

Association of Endothelial Nitric Oxide Synthase (*eNOS*) gene polymorphism (Glu 298 Asp) with coronary artery disease in subjects from Multan, Pakistan

Asia Taqddus¹, Abu Bakar Ali Saad², Burhan Pasha³, Muhammad Latif¹, Sohail Safdar⁴, Rehan Sadiq Shaikh⁵, Muhammad Ali⁵ and Furhan Iqbal^{1*}

¹Institute of Pure and Applied Biology, Zoology Division, Bahauddin Zakariya University, Multan, Pakistan

²Chaudry Pervaiz Ellahi Institute of Cardiology, Multan, Pakistan

³Cardiology Ward, Bakhtawar Amin Memorial Hospital, Multan, Pakistan

⁴PMRC Research Center, Nishtar Medical College, Multan, Pakistan

⁵Institute of Biotechnology, Bahauddin Zakariya University, Multan, Pakistan

Abstract: Acute Coronary Syndrome (ACS) is the most common disease and cause of mortality in both genders across the world and certain risk factors i.e. age, gender, smoking, diabetes, hypertension, drugs usage, weight etc are known to be associated with the disease. The aim of this study was to find if there is any correlation exists between ACS and hereditary genetic defect in endothelial nitric oxide synthase (*ecNOS*) gene as *eNOS* generates Nitric oxide in blood vessels and regulates the vascular tone hence directly affecting the cardiovascular function. Single nucleotide polymorphism (SNP) (Glu 298 Asp) in *ecNOS* was determined in 280 subjects, from Southern Punjab (in Pakistan) population, including (160 ACS patients and 120 healthy controls) by PCR-RFLP method and genotype was correlated with various risk factors as well as with serum cholesterol and triglyceride levels. Our results indicated that the genotype Glu 298 Asp was not associated with ACS but when various studied parameters were compared among patients suffering from various forms of ACS and their healthy controls, it was observed that age (45-55 years) ($P = 0.05$), gender (male) ($P < 0.001$), education ($P < 0.001$), family history ($P = 0.03$), hypertension ($P < 0.001$), diabetes ($P < 0.01$) and smoking habit ($P = 0.03$) were the significantly different parameters among them and may be associated with the incidence of cardiovascular disease. Cholesterol (161.5 ± 79 mg/dL) level was found to be higher in patients ($P = 0.04$) than controls while triglyceride remained unaffected ($P = 0.87$) in both groups.

Keywords: Endothelial nitric oxide synthase, coronary artery disease, SNP, Glu 298 Asp, Risk factors.

INTRODUCTION

Atherosclerosis leads to acute coronary syndromes (ACS) which is complex pathological condition including angina (chest pain), myocardial ischemia (heart attack) and stroke (Pillariseti and Saxena, 2008). Rapture of plaque and thrombosis with or without distal embolization are among the most common pathophysiological features of ACS (Gotlieb, 2005). One of the major causes of death worldwide is the cardiovascular disease and myocardial infarction is one of its most dangerous complications (Jilani *et al.*, 2009). This disease accounts for 25% of the total deaths in developed countries (Yarmohammadian *et al.*, 2009). Several studies have investigated the pattern of coronary heart disease in South Asians and recent reviews have concluded that they are at increased risk (McKeigue, 1989; Nishtar, 2002; Samad, 2003). Pakistani population is more prone to ACS and at an earlier age as compared to population residing in the technologically advanced countries (Balarajan, 1991; Memon and Samad, 1999). There are number of risk factors that are reported to be associated with ACS. On the basis of whether we can alter these risk factors or not, they are classified as modifiable

(high blood pressure, smoking, obesity, abnormal lipids and high blood sugar) and unmodifiable (age, gender and genetics) (Deviprasad *et al.*, 2009).

