

Association Analysis of Endothelial Nitric Oxide Synthase G894T Gene Polymorphism and Erectile Dysfunction Complaints in a Population-Based Survey

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ABSTRACT

Introduction. Erectile dysfunction (ED) is a common disorder leading to a serious, negative impact on the quality of the patient's life. The gene encoding endothelial nitric oxide synthase (eNOS) is an interesting candidate gene for understanding the physiopathology of ED, as it is involved in the catalytic production of nitric oxide (NO), the neurotransmitter that plays a critical role in penile tumescence and erection.

Aim. To evaluate a potential association between the G894T polymorphism in the eNOS gene and ED complaints in a population-based sample in São Paulo, Brazil.

Main Outcome Measures. The prevalence of ED complaints was estimated according to the answer to the question "How would you describe your ability to get and keep an erection that is adequate for satisfactory intercourse?" ED was considered to be present if the response was "sometimes" or "never."

Methods. A total of 449 men were enrolled in the study and answered an eight-item questionnaire to ascertain sexual performance/ED and satisfaction. The eNOS G894T polymorphism was genotyped using a standard polymerase chain reaction method.

Results. Univariate analysis demonstrated that ED was associated with diabetes, hypertension, sleep apnea severity, increasing age and body mass index, as well as testosterone levels ($P < 0.05$). Forward multiple regression models indicated that age was the only independent factor associated with ED in this population (odds ratio = 1.09; 95% CI 1.06–1.11; $P < 0.0001$). Genotypic and allelic analyses provided no evidence for an association between this polymorphism and the risk for ED complaints in this sample. Population stratification did not affect the association test results.

Conclusions. This is the first study to examine the effect of polymorphisms in the eNOS gene and the risk for ED utilizing a case-control approach in the Brazilian population. Our results do not support a major role for eNOS gene polymorphisms in ED in this population. **Andersen ML, Guindalini C, Santos-Silva R, Bittencourt LRA, and Tufik S. Association analysis of endothelial nitric oxide synthase G894T gene polymorphism and erectile dysfunction complaints in a population-based survey. J Sex Med 2010;7:1229–1236.**

Key Words. Erectile Dysfunction; Polymorphism; eNos Gene; No Synthase; Sleep Apnea; G894T; Diabetes; Hypertension; Testosterone; Population Study of Erectile Dysfunction

Introduction

Erectile dysfunction (ED) is a common disorder generally characterized by the inability to achieve or maintain an erection sufficiently rigid

for satisfying sexual intercourse. According to epidemiological data, the prevalence of ED is approximately 5–35% [1], and the condition is known to have a serious, negative impact on the quality of the patient's life, inducing fear, decreased self-confidence, and depression [2,3]. Moreover, recent findings have demonstrated significant associations between ED and all-cause

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mortality, mainly through its connection with cardiovascular disease mortality [4].

A number of medical conditions have been demonstrated to influence erectile ability, including vascular diseases, neurological deficits and endocrinological conditions [1]. Indeed, a growing body of literature has reported that the incidence of ED is higher in patients with diabetes [5,6], sleep apnea [7,8], stroke [9,10], and obesity in both men [11] and animal models [12,13]. However, the physiological mechanism by which these conditions may induce sexual dysfunction is poorly understood. For that reason, a great deal of work has been performed in recent years in an effort to identify candidate genes and single-nucleotide polymorphisms (SNPs) in genes that are associated with ED [14–17]. This approach is reflective of the new era of personalized medicine in which detailed information concerning a patient's genotype can be used to assess the risk and plan treatment and prevention programs in clinical practice.

Nitric oxide (NO) plays an important role in normal penile erection because of its ability to relax corporal smooth muscle cells in the penis [18]. The catalytic production of NO requires NO synthase (NOS), which uses L-arginine as its substrate and is expressed in many biological tissues as three main isoforms: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) [19]. Decreased expression and/or activity of these enzymes have been demonstrated in a number of pathologic disease processes to be associated with the manifestation of ED [20–23].

