

ORIGINAL STUDY

Investigation of eNOS gene polymorphism exposes a genetic association between endothelial dysfunction and osteoporosis in postmenopausal women

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Abstract

Objectives: To investigate the association of genetic polymorphisms of endothelial nitric oxide synthase (eNOS) gene with endothelial dysfunction associated osteoporosis in postmenopausal women of Punjab, India.

Methods: The study involved 456 postmenopausal women having endothelial dysfunction categorized according to women with (n = 236) and without osteoporosis (n = 220). Bone mineral density (BMD) and reactive hyperemia index (RHI) were evaluated together with six single-nucleotide polymorphisms (SNPs) within the eNOS gene (rs2070744, rs1799983, rs1800780, rs3918181, rs891512, and rs1808593).

Results: A moderate association between RHI and BMD at femoral neck ($r^2 = 0.213$, $P = 0.002$) and lumbar spine ($r^2 = 0.267$, $P < 0.001$) was observed. Minor alleles C and T of SNPs rs2070744 and rs1799983 were associated with chances of osteoporosis in both co-dominant (odds ratio [OR] 2.13, $P = 0.017$; OR 2.77, $P = 0.009$) and dominant (OR 2.10, $P = 0.011$; OR 2.45, $P = 0.007$) modes, whereas minor allele A of SNP rs891512 showed marginal probability in dominant model (OR 1.68, $P = 0.047$). A susceptibility haplotype (CTAAAT) was observed within the eNOS gene which conferred 2.32 times higher chances of osteoporosis (OR 2.32, 95% confidence interval 1.18-4.54, $P = 0.021$) after adjusting for the effect of confounders. Genetic model analysis revealed that each copy of susceptibility haplotype increased the possibility of osteoporosis by a factor of 2.11 ± 0.63 ($P < 0.001$). RHI was significantly associated with susceptibility haplotype CTAAAT in a dose-dependent manner, whereby the severity of endothelial dysfunction increased significantly in women having two copies over women having one copy or no copy ($\beta = 2.13$, $P < 0.001$) of susceptibility haplotype.

Conclusion: A susceptibility haplotype CTAAAT within the eNOS gene is associated with double the possibility of endothelial dysfunction affiliated osteoporosis in postmenopausal women of Punjab, India.

Key Words: BMD – Endothelial dysfunction – eNOS gene – India – Osteoporosis – Punjab.

Osteoporosis is a complex disease identified by decreased bone mineral density (BMD) that results in fragile, frail, and fractured bones. During menopause, abrupt hormone and endocrine changes affect physiology that makes the body vulnerable to several health problems including osteoporosis. Clinical literature has

revealed that the first and foremost threat in the series of adverse events that happens after menopause is endothelial dysfunction.¹⁻³ Endothelial dysfunction is a systemic pathological state resulting due to an imbalance between vasodilating and vasoconstricting substances in the endothelium, which can occur as a consequence of low bioavailability of nitric oxide (NO). It has been observed that the free flow of blood to the bone improves bone formation and mineralization, whereas inhibition of NO production (endothelial dysfunction) results in marked bone loss.^{3,4} Healthy endothelial function impacts bone remodeling, which is an imperative component for maintaining bone strength, its structure, and resistance to fractures.^{5,6} Moreover, it not only provides nutrients and immune cells to bone, but also maintains a balance between osteogenesis and angiogenesis.⁷ It has been shown that estrogen receptors are present on endothelial beds which stimulate osteoclast differentiation and proliferation, suggesting a direct effect of estrogen on endothelium.⁸

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Reduced estrogen levels during menopause are significantly associated with endothelial dysfunction, whereas hormone therapy (HT) improves endothelial function and BMD, suggesting a possible association between endothelial dysfunction, estrogen, and BMD.³⁻⁹ Because dysfunction in the endothelium triggers silently and supplements pathological events years before its manifestation, it has evaded the attention of scientists, and as a result, the investigation of its link with osteoporosis has largely been overlooked.

Clinical revelations have gradually started indicating that endothelial dysfunction is genetically regulated,¹⁰ whereby genetic variants within the endothelial nitric oxide synthase (eNOS) gene affect expression levels and are responsible for NO bioavailability in the endothelium.¹¹ The eNOS gene is 21 kb in size, contains 26 exons, and localizes on chromosome position 7q36.1. It has been observed that the T allele of -786T/C (rs2070744) polymorphism is associated with additional binding sites of essential transcriptional proteins, thus increasing NO expression and affording a protection against endothelial dysfunction.¹² On the contrary, T allele of G894T (rs1799983) polymorphism attenuates NO expression and causes coronary vascular resistance and vasomotor dysfunction.¹³ In Chinese postmenopausal women, heterozygotes (GT) of this single-nucleotide polymorphism (SNP) (rs1799983) were observed to have higher plasma testosterone and osteocalcin levels, whereas GG homozygotes had decreased BMD.¹⁴ Incongruity in genetic association studies is because most of the studies have tried to examine the role of eNOS gene polymorphism individually. However, SNPs within eNOS or adjacent genes interact themselves and participate, especially when they are nonrandomly associated.¹⁵ Therefore, the present study has examined the association of six pertinent SNPs of eNOS gene (rs2070744, rs1799983, rs1800780, rs3918181, rs891512, and rs1808593) to explore whether endothelial dysfunction affecting osteoporosis is genetically mediated in postmenopausal women of Punjab, India.

