, clinical evidences suggest that several eNOS polymorphisms might have differential effects according to race [2,17,25]. In addition, Asp298 carrier condition was not associated with change in either eNOS mRNA or protein expression in myocardial tissue obtained from patients with advanced end-stage HF [10], indicating that the associated changes in eNOS expression might be dependent on the temporal stages of HF.

We have demonstrated that African–Brazilian HF patients who carried the -786C/4b/Asp298 haplotype had a better long-term prognosis. This protective effect remained statistically significant after adjustment for other known clinical variables of risk in a Cox regression analysis. Although both -786C and Asp298 alleles have been associated with several cardiovascular disease states [3,23], their impact on HF progression could be dependent on distinct biological processes. As eNOS is expressed in several cell types, not just in the endothelium, these differential effects are biologically plausible.

It is well recognized that reactive oxygen species (ROS) may induce oxidation and damage of macromolecules, membranes, and DNA and thus detrimental for cellular function and viability. In HF patients, the production of ROS is increased and the NO system is disrupted leading to the interruption of signaling pathways and homeostatic mechanisms. One of the major controversies surrounding NO in the heart is derived from the observation that in HF and in cardiac injury due to myocardial infarction, NOS isoforms have been ascribed both protective and detrimental roles [26]. Some studies have shown that endothelial overexpression of eNOS attenuates LV dysfunction in mice after myocardial infarction suggesting that NO was beneficial in HF [27]. However, this effect might be lost when eNOS uncoupling occurs in the presence of ROS, stimulating cardiac pathologic remodeling from chronic pressure overload. One proposed mechanism of cardiac
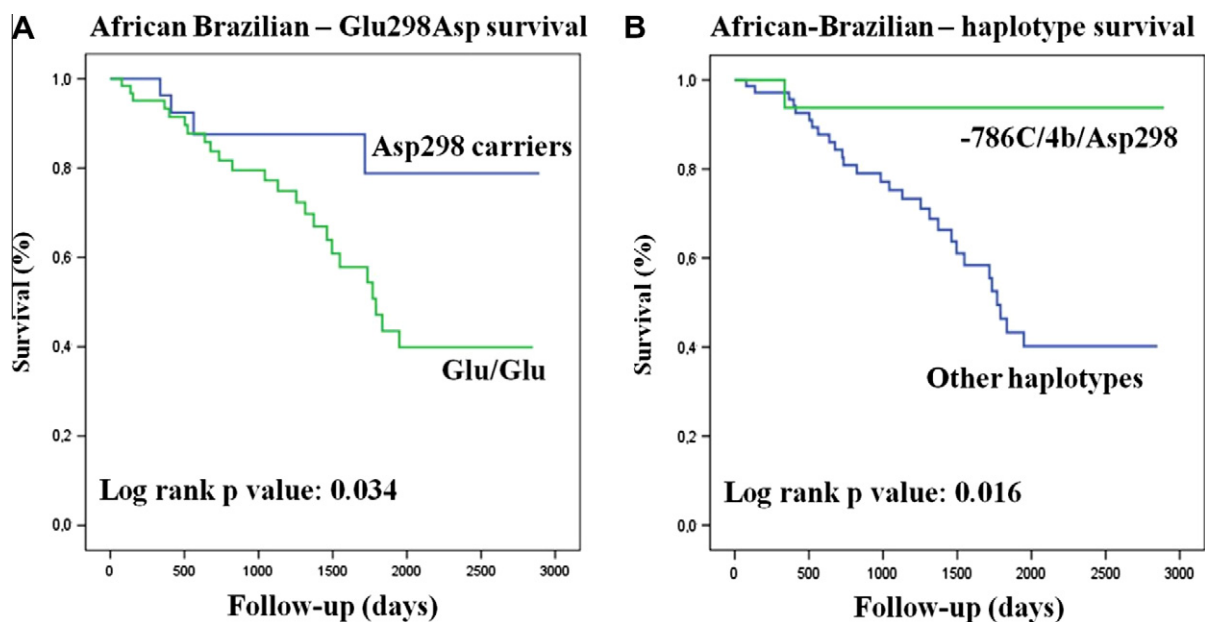

Fig. 2. Kaplan–Meier survival curves comparing African–Brazilian patients who are Asp298 carriers and -786C/4b/Asp298 haplotype carriers (A – Glu298Asp; B – eNOS haplotype).