Polymorphisms in the eNOS gene have been associated not only with ED, but also with its associated risk factors, such as diabetes, cardiovascular diseases, and sleep apnea [24–26]. Recently, a number of studies have evaluated the influence of a polymorphism located in exon 7 of the eNOS gene (G894T), which corresponds to a substitution of glutamate by aspartate at amino acid position 298 of the protein, on the susceptibility to ED [17,27–29]. More specifically, it has been reported that eNOS T allele carriers had a significantly higher risk of ED than G allele carriers in Mexican, Taiwanese, and Turkish populations [17,27–29]. In contrast, a study evaluating ED patients from Germany and their response to sildenafil demonstrated that genotype distributions of eNOS polymorphism did not differ between the patient and healthy control groups [30].

To fully characterize the influence of the G894T polymorphism on the susceptibility of ED and to provide progress towards the understanding

of the role of genetic factors in the physiopathology of this condition, further studies are needed in large, standardized and ethnically diverse populations. Thus, the aim of this study was to explore the association between the G894T polymorphism and ED, as well as its associated risk factors in a population-based sample from the city of Sao Paulo, Brazil.

Methods

Study Population and Sampling Procedures

The Sao Paulo Sleep Epidemiologic Study (EPISONO) is a population-based study of sleep disturbances and their risk factors in the city of Sao Paulo, the largest city in the Southern Hemisphere and the fifth largest metropolis in the world. Its population reached 10,886,518 inhabitants living within an area of 1524 km² in January 2008, corresponding to a population density of 7,233 inhabitants/km². Participants 20 to 80 years of age underwent baseline examinations from July to December, 2007. The design, survey methods, and laboratory techniques have been described elsewhere [31]. Briefly, our single-center study involved 1,101 individuals in the city of Sao Paulo. This sample size was established to allow for prevalence estimates with 3% precision [32]. To obtain a representative sample of the inhabitants of Sao Paulo, we used a three-stage cluster sampling technique with unequal selection probability [33], generating sample weights for each volunteer for all representative estimates. The study protocol was approved by the Ethics Committee for Research of the Universidade Federal de Sao Paulo (CEP 0593/06) and was registered with ClinicalTrials.gov (Identifier NCT00596713).

Clinical and Biochemical Measurements

Physical measurements were taken by physical education instructors and included body weight (kg), height (m) and blood pressure (mm Hg). All participants were asked to declare their diabetes status and cardiovascular history and a panel of specialists analyzed the use of medication. Fasting blood was taken in the morning for lipid and hormone profiling, using Advia[®] 1650/2400 and Immulite[®] 2000 (Siemens Healthcare Diagnostics, Inc., Deerfield, IL, USA). Hypertension was defined by current use of hypertensive medication and/or systolic blood pressure (SBP) of >140 mm Hg and/or a diastolic blood pressure (DBP) of >90 mm Hg; diabetes was defined by use of medication and/or

fasting glucose levels ≥ 126 mg/dL; and hyperlipidemia was defined as a total cholesterol level of 240 mg/dL and/or triglyceride levels of 200 mg/dL. A complete full-night PSG was performed on a digital system (EMBLA[®] S7000, Embla Systems, Inc., Medicare Flaga, Reykjavik, Iceland) at the Sleep Institute/AFIP. Sleep parameters were scored in accordance with the criteria established by the American Academy of Sleep Medicine Manual for Scoring Sleep and Associated Events [34]. OSA was defined by an apnea-hypopnea index (AHI) ≥ 5 .

Definitions of Sexual Problems

To verify the standardization of the data-gathering techniques and reproducibility of the questions, all questionnaires applied in the EPISONO study were initially given to a small group of participants (N = 17) by a well trained group of interviewers. These questionnaires covered demographic details, health, relationships, and sexual behavior. After this validation, the data were collected using the questionnaires and objective methods (additional information published elsewhere [31]). At the Sleep Institute, 449 from the total of 467 men included in the epidemiological survey agreed to answer the 8-item questionnaire to ascertain sexual performance/ED and satisfaction. The questionnaire included the following questions: (i) "Do you have a sexual problem?"; (ii) "Is your sexual desire or sexual interest lower than before?"; (iii) "Are you able to maintain an erection long enough to have intercourse?"; (iv) "Have you sought medical assistance for treatment of erectile dysfunction?"; (v) "Do you have pain or discomfort during intercourse?"; (vi) "Do you sleep better after having intercourse or masturbating?"; (vii) "Do you frequently awake with an erection during the night or early morning?"; and (viii) "When was your last ejaculation?". Six questions had "yes" or "no" as options, and one question was graded by the subject on a scale of (never; sometimes; usually; always). Question 8 was completed manually.

