

## Genetic Polymorphism G894T and the Prognosis of Heart Failure Outpatients

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### Abstract

**Background:** Previous studies have analyzed the role of the genetic polymorphism of endothelial nitric oxide synthase on heart failure prognosis. However, there are no studies relating the G894T and heart failure in Brazil.

**Objective:** To evaluate the association between G894T GP and the prognosis of a sample of Brazilian outpatients with heart failure.

**Methods:** Cohort study included 145 patients with systolic heart failure, followed for up to 40 months (mean = 22), at two university hospitals, in the State of Rio de Janeiro. We evaluated the relationship between G894T and the following outcomes: reverse remodeling, improvement in functional class (NYHA), and mortality and hospitalization rates. The diameters of the left atrium and ventricle, as well as the ejection fraction of the left ventricle, were evaluated at baseline and at 6 months to assess reverse remodeling. The improvement in functional class was evaluated after 6 months, and mortality rate and hospitalization were evaluated during follow-up. Race was self-declared. G894T polymorphism was analyzed by polymerase chain reaction and restriction fragment length polymorphism.

**Results:** The genotypic frequencies were GG (40%), GT (48.3%) and TT (11.7%). The allele frequency was guanine (64.1%) and thiamine (35.8%). There were no differences between the genotype or allelic frequencies according to self-declared race, either as baseline characteristics. There was no relationship between genotype or allele frequency and the outcome measures.

**Conclusion:** No association was observed between the G894T polymorphism (Glu298Asp) and prognosis in this sample of Brazilian outpatients with systolic heart failure. (Arq Bras Cardiol. 2013;101(4):352-358)

**Keywords:** Heart Failure; Polymorphism, Genetic; Nitric Oxide; Ethnicity and Health.

### Introduction

Heart failure patients' evolution, prognosis and therapeutic response are not uniform. The interpersonal variation in disease behavior has multiple causes, genetic factors among them<sup>1-7</sup>. Various genetic polymorphisms have been implicated; among the most studied is G894T (Glu298Asp), in which changing the nucleotide guanine (G) by thiamine (T) at the position 894 of the exon 7 of the gene leads to the substitution of aspartic acid (asp) for glutamic acid (Glu) in the position 298 of the enzyme endothelial nitric oxide synthase (eNOS), responsible for nitric oxide production (NO) in endocardium and endothelium<sup>8-16</sup>.

The study GRACE, whose population sample was composed on European white, evaluated the impact of Glu298Asp polymorphism of eNOS on heart failure patients' survival, and the Asp298 variant was associated to the worst event-free survival, particularly in patients with non-ischemic cardiomyopathy<sup>17</sup>.

In the A-HeFT study, Afro-American patients with systolic heart failure showed a reduction in mortality and admission rates in response to the combination isosorbide dinitrate plus hydralazine (ISDN/HYD), which acted as a NO donor<sup>18</sup>. The GRAHF study, a genetic sub study of A-HeFT, showed that only patients homozygous for Glu298Glu achieved an improvement in the compound score: admission, mortality and better quality of life<sup>19</sup>.

Of note, these studies' population samples show strong ethnic predominance, and a genotype distribution intimately related to skin color or self-declared race. In a Brazilian study, Velloso et al<sup>20</sup> evaluated samples of heart failure patients and healthy controls and found no difference in allelic frequency distribution (G/T) according to self-declared skin color<sup>20</sup>

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There are no studies associating G894T (Glu298Asp) and heart failure in the Brazilian population, allowing us to think that, maybe, the international studies' results may not be applicable directly to Brazil, with its heavily miscigenated population.

The present study aimed to evaluate the association of G894T (Glu298Asp) polymorphism and prognosis in Brazilian outpatients with systolic heart failure.

