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Endothelial nitric oxide synthase Glu298Asp gene polymorphism in a multi-ethnical population with heart failure and controls

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ABSTRACT

Brazilian population has a multi-ethnical profile and the prevalence of endothelial nitric oxide synthase enzyme (eNOS) polymorphism in heart failure (HF) has not been previously studied. Therefore the present study assessed the association of eNOS Glu298Asp polymorphism in patients with HF and controls. In a crossover study, was analysed the distribution of the Glu298Asp in 100 outpatients with HF, and 103 healthy controls. Self-reported race were analyzed. Left atria and left ventricle diameters and ejection fraction were evaluated in patients group. Glu298Asp was analysed by polymerase chain reaction and restriction fragment length polymorphism. The patient's average age was 59 years, 66% males, 49% Afro-descendants. The allelic frequency in patient group was Glu298 = 72%/Asp298 = 28% and the genotype frequency (GF) was Glu298Glu:49%; Glu298Asp:47%; Asp298Asp:4%. In control group, 60% Glu298 and 40% Asp298; 35% Glu298Glu, 49.5% Glu298Asp and 15.5% Asp298Asp. The prevalence of allele Glu298 was significantly higher in patients ($p = 0.009$) as genotype Glu298Glu ($p = 0.03$). The Glu298 in Afro-Brazilians (79%) and white patients (67%) were similar, although there was significant difference ($p = 0.03$) in GF Glu298Glu between Afro-Brazilians and whites. There was an increased prevalence of hypertension and increased atria in Glu298Glu patients comparing with combined genotype Glu298Asp and Asp298Asp. This study suggests a regional variation in the distribution of Glu298Asp. The comparison of this distribution in African-Brazilian suggests a synergistic effect of African-descendent, Glu298Glu genotype and HF. Also demonstrated an increased frequency of Glu298 and Glu298Glu, suggesting interaction of them with HF. In HF patients, the clinical, echocardiograph and genotype analysis suggests an association of Glu298 allele and hypertension.

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Background

Nitric oxide (NO) is well known as an important mediator of many physiologic functions, and its role in the pathogenesis of heart failure (HF) is gaining recognition [1]. Its now well established that NO is produced within the heart, not only vascular endothelium, but by the myocytes themselves [2] and that constitutive NO production exerts a significant role in the regulation of cardiac function both under physiological conditions and in disease states [3].

Nitric oxide has been shown to be a key regulator of excitation–contraction coupling [4], coronary vessel tone [5], modulation of inflammation, promotion, and inhibition of vascular growth [6] and modulation of platelet aggregation [7]. Nitric oxide is synthesized upon the cleavage of L-arginine into L-citrulline by three distinct isoforms of NO synthases (NOS) within the myocardium [8]. Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutive expressed in cardiac myocytes and inducible nitric oxide synthase (iNOS) is only expressed during inflammatory responses [9].

Nitric oxide plays an important role in pathophysiology of several cardiac diseases includes heart failure [10]. Clinical investigations in patients with heart failure demonstrate a decrease in the L-arginine NO metabolic pathway [11]. Studies have consistently reported that eNOS signaling decreases the cardiac functional response to beta adrenergic receptor (β-AR) stimulation and protects from arrhythmias. For example, eNOS knockout myocytes exhibited a greater β-AR-stimulated (Ca^{2+}) transient, cell shortening amplitude and a substantially large increase in action potential waveform duration compared to wild type [8,12].

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Endothelial nitric oxide is encoded by a 26-exon gene located on chromosome 7 [12]. A common polymorphism exists in nucleotide 894 (G–T) that results in the conversion of glutamate (Glu) to aspartate (Asp) for codon 298 [13].

Previous studies associated Glu298Asp variant with systemic arterial hypertension [14], atherosclerosis [15], coronary artery disease [16] and a poor outcome in idiopathic dilated cardiomyopathy [17]. The Glu298 allele is prevalent in Afro-Americans and previous studies have demonstrated that self-identified black persons may product less NO and dysfunctional eNOS [18].