The prevalence of ED complaints was estimated according to the answer to the question "How would you describe your ability to get and keep an erection that is adequate for satisfactory intercourse?" Possible responses were "almost always/always," "usually," "sometimes," or "never." ED was considered to be present if the response was "sometimes" or "never." This question, based on recommendations from the National Institutes of Health Consensus Develop-

ment Panel on Impotence (1993) [35], has demonstrated acceptable accuracy in detecting ED [36]. In designing the study plan and analyses, this single question was chosen in lieu of the International Index of Erectile Function-5 (IIEF-5) because the IIEF-5 had been validated only for clinical studies and not for epidemiologic studies.

Genetic Analysis

DNA was extracted from peripheral whole blood using a standard protocol [37]. The G894T polymorphism located in exon 7 of the eNOS gene was selected for analysis. To correct for the presence of population stratification in our sample, we selected a total of 31 ancestry informative markers, e.g. markers that exhibit large allele frequency differences among the three main Brazilian ancestral populations (Europeans, Africans, and Native American). Using the genotype data, the number of ancestral populations (K) in the sample and individual admixture proportions were estimated using the Bayesian Markov Chain-Monte Carlo method implemented in the program Structure 2.1 [38,39].

The genotyping of all SNPs selected for this study was performed blind to status using allele-specific PCR with molecular beacons assay, and was performed under contract by Prevention Genetics (Marshfield, WI, USA; <http://www.preventiongenetics.com>). Names, primers, and conditions for the markers used can be provided upon request.

Statistical Analysis

Hardy-Weinberg equilibrium, genotype, and allele frequencies, as well as risk factor prevalence, were compared using Fisher's exact test. The Student *t*-test was used to compare quantitative variables between groups. To determine the independent risk factors for ED, stepwise forward multivariate logistic analyses were performed. A *P* value of 0.05 was established as statistically significant. All analyses were performed using the Statistical Package for Social Sciences v.16 (SPSS, Inc., Chicago, IL, USA).

Results

A total of 449 men answered an 8-item questionnaire to ascertain sexual performance/ED and satisfaction and were enrolled in the study. The percentage of individuals reporting ED complaints in this study was 17.6% (N = 79). The mean age was 52.7 ± 16.0 years in the patient group

and 38.1 ± 12.3 years in the healthy group ($P < 0.0001$). Univariate analyses have demonstrated that individuals reporting ED complaints showed a significantly higher prevalence of diabetes (17.9%), hypertension (60.3%), and AHI > 15 (40.5%), compared with controls (6.1%, 31.1%, and 19.5%, respectively) ($P < 0.01$). Body mass index (BMI) was higher and total testosterone levels were lower in the ED group (27.9 ± 6.1 kg/m² and 538.8 ± 214.8 ng/dL, respectively) compared with the control group (26.3 ± 4.6 kg/m² and 602.7 ± 228.8 ng/dL, respectively). No significant differences between groups were found for smoking and alcohol status or for the prevalence of hyperlipidemia ($P > 0.05$) (Table 1).

Genotype frequencies were in Hardy–Weinberg equilibrium in terms of both ED complaints and control groups for the G894T polymorphism

($P > 0.05$). Admixture analysis demonstrated that the proportions of European, African, and Native American ancestry for the sample as a whole were $76.0 \pm 16.9\%$; $17.9 \pm 15.5\%$ and $6.1 \pm 8.6\%$, respectively. The ancestry proportions did not differ between groups ($P > 0.05$).

