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Effects of Polymorphisms of the Sex Hormone-Binding Globulin (SHBG) Gene on Free Estradiol and Bone Mineral Density

Nicola Napoli[1,2], **Ana Varadharajan**[3], **Giovam Batista Rini**[4], **Romano Del Fiacco**[4], **Jayasree Yarramaneni**[1], **Steven Mumm**[1], **Dennis T. Villareal**[5], and **Reina C. Armamento-Villareal**[1]

[1]Division of Bone and Mineral Diseases at Washington University School of Medicine, St. Louis, MO

[2]Division of Endocrinology, University Campus Bio-Medico, Rome, Italy

[3]Internal Medicine, St. Luke's Hospital, St. Louis, MO, USA; Department of Internal Medicine, University ''La Sapienza", Rome, Italy

[4]University Of Palermo, Palermo, Italy

[5]Division of Geriatrics and Nutritional Science at Washington University School of Medicine, St. Louis, MO

Abstract

Background—Polymorphisms of the sex hormone binding globulin (SHBG) gene are associated with differences in SHBG levels, influencing the risk for breast cancer and polycystic ovarian syndrome, but no association has been reported for osteoporosis in post-menopausal women.

Objective—To determine the effect of G to A substitution in the 5′UTR (rs1799941) and the Asp356Asn (rs6259) polymorphisms of the SHBG gene on bone mineral density (BMD).

Methods—This is a cross-sectional study in a university-based research center from May, 2002 to December, 2007. A total of two hundred and thirteen healthy postmenopausal Caucasian women > one year from last menstrual period participated to this study. Serum estradiol by ultrasensitive radioimmnunoassay, serum sex hormone-binding globulin by immunoradiometric assay, and urinary NTx by enzyme-linked immunoassay were measured. BMD measurements were performed by dual energy X-ray absorptiometry and genotyping by Pyrosequencing.

Results—There were no significant differences in SHBG levels associated with either rs1799941 or rs6259. Using a p value of <0.00625 for significance, we found that subjects with the A allele (GA+AA) for the rs1799941, had a trend for lower free estradiol index (FEI) compared to the GG genotype (p=0.04). They also had significantly lower BMD at the intertrochanter (p=0.003) and trend for lower BMD at the total hip (p=0.02). There was no significant difference in FEI levels between the genotypes for the rs6259 polymorphism, but women with the Asn allele (Asp/ Asn +Asn/Asn), had significantly lower BMD in the total femur (p=0.004) and intertrochanter (0.002) compared to those with the Asp/Asp genotype.

Address all correspondence and reprint requests to: Reina C. Armamento-Villareal, MD Division of Bone and Mineral Diseases Washington University School of Medicine Campus Box 8301 660 South Euclid Ave. St. Louis, MO 63110 Phone: 314-454-7987 Fax: 314-454-5047 rvillare@im.wustl.edu.

Conclusions—Our data suggest that polymorphisms of the SHBG gene are associated with significant differences in BMD at the proximal femur sites. Thus, genetic variations in the SHBG gene may influence BMD at the hip in postmenopausal women.

Keywords

sex hormone-binding globulin; free estradiol; bone mineral density; osteoporosis

INTRODUCTION

Sex hormone-binding globulin (SHBG) binds and transports sex steroids, thus, controlling the levels of free hormones in the circulation and their bioavailability to their target tissues (1;2). By regulating the levels of active hormones, its concentration in the body has been linked to the risk of hormone-related disorders. For example, previous reports have indicated that women with increased levels of SHBG are at a reduced risk for breast cancer, presumably from a relatively low estrogenic state (3;4). On the other hand, these women are also at an increased risk for osteoporosis for the same reason (5-7); while the converse is true when SHBG is low (8;9).

