ISSN 0975-6299 Vol.1/Issue-4/Oct-Dec.2010

International Journal of Pharma and Bio Sciences

THE ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE (ENOS) GENE GLU298ASP POLYMORPHISM IN POSTMENOPAUSAL CORONARY ARTERY DISEASE RISK- A PILOT STUDY IN NORTH INDIA

AUTHORS: PRADEEP K. DABLA[1*], SHUBHA S. TRIVEDI[2], NIBHRITI DAS[3] AND JAYASHREE BHATTACHARJEE[1]

[1]Department of Biochemistry, Lady Hardinge Medical College, New Delhi-110001, India,
[2]Department of Obstetrics & Gynecology, Lady Hardinge Medical College, New Delhi-110001, India and
[3]Department of Biochemistry, All India Institute of Medical Sciences, New Delhi-110029, India

Corresponding Author pradeep.dabla@gmail.com

ABSTRACT

Background
This study evaluates the endothelial dysfunction in postmenopausal women in relation to eNOS Glu298Asp gene polymorphism and its effect on other proatherosclerotic markers to increase CAD risk.
Methods
Fifty postmenopausal women were compared with fifty premenopausal women in 45-55 years of age group. We studied eNOS Glu298Asp gene polymorphism and estimated different cardiac risk parameters: nitric oxide, malondialdehyde, platelet factor-4, estriol and lipids.
Results
The postmenopausal women had significantly low estriol and low NO levels whereas triglyceride levels were high ($p<0.001$) compared to premenopausal women. The NO level found to be significantly lower (<0.05) whereas PF4 and MDA have shown significant higher ($p<0.001$) levels in GT genotype.
Conclusion
These observations suggest that eNOS Glu298Asp gene polymorphism and thus, low NO levels may increase the association of future CAD risk in postmenopausal women. However, further studies and follow up of the patients are required to improve the therapeutic strategies.

KEYWORDS

Postmenopause, eNOS gene polymorphism, Coronary Artery Disease, Nitric Oxide, Platelet Factor-4, Malondialdehyde

INTRODUCTION

Heart disease in women is the third leading cause of death in India with prognosis in women is worse than in men. India topped the world with 1531534 CVD related deaths in 2002[1]. Menopausal transition causes a decrease in estrogen and a fluctuating rise in serum follicle stimulating hormone (FSH) levels[2], which plays a role in shift to an atherogenic lipid and lipoprotein profile[3].

Genetic variation in endothelial cells is critical to atherosclerotic process. The specific genetic predisposition can make further advancement as it is modified throughout life by environmental influences[4]. One of the most important products of endothelial cells is Nitric Oxide (NO). It acts as a critical factor in the pathophysiology of the vascular system through its various actions such as preventing the oxidation of lipoproteins, down-regulating inflammatory mediators and controlling the expression of proteins involved in atherogenesis. It is synthesized from L-arginine through catalytic activity of endothelial Nitric Oxide Synthase (eNOS or NOS 3: chromosome 7q)[5]. Therefore, eNOS is a potential candidate gene for atherosclerosis. Different polymorphisms in eNOS gene exists and among them one results from guanine to thymine substitution at nucleotide 1917 in exon 7 of eNOS gene causing an amino acid substitution (glutamate to aspartate) at the codon 298. So far, it is only single exonic polymorphism identified, whereas the rest of the polymorphism to date is intronic[6].

Vascular endothelium modulates platelet activity by exerting a protective antithrombotic effect through the production of NO and prostacyclin (PGI_2)[7]. Platelet factor-4 (PF4) released from α-granules of platelets can serve as a useful marker for platelets in serum or plasma because of its platelet specificity due to synthesis in megakaryocytes. It binds to heparin and neutralizes its anticoagulant activity and promotes platelet aggregation and inhibition of contact activation [8]. Lipid peroxidation occurs in response to elevated levels of reactive oxygen species (ROS) with the release of reactive aldehydes, such as malondialdehyde (MDA), which is used as a biomarker to measure the level of oxidative stress in an organism. It forms covalent protein adducts which are referred to as advanced lipoxidation end-products[9].