The constitutive endothelial nitric oxide synthase (*eNOS*) is encoded by 26-exons located on chromosome 7q35 to 36; with a total size of 21kb (Janssens *et al.*, 1992) and encodes a mRNA of 4052 nucleotides (Marsden *et al.*, 1993). Nitric oxide (NO) protein is a small molecule having molecular weight of 30 Daltons (Palmer *et al.*, 1988) and is an important endothelium-derived relaxing factor (EDRF) that is synthesized from L-arginine and molecular oxygen by these three isoforms of the NO synthase (*eNOS*, *iNOS*, *nNOS*) (Moncada and Higgs, 1993). The constitutive endothelial NO synthase (*eNOS*) has been reported in platelets (Sase and Michel, 1995), vascular endothelium (Lamas *et al.*, 1992; Marsden *et al.*, 1993) and in all those cell types in which a continuous modest produce of nitric oxide (NO) is in progress (Loscalzo and Welch, 1995). NOS is reported to be expressed in macula densa of kidney as well as in the central and peripheral nervous systems (Shibuki and Okada, 1991; Kihara *et al.*, 1997). Certain cytokines and endo toxin, like lipopolysaccharides, are capable of inducing NOS in vessel walls and macrophages under

*Corresponding author: e-mail: furhan.iqbal@bzu.edu.pk

pathological conditions (Moncada, 1992). The aim of this study was to find out if there is any correlation existing between ACS and hereditary genetic defect in endothelial nitric oxide synthase (*ecNOS*) gene at (Glu 298 Asp), risk factors and serum lipid profile of the subjects.

MATERIAL AND METHODS

Study design

160 blood samples of patients suffering from acute coronary artery syndrome (ACS) were collected from two local hospitals in Multan. Among 160 ACS patients, 132 were suffering from acute myocardial infarction, 20 had unstable angina and remaining 8 were clinically diagnosed as stable angina patients. Age matched 120 control blood samples were randomly collected from different areas of Multan city including Bahauddin Zakariya University Multan. The enrolled subject hailed from different cities of Southern Punjab and had different ethnic origins, gender and age ranges. Informed written consent was obtained from all the enrolled subjects and data (gender, age, family history of ACS, diabetes, hypertension, smoking habit, alcohol consumption, body weight and drugs addiction) from each subject was collected through a questionnaire. All the experimental protocol and procedures were approved by ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan.

DNA extraction

Blood samples (3-5 ml) from each subject was preserved by adding 400-500 μ l of EDTA and stored at -4°C . DNA extraction was carried out by inorganic method following Shaikh *et al.* (2005). Briefly, samples were lysed with lysis buffer (Tris EDTA buffer) and centrifuged to washout red blood corpuscles. TE buffer was used to washout proteins. TNE buffer. SDS and proteinase K was used for the breakdown and separation of blood proteins following an overnight incubation at 37°C concentrated NaCl and isopropanol were used for protein precipitation and purification. DNA pallet was washed in ethanol and dissolved in TE buffer for further use (Shaikh *et al.*, 2005).

PCR amplification of G 894 T SNP (Codon 298)

Genotype of *ecNOS* at codon 298 was determined by polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) technique following Yamamoto *et al.* (2007). Glu 298 Asp was amplified in a final reaction volume of 50 μ l. PCR reaction mixture contained 1X buffer S (160mM $(\text{NH}_4)_2 \text{SO}_4$, 500 mM Triss HCL, 17.5mM MgCl_2 , 0.1% TritonTM X 100), 250 ng genomic DNA, 0.2mM of dNTPs, 3 U Taq DNA polymerase (Vivantus, UK), 6mM Magnesium chloride (MgCl_2) and 40pM of each primer, *ecNOS*-F 5'-TCCCTGAGGAGGGCATGAGGCT-3', *ecNOS*-R 5'-TGAGGGTCACACAGGTTTCCT-3'.

DNA amplification was carried out in a DNA thermal cycler (Applied Bio systems, UK). The thermal profile consist of an initial denaturation carried out at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1 min and elongation at 72°C for 1 min and final extension was carried out at 72°C for 10 min.

Restriction analysis of G 894 T SNP

Amplified PCR products were digested with *FriO1* (Vivantis, UK) restriction enzyme at 37°C for 12-16 hours in a final reaction volume of 20 μ l consists of 15 μ l amplified PCR product, 2 μ l of 10X buffer V5 and 0.5 μ l *FriO1* (Vivantis, UK). Restriction products were electrophoresed on 3% agarose gel.

