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# Polymorphisms in the Endothelial Nitric Oxide Synthase Gene and Bone Density/Ultrasound and Geometry in Humans

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## Abstract

Nitric oxide (NO), produced by endothelial cells, is a signaling molecule synthesized from L-arginine by nitric oxide synthases (NOS). NO is known to reduce the ratio of Receptor Activator of Nuclear factor KappaB (RANKL)/Osteoprotegerin (OPG), leading to decreased osteoclastogenesis and a reduction in bone resorption. Endothelial nitric oxide synthase (*eNOS* or *NOS3*) is the predominant constitutive isoform of nitric NOS within bone. Recently, a *NOS3* polymorphism, Glu298Asp, previously implicated in osteoporosis, failed to demonstrate an association with bone mineral density (BMD), although there was some indication of an association with selected geometry indices. Since a single polymorphism does not capture all of the potential variants in a given gene, we investigated a broader coverage of the *NOS3* gene with bone density/ultrasound and geometry indices in a sample of unrelated individuals from the Framingham Offspring Study. Our results indicated that the Glu298Asp polymorphism was not associated with BMD but suggested some haplotype-based associations in the linkage disequilibrium (LD) region that included the Glu298Asp polymorphism with several geometry indices. Although our findings exhibited several associations with selected bone density/ultrasound and geometry indices, the nominally significant associations are regarded as primarily hypothesis generating and suggest that replication in other samples is needed. Thus, *NOS3* genetic variation does not appear to be a major contributor to adult bone density/ultrasound and geometry in our sample.

### Keywords

Nitric oxide synthase; Bone density/ultrasound; Bone geometry; Genetic polymorphisms; Osteoporosis

## INTRODUCTION

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing an individual to increased risk of fracture [1]. There are over 1.5 million osteoporotic fractures annually in the United States, with hip fracture remaining the most severe clinical outcome of

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age-related osteoporosis due to its high prevalence, serious effects on quality of life and excessive economic costs [2–6].

Strength of osteoporotic bones is compromised mainly by age-related changes in the amount and distribution of bone tissue, determinants of fracture risk collectively referred to as bone quality. These aspects of bone quality are quantified by bone mineral density (BMD) and structural geometry, which determine the way stresses produced by loading forces are transmitted through the bone. Both bone mass and geometry may be assessed non-invasively by dual x-ray absorptiometry (DXA) based techniques, which, along with quantitative ultrasound (QUS) [7], provide measures of bone size and mineral distribution that allow for indirect evaluation of influences on bone mechanical properties. There is substantial evidence suggesting that bone mass and geometry are largely determined by genes in both humans [8, 9] and animals [10].

Nitric oxide (NO) is a signaling molecule synthesized from L-arginine by nitric oxide synthases (NOS) [11]. In bone, NO inhibits osteoclastic bone resorption *in vitro* and regulates bone remodeling *in vivo*; thus, drugs and conditions that cause local increase in NO formation in bone may have positive effects on bone remodeling. Among other isoforms, endothelial NOS (*eNOS* or *NOS3*) is the predominant constitutive isoform of NOS within bone. *NOS3* also plays a role in mediating estrogen-induced bone formation in female mice, possibly as a consequence of post-transcriptional regulation of *NOS3* activity by estrogen [12]. NO also decreases the ratio of Receptor Activator of Nuclear factor KappaB (RANKL)/Osteoprotegerin (OPG) leading to a decreased recruitment of osteoclasts and a reduction in bone resorption [13]. The observation that experimental fractures in mice increase the levels of mRNA for NOS [14] supports a recent study by Baldik and colleagues [15] showing the importance of NOS in fracture healing.

A recent study reported a lack of consistent associations between the most studied *NOS3* polymorphism, Glu298Asp (rs1799983), and bone phenotypes among older women [16]. Since this SNP may not explain the variation of the entire gene, we therefore investigated the *NOS3* gene region with broader coverage by eighteen polymorphisms, including Glu298Asp, in a large sample of men and women. We hypothesized that by increasing coverage of the gene, we would be able to discern associations between SNP variants and bone density/ultrasound and geometry at the lumbar spine, proximal femur, and heel.