No single risk factor can be conclusively responsible for entire process of CAD, but a multitude of causes might be responsible. Hence, there is need to understand the various risk factors of CAD especially in postmenopausal women for prevention and early intervention. However, no data is available for eNOS Glu298Asp gene polymorphism in postmenopausal women and its effect on estrogen, PF4, MDA and lipids in Indian women.

METHODS

Study population

The study group consisted of 100 women with informed consent. They were selected from gynaecology clinics and wards of a tertiary care hospital in India. The women were divided into study group consisting of 50 postmenopausal women and control group consisting of 50 premenopausal women in age group of 40-55 years. The reproductive history questionnaire was taken and serum FSH levels were measured to establish postmenopausal status. The study was approved by the Institutional Ethical Committee.

Selection Criteria

Inclusion criteria were

1. Women in age group of 40-55 years with either natural or surgical menopause (hysterectomy with bilateral salpingo-oopherectomy) as per confirmation by treating gynecologist.
2. Premenopause in age group of 40-55 years without any major gynecology complaints (tumors, dysfunctional uterine bleeding).

3. FSH levels ≥ 40 mIU/ml.

Exclusion criteria were

1. Women diagnosed with any malignancy.
2. Women who have had HRT (hormone replacement therapy).
3. Women with diagnosed CVD.

Sample collection and processing: The history was taken and clinical examination was carried out before sample collection. Abdominal Ultrasound for pelvic region was carried out in all women at the time of enrolment to rule out any organic illness.
Venous blood was collected under sterile conditions after overnight fasting. Seven milliliters of blood was collected in sterile screw capped vials containing 5% anticoagulant (1 unit of 5% EDTA for each 9 units of blood). Platelet rich plasma (PRP) was prepared by centrifugation of collected blood at 1000 rpm for 15min at 37°C for NO, estriol and MDA estimation and aliquoted. Remaining PRP was centrifuged at 3000 rpm for 15 min at 37°C to get platelet poor plasma (PPP) for measuring PF4 levels. The plasma obtained was transferred to sterile screw capped vials and stored at -20°C till batch analyzed. The remaining cell aggregate was transferred to polyethylene container and stored at -20°C till analyzed for PCR and RFLP.

Nitric Oxide estimation: Nitric oxide was estimated in platelet rich plasma by modified Griess reaction[10]. Griess reaction involves the formation of a chromophore during the reaction of nitrite (NO_2^-) with sulfanilamide and heterocyclic amine of N (1-napthyl) ethylenediamine (Griess reagent) under conditions of low pH. In an oxygenated solution, NO decomposes to form nitrite (NO_2^-) and nitrate (NO_3^-).

Determination of Estriol: Plasma estriol level was measured by competitive EIA (enzymatic immunoassay)[11], which is based on competitive interaction of estriol and the hormone-enzyme conjugate for a limited number of immobilized anti estriol antibodies (rabbit). After incubation, the bound/total estriol separation was performed by solid phase washing. When substrate solution is added, a blue color develops changing to yellow after stopping the reaction. The intensity of color is inversely proportional to the amount of estriol in the specimen.

Estimation of Platelet Factor-4: It is based on the principle of sandwich ELISA in which the plasma PF4 to be measured reacts with specific antihuman PF4 antibodies coated on the wells[12]. A rabbit anti PF4 antibody coupled with peroxidase reacts with another epitope of the PF4. Upon addition of substrate OPD (orthophenylenediamine) in the presence of H_2O_2, the intensity of color reaction bears a direct relationship with the PF4 concentration.

Malondialdehyde estimation: Plasma MDA was measured using Thiobarbituric acid (TBA). TBA reacts with MDA at low pH to produce a pink chromogen with absorption maximum at 530—535 nm[13].

Estimation of Serum Cholesterol and Triglyceride: These chemistries were carried out in automated Beckman CX9 autoanalyzer using standard reagent kits, calibrators and controls from Randox (UK).