The overall genotype frequency for the G894T polymorphism in the Brazilian population was 52.4% for the GG genotype, 40.5% for the GT, and 7.1% for the TT genotype. No significant differences were observed regarding the genotypic frequency among groups ($P = 0.86$) (Table 1). Allelic distribution did not provide any evidence for association between the marker and ED complaints (odds ratio [OR] = 1.05; 95% confidence interval [CI] = 0.69–1.60). There was no evidence for independent association of risk factors for the manifestation of ED, including diabetes, alcohol

Table 1 Univariate analyses between erectile dysfunction, genotypes of the G894T polymorphism of the endothelial nitric oxide synthase (eNOS) gene, and clinical parameters in a population-based sample of São Paulo city

			Control (N = 370)	Erectile dysfunction (N = 79)	P value
G894T genotypes	GG	N	174 52.9%	32 50%	0.86
	GT	N	131 39.8%	28 43.8%	
	TT	N	24 7.3%	4 6.3%	
Apnea–hipopnea Index (AHI)	<5	N	201 54.3%	32 40.5%	<0.0001
	5–15	N	97 26.2%	15 19.0%	
	>15	N	72 19.5%	32 40.5%	
Smoking	No	N	236 63.8%	44 55.7%	0.20
	Yes	N	134 36.2%	35 44.3%	
Alcohol	No	N	249 72.6%	56 76.7%	0.56
	Yes	N	94 27.4%	17 23.3%	
Hypertension	No	N	252 68.9%	31 39.7%	<0.0001
	Yes	N	114 31.1%	47 60.3%	
Diabetes	No	N	336 93.9%	64 82.1%	0.002
	Yes	N	22 6.1%	14 17.9%	
Hyperlipidemia	No	N	271 75.5%	54 69.2%	0.26
	Yes	N	88 24.5%	24 30.8%	
Age (years)	Mean		38.1	52.7	<0.0001
	SD		12.3	16.0	
Body mass index (kg/m ²)	Mean		26.3	27.9	0.03
	SD		4.6	6.1	
Total testosterone (ng/dL)	Mean		602.7	538.8	0.03
	SD		228.8	214.8	

SD = standard deviation.

and tobacco use, hypertension, hyperlipidemia, and total testosterone levels, with eNOS G894T genotypes ($P > 0.05$; data not shown). The correction for population stratification, using admixture proportions as covariates in the regression model, as well as the correction for other potential confounders, including age, diabetes, hypertension, BMI, AHI, and testosterone levels, did not affect the allelic association test (OR = 0.98; 95% CI = 0.59–1.62; $P = 0.93$), demonstrating that population substructure and other risk factors were not responsible for the negative finding. Further genotypic analyses, evaluating different models considering a dominant and a recessive effect for the T allele, also showed no difference between cases and controls, after correction for confounding variables (OR = 1.01; 95% CI = 0.52–1.96; $P = 0.98$ and OR = 0.85; 95% CI = 0.23–3.07; $P = 0.80$, respectively).

Multiple regression analyses including variables significantly associated with ED were performed to assess independent risk factors. Diabetes, hypertension, AHI, age, BMI, and testosterone levels were included. Forward regression models indicated that age is the only independent factor associated with ED in the population evaluated in this study (OR = 1.08; 95% CI = 1.05–1.11; $P < 0.0001$). Further exploratory analyses have shown that, when the samples is stratified according to age, using 50 years old cutoff, diabetes emerges as an independent factor for ED in the younger group (OR = 6.13; 95% CI = 1.58–23.79; $P = 0.01$), even after controlling for the effect of age. In the group of older individuals, age was maintained as the only independent factor associated with ED complaints (OR = 1.14; 95% CI = 1.07–1.21; $P < 0.001$).

Discussion

In this study we hypothesized that eNOS gene polymorphism could influence susceptibility to ED. The eNOS gene is an interesting candidate for the understanding of the biological processes involved in ED since it is involved in the catalytic production of NO, the neurotransmitter that plays a critical role in the physiological induction and maintenance of erections. Recently, a number of studies have shown that the polymorphism G894T located in exon 7 of the eNOS gene is significantly associated with a higher risk of manifesting ED [17,27–29]. Functional studies reported that eNOS enzyme activity in T allele carriers is 20% lower than the activity found in individuals homozygous

for the G allele [40–42] suggesting that this polymorphism would influence the risk for ED through eNOS dysfunction. Importantly, one study evaluating a potential association between the response to the phosphodiesterase type 5 inhibitor sildenafil and eNOS G894T genotypes in patients with ED demonstrated that although T allele carriers show a reduced response to sildenafil compared with individuals homozygous or heterozygous for the G allele, the distribution of genotypes in the patient group was similar to that of the healthy control group [30]. The latter suggests that, as reported for a number of published data on genetic polymorphisms and different phenotypic traits, population-specific factors may influence the physiological effect of a particular genetic variant.