METHODS

Study population

The present case control study screened postmenopausal women who attended orthopedic outpatient wards of prominent tertiary health care hospitals of Punjab. To verify their vascular health (endothelial dysfunction), these women were tested with the EndoPAT 2000 device (Itamar Medical Ltd., Caesarea, Israel) and for severity of bone loss (BMD) by dual-energy x-ray absorptiometry (DXA) at hip (femoral neck) and lumbar region (L1-L4 vertebrae). After applying inclusion and exclusion criteria stringently, 456 women were enrolled to participate in the study. All these participants were having endothelial dysfunction confirmed with the values of reactive hyperemia index (RHI < 1.67). Out of these 456 women, 236 were diagnosed with osteoporosis (T score \leq -2.5) and 220 presented with normal bone mass (T score \geq -1 SD). Inclusion criteria were: consenting women fell within the age range of 45 to 65 years, original residents of Punjab, and have confirmed postmenopause phase (menses

ceased from the past 1 year). Exclusion criteria were: non-consenting, history of smoking, alcohol use, having cardiovascular disorders, cerebrovascular pathology, diabetes, hypertension, liver disorders, family history of osteoporotic fractures, lupus, chronic kidney disease, taking hormone therapy or any medication affecting blood pressure or lipoprotein metabolism, and taking psychotropics or multivitamins/antioxidants. All participants gave their written consent. The disease status of each participant was kept confidential and blinded to the researchers to avoid any bias. The study protocol was approved by the Institutional Ethical Committee (Punjabi University, Patiala) and adhered strictly to the ethical guidelines given by the Indian Council of Medical Research (ICMR) and rules specified within the declaration of Helsinki.

Definition of variables

Comprehensive history of years since menopause (YSM) and age were noted down from medical records or via interview. Weight and height were measured and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). The mean of the two blood pressure checks, taken 3 minutes apart from seated participants (seated for minimum of 15 minutes) were taken as systolic (SBP) and diastolic blood pressures (DBP). Lipid levels comprising total cholesterol (TC), high-density lipoprotein (HDL) and triglycerides (TG) were examined with a one step enzymatic method using an assay kit (Erba Mannheim, London, UK) on a microplate reader (Lisa Scan, Erba Mannheim, London, UK) with minimum detection limit of 0.1 mg/L. Friedewald equation was applied to evaluate low-density lipoprotein cholesterol (LDL-C). The intra-assay and interassay coefficients of variation (CVs) for measurement of lipid variables were less than 5%.

Endothelial dysfunction testing

Endothelial dysfunction was examined non-invasively by reactive hyperemia-peripheral arterial tonometry (RH-PAT) using an EndoPAT 2000 device (Itamar Medical Ltd. Caesarea, Israel). Participants were advised not to consume any food or beverages having considerable amounts of methylxanthine (chocolate, kola nuts, guarana berries, banana, caffeine, and tea) 5 hours before examination. The testing room temperature was kept at optimal temperature (\sim 20-25°C). Two tests of blood pressure were taken before the test and their mean value was recorded. Finger probes were put on to the index finger of each hand and were inflated. First baseline measurement of pulse amplitude was recorded for 6 minutes and after that the brachial artery was occluded by inflating the cuff manually up to supra systolic blood pressure (at least 60 mm Hg above baseline SBP or up to a maximum of 300 mm Hg). After occlusion, the cuff was released and the pulse amplitude tone (PAT) signals were recorded for 5 minutes by the built-in software of the machine, which shows RHI. Women having RHI < 1.67 were considered to have endothelial dysfunction. The analysis was automatically performed by algorithm-based dedicated software, hence measurements were operator-independent.

BMD measurements

Bone mineral density was measured with Hologic QDR 4500 system (Hologic Inc. Waltham, MA) using DXA. On the basis of T scores calculated by the software, women were characterized as osteoporotic when the T score was ≤ -2.5 standard deviation (SD) and normal (without bone loss), when the T score was ≥ -1 SD from the optimal peak bone density of healthy young adults of the same sex. The DXA system was calibrated in vitro as per the guidelines of the manufacturer before testing. The CV for the measurements of the BMD at the hip and spine were less than 2%.