## METHODS

### Study Participants

The Framingham Heart Study (FHS) Original Cohort began in 1948 with the primary goal of evaluating risk factors for cardiovascular disease. The FHS Original Cohort participants, initially aged 28–62 years, represented two thirds of the households of the Framingham, MA population and have been examined every 2 years since baseline. In 1971, the Framingham Offspring Cohort Study was initiated mainly to evaluate the role of genetic factors in the etiology of coronary artery disease. The design of the FHS Offspring Cohort has been described previously [17]. The Offspring Cohort is comprised of 2616 adult offspring of couples from the Framingham Original Cohort (51%), 898 adult offspring of one parent in the Original Cohort at greater risk of cardiovascular disease (17.5%), 34 stepchildren (<1%) and 1576 spouses of these individuals (30.8%). Nearly all (96.4%) of the Offspring Cohort are Caucasians, with origins in Eastern and Western Europe. Neither the Framingham Original nor Offspring Cohort was selected on the basis of cardiovascular diseases or osteoporosis. The Offspring Cohort members participated in the Framingham Osteoporosis Study between 1996 and 2001, as described elsewhere [18]. Details and descriptions of the Framingham Osteoporosis Study have also been reported [19,20]. The study was approved by the

Institutional Review Board for Human Subjects Research of Boston University and the Institutional Review Board at Hebrew SeniorLife.

Participants in the current study included a subset of unrelated individuals from the Framingham Offspring Cohort who provided blood samples for DNA and who had bone mineral density/ultrasound or geometry measurements. Among these, 1451 (men 47%, 61.4 ± 9.2 years and women 53%, 60.4 ± 9.0 years) not using nitrate and/or osteoporosis medications also had *NOS3* genotyping completed and were included in the analysis of bone density/ ultrasound/hip geometry. Among these 1451 participants with *NOS3* genotyping, 1450 had bone density by DXA, 1404 had bone ultrasound, 1264 had hip geometry, and 1403 of these participant had both bone density and ultrasound.

## Bone Mineral Density (BMD) and Quantitative Ultrasound (QUS)

From 1996 to 2001, the participants underwent bone densitometry of the spine and hip (femoral neck and trochanter) by DXA (Lunar DPX-L) to measure BMD ($g/cm^2$). The coefficients of variation (CV) in normal subjects for the DPX-L were 0.9% (spine), 1.7% (femoral neck) and 2.5% (trochanter) (7). Using a portable QUS device, the Sahara bone sonometer (Hologic, Inc., Waltham, MA), we measured right calcaneal broadband ultrasound attenuation (BUA). Based on duplicate, same-day measurements on 29 subjects, the CV for BUA was 5.3% [21].

## Femoral Geometry by Hip Structural Analysis (HSA)

An interactive computer program, HSA, developed by Beck and colleagues was used to derive a number of structural variables from the femoral DXA scans [22,23]. The following four regions were assessed: the narrowest width of the femoral neck (NN), which overlaps or is proximal to the standard Lunar femoral neck region; an intertrochanteric (IT) region located along the bisector of the neck-shaft angle (NSA); and the femoral shaft (S), distal to the lesser trochanter. HSA provided measures of bone cross-sectional area (CSA), section modulus (Z), subperiosteal width (WID), and average buckling ratio (AvgBR) at each of the 3 femoral regions (NN, IT, and S), as well as NSA and femoral neck length (FNL), defined as the distance from the center of femoral head to intersection of neck and shaft axes). The cortices of the shaft and narrow neck regions were modeled as previously described [22] by concentric circular annuli with 60% and 100% of the measured mass in the cortex, respectively. The intertrochanteric region assumed a concentric elliptical annulus, with 70% cortical mass and the anteroposterior diameter used the measured outer diameter of the shaft region. Thus, there were 14 phenotypes overall. Coefficients of variation for the different geometric measures were previously reported to range from 3.3 (NN_WID) to 9.1% (FNL) [23].

## Other Variables

At the time of bone density/ultrasound and geometry measurements, other variables were obtained including age, sex, weight, height, and for women, estrogen use and menopausal status. These variables along with overall medical history have been described previously [24,25]. Weight was measured using a standardized balance beam scale [26]. Height was measured to the nearest ¼ inch using a stadiometer. Body mass index (BMI) was then calculated in $kg/m^2$. Each woman was assigned to one of the two estrogenic status groups: 1) premenopausal or postmenopausal on estrogen (estrogen-replete) or 2) postmenopausal not on estrogen (estrogen-deplete) where menopause was defined as having no menstrual period for at least one year. Of the total 762 women, there were 368 estrogen-replete women.

## Genotyping Methods

The genotyping protocol has been described in a previous study [27]. Genotyping was performed at the Broad Institute of the Massachusetts Institute of Technology/Harvard

University by using matrix assisted laser desorption ionization–time of flight mass spectrometry (Sequenom) to resolve allele-specific single-base extension products.