Identification of genotype and genotyping: Genomic DNA isolation was done from stored cell aggregate. RBC's were lysed using RBC lysis buffer (Tris, MgCl2, NaCl, pH-7.6) and DNA extraction was done by salting out method[14]. The DNA was amplified for eNOS gene polymorphism by PCR using flanking intron primers 5'-TCC CTG AGG AGG GCA TGA GGCT (sense) and 5'-TGA GGG TCA CAC AGG TTC CT (antisense), which was later screened for RFLP using BanII restriction endonuclease digestion for 20hrs at 37° C[15]. The restricted fragments were resolved on 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis Statistical analysis was carried out using SPSS for windows 12.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed in Mean ± Standard Error of Mean. The difference between groups was compared by independent sample t test or Mann- Whitney test for continuous variables. Spearman's rank correlation was applied to test for association between continuous variables. A two-tailed p value<0.05 was considered statistically significant. Endothelial NOS gene G894T polymorphism genotypes (GG, GT, TT) and alleles (G, T) frequency was analyzed by using Fisher's exact and chi-square tests.

Mean age in the study group was 51.76±0.697 years and among the controls was 45.60±0.622 years. The mean systolic blood pressure was 132.61±2.77 mm Hg among the cases and diastolic blood pressure was 78.10±1.15mm Hg. Among the controls mean systolic blood pressure was 130.70±2.65mm Hg and diastolic blood pressure was 78.30±1.55mm Hg.
The observed levels of NO and estriol were significantly lower (p<0.001) whereas triglyceride level found to be higher (p<0.001) in postmenopausal women compared to premenopausal women. The MDA, PF4 and cholesterol levels difference was insignificant in both groups (Table 1).

RESULTS

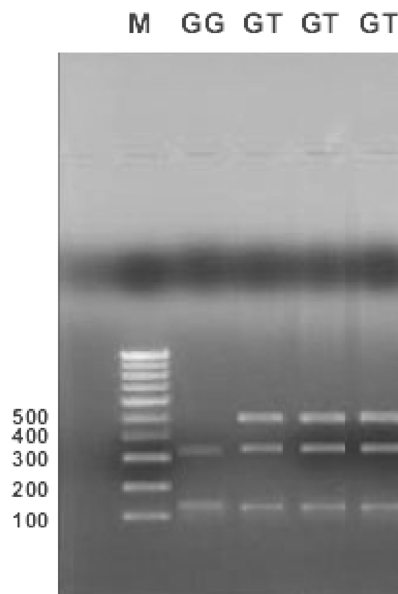
Table1

Plasma Estriol, Nitric Oxide, Platelet Factor-4, Malondialdehyde and serum lipids levels in postmenopausal and premenopausal women

Parameters	Postmenopausal women (n=50) (mean±S.E.M)	Premenopausal women (n=50) (mean±S.E.M)	p value
Estriol (ng/ml)	2.56±0.14	5.58±0.27	<0.001
Nitric Oxide (µmol/L)	14.90±1.08	24.91±1.75	<0.001
PF4 (IU/ml)	10.31±0.64	9.49±0.44	>0.05
MDA (µmol/L)	1.01±0.08	0.93±0.12	>0.05
Cholesterol (mg/dl)	162.86±5.57	160.74±4.56	>0.05
Triglyceride (mg/dl)	173.32±16.35	111.68±6.60	<0.001

A total of 100 participants were genotyped for Glu298Asp polymorphism (Dabla.Fig1). GG genotype was found in 41 subjects (82%) of study group and 45 subjects (90%) of control group whereas, 9 subjects (18%) of study group and 5 subjects (10%) of control group have shown GT genotype. No TT was found in any group. The genotype frequencies were as predicted by Hardy-Weinberg equilibrium (chi-square=1.329, df=1, P=0.05, Not Significant). Study group has shown allelic frequencies of G and T allele as 0.91 and 0.09 respectively. The frequency of G and T alleles in control group was 0.95 and 0.05 (Table 2).