Recent studies have indicated that differences in the levels of SHBG may result from polymorphisms in the gene encoding this protein, which may either lead to an increase in production or decreased clearance of SHBG (10-12). Interest in the role of polymorphisms of the SHBG gene has emerged from breast cancer studies (13-15). Women with the Asn allele (Asp/Asn+Asn/Asn) for the amino acid change from aspartic acid (Asp) to asparagine (Asn) in codon 356 (rs6259) at exon 8 have higher levels of SHBG, lower free estradiol and reduced risk for breast cancer (14). Another polymorphism, the G to A base change in the 5′UTR (rs1799941), has been shown to be associated with significantly increased levels of SHBG in those with the A allele and surprisingly high bone mass in male participants in the GOOD study (16). In a study among postmenopausal women of Spanish ancestry, although there were significant differences in SHBG levels among variants of rs6257 and rs1799941 polymorphisms, these did not result in significant differences in BMD (17). Since bone density (and the risk for osteoporosis) in post-menopausal women is also influenced by circulating hormone levels, and SHBG may influence free estradiol levels, we hypothesize that polymorphisms in the SHBG gene that are associated with differences in SHBG concentration will result in significant differences in bone density and the risk for osteoporosis, by their effect on free estradiol levels.

The objective of this study, therefore, was to determine the effect of polymorphisms of the SHBG gene on bone density, another hormone-dependent health issue. In this study, we investigated polymorphisms that have been reported to result in altered free estradiol level and differences in the risk for hormone-related conditions (14;16) i.e. the G to A substitution in the 5′UTR (rs1799941), and the Asp356Asn (rs6259) polymorphism in exon 8 (rs 6259).

METHODS

Study population

This is a cross-sectional study conducted on 213 community-dwelling, otherwise healthy Caucasian women, who were at least 1 year from the last menstrual period and were living in the St. Louis, MO, metropolitan area. Participants were recruited through advertisements or direct mailing. This study was conducted in accordance with the guidelines in the Declaration of Helsinki for the appropriate treatment of human subjects. The protocol was approved by the Washington University School of Medicine institutional review board, and written informed consent was obtained from each participant. Subjects who were taking any

medication that affects bone metabolism, such as estrogen, selective estrogen receptor modulators (including raloxifene and tamoxifen), bisphosphonates (alendronate, risedronate, pamidronate, or zoledronate), aromatase inhibitors, GnRH analogs, glucocorticoids (>5 mg daily for >1 month), or phenytoin, were excluded from the study, as were subjects with diseases or conditions known to interfere with bone metabolism, including hyperthyroidism, osteomalacia, chronic liver disease, renal failure, hypercortisolism, malabsorption, immobilization, and alcoholism. Current tobacco users were excluded. Past smokers who had stopped smoking for at least 6 months were allowed into the study.

Clinical, dietary, and anthropometric data

Dietary calcium and vitamin D intake were estimated from a 7-day dietary record, which was mailed to the participants at least 1 wk before the study visit. The record contains a list and serving sizes of common dietary sources of calcium. The participants were asked to record daily intake of these foodstuffs, and the average daily intake was determined for 7 days. Alcohol intake was expressed as the average number of alcoholic drink-equivalents consumed over a 1-wk period. A can of beer (336 ml), a glass of wine (112 ml), and 28 ml of a heavy alcoholic beverage were considered one drink-equivalent. Previous smoking was expressed in pack-years and was estimated as the number of 20-cigarette packs smoked per day multiplied by the number of years of smoking. Physical activity was expressed as a numerical score and was defined as: sedentary (sitting or lying most of the day, score 1), moderately active (being on feet more than half a day, score 2), and very active (engaging in regular physical exercise, score 3) (18).

Family history of osteoporosis was obtained through a questionnaire. A family history of osteoporosis was coded as positive in the presence of a blood relative (first and/or second degree, *i.e.* aunts and grandmothers) diagnosed with osteoporosis, kyphosis, and fragility fractures in the absence of secondary causes. Data on estrogen exposure were assessed through a number of variables, such as age at menarche, average number of periods per year during the reproductive years, number of years of birth control pill use (BCP), total number of pregnancies, number of pregnancies to term, months of lactation, age at menopause, and years since menopause (YSM). Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. The waist to hip ratio was calculated as the ratio between waist circumference, taken at the umbilical level, and hip circumference, measured 6 in. (15.24 cm) below the anterior superior iliac spine.