## SNP Selection and Genotyping

The 18 *NOS3* SNP variants were selected from a previous study conducted by Kathiresan and colleagues [28] in which 33 SNPs were first selected on the basis of the underlying common variation using the LD patterns in the Centre d'Etude du Polymorphisme Humain (CEPH) panel (Coriell Institute for Medical Research, Camden, NJ) [27]. Of the 33 SNPs, 11 SNPs were selected as tag SNPs with customized software (http://www.broad.mit.edu/mpg/tagger/), which represented the minimum subset of SNPs required to predict all common haplotypes (≥5% frequency) within each block, with $r^2 \geq 0.9$. This set of 11 tag SNPs was then genotyped in the FHS samples. In addition, 7 other SNPs were genotyped in the Framingham samples: the previously reported common missense variant, Glu298Asp (rs1799983, SNP 8), [29–33] the previously studied $T^{-7863} \rightarrow C$ promoter variant (rs2070744, SNP 3), and 5 SNPs that were redundant in CEPH pedigrees (to help assess LD block structure similarity between CEPH and FHS samples; data not shown). Thus, this set of 18 *NOS3* SNPs genotyped in FHS was used in our study.

There were three "haplotype blocks" (Table 1) with evidence of strong LD defined among the 18 SNPs capturing 97%, 83%, and 98% of common haplotypic variation, respectively [28]. Several common haplotypes with the haplotype frequency exceeding 5% were observed in the sample: 4, 6 and 3 common haplotypes in LD regions 1, 2 and 3, respectively, SNPs 1–5 made up LD region 1, but the four common haplotypes were composed by SNPs 1–4 and thus only these were considered in the analysis. LD region 2 consisted of SNPs 6–14; however, only SNPs 7, 9–11, 13 and 14 were analyzed since these contributed in the six resulting common haplotypes. The third LD region was defined with SNPs 15 and 16.

## Statistical Analysis

Sex-specific analyses were performed using analysis of covariance (ANCOVA) methods (GLM procedure, SAS Inc), with bone density/ultrasound and geometry measures adjusted for age, BMI, height and estrogen status (women). For the association of *NOS3* SNPs with bone density/ultrasound measures, we compared three genotype groups (2 degrees of freedom) for each SNP. In the analysis with geometry traits, we compared three genotype groups (2 degrees of freedom) for all SNPs, except for SNP 14 (rs3918196; minor allele frequency 6%), where the minor allele homozygotes were not observed in the study sample due to fewer individuals having bone geometry measures.

Haplotype-based association analyses for bone density/ultrasound and geometry measures, adjusted for age, BMI, height and estrogen status (women), were performed using HAPLO.STATS (http://www.mayo.edu/hsr/people/schaid.html). The HAPLO.SCORE function in HAPLO.STATS estimates haplotype frequencies with the expectation-maximization (EM) algorithm and provides global score statistics that test whether trait differences exist among all haplotypes simultaneously. Haplotype-specific score statistics are also obtained to test whether trait differences exist between a single haplotype vs. all other haplotypes combined.

To adjust for the number of statistical tests in our analysis, we performed sex-specific permutation tests for the bone density/ultrasound and geometry measures, separately. The set of phenotypes and covariates for each individual was randomly permuted while keeping the genotype LD information and the phenotype correlation intact, then the permuted dataset was analyzed. This procedure was repeated 10,000 times. We calculated adjusted p-values based on the number of minimum permuted p-values less than the observed p-value.

# RESULTS

## Single Nucleotide Polymorphisms

Table 1 displays the eighteen *NOS3* SNPs evaluated in the current analyses. All SNPs in this study were in Hardy-Weinberg equilibrium [28]. Minor allele frequencies of SNPs ranged from 6% to 46%, with no significant differences in the genotype frequencies by sex.

## Subject Characteristics

Table 2 displays descriptive characteristics of the Framingham Osteoporosis study participants by sex for the measures of bone density/ultrasound and geometry indices. Both men and women were of similar age (mean age 61 years) and had similar BMI measures (mean of 28 $kg/m^2$). Among 762 women, 47.8% were classified as "estrogen replete" (either premenopausal or postmenopausal women taking estrogen). In general, compared to women, men had higher density/ultrasound measures by 0.11–0.16 $g/cm^2$ for femoral neck, trochanter, and spine BMD and by 11 db/mHz for BUA. For the hip geometry traits, men generally had greater values as well, such as with CSA, Z and WID.