Fig1



Ethidium bromide-stained agarose gel used for genotyping PCR products(457 bp) with primers flanking Glu298 Asp polymorphic region of e NOS gene digested with Ban II. Homozygotes with G at with position (G/G) showed 2 bandsat 320 bp and 137 bp. Heterozygotes for this mutation (G/T) showed 3 bands at 457 bp, 320bp, and 137 bp. M= molecular wt. marker (Promega USA).

Table 2

Distribution of genotypes and alleles of eNOS Glu298Asp polymorphism in postmenopausal and premenopausal women:

Genotype	Postmenopausal women (n=50)		Premenopausal women (n=50)		Level of Significance
	n	Frequency (%)	N	Frequency (%)	
GG	41	82	45	90	
GT	9	18	5	10	X^2 =1.329 df=1, p>0.05
TT	0	0	0	0	
Alleles					
G		0.91		0.95	
T		0.09		0.05	

The mean plasma NO level was significantly lower (p<0.05) whereas significant higher mean plasma PF4 and MDA levels (p<0.001) were observed in GT genotype compared to GG genotype. The difference between estriol levels was found to be statistically insignificant (p>0.05). The levels of

cholesterol and triglyceride were higher in GT genotype as compared to the GG genotype but the difference was statistically not significant ($p>0.05$) (Table 3).

Table 3
Level of plasma Estriol, Nitric Oxide, Platelet Factor-4, Malondialdehyde and serum lipids in eNOS gene Glu298Asp polymorphism genotypes

Parameters	GG genotype (n=86) (mean±S.E.M)	GT genotype (n=14) (mean±S.E.M)	p value
Estriol (ng/ml)	3.97+1.79	4.47±1.70	>0.05
Nitric Oxide (μmol/L)	22.47±13.92	15.18±7.54	<0.05
PF4 (IU/ml)	9.09±3.83	14.42±2.68	<0.001
MDA (μmol/L)	0.85±0.70	1.72±0.56	<0.001
Cholesterol (mg/dl)	163.07±33.89	154.00±46.90	>0.05
Triglyceride (mg/dl)	140.63±95.57	136.29±59.79	>0.05

DISCUSSION

In the present study, we aimed to focus on possible synergistic effect of eNOS Glu298Asp gene polymorphism on estrogen, platelet function and lipid peroxidation with respect to endothelial dysfunction and CAD risk in postmenopausal women. We found significant decrease in nitric oxide levels with increase in platelet factor-4 and malondialdehyde levels in eNOS Glu298Asp gene polymorphism GT genotype compared to GG genotype.

The eNOS is an important enzyme that regulates NO production in endothelial cells causing vasodilatation. In our study, the observed level of NO was found significantly lower in postmenopausal women and in GT genotype which correlates with inverse relationship of NO with eNOS Glu298Asp gene polymorhism. Previous studies has verified that impaired arterial endothelium dilatation to a flow stimulus and vascular dysfunction in postmenopausal women is due to defect in NO synthesis at the level of endothelial cell[16]. Further, Tempfer et al[17] showed that the female reproductive tract demonstrates a menstrual cycle specific and age specific pattern of eNOS expression. Studies have suggested the association of late menarche, reduced ovulation rates, fewer delivery rate and early onset of menopause with deficiency of gene expression encoding eNOS in mouse model[18]. Although, the study is female gender biased, the allelic frequencies of G and T allele were 0.91% and 0.09% in postmenopausal women as compared to 0.95% and 0.05% in premenopausal women. However, Singh et al[19] in our department demonstrated the frequency of G and T allele as 0.97% and 0.03% in pre-eclampsia patients compared to 0.98% and 0.02% in normotensive women. So, further follow-up studies are requiring at large scale.