The African-American Heart Failure Trial (A-HeFT) evaluated the benefit of fixed-dose combination isosorbide dinitrate-hydralazine (I/H), which may enhance NO bioavailability in African-American patients with advanced heart failure and showed a 43% reduction in mortality [18]. After A-HeFT results, the Food and Drug Adminstration (FDA) approved the use of the first race-specific drug as an adjunct to current standard heart failure therapy in self-identified black patients. The Genetic Risk Assessment in Heart Failure Trial (GRAHF), a prospectively defined genetic analysis of A-HeFT patients, was developed to help identity specific, shared biomarkers within patient cohorts to delineate additional subjects where BiDil may would be also effective [19]. In GRAHF, researchers examined the genetic variation of a number of genes important for cardiovascular diseases, collected from A-HeFT patients and compared with white heart failure subjects from the Genetic Risk Assessment of Cardiac Events (GRACE) study [19].

Results from GRAHF indicated that a marked difference exists in the make up of the endothelial nitric oxide synthase (eNOS) gene in self-identified black patients versus white patients. In the GRAHF subset treatment with BiDil was associated with a trend toward improved composite score (mortality, heart failure hospitalization and change in quality of life, at 6 months) only in Glu298Glu homozygotes.

Brazilian population has a multi-ethnical profile and the prevalence of eNOS polymorphism in heart failure has not been previously studied. Therefore the present study assessed the association of eNOS Glu298Asp polymorphism at exon 7 in patients with heart failure and controls.

Methods

Study population

The investigation conforms to the principals outlined in the declaration of Helsinki, the protocol has been approved by the Institutional Ethics Committee. Following a prospective transversal protocol, we included one hundred outpatients from December 2006 to December 2007, 66 males, with systolic heart failure admitted to Federal Fluminense University. A total of 103 healthy controls, 48 males, were enrolled. The control group consisted of healthy individuals confirmed by questioner, clinical examination and electrocardiogram.

All subjects signed an informed consent form before entering the study and peripheral blood was drawn for DNA isolation by leukocyte centrifugation and cell lyses and genotyping. The genetic factor and the self-reported race of volunteers were analyzed.

A computer search using the hospital databases identified patients who fitted the inclusion/exclusion criteria. Patients with chronic heart failure (CHF) satisfied the following inclusion criteria: clinical diagnosis of heart failure (based on Boston Criteria); standard background therapy with neurohormonal blockage, angiotensin-converting enzyme inhibitors or angiotensin receptor agonists, and β-blockers according to ACC/AHA Guideline update diagnosis and management of chronic heart failure in the adult (2005); age $\geqslant$18 years; echocardiography showing LVEF $\leqslant$50% (Simpson). Patients were excluded from the study if they had: cardiac resynchronization therapy (CRT) device; acute myocarditis; aborted sudden death episode or implanted defibrillator device; indication of cardiac revascularization or angioplasty scheduled within the next 12 month, infarction in the last 3 months. The diagnosis of coronary artery disease was considered in patients with a previous history of AMI, angioplasty, coronary artery bypass grafting, and angina pectoris.

eNOS Asp298Glu polymorphism

For the eNOS Glu298Asp polymorphism, primers 50-AAG GCA GGA GAC AGT GGA TGG A-30 and 50-CCC AGT CAA TCC CTT TGG TGC TCA-30 were used to amplify a 248-bp DNA fragment from exon 7 including the G/T polymorphism at position 894 (codon 298). Polymerase chain reactions were run for 35 cycles: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The product (20 mL) was digested with 3 U Ban II, which cuts the G (Glu298), but not the T allele (Asp298), at 37_C for greater than 4 h, then subjected to gel electrophoresis for genotyping. The eNOS G allele gives 2 fragments of 163 and 85 base pairs, and the eNOS T allele yields a single 248 base-pair fragment.

Statistical analysis

Categorical variables were compared with the case-control situation (case: Patients with Heart Failure – HF), using contingency tables and applied them the Chi-square–Pearson to compare proportions. The Fisher exact test was used when the expected values in the table cells are less than 5.

For quantitative variables we used the Mann–Whitney test, comparison of medians, with the objective to compare the independent groups of different genotypes in relation to cardiac cavities and ejection fraction observed in the group of patients with HF.

The level of null hypothesis rejection was fixed at 0.05 or 5%.

Hardy–Weinberg equilibrium was performed on the distribution of genotypes of the Glu298Asp polymorphism using the Chi-square test.