Measurement of serological parameters

For the separation of serum from the blood cells, 800 μ l of blood samples were centrifuged at 13000 rpm for 10 minutes in 1.5/ml Eppendorf tubes Serological determination of cholesterol and triglyceride was carried out following the standard procedure provided with the diagnostic kits (Merck, Germany).

STATISTICAL ANALYSIS

Statistical package Mini Tab (version 13) was used for statistical analysis of the data. Correlation between acute coronary artery syndrome and all the risk factors (Gender, age, cast, education, marital status, family history, diabetes, hypertension, smoking, alcohol, drug consumption and body weight) associated with the disease were drawn by using binary logistic regression. Chi square test was conducted to calculate the frequency of each risk factor in control and ACS patients. 2-Sample t-test was carried out to compare the triglyceride and cholesterol levels between control and ACS patients.

RESULTS

The oligonucleotide primers, used in this study, amplified a 457 bp fragment of *ecNOS* containing codon 298. When the amplified product was digested with *FriO1* restriction enzyme, a single DNA fragment of 457 bp was produced and observed on agarose gel in all the subjects as they all were having wild genotype (Glu/Glu) for this codon (fig. 1).

We have correlated various risk factors with both phenotypes (healthy and diseased) by applying chi-square test. Age was divided into four different categories and when compared between the control and patient groups and our results indicated that age has a significant correlation ($P = 0.05$) with the incidence of coronary disease. It was observed that majority of patients (33%) were within the age range of 45-55 years, while the minimum number of enrolled subjects were present

within the age range of 65-75 years highlighting a relationship of age with the incidence of coronary diseases (table 1). Association studies of gender with the phenotype of enrolled subjects revealed that gender is highly significantly associated ($P < 0.001$) with the disease as 75% of the diseased subjects were male. Education level had a highly significant correlation ($P < 0.001$) with coronary artery disease as most of the patients were uneducated. Our results revealed that there is a significant association ($P = 0.03$) between ACS and marital status as 92% of the diseased subjects were married. Family history ($P = 0.03$) and Diabetes ($P = 0.03$) were also significantly correlated with ACS (table 1).

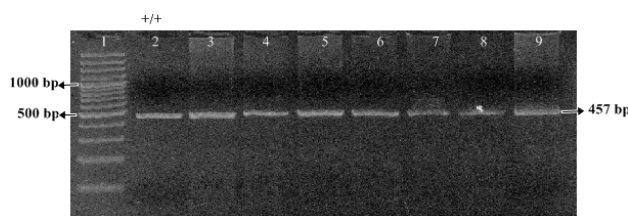


Fig. 1: Agarose gel electrophoresis of restriction digestion products for *ecNOS* codon 298. Lane 1, 100 bp DNA ladder. Lane 2-9, restricted PCR products of ACS patients and controls.

Serum parameters, cholesterol and triglyceride were determined in both patients and control, we found high cholesterol level among ACS patients ($P = 0.048$) as compared to healthy subjects while triglyceride levels remained unaffected (Table 2).

DISCUSSION

NO plays a major role in the regulation of vascular tone as it is among the most powerful endogenous vasodilators (Moncada *et al.*, 1991). EDRF also acts on diverse processes involved in the pathogenesis of atherosclerosis and thrombosis (Besler *et al.*, 2011). Nitric oxide are also known for their inhibitory effect on platelet aggregation and adhesion to the vascular endothelium (Ho *et al.*, 2009; Radomski *et al.*, 1987) and EDRF and exogenous NO also cause platelet disaggregation (Radomskii *et al.*, 1987). Nitric oxide may act on the blood coagulation system through the regulation of the expression of heparin sulfate by endothelial cells (Irokawa *et al.*, 1994). NO also inhibits a variety of functions, such as chemotaxis and the synthesis and release of superoxide radical, in polymorphonuclear leukocytes (Marcucci *et al.*, 2009). Furthermore, NO plays a fundamental role in the control of vascular homeostasis by inhibiting the adhesion of polymorphonuclear cells with the vascular endothelium (Gaboury *et al.*, 1993). All the above mentioned actions are due to granulate cyclase activity resulting in increased concentration of cyclic GMP in target cells (Waldma and Murad, 1988). Failure of this mechanism may promote atherogenesis as it exposes the arterial wall to vasospasm inducing factors and hence increases the risk of

thrombosis which results in acute myocardial infarction (AMI) (Hibi *et al.*, 1998). Endo toxic shocks and intensified inflammatory responses has been reported following a marked increase in NO which may lead to acute hepatic dysfunction and do have potential to cause asthma and cardiomyopathy in patients (Kone, 2002; Yoshimura *et al.*, 1998).