Statistical analysis

All differences in the baseline data were analyzed by applying either the Student's *t* test or Wilcoxon rank-sum test for continuous variables, whereas chi-square test was performed for checking proportions or categorical data. Allele frequencies were calculated by gene counting method, and deviations from Hardy-Weinberg were tested by chi-square with Bonferroni corrections. Linear regression and whisker plots were analyzed by taking BMD as the target variable and RHI as the predictor variable using SPSS with Bonferroni corrections. Collinearity was checked between explanatory variables by analyzing variance inflation factor (VIF). For analyzing the association between variables and BMD, all independent variables were tested in univariate regression analysis (General Linear Model procedure), and those variables which showed association with dependent variables were then analyzed in binary logistic regression analysis, using the conditional backward method (SPSS Ver 18.0, IBM, NY). Cochran-Armitage test for trend was applied to examine the association of any designated allele with chances of osteoporosis under codominant, dominant, and recessive models of inheritance. Pairwise SNP-SNP interactions in relation to various variables were analyzed using an extended Kempthorne model in software *epiSNP*.¹⁶ Genotype data of the respective SNPs were imputed to determine haplotypes by using *Arlequin* software ver. 3.5.¹⁷ For checking the associations between osteoporosis and haplotypes, binary logistic linear regression was used and odds ratios (ORs) were calculated by analyzing an unadjusted model and a model with adjusted effects of associated factors after taking the most common haplotype as referent. The association of the significant haplotype deduced in the analysis was further tested to identify which genetic model (dominant, recessive, multiplicative, or general) best explained its impact on the probability of osteoporosis. Best fit model was identified by using Akaike information criterion ($AIC = -2 \times \ln \text{likelihood} + 2 \times \text{number of parameters}$) and Wald statistics. Haplotype uncertainty measure (R^2_h) was investigated by the method of Stram et al.¹⁸ The association of susceptibility haplotype with reactive hyperemia was analyzed according to the haplotype dosage (0 copy, 1 copy, or 2 copies) after using logistic regression analysis considering two-tailed $P < 0.05$; however, avoid significance level was set at 0.01 for multiple comparisons.

Selection of the respective SNPs and genotyping

The SNPs within eNOS gene were selected on the basis that all these SNPs were previously validated by submitted reports on the refSNP cluster at dbSNP database (<http://www.ncbi.nlm.nih.gov/snp>). Secondly, these SNPs were previously identified as having an association with either endothelial dysfunction or osteoporosis.¹⁰⁻¹⁴ All the selected SNPs were polymorphic with minor allele frequency > 0.05 . Following these criteria, six SNPs of eNOS gene (rs2070744, rs1799983, rs1800780, rs3918181, rs891512, and rs1808593) were finally chosen and genotyped. Deoxyribose nucleic acid (DNA) was extracted from the whole blood using the salting out method. Reaction mixture (25 μ L) was used to amplify the extracted DNA by employing polymerase chain reaction (PCR). Amplified product was digested with respective high fidelity restriction endonucleases (New England BioLabs Inc, Hertfordshire, UK). The genotypes were scored and visualized on 2% to 3% agarose gel electrophoresis depending upon the size of the product. The experiment was anonymized for the case control status to avoid any bias, and approximately 15% of samples were reanalyzed to confirm and validate genotyping procedure.

Feasibility of genetic association and population structure analysis

A priori investigation was conducted by using population genetic association (PGA) software according to the method given by Menashe et al,¹⁹ which suggested that a sample size of 456 participants (236 osteoporotic and 220 nonosteoporotic women) would be able to provide at least 85% power to discriminate a minimum genotype relative risk (MGRR) of 2.0 with major allele frequency of at least 0.5 at a 5% significance level. Analysis also revealed that it would provide minimum detectable relative risk (MDRR) of 1.5 for haplotypes with more than 90% power.

It is well known that a case control design can incorporate population stratification (PS) in the data due to self-reported ethnicity or ancestry, possibly leading to false-positive inferences and spurious conclusions. PS was examined by the method of Devlin et al,²⁰ employing *Helix Tree* Software version 6.4.0 (Golden Helix Inc, Bozeman, MT). The inflation factor λ was calculated to be 0.92, which suggested that no considerable PS existed in the participants of this study.

RESULTS

Variables at baseline and their differences

Demographic, biochemical, and genetic characteristics of the postmenopausal women having endothelial dysfunction with and without osteoporosis are given in Table 1. It was revealed that BMI in women with osteoporosis was significantly higher ($P < 0.001$) than their normal counterparts. Values for YSM, DBP, TC, HDL, and LDL-C were observed to be similar ($P > 0.05$) between both the groups. SBP ($P < 0.001$) and TG ($P = 0.005$) were observed to be significantly dissimilar between both the groups. It was observed that although both the groups had endothelial dysfunction (RHI < 1.67), its severity was more pronounced in women with

TABLE 1. Demographic, biochemical, and genetic characteristics of the study group