For the Glu298Asp polymorphism 2-group comparison based on the presence or absence of the Asp298 variant was used given the rarity of the Asp298Asp genotype.

Results

The patient group was 66% male, 49% self-designated Afro-Brazilians, 35%, 42%, 21%, 2% New York Heart Association Class (NYHA) I, II, III, IV, respectively, with a mean age of 59 ± 12. Clinical history of hypertension was present in 74%, diabetes in 24% and 43% had ischemic etiology. The clinical characteristics of the 100 patients enrolled in the study are summarized in Table 1.

Healthy subjects and patient groups were matched for ethnicity, which is a known factor influencing the distribution of eNOS genotype. The distribution of Glu298Asp in the sample of 100 patients and 103 controls did not deviate from the Hardy–Weinberg equilibrium.

eNOS allele and genotype frequencies

In terms of the eNOS Glu298Asp polymorphism, the Glu298 allele was much more prevalent in patient group (72.5%) than controls (59.7%), $p = 0.009$. The Glu298Glu genotype were more frequent in the patient group, $p = 0.030$ and Asp298Asp in control group, $p = 0.05$. There was no differences between the heterozygotes (% Glu298Glu/Glu298Asp/Asp298Asp patients = 49%/47%/4%; controls = 35%, 49%, 16%) (Table 2).

Table 1
Baselines characteristics.

	Patients (n = 100)
Age (years)	59 ± 12
Self-reported race (%)	
Afro-Brazilian	49
Whites	51
Male (%)	66
NYHA (%)	
I	35
II	42
III	21
IV	2
Ischemic etiology (%)	43
Hypertension (%)	74
Diabetes (%)	24

NYHA, New York Heart Association.

Table 2
eNOS genotype distribution and allelic frequencies of heart failure patients and controls.

Variables	Patients – n = 100 n (%)	Controls – n = 103 n (%)	p
Genotypes			
Glu298Glu	49 (49)	36 (35)	0.030
Glu298Asp	47 (47)	51 (49.5)	0.414
Asp298Asp	4 (4)	16 (15.5)	0.005[a]
Allelic frequencies			
Glu298	0.725	0.597	0.009[a]
Asp298	0.275	0.403	0.009[a]

[a] Fisher exact test.

eNOS allele and genotype frequencies by race

In patient group, comparison of race revealed no significant differences in allele frequencies for the self-report Afro-Brazilian and white. For the genotype frequency, Glu298Glu was markedly prevalent in Afro-Brazilian patients. The allele and genotype frequencies in control group failed to reach significance.

For the analysis of the impact of the polymorphism in race, were compared allele and genotype frequencies in Afro-Brazilian, patients versus controls and white-Brazilians, patients versus controls and find no relevant difference into the two groups.

For Afro-Brazilian, allele Glu298 was evident in 78.6% patients and 65.2% in control group. The genotype frequencies in patients were 29 (59.2%) Glu298Glu and 20 (40.82%) Glu298Asp + Asp298Asp. The Glu298 homozygote was present in 19 (41.3%) and combined genotype (Glu298Asp and Asp298Asp homozygote) in 27 (58.7%) controls.

In white subjects, the distribution of the Glu298 allele frequency was 66.7% for patient and 55.3% for control group. Glu298Glu and combined genotype was 20 (39.2%) and 31 (60.8%), respectively, in patient group. In controls, Glu298Glu was present in 17 (29.8%) and 40 (70.2%) were Asp carries.

eNOS genotype and clinical characteristics

Comparison of baseline clinical characteristics between Asp carries and no carries (sex, NYHA class, ischemic etiology, blood pressure level, coronary arterial disease and atrial fibrillation; Table 3) revealed no significant differences in phenotype for the codon 298. There were significantly higher prevalent of hypertension patient in Glu289Glu group. With regards to heart failure therapy, the proportions of patients taking inhibitors of angiotensin-converting enzyme, angiotensin II receptor antagonist, Isosorbide/hydralazine, digoxin, loop diuretic and aldosterone receptor antagonist are not significantly different (Table 4).

eNOS genotype and echocardiography parameters

There was no relationship between measures of ejection fraction, the left atrium and left ventricle diastolic diameter and self-reported race. However, considering the analysis of genotype and left atria diameter, the number of individuals Glu298Glu with left atrium >5 were significantly larger in relation to the Asp carries (p = 0.049).