## Methods

### Study population

This was an observational, multicenter, prospective cohort study developed in the heart failure clinics of the Antonio Pedro University Hospital of the Fluminense Federal University (Universidade Federal Fluminense - UFF) and the General Hospital of Valença Medical School, both in Rio de Janeiro state, from December 2005 to March 2009. The study included 145 outpatients with predominantly systolic heart failure from the Unified Health System (Sistema Único de Saúde - SUS). Convenience sample

The inclusion criteria were: age  $\geq 18$  years, history and physical examination compatible with heart failure and echocardiogram with left ventricle ejection fraction (LVEF)  $\leq 50\%$  (Simpson). The exclusion criteria were: active myocarditis, myocardial infarction (MI)  $< 3$  months, cardiac resynchronization therapy (CRT), aborted sudden death episode or implantable defibrillator, angioplasty or heart surgery scheduled for the next 12 months.

Race was self-declared. Patients with past MI, confirmed by electrocardiography, functional tests compatible with ischemia or coronary angiography showing an epicardial vessel with  $\geq 50\%$  stenosis were considered to have an ischemic etiology.

After inclusion, patients had appointments scheduled with cardiologists every 3 months or less, if the team judged necessary, and were followed for 12 to 40 months (mean = 22 months). Heart failure was treated according to the II Guideline on Diagnosis and Treatment of Heart Failure of the Brazilian Society of Cardiology<sup>21</sup>. On admission, blood was sampled for lab exams (hemoglobin, glucose, creatinine and sodium) and genetic analysis. Echocardiography was performed on admission and after 6 months.

The following endpoints were analyzed: death during follow-up, admission during follow-up, functional class (NYHA) improvement after 6 months and reverse remodeling after 6 months. Admission were counted on subsequent clinical appointments, and deaths were confirmed by calling relatives and/or patient record review. Functional class (NYHA) was evaluated on admission and after 6 months of follow-up. The 6-month echocardiograms were compared to admission, and the presence of one or more of the following parameters was considered evidence of reverse remodeling (echocardiographic improvement): (1) LVEF increase  $\geq 20\%$ ; (2) LVEF increase  $\geq 10\%$ ; or (3)  $\geq 5\%$  reduction in left atrium (LA) diastolic diameter and/or left ventricle diastolic diameter (LVDD).

The study was approved by the Ethics Committees of the Institutions involved, in compliance with the Declaration of Helsinki. All participants signed an informed consent document.

### eNOS gene polymorphism molecular analysis

The Glu298Asp (G894T) polymorphism, caused by a G-to-T transversion, located at the exon 7 of the eNOS gene, was analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The PCR reaction was performed in a total blood volume of 25 mL, using the following: 50 to 100 ng of genomic DNA, after adjusting for concentration, 15 pmol of each oligonucleotide sense 5'-AAGGCAGGAGACAGTGGATGG-3' and antisense 5'-CCCAGTCAATCCCTTTGGTGCTCA-3', 1 U of *Taq* DNA polymerase, Fermentas reaction buffer (KCl 50 mM, MgCl<sub>2</sub> 1,5 mM, Tris-HCl 10 mM), 200mM of each desoxynucleotide (dATP, dCTP, dGTP, dTTP). After denaturation at 94°C for 5 minutes, the samples were amplified in a program with 35 1-minute cycles at 94°C (denaturation) 1 minute at 58°C (annealing) and 1 minute at 72°C (elongation) in a Progene Techne thermocycler. After that, the samples were submitted to final elongation in a 7-minute cycle at 72°C. The amplicons were evaluated in 2% agarose gel using a 100 base pair marker as length indicator.

The amplified 248 base pairs fragment was digested with the restriction enzyme (RFLP) Ban II, which recognizes the G base at codon 298, generating two fragments (with 163 and 85 base pairs) in the presence of the amino acid Glu (E). The genotype pattern defined in 2% agarose gel was: two fragments, with 163 and 85 bp, for the Glu298 homozygous, and one single 248 base-pair fragment identifying the Asp298 homozygous. The heterozygous subjects were identified by the presence of 248-, 162- and 85-base pairs fragments.