Variables	Endothelial dysfunction		P
	With osteoporosis	Without osteoporosis	
Number	236	220	—
Age (y)	59.8 ± 11.2	60.0 ± 9.70	0.934
Body mass index (kg/m ²)	29.6 ± 2.82	25.7 ± 3.53	<0.001
Years since menopause (y)	9.71 ± 4.30	8.92 ± 3.38	0.106
Diastolic blood pressure (mm Hg)	99.0 ± 11.10	96.70 ± 10.23	0.087
Systolic blood pressure (mm Hg)	142.26 ± 14.40	126.72 ± 12.35	<0.001
Total cholesterol (mg/dL)	224.12 ± 22.70	219.09 ± 19.78	0.061
Low-density lipoprotein (mg/dL)	197.28 ± 22.41	195.18 ± 20.90	0.441
High-density lipoprotein (mg/dL)	47.3 ± 3.56	46.9 ± 2.88	0.189
Triglyceride (mg/dL) ^a	219 (112, 247)	202 (100, 242)	0.005
Reactive hyperemia index ^a	1.29 (0.9, 1.65)	1.48 (1.04, 1.60)	<0.001
BMD_FN (g/cm ²) ^b	0.71 ± 0.11	0.89 ± 0.10	<0.001
BMD_LS (g/cm ²) ^b	0.82 ± 0.12	0.92 ± 0.07	<0.001
rs2070744 (MAF ± SE) ^c	0.28 ± 0.04	0.18 ± 0.02	0.033
rs1799983 (MAF ± SE) ^c	0.21 ± 0.03	0.09 ± 0.01	0.004
rs1800780 (MAF ± SE) ^c	0.44 ± 0.05	0.42 ± 0.04	0.759
rs3918181 (MAF ± SE) ^c	0.33 ± 0.04	0.29 ± 0.03	0.434
rs891512 (MAF ± SE) ^c	0.24 ± 0.03	0.16 ± 0.02	0.032
rs1808593 (MAF ± SE) ^c	0.21 ± 0.02	0.15 ± 0.01	0.010

BMD_FN, bone mineral density at femoral neck; BMD_LS, bone mineral density at lumbar spine; MAF, minor allele frequency.

^aValues are median (25th-75th interquartile range).

^bValues are corrected with age, body mass index, and years since menopause.

^cValues are mean ± SD except values which are minor allele frequency ± standard error.

osteoporosis than in women without osteoporosis (1.29 vs 1.48; $P < 0.001$). Adjusted BMD values at femoral neck and lumbar spine were detected to be significantly lower ($P < 0.001$) in osteoporotic women than in women without it. Minor allele frequencies of eNOS SNPs rs2070744, rs1799983, rs1800780, rs3918181, rs891512, and rs1808593 were 0.28, 0.21, 0.44, 0.33, 0.24, and 0.21 in osteoporotic women; and 0.18, 0.09, 0.42, 0.29, 0.16, and 0.15 in women with normal bone mass, respectively. All genotype frequencies of these SNPs were within Hardy-Weinberg equilibrium ($P > 0.05$). Minor allele frequency of rs2070744 ($P = 0.033$), rs1799983 ($P = 0.004$), rs891512 ($P = 0.032$), and rs1808593 ($P = 0.010$) were observed to be significantly higher in osteoporotic women ($P < 0.05$) when compared with women without osteoporosis.

Multivariate analysis of variables and allele frequency models

Logistic regression analysis of the variables between both the groups is presented in Table 2, which revealed that BMI

(≥ 30 kg/m²), SBP (> 120 mm Hg), TG (> 100 mg/dL), BMD_FN (< 0.7 g/cm²), and BMD_LS (< 0.8 g/cm²) were significant univariate covariates. All these variables which showed significance ($P < 0.05$) in univariate testing were examined in a multivariate model which confirmed that these variables were independent predictors for endothelial associated osteoporosis.

Genotype-specific associations were modeled into codominant, dominant, and recessive models by taking 1 (AA), r (AB), and r^2 (BB) genotypes, whereby an increase of the chance was depicted by the factor r for each B allele carried (Table 3). It was observed that allele frequencies, after adjusting for the effects of variables and Bonferroni correction, showed an association of C allele of rs2070744 in both codominant (OR 2.13, 95% confidence interval [CI] 1.34-3.56, $P = 0.017$) and dominant modes (OR 2.10, 95% CI 1.22-3.47, $P = 0.011$). Minor allele T of SNP rs1799983 conferred higher chances of osteoporosis in codominant (OR 2.77, 95% CI 1.22-5.10, $P = 0.009$) and dominant (OR 2.45, 95% CI

TABLE 2. Univariate and multivariate analysis for various variables associated with osteoporosis

Variables	Input parameters	Univariate analysis		Multivariate analysis	
		OR (95% CI)	P	OR (95% CI)	P
Body mass index (kg/m ²)	<25 vs ≥ 30	1.89 (1.92-2.90)	0.008	1.32 (1.02-2.78)	0.013
Years since menopause (y)	≤ 5 vs > 5	1.96 (0.92-3.18)	0.192	—	—
Diastolic blood pressure (mm Hg)	≤ 80 vs > 80	1.52 (0.78-2.78)	0.177	—	—
Systolic blood pressure (mm Hg)	≤ 120 vs > 120	2.00 (1.16-3.18)	0.007	1.12 (1.33-2.89)	0.002
Total cholesterol (mg/dL)	≤ 200 vs > 200	1.33 (0.82-3.29)	0.199	—	—
Low-density lipoprotein (mg/dL)	≤ 100 vs > 100	2.12 (0.69-2.91)	0.167	—	—
High-density lipoprotein (mg/dL)	≥ 40 vs < 40	1.48 (0.65-2.58)	0.162	—	—
Triglyceride (mg/dL)	≤ 150 vs > 150	2.56 (1.86-4.23)	<0.001	1.88 (1.34-3.47)	0.007
BMD_FN (g/cm ²)	≥ 0.7 vs < 0.7	1.59 (1.11-3.45)	0.003	1.12 (1.00-2.15)	0.032
BMD_LS (g/cm ²)	≥ 0.8 vs < 0.8	1.66 (1.21-3.33)	0.002	1.23 (1.09-2.89)	0.024

BMD_FN, bone mineral density at femoral neck; BMD_LS, bone mineral density at lumbar spine; CI, confidence interval; OR, odds ratio. Values in bold face show significant associations.