Accordingly, in our study, genotypic and allelic distribution, as well as the evaluation of recessive or dominant models for the variant in a Brazilian population involving a total 449 individuals, did not provide evidence for an association between genotypes and the trait under study. Population stratification was evaluated and did not affect the association test results, indicating that a false positive effect caused by this is unlikely. However, it is possible that further undetected or cryptic population stratification may serve to mask a positive association and thus we cannot rule out a minor role of the gene or a role for a polymorphism not in significant linkage disequilibrium with the polymorphism under study. One possible explanation for the negative finding is lack of power caused by sample size. However, previous studies reporting significant associations between the G894T polymorphism and ED have demonstrated frequency differences between studied groups up to 24.3%, suggesting that the genetic variant has a large magnitude of effect [17,27–29]. Power calculation revealed that our sample has 80% of power to detect differences in genotypic frequency $>17\%$, assuming the frequency of 47% for the GT+TT genotypes in the control group, as identified in this sample.

The discrepancy among the results is likely to be related to methodological differences, heterogeneity in the ethnic compositions of the studied groups and diagnostic criteria. Indeed, the great variability observed for the frequency of the GT/TT genotypes is shown in Table 2, and highlighting that validation in ethnic different population and correction for population specific effects is essential for an adequate interpretation of the genetic association results. Differently from other published data, our study used a single-

Table 2 Review of published genetic associations studies evaluating the role of endothelial nitric oxide synthase (eNOS) gene polymorphism on erectile dysfunction

Publication	N		Age		Nationality	Overall GT/TT (%)	Positive association?
	Cases	Controls	Cases	Controls			
Eisenhardt et al. 2003	113	108	53.1	57.1	German	55.7	No
Rosas-Vargas et al. 2004	53	62	46.9	44.3	Mexican	34.8	Yes
Lee et al. 2007	151	77	65.5	54.8	Taiwanese	17.5	Yes
Erol et al. 2009	64	82	51.3	50.2	Turkish	36.3	Yes
Lee et al. 2009	235	137	63.0	55.4	Taiwanese	15.9	Yes
Current study	370	79	52.7	38.1	Brazilian	47.6	No

question self-report of ED. This decision was based on recommendations from the National Institutes of Health Consensus Development Panel on Impotence (1993) [35], as the question chosen has been demonstrated to have acceptable accuracy in detecting ED. Moreover, it has been formerly verified that this single question shows advantages for use in clinic-based screening and in large, population-based studies in which ED is one of many outcomes of interest [36]. Recent results from our group regarding the prevalence of ED in the same population sample suggest that the method used for determining ED complaints was not flawed or biased, as prevalence estimates were similar to other studies, as well as associations with previously identified risk factors have been found [42]. Nevertheless, we recognize that the approach chosen is not a definitive determinant of ED prevalence and that, by using this definition, men with mild or moderate ED may have been neglected. It is not clear whether this would have affected the genetic association data, therefore the results of the current study should be interpreted cautiously. Finally, one last point that may be contributing to the divergence in the present results is the mean difference in age between ED and control groups, which is much higher than observed for the other reports (Table 2). Indeed, although univariate analyses have showed significant associations between ED and diabetes, hypertension, BMI, age, and testosterone levels, the lack of significance for these variables in the multivariate analysis is because of the strong influence of the variable age in our study, which is accounting for the majority of the variance in the model. The key contribution of age is also observed when diabetes emerges as an independent risk factor for ED, after the stratification of the sample by age groups. Nevertheless, it is unlikely that the age difference has contributed to the negative association between the polymorphism and ED complaints, since logistic regression analysis to control

for this and other confounding variables has been performed and no impact on the negative results has been identified.

In summary, our results do not support a specific role for the G894T polymorphism in ED complaints in the Brazilian population. However, our study does not exclude a minor role for the eNOS enzyme or its related pathway in the development of ED and suggests that the examination of other variants within this gene not in close LD with the G894T polymorphism in ethnically different populations is necessary to fully characterize the role of this gene in the development of ED.

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