In the present case control study, we have analyzed the genotypic combination at codon 298 of *ecNOS* gene in enrolled subjects and analyzed its correlation with ACS. Genetic analysis at codon 298 of *ecNOS* gene revealed that both patient and control groups had only Glu/Glu (wild) genotype while Glu/Asp (heterozygous) and Asp/Asp (homozygous mutant) genotypes were absent in subject under study, indicating that this SNP is not associated with ACS. Our results are contradictory to Salimi *et al.* (2010) who had reported that genotypic frequencies of Glu/Glu (wild), Glu/Asp (heterozygous) and Asp/Asp (homozygous mutant) were significantly different between individuals with and without ACS and the frequencies of Glu/Asp and Asp/Asp genotypes were significantly higher in patients indicating their association with the disease. For mutant genotype (Asp/Asp) our results are in agreement with Nishevitha *et al.* (2009) who had reported that the homozygous mutant (Asp/Asp) were completely absent among the studied South Indian population. In a previous study, Wang *et al.* (2001) had found no evidence of an association between the Glu 298 Asp variant of the *eNOS* gene and ACS in Taiwanese population. Our findings are in agreement with them and those results indicated that same genotypic combinations in *ecNOS* at codon 298 may have different phenotypic expression in different populations.

The genetic risk factors play an important role in the pathogenesis of coronary atherosclerosis (Umbach and Weinberg, 1997). We have correlated various risk factors with both phenotypes (healthy and diseased) by applying chi-square test. It was observed that majority of patients (33%) were within the age range of 45-55 years highlighting a relationship of age with the incidence of coronary diseases (table 1). Association studies of gender with the phenotype of enrolled subjects revealed that gender is highly significantly associated ($P < 0.00$) with the disease as 75% of the diseased subjects were male. One of the justifications for this finding could be that males, in our society, are more exposed to environmental pollution, smoking and stress which are the major risk factor for heart diseases. Our results are in agreement with Rosengern *et al.* (2004) who had reported high prevalence of ACS among males as compared to females at younger age while in older age both males and females had equal incidence of ACS. This clear trend of ACS towards male is also due to the difference in estrogen levels between the two genders. As it has been an established fact that high estrogen levels, in case of premenopausal females,

Table 1: Analysis of the risk factors associated with acute coronary artery syndrome. P value indicates the results of chi-square test when each parameter was compared between control and patients. N indicates the number of samples in each treatment.

Risk Factors	Category	Control N = 120	ACS Patients N = 160	P-value
Age	35-45	20 (33%)	22 (27.5%)	0.05*
	45-55	16 (26%)	26 (32.5%)	
	55-65	24 (40%)	24 (30%)	
	65-75	0	8 (10%)	
Gender	Male	60 (100%)	60 (75%)	0.000***
	Female	0	20 (25%)	
Marital status	Married	60 (100%)	74 (92.5)	0.03*
	Unmarried	0	6 (7.5%)	
Cast	Arain	10 (16.6%)	16 (20%)	0.752 ^{ns}
	Jutt	12 (20%)	18 (22.5%)	
	Rajpoot	10 (16.6%)	16 (20%)	
	Others	28 (46.6%)	30 (37.5%)	
Education	Nil	16 (26.6%)	54 (67.5%)	0.000***
	Upto matric	24 (40%)	16 (20%)	
	Upto graduation	14 (23.4%)	8 (10%)	
	Above garduation	6 (10%)	2 (2.5%)	
Family history	Yes	10 (16.6%)	26 (32.5%)	0.034*
	No	50 (83.4%)	54 (67.5%)	
Diabetes	Yes	10 (16.6%)	28 (35%)	0.016*
	No	50 (83.4%)	52 (65%)	
Hypertension	Yes	11 (18.4%)	44 (55%)	0.000***
	No	49 (81.6)	36 (45%)	
Smoking	Yes	18 (30%)	38 (47.5%)	0.036*
	No	42 (70%)	42 (52.5%)	
Alcohol*	Yes	-	-	-
	No	-	-	-
Drugs*	Yes	-	-	-
	No	-	-	-
Weight	40-50	6 (10%)	8 (10%)	0.066 ^{ns}
	50-60	16 (26.6%)	38 (47.5%)	
	60-70	16 (26.6%)	20 (25%)	
	70-80	10 (16.6%)	6 (7.5%)	
	80-above	12 (20%)	8 (10%)	