## Individual SNP Associations with Bone Density/Ultrasound or Geometry Traits

Table 3 contains the significant ($p<0.05$) sex-specific associations in either men or women between bone density/ultrasound and geometry traits and the *NOS3* SNPs. Among men only, we found a significant association between femoral neck BMD and SNP 15 (rs753482; T>G). Participants homozygous for the minor allele of SNP 15 had lower femoral neck BMD than the major allele homozygotes (0.99±0.01 and 0.93±0.02 for TT and GG genotypes, respectively; p=0.031). The heterozygote group (GT) did not differ significantly from the other two genotypes (p=0.15 and p=0.08 for GG and TT, respectively). None of the other SNPs was significantly associated with bone density/ultrasound traits in men. In women, we observed significant associations between BUA and SNP 1 (rs10952296; C>A), SNP 2 (rs1800783; T>A), and SNP 3 (rs2070744; T>C). Among the three genotype groups, the heterozygote group had significantly lower BUA measure compared to the other two homozygote groups for SNP 1. For SNPs 2 and 3, the significant associations were driven by the difference between the heterozygote and major homozygote genotype group with higher measures of BUA. The minor homozygote groups did not differ significantly from the other two groups. These three SNPs are located in the 5' upstream and intron 1 area (Table 1).

We also observed several significant associations with geometry traits in men and women (Table 3). Among men, we observed significant associations between SNP 16 (rs1065299; G>T) and IT_Z and between SNP 12 (rs2853795; A>G) and NSA. In both cases, men either heterozygous or homozygous for the major allele ($p<0.05$) had higher trait mean values compared to those with minor allele homozygote group. In addition, SNP 14 (rs3918196; A>G) was significantly associated with S_CSA (p=0.018) and S_AvgBR (p=0.043), where men homozygous for the major allele had higher trait mean values compared to the heterozygote group. In women, we found significant associations between SNP 4 (rs1800781; G>A) and IT_WID and NN_WID (p=0.013 and p=0.020) and between SNP 17 (rs11760487; G>A) and IT_Z (p=0.026); these associations were driven by the minor allele homozygote group with genotype frequency of only 2% in the sample. In addition, a significant association was observed between SNP 10 (rs1800780; G>A) and FNL, where women either heterozygous or homozygous ($p<0.05$) for the major allele of SNP 10 had higher FNL mean values compared to those with minor allele homozygote group (5.20±0.06, 5.40±0.04 and 5.30±0.05 for AA, AG, and GG genotypes, respectively; p=0.014).

### Haplotype Associations with Bone Density/Ultrasound or Geometry Traits

Using the haplotype block (LD region) definitions of Kathiresan et al. [28], we summarized selected results from haplotype analysis in Tables 4 and 5. Table 4 displays selected haplotype analysis results for bone density/ultrasound. We performed individual haplotype analyses comparing to all other haplotypes combined since the global test tends to under-represent small but significant effects in association. In women we found significant associations between haplotype 1A (CTTA, 36%) in LD region 1 and BUA (p=0.011) (Table 4a). This region included the three SNPs that were shown to be individually significant with the same phenotype. In men, haplotype 3C (GG, 23%) in LD region 3 (Table 4b), which consisted of SNPs 15 and 16, exhibited a significant association with femoral neck BMD (p=0.009). As seen from the individual SNP analysis in men, this association was driven by the association of SNP 15. Thus, despite several significant results obtained from haplotype analyses, these results did not provide additional information beyond those obtained from individual SNP analyses of bone density/ultrasound indices.

Table 5 presents selected haplotype analysis results for geometry traits. In men, four geometry indices showed significant haplotype-specific associations in LD region 2, which consisted of SNPs 7, 9–11, 13 and 14. In this region, the common haplotype 2A (AAACGG, 29%) was significantly associated with IT_CSA (p=0.003) and IT_Z (p=0.011). Additionally, we observed significant associations of two other rare haplotypes, 2C (GAGAAG, 8%) and 2E (AGGCAG, 7%), with IT_AvgBR (p=0.039) and FNL (p=0.008), respectively. No other significant relationships were observed.

### Accounting for Multiple Testing

To correct for multiple testing, we constructed 10,000 permuted data sets and found that empirical probability values exceeded 0.05 for all bone density/ultrasound and geometry indices in both men and women. In the analysis of bone density/ultrasound, the minimum nominal p-value was 0.001 in women for BUA and SNP 1; however the corresponding adjusted p-value was 0.07. For geometry measures, the minimum nominal p-value was 0.003 in men for IT_CSA in LD region 2 with its corresponding adjusted p-value of 0.28. All other corresponding adjusted p-values for nominally significant p-values are presented in the Tables 3–5.