Table 3
Univariate analysis and Cox proportional hazard models for all-cause mortality in African–Brazilian patients.

Clinical characteristics	Univariate analysis			Adjusted model 1[*]			Adjusted model 2[*]		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Sodium, each 1 mEq/L	0.80	0.71–0.90	0.023	0.77	0.67–0.89	<0.001	0.77	0.67–0.89	<0.001
QRS duration, each 10 ms	1.16	1.05–1.28	0.003	1.17	1.05–1.29	0.005	1.17	1.05–1.29	0.005
Beta-blocker	0.36	0.16–0.81	0.014	0.19	0.08–0.49	0.001	0.19	0.08–0.49	0.001
LVEF, each 1%	0.95	0.91–1.00	0.032	0.97	0.92–1.02	0.25	0.97	0.92–1.78	0.18
Haplotype -786C/4b/Asp298	0.13	0.02–0.93	0.042	0.11	0.01–0.83	0.03	0.11	0.01–0.83	0.03
LVDD, each 1 mm	1.35	0.89–2.03	0.156	0.91	0.51–1.64	0.76	1.11	0.70–1.78	0.65
AMI	1.96	0.92–4.19	0.083	2.37	1.08–5.21	0.03	2.37	1.08–5.21	0.03
NYHA Class III or IV	1.84	0.86–3.97	0.12	0.82	0.33–2.01	0.66	0.84	0.33–2.09	0.70
Age, each year	1.00	0.97–1.04	0.84	0.99	0.96–1.03	0.66	–	–	–
Sex (female as reference)	0.63	0.26–1.54	0.31	0.47	0.18–1.27	0.14	–	–	–

NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LVDD, left ventricular diastolic diameter; AMI, acute myocardial infarction.
[*] Model 1 was adjusted for all variables. Model 2 was not adjusted for age and sex.

dysfunction in chronic HF attributes a major role to an excessive production of NO in the heart, particularly in cardiac myocytes. It has been demonstrated that NO donors increase cardiomyocyte cell death and switch cell death form from apoptosis to necrosis in a concentration dependent manner [28]. The detrimental effect of excess NO is attributed to its action on the mitochondria, by inhibiting the mitochondrial respiratory chain, resulting in increased oxidant production and increased susceptibility to cell death [29]. Moreover, elevated levels of circulating cytokines could stimulate the expression of inducible NOS, with consequent overproduction of NO which has been shown to have negative inotropic effects on failing hearts that have undergone β-adrenergic stimulation [30]. Taking into account that -786C and Asp298 alleles are related to low levels of mRNA and enzyme activity [6,7,9,10], the above data could explain, in part, how the -786C/4b/Asp298 haplotype might have a protective role in HF.

We must consider some methodological aspects of our study. First, our African–Brazilian patients and controls could be considered a mixed population and this could be a confounding variable. Second, previous studies that analyzed eNOS haplotypes were carried out in patients with heterogeneous disease states, such as coronary artery disease and hypertension [1,22]. These entities have distinct phenotypes and different progression profiles and thus should be cautiously compared to HF cohorts. Also, our results are derived from a relatively small sized cohort of HF patients, based on the analysis of specific pre-defined polymorphism (and not genome-wide analysis); as such our findings must be interpreted with caution and be considered hypothesis generators. In addition, because our sample size we were underpowered to perform an interaction analysis of ethnicity and clinical outcomes. Finally, the VNTR intron 4 polymorphisms typically would not be suitable for haplotype analysis. However, as the c allele was very rare, carriers of this allele were excluded from haplotype analysis and the VNTR intron 4 a/b polymorphism was considered as a bi-allelic marker. This approach has been consistently used "successfully" by several groups of investigators [31,32].

In conclusion, we showed that the -786C/4b/Asp298 haplotype had a protective effect on HF susceptibility among African–Brazilian subjects, as well as an important beneficial impact on HF outcome in African–Brazilian patients. On the other hand, the distribution of individual eNOS polymorphisms had no major differences between HF patients and controls in both Caucasians and African–Brazilians. Haplotype analyses may help to underscore the final effects of genetically determined eNOS variability in HF.

Conflict of interest

None to declare.

Funding

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.niox.2012.01.003.

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