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Glu298Asp polymorphism in the *NOS3* gene is not associated with susceptibility to chronic heart failure in a Russian population

E.S. Goppe[1], P.E. Maltese[2], A. Chernova[3], S.Y. Nikulina[1], A. Semenchukov[3], A. Matveeva[1], E. Manara[4] and M. Bertelli[4]

[1] Department of Internal Diseases 1, Krasnoyarsk State Medical University, Krasnoyarsk, Russia
[2] Magi's Lab, Rovereto, Italy
[3] Russia-Italy Laboratory of Medical Genetics MAGI-Russia, Research Institute for Molecular Medicine & Pathobiochemistry, Krasnoyarsk State Medical University V.F. Voino-Yasenetsky, Krasnoyarsk, Russia
[4] MAGI Euregio, Bolzano, Italy

Corresponding author: E. Manara
E-mail: elena.manara@assomagi.org

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ABSTRACT. The eNOS Glu298Asp (rs1799983) polymorphism of the *NOS3* gene has been implicated as a risk factor for cardiovascular diseases; however, not all studies find significant associations. We examined this possibility in a Russian (Siberian) population. One hundred patients with chronic heart failure and 40 controls were investigated. PCR analysis was performed on DNA samples. The aim was to evaluate a possible association between the (Glu298Asp) polymorphism (rs1799983) of the *NOS3* gene and susceptibility to chronic heart failure in the Russian population. We evaluated genotype distributions in patient and control groups and assessed the relationship between genotypes and chronic heart failure. We found that this polymorphism is not associated with increased risk of chronic heart failure in our study cohort. In conclusion, testing of the *NOS3* gene polymorphism does not seem useful for evaluating predisposition for chronic heart failure or its diagnosis and prognosis.

Key words: Polymorphism; Chronic heart failure; Genotype; Glu298Asp; *NOS3* gene

INTRODUCTION

Chronic low heart performance leads to chronic heart failure (CHF), a syndrome with typical symptoms (shortness of breath, swelling of ankles, fatigue) and signs (elevated jugular vein pressure, lung rales, peripheral edema) caused by disruption of heart structure and/or function, reduced cardiac output and/or increased intra-cardiac pressure at rest or during exercise. This complex syndrome is a significant health care issue with high incidence and mortality. Prevalence is estimated at more than 10 million patients in Europe and 23 million worldwide (Jessup and Brozena, 2003). More than 612,000 persons per year are diagnosed for the first time with heart failure in Russia. One in every eight death certificates mentions heart failure, with 12% as the primary cause of death (usually coronary heart disease, heart attacks, high blood pressure).

These figures indicate that substantial efforts are needed to identify and treat factors that predispose to CHF to reduce the risk of heart failure by preventing its occurrence, and to obtain better overall survival. Research into polymorphisms identifying patients at risk of heart failure could be a useful contribution to this effort (Mosterd and Hoes, 2007).

Genetic polymorphisms in genes belonging to pathways involved in the regulation of basal vascular tone and cardiac myocyte function have been the focus of many studies designed to unravel the contribution of germline genetics in patients with heart failure.

The endothelial synthase of the nitric oxide gene, also known as the nitric oxide synthase 3 (*NOS3*) gene, encodes the enzyme that converts L-arginine to nitric oxide (NO). It is expressed in the early stages of cardio-genesis and plays an essential role in normal heart development. Nitric oxide is a small gaseous lipophilic molecule involved in various biological processes important for the cardiovascular system, such as regulation of the smooth muscle tone of blood vessels and platelet aggregation in the clotting process. Nitric oxide causes vasodilation, prevents migration and proliferation of vascular smooth muscle cells, and inhibits adhesion of platelets and leucocytes (Yang et al., 1993; Jeremy et al., 1999).