## DISCUSSION

In our sample from the Framingham Osteoporosis Study, we evaluated multiple polymorphisms in the region of the *NOS3* gene as they related to a range of bone phenotypes, including BMD of the hip and spine, heel ultrasound, and proximal hip geometry indices. Prior association studies have mainly focused on the Glu298Asp polymorphism (rs1799983, SNP 8 in our study) of the *NOS3* gene and bone phenotypes. Thus, a recent study by Taylor and colleagues [16] implicated the Glu298Asp polymorphism in osteoporotic fracture yet failed to demonstrate an association with BMD, although there was some indication of an association with selected geometry indices. The magnitude of the differences in calcaneal BMD loss and hip geometric indices attributed to this SNP was small, and adjustment for age, BMI, height and estrogen status (women) did not explain the fracture results. Thus, although the non-synonymous SNP, Glu298Asp, may have importance in non-skeletal phenotypes, there was no evidence that it contributed to pathways involved in the determination of bone mass or bone quality/strength. Also, the magnitude of the association between the *NOS3* genotypes and hip fracture was relatively small *per se*.

Despite the similarities in the design and phenotypes studied by us to those of Taylor et al. [16], there are several differences. Their study population included only older white women

and evaluated a single polymorphism. We investigated a broader coverage of the *NOS3* gene with eighteen SNPs (including the Glu298Asp polymorphism). In addition, our study sample consisted of both men and women, unrelated individuals from a general population of Caucasians (Framingham Heart Study), which afforded a comparison of *NOS3* actions in men and women. Furthermore, in our study, we excluded participants who used nitrate and/or osteoporosis medications and adjusted bone density/ultrasound and geometry measures for age, BMI, height and estrogen status (in women), while Taylor et al. [16] did not include similar variables in their models of BMD and bone geometry. Also, in Taylor's study, there was no attempt to adjust for multiple comparisons.

Our findings did not confirm the association for the Glu298Asp (SNP 8) polymorphism with BMD; however, an association between a different *NOS3* polymorphism, SNP 1, and BUA in LD region 1 in women, was found. This observed association with SNP 1 in women was further investigated after stratifying women into an estrogen-replete group (premenopausal women and postmenopausal women taking estrogen) and an estrogendepleted group (postmenopausal women not taking estrogen). We found that the results were driven by the estrogen deplete group, suggesting the possibility of an interaction with endogenous estrogen or estrogen therapy; however considering multiple testing and the reduced power of this stratified analysis, these results should be replicated in a larger sample of women. LD region 3, which consists of SNPs 15 and 16, exhibited associations with femoral neck BMD in men. Also, SNP 16 in men and nearby SNP 17 in women were both significantly associated with IT_Z. Additionally in men, we observed several haplotype-based associations in LD region 2, which included Glu298Asp polymorphism, with four geometry traits, FNL, IT_AvgBR, IT_CSA and IT_Z. The common haplotype in this region was associated with IT_CSA and IT_Z, while the rare haplotype was associated with IT_AvgBR; thus these results indicated a localized association of LD region 2 with intertrochanteric geometry in men. Despite our significant associations with geometry indices, these results are not identical to the recent findings of Taylor et al., who found in older women that NN_Z and NN_CSA were the only traits associated with the Glu298Asp polymorphism [16]; however, they point in the same direction. Taylor et al. [16] concluded that variations in the Glu298Asp polymorphism were associated with lower bone strength indices, but not BMD; we observed association between *NOS3* LD region 2, which includes Glu298Asp polymorphism, to hip geometry rather than to BMD or QUS measures.

We interpret our results with caution, as our findings provide only nominally significant relationships and fail to retain the level of significance when accounting for multiple testing. Although none of the adjusted p-values reached statistical significance, we took the most conservative approach of permutation without a priori hypothesis of the relations of any specific SNPs and phenotypes in our study. As a result, these nominally significant results should not be completely overlooked but rather be considered as hypothesis-generating. We provide both nominal and multiple-testing adjusted p-values so that the reader may interpret these results for themselves.

As noted above, we performed analyses stratified by sex and found that the different regions of the gene were associated with various bone traits in men and women. This finding is not surprising given previous reports of gender-specific linkage of osteoporosis-related phenotypes [34], including a recent meta-analysis of BMD linkage studies [35]. Furthermore a large body of literature has demonstrated that there are genetic and gene-by-environment differences between the genders [36–38].