TABLE 3. *Cochrane-Armitage test for trend to examine genetic effects of eNOS gene SNPs for the endothelial dysfunction associated osteoporosis possibility in postmenopausal women*

eNOS SNPs	Genetic models	Unadjusted OR (95% CI)	P	Adjusted OR (95% CI)	P
rs2070744	TT	Referent	—	Referent	—
Codominant	TT vs TC	1.87 (1.08-3.23)	0.034	2.13 (1.34-3.56)	0.017
Codominant	TT vs CC	2.27 (0.81-6.36)	0.178	2.44 (0.92-7.02)	0.181
Dominant	TT vs TC + CC	1.94 (1.16-3.24)	0.016	2.10 (1.22-3.47)	0.011
Recessive	TT + TC vs CC	1.84 (0.67-5.06)	0.343	1.92 (0.81-6.28)	0.442
rs1799983	GG	Referent	—	Referent	—
Codominant	GG vs GT	3.15 (1.68-5.89)	<0.001	2.77 (1.22-5.10)	0.009
Codominant	GG vs TT	3.56 (0.70-18.12)	0.206	2.77 (0.67-16.19)	0.198
Dominant	GG vs GT + TT	3.19 (1.75-5.82)	<0.001	2.45 (1.34-5.49)	0.007
Recessive	GG + GT vs TT	2.72 (0.54-13.75)	0.368	2.10 (0.42-12.83)	0.273
rs1800780	GG	Referent	—	Referent	—
Codominant	GG vs GA	0.79 (0.45-1.38)	0.488	0.90 (0.56-1.81)	0.427
Codominant	GG vs AA	1.29 (0.64-2.60)	0.601	1.54 (0.79-2.73)	0.654
Dominant	GG vs GA + AA	0.91 (0.54-1.53)	0.829	0.91 (0.54-1.53)	0.829
Recessive	GG + GA vs AA	1.48 (0.79-2.76)	0.280	1.61 (0.84-2.89)	0.341
rs3918181	GG	Referent	—	Referent	—
Codominant	GG vs GA	1.06 (0.62-1.80)	0.935	1.21 (0.84-2.12)	0.978
Codominant	GG vs AA	1.51 (0.68-3.37)	0.416	1.72 (0.77-3.79)	0.578
Dominant	GG vs GA + AA	1.16 (0.71-1.89)	0.653	1.32 (0.82-1.92)	0.701
Recessive	GG + GA vs AA	1.47 (0.68-3.18)	0.423	1.56 (0.75-3.33)	0.566
rs891512	GG	Referent	—	Referent	—
Codominant	GG vs GA	1.79 (1.02-3.15)	0.059	1.22 (0.48-2.93)	0.083
Codominant	GG vs AA	1.79 (0.62-5.15)	0.406	1.19 (0.41-4.76)	0.321
Dominant	GG vs GA + AA	1.79 (1.06-3.03)	0.041	1.68 (1.01-2.97)	0.047
Recessive	GG + GA vs AA	1.51 (0.53-4.28)	0.605	1.44 (0.49-3.99)	0.533
rs1808593	TT	Referent	—	Referent	—
Codominant	TT vs TG	1.52 (0.85-2.70)	0.202	1.44 (0.72-2.57)	0.187
Codominant	TT vs GG	1.82 (0.59-5.65)	0.440	1.71 (0.41-4.56)	0.389
Dominant	TT vs TG + GG	1.57 (0.91-2.69)	0.134	1.29 (0.78-2.10)	0.110
Recessive	TT + TG vs GG	1.63 (0.53-5.01)	0.558	1.33 (0.42-4.89)	0.491

Genotype-specific risks were modeled as 1 (AA), *r* (AB), and *r*² (BB), increasing by factor of *r* for carriage of each B allele. Dominant model (carrying a single copy of the B allele increases the risk), recessive model (carrying two copies of the allele B increases the risk), codominant model (increase risk of *r* for AB genotype and 2*r* for BB genotype).

Values in bold face show significant associations.

CI, confidence interval; eNOS, endothelial nitric oxide synthase; OR, odds ratio.

1.34-5.49, *P*=0.007) models when compared with major allele G. Minor allele A of SNP rs891512 showed a marginal possibility for osteoporosis (OR 1.68, 95% CI 1.01-2.97, *P*=0.047) over major allele A. All other SNPs (ie, rs1800780, rs3918181, and rs1808593) did not show any association with osteoporosis.