### Statistical analysis

The observed data were described in tables as mean  $\pm$  standard deviation (numerical variables) and frequency and percent values (categorical variables). For analyzing the echocardiographic variables, the Student t-test (paired, for functional class - categorical data) and the McNemar test were used.

The relationship among clinical, laboratorial and echocardiographic variables and the genotype (GG, GT or TT) was accessed by the Kruskal-Wallis univariate variance analysis (non-parametric ANOVA).

The relationship among clinical, laboratorial and echocardiographic variables, as well as survival and event-free (admission) survival rates was accessed by the following methods:

Chi-square or Fisher's exact test was used for comparing with categorical data;

Student t-test for independent samples or Mann-Whitney test was used for comparing with numerical data; The Bartlett test was used for analyzing subgroup variance homogeneity;

Logistic regression analysis was used for identifying variables that could predict (or explain) the outcomes or therapeutic responses.

Non-parametric methods were used for non-normal variables due to data dispersion and rejection by the Kolmogorov-Smirnov test. The criteria for determining significance was 5%. The statistical analysis was performed by the software SAS 6.11 (SAS Institute Inc., Cary, NC).

## Results

### General sample profile

The study population was in Hardy-Weinberg equilibrium ( $\chi^2 = 0.387$ ;  $p = 0.53$ ). The baseline characteristics are summarized in Table 1.

### Clinical and genotypic characteristics

There were no differences in genotypic distribution or allelic frequency according to self-declared race. Also, no differences were found regarding clinical, laboratorial or echocardiographic characteristics on admission regarding genotype (Table 2). Ischemic etiology tended to be more frequent in TT patients.

### Genotype and therapeutics

There were no differences regarding standard heart failure therapy among the genotype subgroups. However, TT patients were more frequently taking aspirin on admission (Table 3).

### Genotype and reverse remodeling

There was no relation between genotype and echocardiographic features observed throughout the study.

Analyzing the variables simultaneously and using logistic regression, we observed that larger LA ( $p = 0.0005$ ) and smaller LVDD ( $p = 0.009$ ) on admission were associated to LA diameter reduction  $\geq 5\%$ . Atrial fibrillation ( $p = 0.0002$ ) and smaller LA ( $p = 0.004$ ) on admission were associated to LA diameter reduction  $\geq 5\%$  after 6 months.

Functional class (NYHA) II ( $p = 0.006$ ) and III/IV ( $p = 0.030$ ), as well as smaller LVEF ( $p = 0.023$ ) on admission were associated to a  $\geq 10\%$  LVEF increase after 6 months. Smaller LVEF ( $p = 0.0001$ ) and no hyponatremia ( $p = 0.02$ ) on admission were associated to a  $\geq 20\%$  LVEF increase after 6 months.

### Genotype and HF improvement (NYHA)

HF improvement (NYHA) after 6 months was not related to genotype or allelic frequency.

HF improvement after 6 months was only associated to creatinine levels  $> 1.5$  mg/dL and functional class III/IV on admission ( $p = 0.25$  and  $0.0001$ , respectively).

### Genotype impact on survival or admission

Fifteen patients (10.3%) died during follow-up. Thirty-four patients (23.4%) were admitted once; nine patients (6.2%) were admitted twice and three patients (2.1%) were admitted three times or more.

**Table 1 - Baseline characteristics**

Age (years)	58.8 ± 13.0
Left atrium (mm)	46.4 ± 7.8
LVDD (mm)	67.9 ± 9
LVEF (%)	35.5 ± 9.2
Body mass index(kg/m <sup>2</sup> )	25.8 ± 5.1
Hemoglobin (g/dL)	13.5 ± 1.8
Creatinine (mg/dL)	1.24 ± 0.73
Sodium (mEq/L)	139.2 ± 3.6
Male (%)	67.8
<b>Self-declared race (%)</b>	
Non afro-Brazilian	50.7
Afro-Brazilian	49.3
Diabetes (%)	34.9
Atrial fibrillation (%)	15.8
Ischemic etiology (%)	46.6
Arterial hypertension (%)	74.0
<b>NYHA (%)</b>	
I	33.6
II	41.8
III	23.2
IV	1.4
<b>Genotype (%)</b>	
GG	40.0
GT	48.3
TT	11.7
<b>Allele frequency (%)</b>	
G	64.1
T	35.8

LVDD: left ventricle diastolic diameter; LVEF: left ventricle ejection fraction; G: guanine; T: thiamine.