Platelet function is regulated by platelet-derived NO which prevents endothelial dysfunction by increasing P-selectin expression, thus inhibits platelet activation and thrombus formation[20]. The plasma PF4 level was higher in GT

genotype which is supported by the observation that, elevated eNOS expression in endothelial cells reduces platelet and endothelial activation both in vitro and vivo. Also, downregulation of eNOS enzyme in endothelial cells enhances platelet aggregation as a result of an activation of glucorticoid receptors[21]. Lipid peroxidation and reactive aldehydes are associated with aging and ischemia[22]. We found higher MDA levels in GT genotype which correlates with the finding of Imamura et al[23] who showed the association of Glu298Asp polymorphism with atherogenic lipid levels including significantly higher plasma malondialdehyde-modified LDL (MDA-LDL). In another study, Nosratola et al[24] have shown that the lead-treated hypertensive patients exhibited a rise in blood pressure and plasma MDA concentration with fall in urinary NO metabolites excretion, and a paradoxical rise in vascular and renal tissue eNOS and iNOS expression. We observed higher triglyceride level in postmenopausal women compared to premenopausal women. Similarly, Ikeneove et al[25] has reported significantly higher values of triglyceride and apo-B in postmenopausal women. Our results comply with the finding of Berg G et al[26] who reported higher level of triglyceride, IDL-cholesterol and Lp(a) in postmenopausal women in comparison with premenopausal women. Menopause has an unfavorable effect on lipid profile especially triglyceride levels which may contribute to increase risk of CAD due to impaired nitric oxide synthesis and function[27].

To our knowledge, this is the first study of its own kind in which correlative observations were made to explore the synergistic effect of eNOS Glu298Asp gene polymorphism on various endothelial functions determinants with respect to postmenopausal women.

However, the shortcoming of the present study is that, it was a short hospital based case-control study. Due to cost and time constraints, follow-up of the patients could not be done, which could have provided more mechanistic evaluation of correlations and observations over the period.

CONCLUSIONS

Thus, authors conclude that although natural menopause does not cause an immediate increase in risk of heart disease, it does signal a period of increasing risk that is related to both estrogen deficiency and low NO levels. In addition to established risk factors, the reduced bioavailability of NO may result from down regulated protein expression, depressed activation or reduced enzymatic function of eNOS and the postmenopausal status is one of the causes. Thus, eNOS gene Glu298Asp polymorphism may have important role in the pathogenesis of Coronary Artery Disease in postmenopausal women. The postmenopausal women those who were observed with Glu298Asp variant may accelerate those mechanisms common to both the early and late manifestations of CAD which may helps further in early intervention and treatment.

Declarations
Competing Interests: None
Disclosures: None
Funding: No competing financial interests exist.
Contribution to authorship: All authors planned the study. P.K.D participated by coordinating the work, collecting data, main part of analyses and drafting the manuscript. J.B helped in supervising and structuring of the work and manuscript. S.S.T and N.D assisted in analyses and revision of manuscript.

REFERENCES

1. Sharma M, Ganguly NK. Premature coronary artery disease in Indians and its associated risk factors. Vasc Health and Risk Manag, 1(3): 217–225, (2005).
2. Kriplani A, Bannerjee K. An overview of age of onset of menopause in northern India. Maturitas, 52(3–4): 199–204, (2005).
3. Granfone A, Campos H, McNamara JR, et al. Effects of estrogen replacement on

plasma lipoproteins and apolipoproteins in postmenopausal dyslipidemic women. Metabolism, 41: 1193-1198, (1992).