P < 0.05* =Least Significant P < 0.01** =Significant P < 0.001*** = Highly Significant P > 0.05 =Non Significant (ns)

*P-Value was not calculated because of insufficient data regarding the use of alcohol and drugs.

Table 2: A comparison of serum biochemical parameters of control and patients of acute coronary syndrome, Data is expressed as Mean ± Standard Deviation (SD). P-value indicates the results of 2-sample t-test. N indicates the number of samples in each treatment.

Parameters	Control (N = 100)	ACS Patients (N = 100)	P - value
	Mean ± SD	Mean ± SD	
Cholesterol (mg/dL)	142.1 ± 54	161.5 ± 79	0.048*
Triglyceride(mg/dL)	123 ± 48.6	124.2 ± 54.6	0.87 ^{ns}

P < 0.05* =Least Significant P < 0.01** =Significant P < 0.001*** = Highly Significant P > 0.05 =Non Significant (ns)

decreases the incidence of cardiovascular diseases (Mosca, 2000).

Education level had a highly significant correlation (P < 0.001) with coronary artery disease as most of the patients

were uneducated. This can be linked to the lack of awareness of using less cholesterol containing diet and to do regular exercise to change life style in order to avoid the disease in uneducated or less educated subjects. Our results revealed that there is a significant association (P

=0.03) between ACS and marital status as 92% of the diseased subjects were married. Similar observations were reported by Koskenvuo *et al.* (1981) who had reported an association between heart diseases and married men and women and had predicted an association between marital problems and ACS.

In our enrolled subjects, 67% of the patients do not had any family history of heart disease but still a moderate association ($P = 0.03$) was existing between ACS and family history, confirming that the inheritance of the disease do exist. Rich *et al.* (2004) had reported the similar results as they indicated significant correlation between heart disease and family history.

While studying the diabetes in the patient groups, it was found that diabetes had a significant association ($P = 0.03$) with heart disease as 35% of the patients were diabetic. Our result are in agreement with Marso *et al.* (2006) as they reported a measurable difference in mortality for diabetic patients compared with non diabetic patients who were suffering from acute coronary disease. Our results indicated that out of 160 patients, 88 were suffering from high blood pressure and ACS was highly significantly correlated ($P < 0.001$) with hypertension. Our results are in agreement with Dessap *et al.* (2008) who had reported that hypertension is significant risk factor associated with prognosis of acute coronary disease. They observed that during severe acute chest syndrome, pulmonary pressures increase and it is associated with the elevation of cardiac biomarker threatens the life of the patient. Any amount of smoking, even light smoking or occasional smoking, damages the heart and blood vessels. Smoking increases the risk of atherosclerosis and limits the flow of oxygen-rich blood to organs in body affecting their normal physiology. We observed that smoking also enhances ($P = 0.03$) the risk of coronary artery disease significantly and a number of the patients were smokers. Similar observations were reported by Wang *et al.* (1996) who had found a strong relationship between smoking and coronary artery syndrome. Role of alcohol and drugs cannot be established as diseased individuals did not admitted if they are addicted or not; an expected outcome in our society, where use of alcohol and drugs are socially, religiously and legally prohibited (table 1).

Serum parameters, cholesterol and triglyceride were determined in both patients and control, we found high cholesterol level among ACS patients ($P = 0.048$) as compared to healthy subjects. Our results are in agreement with Kumar *et al.*, (2011) who had also predicted higher cholesterol level in ACS patients (Table 2). Triglyceride level was also studied among all the subjects but level of triglyceride was lower in patients as compared to control. In case of triglyceride our results are

contradictory to Kumar *et al.* (48) who predicted high triglyceride level in ACS patients.

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