After logistic regression, in only one occasion a higher LVDD was independently related to death ( $p = 0.004$ ), while larger LA ( $p = 0.02$ ) and functional class III/IV ( $p = 0.004$ ) on admission were related to hospitalization.

Mean LVDD was  $75.6 \pm 14.7$  mm in the group who died versus  $67.1 \pm 8.8$  mm in the patients that survived on follow-up ( $p = 0.017$ ). Among the patients who were hospitalized at least once, mean LVDD was  $48.6 \pm 8.6$  mm versus  $45.6 \pm 7.1$  mm in the patients not admitted during follow-up ( $p = 0.017$ ).

No statistically significant difference was observed on survival or hospitalization rates regarding genotype or allelic frequency (Kaplan-Meier curve, long-rank test) (Graph 1).

Table 2 - Baseline characteristics versus genotype

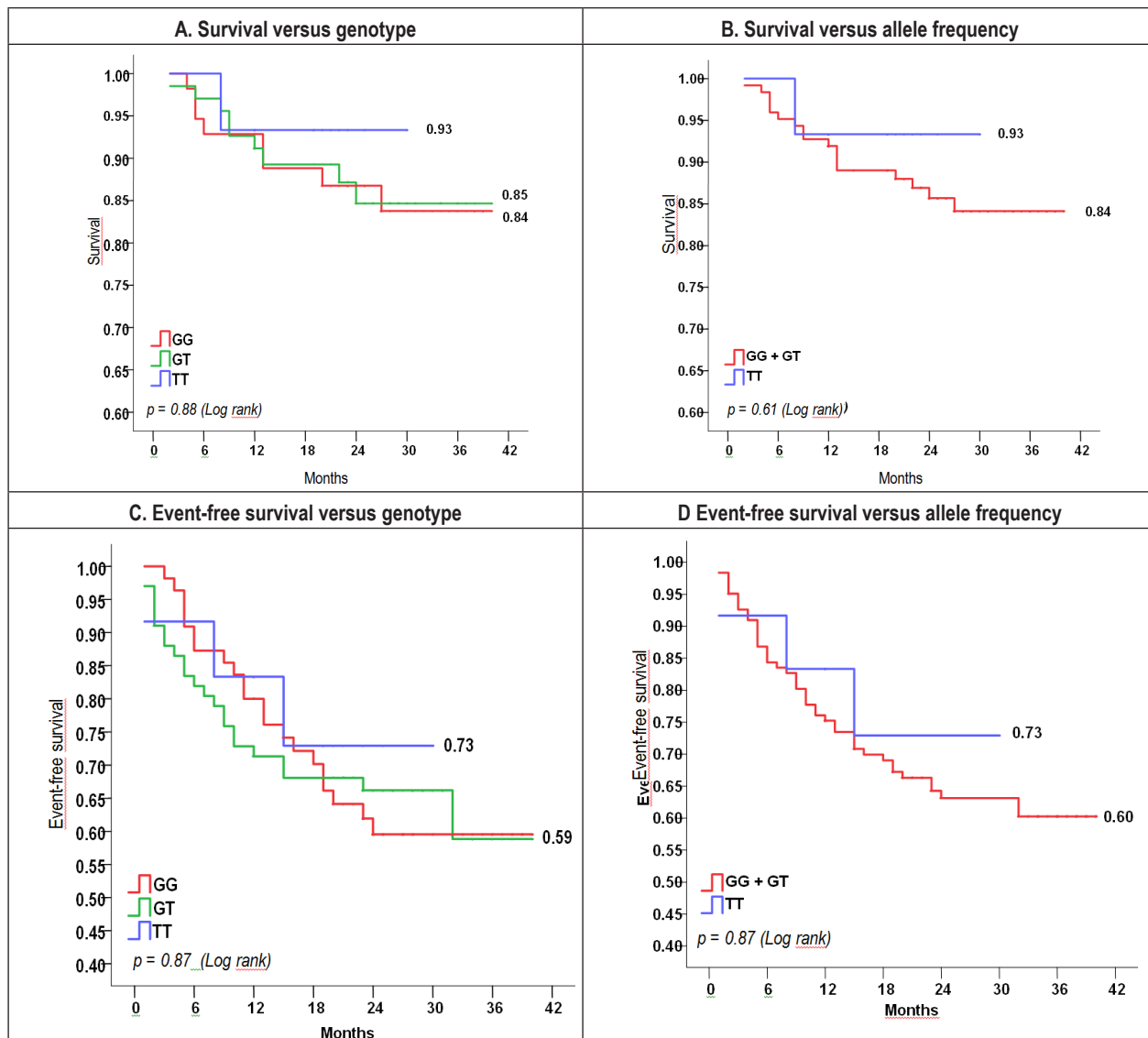
	GG	GT	TT	p value
Age (years)	56.0 ± 12.3	59.9 ± 13.7	63.4 ± 11.7	0.073
Male (%)	58.6	74.3	76.5	NS
<b>Self-declared race (%)</b>				
Non afro-Brazilian	43.1	54.3	64.7	NS
Afro-Brazilian	56.9	45.7	35.3	
Ischemic etiology (%)	41.4	44.3	70.6	0.095
Diabetes (%)	39.7	28.6	41.2	NS
Atrial fibrillation (%)	13.8	15.7	23.5	NS
Arterial hypertension	75.9	70.0	82.4	NS
<b>NYHA (%)</b>				
I	37.9	35.7	11.8	NS
II	37.9	40.0	58.8	
III/IV	24.2	24.3	29.4	
Anemia (%)	30.4	34.8	29.4	NS
Creatinine > 1.5	14.0	21.4	5.9	NS
Sodium < 135 (%)	10.5	5.7	0.0	NS
Left atrium (mm ± SD)	47.3 ± 8.5	45.5 ± 7.2	47.5 ± 7.6	NS
LVDD (mm ± SD)	69.1 ± 9.7	67.6 ± 9.9	64.8 ± 9.2	NS
LVEF (% ± SD)	33.8 ± 9.4	36.3 ± 9.0	38.5 ± 8.7	NS
Body mass index (kg/m <sup>2</sup> ± SD)	26.5 ± 5.4	25.6 ± 5.1	25.0 ± 3.7	NS