Discussion

Several studies have demonstrated possible correlations between the eNOS polymorphism Glu298Asp and cardiovascular diseases: hypertension [20,21], acute coronary syndrome [22], myocardial acute infarction [16] and heart failure [23]. However, others had contradictory results. One important factor that may be related to the contrasting results is the population stratification, as a consequence of ethnic diversity. Investigations have previously showed a variable distribution of eNOS polymorphism in different populations and subpopulations [24–26]. To evaluate the importance of this polymorphism in the cardiovascular disease it could be interesting to analyze its distribution.

In the current study, the analyses of the control group showed a higher prevalence of heterozygote (46.51%) when compared with homozygote for Glu298Glu (34.95%) and Asp298Asp (15.53%). This distribution was similar to that found by Fatini and Cols [27] in the Italian population, where 537 individuals were evaluated. In this study, the frequency of heterozygote was 45.2%, and for the homozygote Glu298Glu and Asp298Asp was 43.9% and 10.8%, respectively. In English population, the study CHAOS [28] showed 42% of heterozygote among 138 subjects studied, 47.8% of homozygote Glu298Glu and 10.2% Asp298Asp. The results of allelic distribution demonstrated the Glu allele in a frequency of 60%, while in Italian and English studies this allele was 67% and 69%.

Table 3
Racial differences in eNOS genotype and allele frequencies patients and controls.

	Patients			Controls		
	A (n = 49)	W (n = 51)	p-Value	A (n = 46)	W (n = 57)	p-Value
Genotypes						
Glu298Glu	59.2	39.2	0.036	41.3	29.8	0.157
Glu298Asp	40.8	60.8		58.7	70.2	
Allelic frequencies						
Glu298	78.6	66.7	0.140	65.2	55.3	0.349
Asp298	21.4	33.3		34.8	44.7	

A, Afro-Brazilian; W, white.
Glu298Asp represents Glu298Asp and Asp298Asp.

Table 4
Demographic and clinical characteristics by codon 298 Glu/Asp genotype.

Variables	Glu298Glu – $n = 49$	Asp298[a] – $n = 51$	p
Age (years)	56.5± 1.7	62.1 ± 1.8	0.034*
Female (%)	31	27	0.449
New York Heart Association			
% III	27	16	0.139
% IV	0	4	0.258
Ischemic etiology (%)	63	35	0.150
Ejection fraction–Simpson (%)	34.7 ± 1.2	34.6 ± 1.2	0.948
LV diastolic diameter (cm)	7 ± 0.2	6.8 ± 0.2	0.294
Left atria (cm)	4.7 ± 0.1	4.5 ± 0.1	0.098
BP systolic (mm Hg)	127.3 ± 3	127.6 ± 3.5	0.544
BP diastolic (mm Hg)	78.2 ± 1.8	78 ± 2.1	0.859
CAD (%)	37	45	0.263
Hypertension (%)	80	67	0.026*
Atrial fibrilation (%)	10	10	0.604
Therapy			
ACEI (%)	90	76	0.093
ARA II (%)	14	10	0.394
β-Blocker (%)	88	71	0.044*
I/H (%)	2	4	0.551
Digoxin (%)	67	53	0.124
Loop diuretic (%)	71	67	0.068
Aldosterone receptor antagonist (%)	65	47	0.090

LVFE, left ventricle ejection fraction; LVDD, left ventricle diastolic diameter; LA, left atria; SBP, systolic blood pressure; DBP, diastolic blood pressure; CDA, coronary artery disease; ACE, angiotensin-converting enzyme inhibitor; ARA II, angiotensin II receptor antagonist; I/H, isosorbide/hydralazine.
* $p < 0.05$.
** Glu298Asp + Asp298Asp.

The Brazilian population studied by Sandrim et al. (98 healthy individuals) showed higher prevalence of the Glu allele (67%) and heterozygous Glu298Asp (49%), 43% of genotype Glu298Glu and 8% of Asp298Asp. Considering the allelic distribution, these results were similar to our population, differing only in relation to the genotypes with lower prevalence of homozygote Asp when compared to our study [29].