The *NOS3* gene contains 26 exons spanning 21 kb on chromosome 7q35-36 (Marsden et al., 1993). Various polymorphisms in the *NOS3* gene have been reported. A common polymorphism is the Glu298Asp substitution in the eNOS enzyme, whose functions are still unclear. The polymorphism does not change the kinetics of the reaction leading to the production of NO, although eNOS Asp298 has been found more susceptible to proteolytic cleavage than eNOS Glu298, and subjects with the Asp298 variant may show a reduction in nitric oxide production (Tesauro et al., 2000; Leeson et al., 2002). The eNOS Glu298Asp (rs1799983) polymorphism has been implicated as a risk factor for various cardiovascular diseases, such as congenital heart defects (Shaw et al., 2005; Van Beynum et al., 2008; Khatami et al., 2017), coronary artery disease (Colombo et al., 2003; Shahid and Rehman, 2017), myocardial infarction (Hibi et al., 1998), hypertension (Miyamoto et al., 1998; Gamil et al., 2017) and heart failure (Velloso et al., 2010). However, other authors report contradictory results (Kato et al., 1999; Nassar et al., 2001; Gamil et al., 2017). This could be due to sample size and/or cohort ethnicity.

It is of utmost diagnostic, preventive and therapeutic importance to understand the molecular events that trigger the development and progression of heart failure in different populations. The aim of this paper was to investigate the relationship between the

Glu298Asp polymorphism (rs1799983) in endothelial nitric oxide synthase and the risk of developing chronic heart failure in a Russian population.

MATERIAL AND METHODS

Patients and healthy subjects

One hundred patients with chronic heart failure of varying severity confirmed clinically and by other investigations, were enrolled in the study in the period March 2016 to April 2017 at Krasnoyarsk Interdistrict Hospital no. 20. The study was approved by and performed in accordance with the protocol of the institutional ethics committee. The study inclusion criteria were: a history of CHF; either gender; any age; informed consent; resident in Krasnoyarsk; ability to perform the necessary procedure. Patients were assessed for overall health status, undergoing palpation, percussion, auscultation, electrocardiography and echocardiography, as indicated by the European Society of Cardiology (Ponikowski et al., 2016). Briefly, we estimated probability of CHF based only on medical history (e.g. coronary heart disease, hypertension, use of diuretics), symptoms (e.g. shortness of breath, bilateral edema, increased jugular vein pressure, apical impulse displacement) and ECG (preserved ejection fraction of left ventricle >50%; low ejection fraction of left ventricle <40%; average ejection fraction of left ventricle 40 - 49%). The control group consisted of 40 subjects without CHF. A blood sample for molecular genetic studies was collected during assessment. Development of research protocols and genetic analysis were conducted in the MAGI genetic laboratory in Italy and in the Russian-Italian laboratory of medical genetics in Krasnoyarsk.

Polymerase chain reaction (PCR)-restriction fragment length screening for the *NOS3* rs1799983 polymorphism

DNA was extracted from 0.5 mL whole blood with E. Z. N. A. DNA blood kits (omega Bio-Tek; NORCROSS, GA, USA), as previously described (Akhmedova et al., 2017).

Screening for the p.(Glu298Asp) polymorphism in the *NOS3* gene was performed by PCR amplification of 100 ng of DNA, followed by ECO24I restriction and fragment length screening. The PCR amplification of a 153 bp fragment encompassing the polymorphism rs1799983 (NM_000603.4:c.894T>G) was performed using the AmpliTaq Gold fast (Thermoscientific, Milan, Italy) and 10 pmol primers 5'GGCTGGACCCCAG GAAAC3’ (forward) and 5'CCACCCAGTCAATCCCTTTG3" (reverse) as follows: 95°C for 10 min (initial denaturation), then 35 cycles of 95°C for 30 s (denaturation), 57°C for 30 s (annealing) and 72°C for 30 s (extension), then final elongation at 72°C for 5 min. The restriction reaction was performed on the amplified fragment using the enzyme ECO24I (Thermoscientific, Milan, Italy) at 37°C for 100 min, followed by enzyme inactivation for 20 min at 65°C. Digestion was resolved on a 2% agarose gel and detected by ultraviolet radiation. The 153 bp fragment was cleaved into 88 and 65 bp fragments in the presence of a T at nucleotide 954 of the *NOS3* gene.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was tested in the control group using an online tool (the Hardy-Weinberg 2-Allele Calculator http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html). We used the chi-square (χ^2) test to evaluate the distribution of polymorphic *NOS3* rs1799983 in patients and controls. χ^2 was calculated using the Cochran-Armitage test for trend. Armitage's trend test is a valid test even in Hardy-Weinberg disequilibrium (Armitage, 1955).