Association of RHI with BMD

To identify an association between RHI and BMD, linear regression analysis (Fig. 1) exposed that both variables were positively correlated, whereby gradual increase of the hyperemia index showed significant increase in BMD at femoral neck (*P*=0.002) and lumbar spine (*P*<0.001) after Bonferroni correction. Multicollinearity was checked and VIF was elucidated as 1.2, indicating that both BMDs were not linearly related. This link between BMD and RHI was further tested with whisker plots, which confirmed the positive association of both BMDs at femoral neck (*P*=0.004) and lumbar spine (*P*<0.001) with RHI.

SNP-SNP interactions, exposure variables, and possibility of osteoporosis

SNP pair epistatic effects on exposure variables for the association of osteoporosis were identified from genotype

data (Table 4). All the single marker effects were further tested for pairwise SNP interactions to identify epistatic relationships. It was observed that SNP rs2070744 showed an epistatic effect on SNPs; rs1799983 (*P*=0.0035) and rs3918181 (*P*=0.0023) affecting RHI through AD (additive × dominant) effect. SNP rs2070744 also exhibited significant effect on TG (*P*=0.0010) in collaboration with SNP rs1808593 for the possibility of osteoporosis. Interactions between SNPs rs1799983 and rs3918181 affected LDL-C levels through AA (additive × additive) mode (*P*=0.0002). SNP combinations of rs891512-rs1800780 and rs1800780-rs1808593 affected TC (*P*=0.005) and BMI (*P*=0.009) in DA (dominant × additive) and I (interactive) effect modes. Interestingly, interactive effects of SNP combinations rs2070744-rs1799983 and rs891512-rs1800780 had a significant impact on RHI (*P*=0.001) and TC (*P*=0.047) in women having normal bone mass.

Haplotypes and their association with osteoporosis

Genotypes within six SNPs of eNOS gene deciphered 37 visible haplotypes. Among these, 30 haplotypes had frequencies <0.05; hence, these were excluded from further analysis. Seven haplotypes were finally determined (Table 5), which explained 85% of the genetic variability in osteoporotic

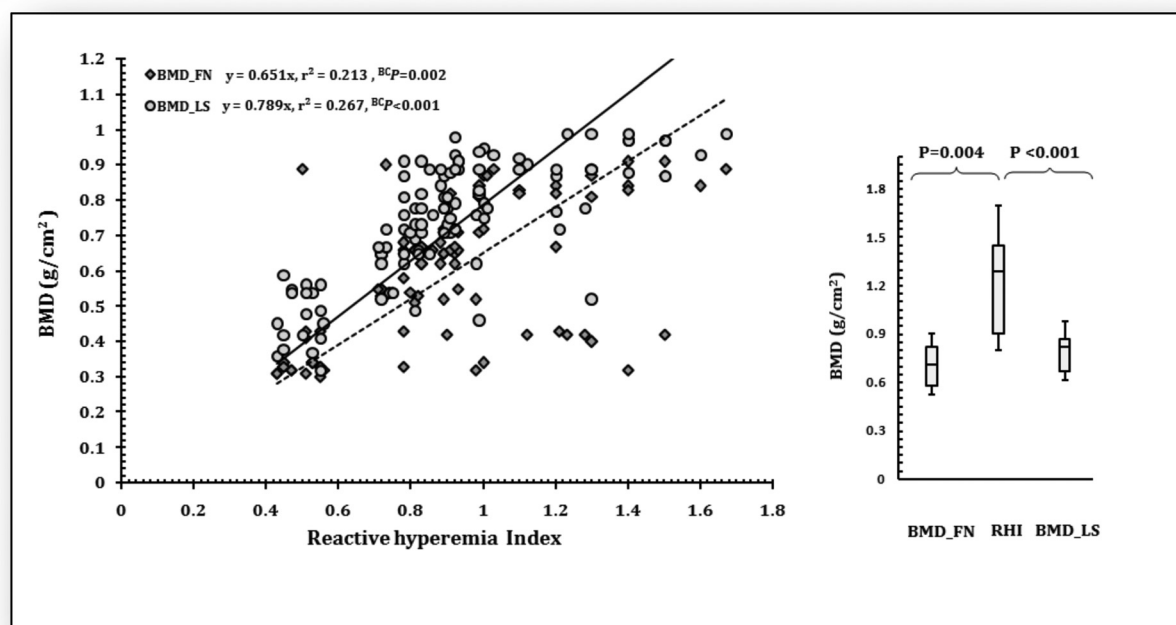


FIG. 1. The trend-line depicts linear regression between bone mineral density; BMD at femoral neck (FN), lumbar spine (LS), and reactive hyperemia index. Correlation coefficients (r^2) and Bonferroni corrected P values (^{BC}P) indicated a possible association between BMD and reactive hyperemia index. BMD, bone mineral density.