NS: non-significant; SD: standard deviation; LVDD: left ventricle diastolic diameter; LVEF: left ventricle ejection fraction.

Table 3 - Genotype and therapeutics

	Admission				After 6 months			
	GG	GT	TT	p value	GG	GT	TT	p value
Angiotensin conversion enzyme inhibitor (%)	80.4	76.2	93.3	NS	76.5	66.7	73.3	NS
Angiotensin receptor blocker (%)	17.7	12.7	0.0	NS	23.5	27.0	20.0	NS
Betablocker (%)	76.5	65.1	73.3	NS	92.2	87.3	93.3	NS
Spironolactone (%)	66.7	50.8	60.0	NS	70.6	68.3	73.3	NS
Furosemide (%)	64.7	65.1	60.0	NS	62.8	63.5	66.7	NS
Hidralazine (%)	7.8	7.9	6.7	NS	13.7	11.1	13.3	NS
Nitrate (%)	35.3	25.4	53.3	NS	33.3	36.5	46.7	NS
Digoxin (%)	64.7	47.6	53.3	NS	54.9	52.4	40.0	NS
Amiodarone (%)	3.9	4.8	0.0	NS	3.9	4.8	0.0	NS
Thiazides (%)	19.6	25.4	40.0	NS	11.8	15.9	20.0	NS
Calcium channel blocker (%)	9.8	15.9	26.7	NS	9.8	15.9	26.7	NS
Aspirin (%)	37.3	54.0	73.3	0.031	47.1	61.9	73.3	NS
Statin (%)	41.2	38.1	66.7	NS	52.9	54.0	80.0	NS
Warfarin (%)	19.6	14.3	13.3	NS	17.7	11.1	20.0	NS

NS: non-significant.