The odds ratio was used to assess the risk of an association between the polymorphism and chronic heart failure. Statistical analysis was conducted with the MedCalc software (Mariakerke, Belgium). Statistical significance was set at P value <0.05.

RESULTS

Analysis of the *NOS3* Glu298Asp polymorphism

We compared the alleles and frequencies of rs1799983 *NOS3* Glu298Asp genotypes in 100 CHF patients and 40 controls. Our group of patients included 57 men and 43 women, with an average age of 68±11.5 years.

The results of restriction enzyme digestion for analysis of the rs1799983 polymorphism of the *NOS3* gene revealed that five of our cohorts of CHF patients were homozygous for the T nucleotide, 48 were homozygous for the G variant and 47 patients were heterozygous. The frequencies of the T, G and T/G variants in the control group were 5%, 60% and 35%, respectively. The genotype frequencies of rs1799983 were in Hardy-Weinberg equilibrium in the CHF cohort (P = 0.16), but they deviated significantly from that equilibrium in the control group (P = 0.04).

There was no significant difference in the genotype distribution between patients and controls (P = 0.23 considering G/G, G/T and T/T groups and P = 0.071, considering the dominant genetic model G/G vs G/T +T/T) (Table 1).

Table 1. rs1799983 *NOS3* Glu298Asp allele and genotype frequencies recorded in chronic heart failure patients and controls.

Genotype (codon change)	Patients (N=100)	Controls (N=40)	χ^2 for trend	P value	OR	95% CI	Significance level
G/G (Glu/Glu)	48 (48%)	14 (35%)			Reference	-	-
G/T (Glu/Asp)	47 (47%)	24 (60%)	1.426	P = 0.2324	1.7508	0.8089 to 3.7894	P = 0.1551
T/T (Asp/Asp)	5 (5%)	2 (5%)			1.3714	0.2396 to 7.8494	P = 0.7227
Dominant model			χ^2	**P value**	**OR**	**95% CI**	**Significance level**
G/G Vs G/T + T/T	52 (52%)	26 (65%)	3.254	P = 0.0712	1.7143	0.8025 to 3.6619	P = 0.1640

Demographic and clinical characteristics of the cohort of patients divided by genotype

Table 2 shows the main clinical characteristics of the CHF group divided by genotype. Clinical and laboratory values were compared between different genotypes with a polymorphic *NOS3* 298 codon; however, no significant differences were noted in the case of wall ischemia (P = 0.28), palpitations (P = 0.67), arrhythmia (P = 0.98), myocarditis (P =

0.94), cardiomyopathy (P = 0.58), hypertensive disease (P = 0.58), valve disease (P = 0.71) or post-infarction cardiosclerosis (P = 0.34) (Table 2).

Table 2. rs1799983 *NOS3* Glu298Asp allele and genotype frequencies according to clinical characteristics of chronic heart failure.

***NOS3* p.(Glu298Asp)**	**Wall ischemia**			**Palpitations**		
	NO	YES	χ^2 (P value)	NO	YES	χ^2 (P value)
Glu/Glu	40 (50.0%)	4 (33.3%)		26 (45.6%)	17 (47.2%)	
Glu/Asp	35 (43.8%)	8 (66.7%)	χ^2 = 2.529	27 47.4%)	18 (50.0%)	χ^2 = 0.782
Asp/Asp	5 (6.3%)	0	P = 0.28	4 (7.0%)	1 (2.8%)	P = 0.67
TOTAL	80	12		57	36	
	Arrhythmia			**Myocarditis**		
	NO	YES	χ^2 (P value)	NO	YES	χ^2 (P value)
Glu/Glu	19 (47.5%)	25 (46.3%)		43 (46.7%)	1 (50.0%)	
Glu/Asp	19 (47.5%)	26 (48.1%)	χ^2 = 0.022	44 (47.8%)	1 (50.0%)	χ^2 = 0.115
Asp/Asp	2 (5.0%)	3 (5.6%)	P = 0.98	5 (5.4%)	0	P = 0.94
TOTAL	40	54		92	2	
	Cardiomyopathy			**Hypertensive disease**		
	NO	YES	χ^2 (P value)	NO	YES	χ^2 (P value)
Glu/Glu	16 (44.4%)	28 (48.3%)		4 (33.3%)	40 (48.8%)	
Glu/Asp	19 (52.8%)	26 (44.8%)	χ^2 = 1.071	7 (58.3%)	38 (46.3%)	χ^2 = 1.083
Asp/Asp	1 (2.8%)	4 (6.9%)	P = 0.58	1 (8.3%)	4 (4.9%)	P = 0.58
TOTAL	36	58		12	82	
	Valve disease			**Postinfarction cardiosclerosis**		
	NO	YES	χ^2 (P value)	NO	YES	χ^2 (P value)
Glu/Glu	38 (48.7%)	6 (37.5%)		13 (44.4%)	31 (52.5%)	
Glu/Asp	36 (46.2%)	9 (56.3%)	χ^2 = 0.671	20 (52.8%)	25 (42.4%)	χ^2 = 2.13
Asp/Asp	4 (5.1%)	1 (6.3%)	P = 0.71	2 (2.8%)	3 (5.1%)	P = 0.34
TOTAL	78	16		35	59	