Our findings thus do not confirm a strong role for the *NOS3* gene in bone density/ultrasound and geometry phenotypes, although the biological role of the nitric oxide synthases is well established. First, NO has been implicated in osteoporosis [11] and bone maintenance, as is evidenced from both animal (39, 44) and human studies (14). Also Loveridge et al. [39]

concluded that the normal regional and osteonal pattern of *NOS3* expression by osteocytes was disrupted in hip fracture, particularly at skeletal sites most loaded by physical activity. A reduction in a number of *NOS3*-expressing osteocytes, coupled to an increase in their remoteness from canal surfaces (and blood supply), may thus contribute to fracture healing and possibly to the fragility of osteoporotic bone [39]. Indeed, hip fracture cases showed *NOS3*-expressing osteocytes only in deep osteonal bone, and 25–35% reduced expression overall in femoral neck cortex [39].

There are several limitations to our study. First, the HSA method conceptualizes a three-dimensional model of the proximal femur, but employs two-dimensional projections produced by DXA technology. Section modulus and AvgBR are relevant for bending resistance and buckling, respectively, only in the plane of the image; thus out-of-plane differences in geometry may be unrecognized. The method assumes that bone tissue mineralization is fixed so that any differences in mineral quantity or distribution are expressed geometrically; there may thus be some problems with bone edge detection particularly in osteoporotic bones. Conceivably, some genetic factors may influence average tissue mineralization, which in turn might confound geometry measured by this method. However, we would capture those seemingly pleiotropic variants in our study, since we also analyzed conventional BMD. Second, our use of DXA, a common skeletal imaging modality that is most suitable for large scale population studies like ours, does not capture intrinsic material or micro-architectural properties of bone. [40]. Third, our findings are generalizable only to Caucasians. Despite these limitations, non-invasive measurements of bone mass and geometry by DXA and other imaging modalities are the only practical approach to genetic studies of risk factors of bone fragility in a general population sample of humans.

An additional limitation of our study was that we used proxies to the osteoporotic fracture phenotype (BMD, QUS and hip geometry) and not the fracture itself. It is known that genetic contribution to a risk factor may differ from the ultimate disease phenotype.(1, 7, 40, 41) At present, we are performing a detailed assessment of osteoporotic fractures in the Framingham Offspring Cohort. Another way to investigate the contribution of the *NOS3* region to osteoporosis includes using longitudinal phenotypic measurements overtime; an approach that we plan for future analyses.

Although our results reveal several nominally significant associations with selected bone mass and geometry indices in a large sample of men and women, as well as contribute additional information about the *NOS3* gene region, these associations are regarded as primarily hypothesis-generating and will require replication in other samples or may contribute to future meta-analyses. Our study suggests that *NOS3* genetic variation does not appear to be a major contributor to adult bone density/ultrasound or geometry in our sample.

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## LIST OF ABBREVIATIONS

***NOS3***

endothelial nitric oxide synthase

**BMD**

bone mineral density

**AvgBR**
average buckling ratio

**BMI**
body mass index

**CSA**
cross-sectional area

**DXA**
dual x-ray absorptiometry

**FNL**
femoral neck length

**HAS**
hip structural analysis

**IT**
intertrochanteric

**NN**
narrowest neck

**NSA**
neck-shaft angle

**QUS**
quantitative ultrasound

**S**
femoral shaft

**WID**
subperiosteal width

**Z**
section modulus

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**Table 1**

***NOS3* 18 SNPs**

| SNP number | SNP rs name | LD Region | Position on Chr7 (UCSC coordinates) | Variant Type | Alternate nucleotides | Minor Allele Frequency |
|---|---|---|---|---|---|---|
| 1 | rs10952296 | 1 | 150076571 | 5′ Upstream | C>A | 0.26 |
| 2 | rs1800783 | 1 | 150081138 | Intron 1 | T>A | 0.38 |
| 3 | rs2070744 | 1 | 150081820 | Intron 1 | T>C | 0.4 |
| 4 | rs1800781 | 1 | 150084185 | Intron 3 | G>A | 0.14 |
| 5 | rs3918169 | 1 | 150086347 | Intron 5 | A>G | 0.14 |
| 6 | rs1549758 | 2 | 150087467 | Coding, Synonymous | C>T | 0.31 |
| 7 | rs1007311 | 2 | 150087749 | Intron 7 | G>A | 0.44 |
| 8 | rs1799983 (Glu298Asp) | 2 | 150087852 | Coding,Nonsynonymous | G>T | 0.34 |
| 9 | rs3918174 | 2 | 150089035 | Intron 9 | A>G | 0.14 |
| 10 | rs1800780 | 2 | 150090620 | Intron 12 | G>A | 0.46 |
| 11 | rs3918188 | 2 | 150094522 | Intron 14 | C>A | 0.37 |
| 12 | rs2853795 | 2 | 150094983 | Intron 14 | A>G | 0.18 |
| 13 | rs891511 | 2 | 150096584 | Intron 17 | G>A | 0.34 |
| 14 | rs3918196 | 2 | 150097583 | Intron 17 | G>A | 0.06 |
| 15 | rs753482 | 3 | 150098124 | Intron 19 | T>G | 0.22 |
| 16 | rs1065299 | 3 | 150101312 | Intron 24 | G>T | 0.34 |
| 17 | rs11760487 | | 150111812 | 3′ Flanking | G>A | 0.14 |
| 18 | rs12666075 | | 150112049 | 3′ Flanking | G>T | 0.16 |