The presence of Glu allele had a greater impact in the United States, Japan, Korea, Mexico, Chile and Turkey, with allelic frequencies of 74% [27], 99.18% [20], 99.1% [22], 88% [28], 85% [30] and 83.1% [31], respectively.

The Brazilian population is formed by an extensive admixture between Amerindian, Europeans and Africans and is one of the most variable in the world [32]. Interethnic differences have been reported on the distribution of the Glu298Asp polymorphism [27].

In this study, in healthy individuals, the Glu allele represented 55% of the alleles in whites and 65% in African-Brazilians, with no significant difference. The racial difference observed in Glu allele frequency was well characterized in an American study [27] that included 721 whites individuals and 300 African-Americans. While in African-Americans the Glu allele occurred in a frequency of 89.5%, the authors found in white individuals a percentage of 67.6% for this allele. In Brazil, Marroni et al. [33] also observed a marked racial difference in Glu298Asp polymorphism. The frequency of Glu allele was 84.9% in the African-Brazilians compared with 67.2% observed in white individuals. However that study included individuals from the city of Ilhéus (Bahia) and Ribeirão Preto (São Paulo), suggesting a regional influence on allelic distribution. An analysis of polymorphism in a group consisting only of individuals of Ribeirão Preto observed no allelic differences between white and African-Brazilians [24]. Comparison of the genotype Glu298Glu with the combined genotype (Glu298Asp and Asp298Asp) frequencies revealed a higher percentage of Glu298Glu in African-Brazilian group (41.30%) compared to white group (29.82%) with no significant difference. For the combined genotypes, the frequency of 70.17% found in whites individuals was no different for African-Brazilians, whose frequency was 58.7%.

Sandrim et al. [35] also observed no difference in genotype distribution of the Glu298Asp polymorphism between whites individuals and African-Brazilians. The genotype distribution in our study was different from that observed by Marroni et al. [34] and Chen et al. [25]. In the study of Ilhéus/Ribeirão Preto the Glu298Glu genotype was found in 71.3% of African-Brazilians while for whites, included in this study, the frequency was 42.2%. A similar pattern was observed in the American study conducted by Chen et al. [25]. These data indicate the importance of considering the ethnic and regional characteristics in the analysis of this polymorphism.

A comparative analysis of allelic distribution of the Glu298Asp polymorphism between patients with HF and healthy subjects, showed that the Glu allele was more frequent in patients (72.5% versus 59.7%, $p = 0.009$). The HF patients Glu298Glu frequency was significantly greater than that observed in the control group (49% versus 34.95%, $p = 0.030$). Until date, no studies compared the prevalence of the Glu298Asp polymorphism between HF patients and healthy subjects.

The increased prevalence of the Glu28Glu genotype and Glu allele in patients evaluated in this study indicates that the Glu298Asp polymorphism may be associated with HF. MacNamara et al. [18] demonstrated, in a previous study that evaluated the association of the Glu298Asp polymorphism with survival of patients with systolic HF, that the heterogeneity of this genetic polymorphism influenced the prognosis in HF. However, in this study, the risk was attributed to the variant Asp298 which is associated with worse survival in patients with non-ischemic dilated cardiomyopathy.

Concerning self-reported race, no difference in allelic frequency was found between white and African-Brazilian patients, however, a significant difference was observed related to genotypes in these groups. The Glu298Glu genotype was more frequent in African-Brazilian patients (59.8%) when compared to whites (39.22%), $p = 0.036$.

The association of polymorphism Glu298Asp with HF and race has being investigated from the A-HeFT study [20]. The beneficial therapeutic effect of I/H association seems to be related with its action in NO pathway, hydralazine conferring protection against the degradation of nitric oxide induced by oxidative stress and isosorbide serve as a nitric oxide donor.

