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More severe clinical course of cardiovascular dysfunction in intensive care unit patients with the 894TT *eNOS* genotype

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ABSTRACT. The endothelial nitric oxide synthase (eNOS) plays an important homeostatic role in the cardiovascular system (CVS) by maintaining appropriate blood pressure through production of nitric oxide. The 894TT genotype of 894G>T (Glu298Asp, rs1799983), a polymorphic variant of *eNOS*, has been associated with several vascular diseases. On the basis of this strong relationship, we monitored daily 585 critically ill adult patients according to their degree of CVS dysfunction and investigated their disease progression by the 894G>T genotype. To obtain information of the general population, we obtained the 894G>T

genotypic and allelic frequencies in a random group of 149 healthy subjects. The patients were genotyped for the *eNOS* 894G>T polymorphism and daily evaluated according to their degree of CVS dysfunction through the Cardiovascular Sequential Organ Failure Assessment (SOFA) score. The mean value of the global CVS dysfunction score was significantly higher in 894TT patients (1.35 ± 0.57) than in non-894TT patients (1.23 ± 0.37; P = 0.035). This score remained significantly higher in 894TT patients, even in different patient clusters (all patients, septic, and non-septic patients) during the 1st week at the intensive care unit (1.86 ± 0.8 versus 1.63 ± 0.62, P = 0.005; 2.32 ± 0.10 versus 2.06 ± 0.08, P = 0.009; 0.84 ± 0.09 versus 0.64 ± 0.08, P = 0.027; respectively). This result shows that the mean values of the cardiovascular SOFA score were higher in 894TT patients in all subgroups. The present study provides evidence that the 894TT *eNOS* genotype is associated with a higher degree of CVS dysfunction in critically ill patients.

Key words: eNOS; 894G>T SNP; Genetic risk factors; SOFA score; Cardiovascular system dysfunction; Critically ill patients

INTRODUCTION

The endothelial nitric oxide synthase (eNOS) plays an important homeostatic role in the cardiovascular system (CVS) by relaxing the endothelium and maintaining appropriate blood pressure through production of nitric oxide (Moncada and Higgs, 1993; Umans and Levi, 1995). Within exon 7 of the *eNOS* gene, there is a single nucleotide polymorphism (SNP) that replaces the 894 guanine to thymine (894G>T; rs1799983), resulting in amino acid replacement at position 298 (Glu298Asp) (Nadaud et al., 1994; Miyamoto et al., 1998). The 894T allele is associated with a reduction in the function of the enzyme (Tesauro et al., 2000), and several studies have addressed positive relationships between the 894TT genotype and vascular disease states such as coronary artery disease (Hingorani et al., 1999; Kerkeni et al., 2006), hypertension (Miyamoto et al., 1998), myocardial infarction (Hibi et al., 1998; Antoniades et al., 2005), and atherosclerosis (Lembo et al., 2001). On the basis of the strong relationship between this *eNOS* polymorphism and the function of the CVS, we monitored daily 585 critically ill adult patients according to their degree of CVS dysfunction (maximum duration of monitoring: 199 days) and investigated disease progression by the 894G>T genotype.

MATERIAL AND METHODS

In this study, we included a total of 585 (304 men and 281 women) critically ill adult patients from the general intensive care unit (ICU) at the São Lucas Hospital, Porto Alegre, RS, Brazil, admitted between January 2003 and December 2007. The patients were monitored from their admission to the ICU until discharge from the hospital or death, for a maximum of 199 days. They were daily evaluated according to their degree of CVS dysfunction. In addition, to determine the allele frequencies in the general population, we collected data from a random group of 149 healthy subjects. All subjects (patients and healthy donors) were from

southern Brazil, which is composed of a singular genetic background: the majority of subjects were of European origin (Portuguese, Italians, Spanish, and Germans ancestry), and there was a small number of individuals with African traits contributing to their genetic pool (Parra et al., 2003). This study was approved by the Ethics Committee of our institute (Protocol #05/02598), and informed consent was obtained from all participants.