## Table 2

### Subject characteristics

| Characteristics | Males N=689 | Females N=762 |
|---|---|---|
| Age (years) | 61.4 ± 9.2 | 60.4 ± 9.0 |
| BMI(kg/m$^2$) | 28.7 ± 4.6 | 27.5 ± 5.5 |
| Height(inches) | 69.1 ± 2.7 | 63.5 ± 2.5 |
| Estrogen status positive† | --- | 368 (48.3%) |
| **Bone Density/Ultrasound** | | |
| Neck BMD (g/cm$^2$) | 0.98 ± 0.14 | 0.87 ± 0.14 |
| Trochanter BMD (g/cm$^2$) | 0.89 ± 0.14 | 0.72 ± 0.13 |
| Spine BMD (g/cm$^2$) | 1.33 ± 0.21 | 1.17 ± 0.20 |
| BUA (db/mHz) | 83.27 ± 19.09 | 71.91 ± 18.16 |
| **Bone Geometry*** | | |
| NSA | 129.5 ± 4.61 | 127.78 ± 5.01 |
| FNL | 6.12 ± 0.75 | 5.33 ± 0.61 |
| IT_AvgBR | 11.79 ± 3.06 | 13.06 ± 3.47 |
| IT_CSA | 5.00 ± 0.82 | 3.81 ± 0.69 |
| IT_Z | 5.52 ± 1.15 | 3.60 ± 0.87 |
| IT_WID | 6.71 ± 0.65 | 5.92 ± 0.55 |
| NN_AvgBR | 15.08 ± 4.23 | 14.34 ± 3.59 |
| NN_CSA | 2.85 ± 0.43 | 2.35 ± 0.37 |
| NN_Z | 1.95 ± 0.39 | 1.43 ± 0.30 |
| NN_WID | 3.95 ± 0.43 | 3.50 ± 0.38 |
| S_AvgBR | 4.15 ± 1.14 | 5.00 ± 1.36 |
| S_CSA | 4.47 ± 0.60 | 3.36 ± 0.53 |
| S_Z | 2.90 ± 0.48 | 2.02 ± 0.40 |
| S_WID | 3.59 ± 0.35 | 3.36 ± 0.31 |

Values are mean ± SD, unless otherwise specified.

† Estrogen positive includes premenopausal women and postmenopausal women taking estrogen.

* There are 187 less individuals (total 610 men and 654 women) due to missing values.