DISCUSSION

Chronic heart failure is a significant worldwide health care issue, and it is of utmost importance to understand the molecular events involved in this chronic disease that in most cases leads to death. We investigated the Glu298Asp polymorphism in endothelial nitric oxide synthase in relation to CHF risk.

Contradictory results have been obtained in other studies on a possible correlation between the eNOS Glu298Asp polymorphism and heart disease (Hibi et al., 1998; Miyamoto et al., 1998; Kato et al., 1999; Nassar et al., 2001; Colombo et al., 2003; Shaw et al., 2005; van Beynum et al., 2008; Velloso et al., 2010; Gamil et al., 2017; Khatami et al., 2017; Shahid and Rehman, 2017). This may depend on group selection, due to variable distribution of the polymorphism in different ethnic groups, and small sample size (Chen et al., 2001; Rosas-Vargas et al., 2003; Sandrim et al., 2006). The Glu allele has been reported to have different allelic frequencies: 74% in the USA (Fatini et al., 2004), 99% in Japan (Kato et al., 1999), 99% in Korea (Park et al., 2004), 88% in Mexico (Stephens et al., 1996), 85% in Chile (Jaramillo et al., 2006), 83% in Turkey (Cam et al., 2005) and 67% in Brazil (Velloso et al., 2010). We therefore evaluated the role of this polymorphism in CHF in a Russian population. To the best of our knowledge, this is the first study performed on a Siberian population. The result of this preliminary study conducted on a relatively small cohort showed relatively low Glu298 allele frequencies of 65 and 71.5% in our control group and patient cohort, respectively.

The rs1799983 *NOS3* polymorphism was not found to be associated with CHF in our population. No significant differences were found when we compared clinical characteristics (i.e. wall ischemia, palpitations, arrhythmia, myocarditis, cardiomyopathy, hypertensive disease, valve disease, post-infarction cardiosclerosis) in groups with different

genotypes. Because of gender differences in NO production (Forte et al., 1998; Tsang et al., 2001), the allele and genotype frequencies of the polymorphism were analyzed separately in men and women. No differences were found (data not shown).

In our study, we observed Hardy-Weinberg disequilibrium in rs1799983 in controls. This has also been described by others (Tang et al., 2008; Serrano et al., 2010; Li et al., 2011). The Hardy-Weinberg model requires several assumptions, such as unlimited population size and that natural selection do not act on the alleles under consideration. However, in a previous study on this polymorphism, it was suggested that Hardy-Weinberg disequilibrium was caused by selection pressure (Dhangadamajhi et al., 2009).

In conclusion, our results show that genetic testing of polymorphism rs1799983 cannot be used to assess risk for CHF in routine screening and did not provide useful clinical information for disease prognosis in our Russian population. Limitations of our study include the small size of the patient and control groups. Larger cohorts of controls and CHF patients are needed to confirm that eNOS Glu298Asp does not play a role in CHF in the Siberian population.

CONFLICTS OF INTEREST

The authors declare that they do not have any conflicts of interest.

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