Phenotyping

We evaluated daily the arterial pressure and the necessity of administration of dopamine, dobutamine, adrenaline, or noradrenaline to normalize the abnormal arterial pressure. We transformed these data to obtain the Cardiovascular Sequential Organ Failure Assessment (SOFA; Vincent et al., 1998, Ferreira et al., 2001) scores as follows: score 0 = mean arterial pressure (MAP) ≥70 mmHg; score 1 = MAP <70 mmHg; score 2 = necessity of administration of dopamine or dobutamine (≤5 mg); score 3 = necessity of administration of dopamine (>5 mg), or adrenaline (≤0.1 mg) or noradrenaline (≤0.1 mg), and score 4 = necessity of administration of dopamine (>15 mg), adrenaline (>0.1 mg), or noradrenaline (>0.1 mg). The cardiovascular SOFA score was evaluated in all 585 patients during the entire stay at the ICU. For the evaluation of the severity of general illness, we used the Acute Physiology and Chronic Healthy Evaluation II (APACHE-II; Knaus et al., 1985) score obtained on the day of ICU admission. Mortality was measured in days until death. For the diagnosis of sepsis and septic shock, we used the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference criteria (Levy et al., 2003). Different phenotypic and genotypic information about these patients was found in Paskulin et al. (2011), Paludo et al. (2013), and Fallavena et al. (2013).

Genotyping

Genomic DNA was isolated from leukocytes according to Lahiri and Nurnberger Jr. (1991) and we followed the 894G>T genotyping protocol described by Hibi et al. (1998). We used a quality control system to ensure genotyping accuracy: sequencing verification of the amplified DNA fragment, black controls, and repetitions. Thus, in order to confirm that the product was correctly amplified by polymerase chain reaction, we performed a sequence analysis on the MegaBace 1000 capillary DNA sequencer (Amersham Biosciences UK Ltd., Chalfont St. Giles, Bucks, UK). Alignment view was performed using the ClustalX program (version 1.8, as described at ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) in multiple alignment modes, with sequences uploaded in the FASTA format. The sequence obtained was submitted to a nucleotide-nucleotide BLAST online alignment (http://www.ncbi.nlm.nih.gov/BLAST/) with the human genome and we found consensus with *Homo sapiens* nitric oxide synthase 3 (endothelial cell: NOS3; GI:48762674, NM_000603). At least 20% of all samples were confirmed by a 2nd and independent genotyping. According to HapMap, the expected prevalence of the 894G>T SNP was sufficient in our patient population: Caucasian/European heritage 894T = 0.34, 894G = 0.66 (http://www.hapmap.org/). These frequencies were compatible with a previous study performed in our population with 156 cardiac patients (894T = 0.36 and 894G = 0.64; Iturry-Yamamoto et al., 2007). The allele frequencies in our control population (healthy individuals) were as follows: 894TT = 0.31, 894GT = 0.33, and 894GG = 0.36; 894T

= 0.48 and 894G = 0.52; chi-square test Hardy-Weinberg equilibrium: P = 0.099. We did not consider healthy subjects as the control group because we assumed that environmental factors had a crucial influence. Genotyping was performed in a blinded fashion, i.e., investigators were unaware of patient data.

Statistical analysis

Statistical analysis was performed using SPSS (Chicago, IL, USA) for Windows (version 14.1, Copyright SPSS Inc.). The multilevel linear regression model was used to evaluate the progression of CVS dysfunction. The number of days that each patient remained at the ICU during these 2 weeks was introduced as random effect. Genotypes were introduced in the model as fixed-effect to detect differences between genotypes, independent of the observation time. Differences in the degree of CVS dysfunction of each patient were introduced in the test as dependent variables. Non-normally distributed variables were analyzed by the Student *t*-test and the Mann-Whitney U-test. For categorical data, we used the Pearson chi-squared test. To test Hardy-Weinberg equilibrium, the chi-squared test was used. A P value <0.05 was considered to indicate statistical significance.