**Table 3**

**Association of selected *NOS3* SNPs and bone density/ultrasound and geometry (p-value<0.05)**

| | Genotype | n | Adjusted Mean ± SE | p-value* |
|---|---|---|---|---|
| **Men** | | | | |
| **Bone Density/Ultrasound** | | | | |
| SNP 15: Neck BMD | TT | 376 | 0.99±0.01 | 0.03 (0.74) |
| | GT | 230 | 0.97±0.01 | |
| | GG | 35 | 0.93±0.02 | |
| **Bone Geometry** | | | | |
| SNP 12: NSA | AA | 373 | 129.11±0.23 | 0.033 (0.57) |
| | AG | 159 | 130.20±0.36 | |
| | GG | 12 | 128.65±1.30 | |
| SNP 14: S_CSA | AG | 77 | 4.61±.0.06 | 0.018 (0.52) |
| | GG | 515 | 4.46±0.02 | |
| SNP 14: S_AvgBR | AG | 77 | 3.90±0.12 | 0.043 (0.57) |
| | GG | 515 | 4.17±0.05 | |
| SNP 16: IT_Z | GG | 271 | 5.44±0.06 | 0.018 (0.52) |
| | GT | 262 | 5.66±0.06 | |
| | TT | 60 | 5.40±0.13 | |
| **Women** | | | | |
| | Genotype | n | Adjusted Mean ± SE | p-value* |
| **Bone Density/Ultrasound** | | | | |
| SNP 1: BUA | CC | 370 | 72.73±0.89 | 0.001 (0.07) |
| | AC | 261 | 69.25±1.06 | |
| | AA | 38 | 79.23±2.79 | |
| SNP 2: BUA | TT | 284 | 74.25±1.01 | 0.006 (0.28) |
| | AT | 377 | 70.09±0.88 | |
| | AA | 91 | 72.37±1.81 | |
| SNP 3: BUA | TT | 259 | 74.43±1.07 | 0.005 (0.23) |
| | CT | 354 | 69.96±0.92 | |
| | CC | 88 | 71.28±1.86 | |
| **Bone Geometry** | | | | |
| SNP 4: IT_WID | AA | 14 | 6.30±0.14 | 0.014 (0.48) |
| | AG | 153 | 5.88±0.04 | |
| | GG | 471 | 5.92±0.02 | |
| SNP 4: NN_WID | AA | 14 | 3.74±0.10 | 0.02 (0.54) |
| | AG | 153 | 3.46±0.03 | |
| | GG | 471 | 3.50±0.02 | |
| SNP 10: FNL | AA | 106 | 5.20±0.06 | 0.013 (0.49) |
| | AG | 226 | 5.40±0.04 | |
| | GG | 163 | 5.30±0.05 | |
| SNP 17: IT_Z | AA | 12 | 3.55±0.18 | 0.026 (0.56) |
| | AG | 142 | 3.48±0.05 | |
| | GG | 456 | 3.65±0.03 | |

* Permuted p-values in parentheses.

## Table 4

### Relations of selected *NOS3* LD regions to bone density/ultrasound indices†

**Table 4a. Relations of *NOS3* LD region 1 haplotypes to BUA in women**

| | SNP 1 (rs10952296) | SNP 2 (rs1800783) | SNP 3 (rs2070744) | SNP 5 (rs3918169) | Haplotype Frequency | Haplotype p-value* | Global p-value |
|---|---|---|---|---|---|---|---|
| Haplotype 1A | C | T | T | A | 0.36 | **0.011** (0.33) | 0.094 |
| Haplotype 1B | A | T | T | A | 0.25 | 0.764 | |
| Haplotype 1C | C | A | C | A | 0.23 | 0.112 | |
| Haplotype 1D | C | A | C | G | 0.14 | 0.463 | |

**Table 4b. Relations of *NOS3* LD region 3 haplotypes to Neck BMD in men**

| | SNP 15 (rs753482) | SNP 16 (rs1065299) | Haplotype Frequency | Haplotype p-value* | Global p-value* |
|---|---|---|---|---|---|
| Haplotype 3A | T | G | 0.45 | 0.206 | **0.034** (0.27) |
| Haplotype 3B | T | T | 0.32 | 0.317 | |
| Haplotype 3C | G | G | 0.23 | **0.009** (0.31) | |

† Bold font where p-values < 0.05.

* Permuted p-values in parentheses.

**Table 5**

**Relations of *NOS3* LD region 2 haplotypes to hip geometry indices in men[†]**

| | SNP 7 (rs1007311) | SNP 9 (rs3918174) | SNP 10 (rs1800780) | SNP 11 (rs3918188) | SNP 13 (rs891511) | SNP 14 ( rs3918196) | Haplotype Frequency | Haplotype p-value[*] FNL | IT_AvgBR | IT_CSA | IT_Z |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype 2A | A | A | A | C | G | G | 0.29 | 0.218 | 0.138 | **0.003** (0.28) | **0.011**(0.68) |
| Haplotype 2B | G | A | G | A | G | G | 0.25 | 0.533 | 0.991 | 0.991 | 0.678 |
| Haplotype 2C | G | A | G | A | A | G | 0.08 | 0.958 | **0.039** (0.96) | 0.386 | 0.792 |
| Haplotype 2D | A | A | G | C | A | G | 0.07 | 0.605 | 0.704 | 0.807 | 0.932 |
| Haplotype 2E | A | G | G | C | A | G | 0.07 | **0.008** (0.57) | 0.992 | 0.314 | 0.060 |
| Haplotype 2F | A | G | A | C | A | G | 0.06 | 0.324 | 0.722 | 0.460 | 0.455 |
| | | | | | | | Global p-value | 0.189 | 0.392 | 0.080 | 0.118 |

[†] Bold font where p-values < 0.05.

[*] Permuted p-values in parentheses.