women and 87% in women having normal BMD. Haplotype TGAAGT was observed to be the most prevalent in both groups, hence served as the reference for disease association analysis. Haplotype CTAAAT was revealed to be strikingly higher ($P < 0.001$) in women having osteoporosis than women without it. After adjusting the effect of variables, it emerged to be the susceptibility haplotype, carriers of which were at approximately double the possibility of osteoporosis (OR 2.32, 95% CI 1.18-4.54, $P = 0.021$). Other haplotypes did not differ between both groups ($P > 0.05$). Functional effect of this susceptibility haplotype and selection of best fit model according to AIC and R^2_h was conducted to evaluate which mode of inheritance described its maximum effect on BMD (Table 6). It revealed that CTAAAT was a susceptibility haplotype, which was substantially associated with the possibility of osteoporosis ($\beta \pm SE$ 2.11 \pm 0.63, $P = 8 \times 10^{-5}$) in

multiplicative mode (women having one copy of this haplotype are at intermediate probability than those having no copy or two copies). Furthermore, the relationship of this susceptibility haplotype with reactive hyperemia was analyzed (Fig. 2), which highlighted a haplotype dose-dependent effect. Women having no copy of susceptibility haplotype had higher RHI values when compared with women having single or double copy of the haplotype (1.59 vs 1.47 and 1.2).

DISCUSSION

This is the first study from India, which has reported that endothelial dysfunction is associated with the possibility of osteoporosis (low BMD) in postmenopausal women. It is well known that hormonal and endocrine disturbances during menopause result in a substantial loss of bone minerals and protein in women, but the imperative cause and consequence

TABLE 4. Significant SNP-SNP cross-talks within eNOS gene influencing variables

SNP	SNP	Trait	Test	P^a	P^b
rs2070744	rs1799983	RHI	AD	0.0035	0.001
rs1799983	rs3918181	LDL	AA	0.0002	1.356
rs2070744	rs1808593	TG	DA	0.0010	2.229
rs891512	rs1800780	TC	DA	0.0057	0.047
rs1800780	rs1808593	BMI	I	0.0090	1.117
rs2070744	rs3918181	RHI	AD	0.0023	3.421

Out of several SNP-SNP interactions, only significant effects in osteoporotic women were compared with nonosteoporotic women. Two-way epistatic effects of these SNP pairs indicate.

AA, additive \times additive; AD, additive \times dominant; BMI, body mass index; DA, dominant \times additive; I, interactive effect; LDL, low-density lipoprotein; RHI, reactive hyperemia index; TC, total cholesterol; TG, triglyceride.

^a P values of SNP-SNP interaction effect on risk covariates in osteoporotic women ($n = 236$).

^b P values in women without osteoporosis ($n = 220$).

TABLE 5. Haplotype analysis of eNOS gene SNPs in the study participants

Haplotype	Endothelial dysfunction		P ^a	Unadjusted OR (95% CI)	P	Adjusted OR (95% CI) ^b	P
	With osteoporosis	Without osteoporosis					
TGAAGT	0.20 (47)	0.21 (46)	0.71	Referent	—	Referent	—
TTGGGG	0.11 (26)	0.14 (31)	0.16	0.82 (0.42-1.59)	0.68	0.82 (0.42-1.59)	0.70
CGAAGG	0.10 (24)	0.13 (29)	0.16	0.81 (0.41-1.59)	0.66	0.78 (0.39-1.53)	0.58
TTAGGG	0.10 (24)	0.14 (31)	0.07	0.76 (0.39-1.48)	0.52	0.73 (0.37-1.43)	0.45
CTAAAT	0.19 (45)	0.07 (15)	<0.001	2.94 (1.44-5.98)	0.004	2.32 (1.18-4.54)	0.021
CGGAGG	0.08 (19)	0.09 (20)	0.57	0.93 (0.44-1.96)	1.00	0.88 (0.44-1.88)	0.90
CTGGAT	0.07 (17)	0.09 (20)	0.30	0.83 (0.39-1.79)	0.78	0.75 (0.35-1.61)	0.58

Values in parenthesis are numbers.

Thirty out of visible 37 haplotypes showed frequencies <0.05; hence, excluded from analysis.

Bold faces show the susceptibility haplotype.

CI, confidence interval; eNOS, endothelial nitric oxide synthase; OR, odds ratio.

^aCorrected for multiple comparisons (Bonferroni adjustment).

^bOdds ratios are adjusted with body mass index, systolic blood pressure, triglyceride levels, and BMDs at femoral neck and lumbar spine.

of restricted supply of blood to the bone remains to be fully investigated. The present study investigated the contribution of six SNPs within the eNOS gene in postmenopausal women according to osteoporosis status. The estrogen and progesterone dip during menopause is strongly correlated with NO deficiency, which can be corrected with the supplementation of HT.²¹ Many studies have highlighted a strong connection between bone mass and NO levels.^{22,23} Genetic variants within the eNOS gene have been confirmed to provide NO in vascular endothelium, thereby regulating the mechanism of endothelial dysfunction.²⁴ It was observed that Chinese postmenopausal women having major allele G of rs1799983 (G894T) had significantly lower BMD values at femoral neck, lumbar spine, and Ward’s triangle ($P < 0.05$).¹⁴ Another study revealed that C allele of SNP rs2070744 and T allele of SNP rs1799983 were significantly associated with osteoporosis.²⁵ The present study exposed that minor alleles C, T, and G of SNPs rs2070744, rs1799983, and rs891512 were significantly associated with osteoporosis in women having endothelial dysfunction. One may reason why polymorphisms within the eNOS gene display a complex and contradictory picture of its association with osteoporosis.^{26,27} It has been clarified earlier that SNPs individually are not able to capture vast genetic variation related to a particular disease, but they interact and participate differentially in different situations.^{28,29} For instance, minor alleles A of SNPs rs1800780 and rs3918181 did not show association individually with