Graph 1 - Survival and event-free survival versus genotype and allele frequency.

## Discussion

The present study population sample showed major differences regarding previous studies involving eNOS genetic polymorphism: the proportion of patients who declared themselves white (50.7%) and Afro-Brazilians (49.7%) was virtually the same, a fact that was not found in previous studies such as GRACE<sup>17</sup>, with a 90.4% whites and A-HeFT<sup>18</sup> with 100% Afro-Americans. Still, different to what was shown in previous studies, on which the G allele was more prevalent among blacks and the T allele was more prevalent among whites<sup>17,19,22</sup>, no association was observed between skin color or self-declared race and genotypic distribution or allelic frequency. Besides, the genotype distribution was 40% for Glu298Glu, 48.7% for Glu298Asp e 11.7% for Asp298Asp, while in the A-HeFT study population it was 9% for Glu298Glu, 20% for

Glu298Asp and 1% for Asp298Asp<sup>19</sup> and, in GRACE, 67% for Glu298Glu, 31% for Glu298Asp and 2% for Asp298Asp, among the black patients, and 41% for Glu298Glu, 45% for Glu298Asp and 14% for Asp298Asp, among whites<sup>17,18</sup>.

The great differences found on distribution according to self-declared race, genotype distribution and frequency between the present and previous studies' samples evaluating the G894T (Glu298Asp) polymorphism allow us to think that the international studies' results may not be directly applicable to Brazilian heart failure patients.

After the results of the A-HeFT study, the FDA approved BiDil® (ISDN/HYD) for black patients with systolic heart failure, the first time a drug was approved for use in a specific race.

Heart failure has a huge social and economic impact in Brazil<sup>23-25</sup>. Hydralazine and nitrate are low-cost drugs, and their addition to standard therapy may potentially improve

the prognosis of systolic heart failure patients harboring the Glu298Glu variant<sup>19</sup>. Thus, the proper quantification of Glu298Asp genetic variability in the Brazilian population and the potential impact of the ISDN/HYD association in Brazilian heart failure patient is important, regardless of the self-declared race.

Other significant finding is the higher tendency to ischemic etiology ( $p = 0.09$ ) found in TT patients, which also showed a higher acetylsalicylic acid use rate ( $p = 0.03$ ), matching the findings in other studies<sup>17,26,27</sup>.

This study has some inherent limitations. The first one is the sample, which is probably small for a genetic study ( $n = 145$ ). Besides, there was a low rate of hydralazine/nitrate use, and a therapy based on NO donors (such as hydralazine and nitrate) could exert some influence on the relationship among the studied polymorphism and the analyzed outcomes. Besides, currently, the genetic studies are not based on the analysis of a single gene, but a set or even thousands of related haplotypes simultaneously, such as the Genome-Wide Association Studies (GWAS), which uses data from HapMap<sup>28-30</sup>.

## Conclusion

This study found no association between genotype or allelic frequency of the G894T (Glu298Asp) eNOS genetic

polymorphism and the death, hospitalization, functional class (NYHA) improvement and reverse remodeling in Brazilian outpatients with systolic heart failure.

## Author contributions

Conception and design of the research, Acquisition of data and Analysis and interpretation of the data: Tardin OMA, Pereira SB, Velloso MWM, Balieiro HM, Costa B, Alves TO, Giro C, Pessoa LP, Ribeiro GS, Mesquita ET; Statistical analysis and Critical revision of the manuscript for intellectual content: Tardin OMA, Pereira SB, Mesquita ET; Writing of the manuscript: Tardin OMA, Mesquita ET.

## Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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## Study Association

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