RESULTS

Demographic and clinical characteristics of the patients by genotype group are shown in Table 1. The genotypic and allelic frequencies were: 894TT = 0.11, 894GT = 0.42, 894GG = 0.47, 894T = 0.32, and 894G = 0.68; these allele and genotype frequencies did not differ significantly from those predicted by the Hardy-Weinberg distribution (chi-square test Hardy-Weinberg equilibrium: P = 0.572). For genotype analysis, a recessive heredity model to 894T, the rarest allele, was assumed (combined 894TT versus the 894GT+894GG genotypes).

Table 1. Critically ill patients' clinical and demographic data according to the *eNOS* 894G>T >T SNP genotype groups.

Characteristic	All (N = 585)	894TT (N = 67)	894GT+GG (N = 518)	P
Frequency [N (%)]	585 (100)	67 (11.5)	518 (88.5)	0.572^{HW}
Female [N (%)]	281 (48)	29 (43.3)	252 (48.6)	0.408$^{\chi 2}$
Age [mean (SD)]	55.4 (19.8)	58.1 (19.4)	55.1 (19.8)	0.236ST
APACHE-II score [mean (SD)]	19.7 (7.8)	20.9 (7.6)	19.6 (7.8)	0.175ST
Global Cardiovascular score [mean (SD)][a]	0.95 (1.5)	1.35 (0.57)	1.23 (0.37)	0.035^{MR}*
Sepsis [N (%)]	413 (70.6)	47 (70.1)	366 (70.7)	0.932$^{\chi 2}$
Septic shock [N (%)]	291 (49.7)	37 (55.2)	254 (49.0)	0.340$^{\chi 2}$
ICU LOS [median (min/max)]	14 (0/125)	15 (2/78)	13 (0/125)	0.218^{MW}
ICU+H LOS [median (min/max)]	36 (1/242)	40 (7/108)	35 (1/242)	0.448^{MW}
Mortality in ICU [N (%)]	190 (32.5)	27 (40.3)	163 (31.5)	0.146$^{\chi 2}$
Mortality in ICU+H [N (%)]	264 (45.1)	33 (49.3)	231 (44.9)	0.496$^{\chi 2}$

[a]Patients' cardiovascular score values measured by sequential organ failure assessment. 894GT+GG = heterozygotes; 894TT = homozygotes; APACHE-II = Acute Physiology and Chronic Health Evaluation II; ICU = intensive care unit; ICU+H = ICU plus hospital; LOS = length of stay; N = number; SD = standard deviation; HW = Pearson chi-square test for Hardy-Weinberg equilibrium; χ^2 = Pearson chi-square test; ST = Student *t*-test; MW = Mann-Whitney *U*-test; MR = multi-level linear regression model test; *P value describes a comparison between 894TT versus no 894TT genotype groups.

There was no significant difference between the genotype groups (894TT versus

894GT + 894GG patients) and the patient characteristics, i.e., gender, age, APACHE-II score, occurrence of sepsis or septic shock, length of stay at the ICU and post-ICU (hospital), and mortality rates. However, the mean value of the global cardiovascular dysfunction score was significantly higher in 894TT patients (P = 0.035). Table 2 presents a discriminated analysis of the effect of the 894TT genotype in different patient clusters (all patients, septic, and non-septic patients) during the 1st week at the ICU (P = 0.005, P = 0.009, and P = 0.027, respectively). In all subgroups, the mean values of the cardiovascular SOFA scores were higher in 894TT patients, but the 894TT effect over CVS dysfunction was most decisive and significant during the 1st week at the ICU. There was no significant difference among the subgroups in the following ICU weeks when analyzed separately. The 894TT genotype influenced the patients' CVS dysfunction independently; even in patients with sepsis and septic shock, the degree of CVS dysfunction was significantly higher in 894TT patients.

Table 2. One intensive care unit week cardiovascular scores (measured by sequential organ failure assessment) according to the 894G>T *eNOS* polymorphism.

	Patients	894TT (N = 67)	894GT+GG (N = 518)	P
All patients [mean (SD)]	1.12 (1.6)	1.86 (0.80)	1.63 (0.62)	0.005^{MR}*
Patients with sepsis [mean (SD)]	1.38 (1.7)	2.32 (0.10)	2.06 (0.08)	0.009^{MR}*
Patients without sepsis [mean (SD)]	0.41 (1.1)	0.84 (0.09)	0.64 (0.08)	0.027^{MR}*

894GT+GG = heterozygotes; 894TT = homozygotes; MR = multi-level linear regression model test; *P value describes a comparison between 894TT versus no 894TT genotype groups. SD = standard deviation.