osteoporosis in the present study (Table 3), but when their collaborative role was examined in the susceptibility haplotype (CTAAAT), these two alleles conferred significant chance (OR 2.32, 95% CI 1.18-4.54, $P = 0.021$) of osteoporosis to the postmenopausal women (Table 5). For the verification of this inference, it was hypothesized that all women in both groups had equal frequencies of this haplotype (null), while rejecting this proposition, it emerged that its effect disappeared statistically (OR 0.75, 95% CI 0.35-1.61, $P = 0.58$), when minor alleles A of SNPs rs1800780 and rs3918181 at positions 3 and 4 in the haplotype CTAAAT were replaced with major alleles G of SNPs rs1800780 and rs3918181 in the haplotype CTGGAT. In the pairwise SNP-SNP interactions, the present study showed that SNP rs2070744 showed an epistatic effect on SNPs rs1799983 and rs3918181 for the association of ED (RHI) in osteoporotic women. This further added to the view point that although these SNPs within the eNOS gene participated, their effect was different when analyzed individually and altogether different when their interactions affected some underlying variables such as RHI, LDL-C, TG, TC, and BMI (Table 4).

First of all, older and weak skeletal vasculature supply reduced levels of minerals, nutrients, and immune cells to the bone for repairing older bone fragments and micro-fractures.³⁰ Secondly, in postmenopausal women, huge losses of sinusoidal and arterial capillaries accelerate bone density loss.¹ Thirdly, during the postmenopausal phase of life

TABLE 6. Functional effect of susceptibility haplotype for the best fit model

Effect	$\beta \pm SE^a$	Susceptibility haplotype, CTAAAT		
		Wald (P)	R ² _h	AIC
Dominant	2.75 ± 0.97	2.85 (0.004)	0.9778	2032.69
Recessive	0.28 ± 0.33	0.84 (0.396)	0.8743	2351.78
Multiplicative	2.11 ± 0.63	3.35 (0.00008)	1.000	1030.51
General	-0.42 ± 0.63	-0.67 (0.500)	0.5759	1730.79
General	2.26 ± 0.77	2.93 (0.0033)	0.5759	1730.79

Model effect with adjusted risk covariates; body mass index, systolic blood pressure, triglyceride levels, and BMDs at femoral neck and lumbar spine.

Multiplicative effect (women having 1 copy of the haplotype are at intermediate risk than women having no copy or 2 copies).

Values in bold face show lowest AIC and highest R²_h values.

AIC, Akaike information criterion.

^aEstimated haplotype effect, P-asymptotic value, R²_h-haplotype uncertainty measure.

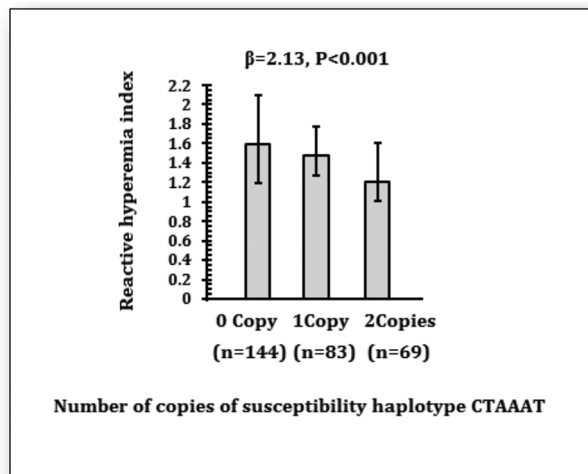


FIG. 2. Association of reactive hyperemia index according to the number of copies of susceptibility haplotype CTAAAT of eNOS gene. Values of reactive hyperemia index were corrected with the effect of body mass index, systolic blood pressure, triglyceride levels, and BMDs at femoral neck and lumbar spine. Error bars represent 95% confidence intervals. All double, triple, and multiple heterozygotes ($n = 139$) were excluded from the analysis. BMD, bone mineral density.

women suffer a marked loss of bone perfusion, which is significantly correlated with reduced BMD.³¹ The present study aligns with the above mentioned studies and confirmed that the progressive increase in endothelial dysfunction is in sync and associated with decreased BMD in postmenopausal women (Fig. 1). Moreover, this link is observed to be genetic as susceptibility haplotype CTAAAT within the eNOS gene is significantly associated with RHI.

CONCLUSIONS

In conclusion, the present study observed a susceptibility haplotype CTAAAT within the eNOS gene, where CTAAAT carriers have almost doubled the possibility of endothelial dysfunction associated osteoporosis compared with non-carriers. Results of the present study also explained that the genetic relationship of endothelial dysfunction and osteoporosis exists in postmenopausal osteoporotic women from Punjab, India.

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