The GRAFH was designed to evaluate whether these differences could, at least in part, reflect differences in genetic profile or the gene–environment interaction [19]. This study assessed whether eNOS polymorphisms (Glu298Asp,-786T/C and intron 4) heterogeneity had any impact on clinical phenotype and evolution of patients in A-HeFT study. For the Glu298Asp polymorphism in GRAFH study, the prevalence in 352 patients was 78% ($n = 274$) for the genotype Glu298Glu, and 22% for Glu298Asp + Asp298Asp (77 heterozygote and 1 homozygote Asp298Asp). There was significant linkage equilibrium between polymorphism-786T/C and Glu298Asp. Genotype distribution analysis concerning race was similar to our study, with higher prevalence of Glu298Glu genotype in African-descendants. Treatment with combination I/H improved quality of life in Glu298Glu homozygote, suggested a tendency to reverse remodeling, and there was no evidence of any impact on Asp carries patients.

To evaluate whether this polymorphism is associated to race, disease or race and disease, the study compared its distribution in African-Brazilian and whites, analyzing the condition of having HF or not. Despite the small sample, the analysis of African-Brazilian patients showed a tendency of higher prevalence of the Glu298 allele and Glu298Glu genotype in relation to African-Brazilians controls. In white subjects there was no significant difference of allele and genotype frequency, considering patients versus controls.

This analysis suggests that the condition of being African-descendants may have a synergistic effect in the association of Glu298Glu genotype and HF.

The I/H therapy is provided by Brazilian Public Health Service and has low cost becoming an accessible drug for the low income population. Regarding the higher prevalence of Glu298Glu genotype in HF patients in our study, the better treatment response among this genotype in African-descendants (GRAFH), and the fact that Brazil have a multi-ethnical population, our data suggest that this treatment may be an important therapeutic use in HF, regardless of self-reported race.

Previous investigations of eNOS genotypes and clinical phenotypes showed Asp298 variant association with reduced eNOS activity [16] and antiatherogenic NO effect [34] caused increasing interest on the investigation of coronary artery disease (CAD) and the Glu298Asp polymorphism.

Our work confirms the finding of Nassar et al. [35] that demonstrated no association between this polymorphism and CAD. Consistent association between the presence, severity and extent of CAD and Asp298Asp genotype were reported by Colombo et al. [36] reported when examined 201 patients with coronary angiography. Similar results were demonstrated by Shimasaki et al. [37] and Colombo et al. [38].

Many studies address the possible association between eNOS polymorphism and hypertension, but it is still a contradictory topic. Our study suggests a relation between Glu298Glu genotype and this disease, similarly to Lacolley et al. [16]. Chen et al. [25] demonstrated that Asp allele carries with insulin resistance had a predisposition to hypertension. Kato et al. [30] found no association between the Glu298Asp polymorphism and hypertension.

The important role of nitric oxide in the regulation of blood pressure resulted in studies that assessed not only the Glu298Asp, but other eNOS polymorphisms and haplotypes. Nejatizadeh et al. [39] analyzed −922A/G, −786T/C, 4b/4a, and Glu298Asp eNOS polymorphisms and concluded that the haplotypes ATaGlu, ATaAsp and GCbGlu were significantly associated with hypertension, suggesting a susceptibility of these individuals to this disease. Sandrim et al. [22] performed a combined analysis of genotypes of three eNOS polymorphisms, −786T/C, Glu298Asp and 4b/4a, the presence of diabetes mellitus type II and the hypertension susceptibility, finding a protective effect for the CGlub haplotype and an increased susceptibility of CAspb haplotype [28]. We found consistent association between the Glu298Glu genotype and left atria than combined genotype Glu298Asp and Asp298Asp. The homozygote Glu298 also showed an increased prevalence of hypertension, that may have contributed to the larger diameter of the cavity.

Conclusion

In conclusion, we found a different prevalence of Glu298Asp polymorphism in the controls when compared with other countries and even in different regions of Brazil, suggesting a significant regional variation in the distribution of this polymorphism. Concerning race, the comparison of the eNOS polymorphism distribution in African-Brazilian, patients and healthy volunteers, suggests a synergistic effect of the condition to be African-descendent, Glu298Glu genotype and HF. Analyses the eNOS polymorphism prevalence in patient group compared with controls, demonstrated an increased frequency of Glu298 allele and genotype Glu298Glu, suggesting a interaction of them with HF. Finally, in HF patients, the clinical, echocardiographic and genotype analysis suggests an association of Glu298 allele and hypertension.

As the magnitude of the genetic effect is influence by the sample size, this finding indicated the need to perform a large study in order to confirm our data.

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