DISCUSSION

Here, we found that the degree of CVS dysfunction was significantly higher in 894TT patients when compared with 894GT+894GG patients during the crucial 1st week at the ICU. This finding provides new information about the effect of the 894G>T polymorphism on the condition of the CVS, suggesting that the 894T variant in homozygous subjects has a negative effect on CVS function when the patient is under critical circumstances.

The present study used the multilevel linear regression model, also called mixed-effect linear model, which is an appropriate model for analyzing longitudinal data to repeated measures of the same patient. The model consists of an analysis that is expressed as a function of both components: fixed (overall prediction) and random (inherited individual predictions). We grouped covariates according to a classification factor (the 894G>T genotype, fixed-effect) and the time between measurements (random-effect) with a continuous dependent variable (CVS dysfunction daily scores). This longitudinal approach was helpful in understanding how subtle was the association between a factor (genotype) and a variable that change over the time.

The 894T allele is associated with worse *eNOS* functionality, i.e., decreased nitric oxide production by endothelial cells, than the 894G variant (Veldman et al., 2002). Since *eNOS* is a constitutive enzyme, the entire endothelium is affected by the 894G>T polymorphism (Veldman et al., 2002; Antoniades et al., 2005), and this directly affects the regulation of the CVS (Dafni et al., 2010). Because of this association, this common, but functional, polymorphic variation has been the focus of interest and pointed out as risk factor and/or potential marker in the pathobiology of cardiovascular diseases in different populations. Dafni et al. (2010) studied 204 patients with myocardial infarction and 218 control subjects; in that population of Greek origin, the risk of developing myocardial infarction was found to be about

2-fold higher for 894TT patients when compared with 894G carriers. The 894TT genotype was also associated with coronary artery disease in a Tunisian population. Kerkeni et al. (2006) studied 100 patients with coronary artery disease and 120 control subjects. In addition to the effect of the 894TT genotype, the Tunisian study found a relationship between the 894T variant and hyperhomocysteinemia and severity of coronary artery disease. In another study that also evaluated the influence of this *eNOS* polymorphism in critically ill patients, Huttunen et al. (2009) found a significant relationship between the 894T allele and hypotension in patients with *Escherichia coli* bacteremia. In addition, this study, which followed the clinical course of 147 patients during the 1st 6 days at the ICU, showed that carriers of the 894T allele had lower blood pressure and higher SOFA scores. The same instrument was used by us to assess the function of the CVS in our sample population. Another finding in Huttunen's study that confirms our results is that the effect of the 894T allele on blood pressure was most prominent in the early stage of the disease: on admission, MAP was lower in carriers of the 894T allele compared with non-carriers. The above mentioned studies, in addition to our study, show that this polymorphic variant affects directly the condition, structure, and proper functioning of the CVS; moreover, the allelic and genotypic frequencies resemble those found in our group.

In the vital 1st week at the ICU, the association between the 894TT genotype and worse CVS scores was powerfully exposed. Interestingly, this inherited influence gradually disappeared from the 2nd week at the ICU by the intensive treatment. We also could note that septic patients presented the highest degree of CVS dysfunction (Table 2), independent of the genotype. Due to several physiological changes caused by the sepsis, e.g., cytokine circulation and altered homeostasis, this clinical condition causes myocardial dysfunction, affecting the CVS individually (Krishnagopalan et al., 2002). However, even in patients with sepsis, the degree of CVS dysfunction was higher in 894TT patients, indicating that the effect of this genotype is an independent factor of cardiovascular dysfunction.

In conclusion, the present study provides evidence that the 894TT *eNOS* genotype is associated with a higher degree of CVS dysfunction in critically ill patients. In addition, this polymorphism can be clinically useful as potent genetic marker for the prognosis of the risk to develop CVS dysfunction and of the outcome in critically ill patients.

Conflict of interest

There is no conflict of interest.

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