4. Lesson CP, Hingorani AD, Mullen MJ, et al. Glu298Asp endothelial nitric oxide Synthase gene polymorphism interacts with environmental and dietary factors to influence endothelial function, 90: 1153-1158, (2002).
5. Odeberg J, Larsson CA, Rastam L, Lindblad U. The Asp298 allele of endothelial nitric oxide synthase is a risk factor for myocardial infarction among patients with type 2 diabetes mellitus. BMC Cardiovasc Disord, 8: 36, (2008).
6. Srivastava K, Biswas UK, Narang R, Varghese JJ, Das N. Prevalence of eNOS Glu298Asp polymorphism in healthy volunteers from a region of northen India. Community Genet, 8: 180-183, (2005).
7. Ware JA, Heistad DD. Seminars in medicine of the Beth Israel Hospital, Boston. Platelet-endothelium interactions. N Engl J Med, 328(9): 628-635, (1993).
8. Capitanio AM, Niewiarowski S, Rucinski B et al. Interaction of platelet factor 4 with human platelets. Biochim Biophys Acta, 839(2): 161-173, (1985).
9. Farmer EE, Davoine C. Reactive electrophile species. Curr Opin Plant Biol, 10(4): 380–386, (2007).
10. Mathew F, Glenda J, Jack L. Quantification of nitric and nitrate in extracellular fluids. Metho Enzymol, 268: 237-246, (1996).
11. Vinning RF, McGinley R, Rice BV. Saliva estriol measurements: an alternative to the assay of serum unconjugated estriol in assessing feto-placental function. J Clin Endocrinol Metab, 56: 454-460, (1983).
12. Files JC, Malpass TW, Yee EK, Harker LA. Studies of human plate granule-release in vivo. Blood, 58(3): 607-618, (1981).
13. Konukoglu D, Akcay T, Erdem T. Susceptibility of erythrocyte lipids to oxidation and erythrocyte antioxidant status in myocardial infarction. Clin Biochem, 31(8): 667-671, (1998).
14. Hibi K, Ishigami T, Tamura K, et al. Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. Hypertension, 32: 521-526, (1998).
15. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucl Acid Res, 16: 1215, (1988).
16. Salhotra S, Arora S, Anubhuti, Trivedi SS, Bhattacharjee J. Influence of menopause on biochemical markers of endothelial - A case control pilot study in North Indian population. Maturitas, 62: 166–170, (2009).
17. Tempfer C, Rene M, Moreno, Anthony R. Genetic contributions of the endothelial nitric oxide synthase gene to ovulation and menopause in a mouse model. Fertil Steril, 73(5): 1025-1031, (2000).
18. Cannon Ro. Role of nitric oxide in cardiovascular disease: focus on endothelium. Clini Chem, 44: 1809-1819, (1998).
19. Singh A, Sharma D, Raghunandan C. Bhattacharjee, J. Role of inflammatory cytokines and eNOS gene polymorphism in pathophysiology of pre-eclampsia. Am. J Reprod Immunol, 63: 244–251, (2010).
20. Blakeley JO, Llinas RH. Thrombolytic therapy for acute ischemic stroke. J Neurol Sci, 261: 55-62, (2007).
21. Limbourg FP, Huang Z, Plumier JC, Simoncini T, Fujioka M, Tuckermann J. Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids. J Clin Invest, 110: 1729-1738, (2002).
22. Pedersen WA, Fu W, Keller JN, et al. Protein modification by the lipid peroxidation product 4-hydroxynonenal in the spinal cords of amyotrophic lateral sclerosis patients. Ann Neurol, 44: 819–824, (1998).
23. Imamura A, Takahashi R, Murakami R, et

al. The effects of endothelial nitric oxide synthase gene polymorphisms on endothelial function and metabolic risk factors in healthy subjects: the significance of plasma adiponectin levels. Eur J Endocrinol, 158(2):189-195, (2008).

24. Vaziri ND, Ding Y, Ni Z. Nitric Oxide Synthase Expression in the Course of Lead-Induced Hypertension. Hypertension, 34: 558-562, (1999).
25. Ikenoue N, Wakatsuki A, Okatani Y. Small low density lipoprotein particles in women with naturally or surgically induce menopause. Obstet Gynecol, 93: 566-570, (1997).
26. Berg G, Mesch V, Boero L, et al. Lipid and lipoprotein profile in menopausal transition. Effects of hormones, age and fat distribution. Horm Metab Res, 36(4): 215-220, (2004).
27. Kincer JF, Uittenbogaard A, Dressman J, et al. Hypercholesterolemia promotes a CD36-dependent and endothelial nitric-oxide synthase-mediated vascular dysfunction. J Biol Chem, 277(26): 23525–23533, (2002).