Biochemical data

Serum samples were collected in non-fasting state. Serum estradiol was measured by ultrasensitive radioimmunoassay technique (Diagnostic Systems Laboratory, Webster, TX.), and sex hormone-binding globulin (SHBG) by immunoradiometric (IRMA) assay (Diagnostic Systems Laboratory, Webster, TX.). The free estradiol index (FEI) was calculated as the molar ratio of the total estradiol to SHBG (19). Urinary N-telopeptide of type 1 collagen (NTx) was measured on a 24-h urine sample by an enzyme-linked immunosorbent assay (Osteomark NTx; Ostex International, Seattle, WA, USA) and expressed as nanomoles of bone collagen equivalent (BCE) per millimolar of creatinine. The inter- and intra-assay coefficients of variability for serum estradiol, SHBG and urinary NTx were < 10% (19).

Genotyping

Genotyping for the SHBG gene polymorphisms: Genomic DNA was extracted from peripheral leukocytes using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) and used as a template for genotyping procedures. All genotyping was done by Pyrosequencing. The G to A substitution in the 5′UTR was detected by, first, amplifying by

PCR using the forward primer 5′-GAG GGG TGA TAG CTG AGT CTTG-3′ and the reverse primer 5′-biotin-GCG GCT CAG ACA ACT CTT GG-3′. The PCR reaction was performed using standard protocols with 1.5 mmol of $MgCl_2$ and 1 μmol of each primer. The PCR reactions started with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles for 0.5 minutes at 94°C, 1 minute at 63°C, and 1 minute at 72°C, with a final extension of 7 minutes at 72°C. PCR amplicons were then analyzed by Pyrosequencing (Upssala, Sweden) using the following sequencing primer: 5′-CCA CCG CCC ACAC-3′.

The G to A substitution at position 1066 in exon 8, which results in an amino acid change from aspartic acid to asparagine at codon 356 (Asp356Asn) was detected by first amplifying by PCR using the forward primer 5′-TTG GAG GAG TGG AAA AGT GG-3′ and reverse primer 5′-biotin-ACA TCC AGC CTC TGA CCT TG-3′. PCR conditions were 35 cycles for 0.5 minutes at 94°C, 0.5 minutes at 63°C, and 0.5 minutes at 72°C. Genotype analysis was then performed by Pyrosequencing (Upssala, Sweden) with the following sequencing primer: 5′-TCC CTC TAG GAG AA-3′.

Bone mineral density (BMD)—Bone mineral density of the lumbar spine and the proximal femur were measured by dual energy X-ray absorptiometry (DEXA) using Hologic QDR 4500 (Hologic Inc., Waltham Ma, U.S.A.). BMD of the lumbar spine was performed using the anteroposterior projection, and was calculated as the average of L1 to L4 vertebrae. The non-dominant hip was used for proximal femur scans and values were calculated on the total femur, femoral neck, trochanter and intertrochanteric areas. The coefficient of variability of this technique using QDR 4500 densitometer is 1.09% for the lumbar spine and 1.2% for the total femur in our center (20). Bone sizes of L1 to L4 lumbar vertebrae and the different regions of the proximal femur were measured by DEXA and expressed in square centimeter (cm^2).

Statistical analysis—Results are expressed as means ± SE. The significance level was set using a more stringent *P value of* < 0.00625. Simple and partial correlation analyses were used to determine the relationship between SHBG with clinical, biochemical, and BMD variables. Group comparisons for BMD of the different genotypes of the G to A substitution at the 5′UTR were adjusted for age, BMI, YSM, and average calcium intake, while comparisons for bone size, SHBG and FEI were adjusted for age, BMI and YSM using analysis of covariance. SHBG was not normally distributed, and therefore, were log-transformed and then used for analysis. The clinical features of the different genotypes were compared using Student's t test for continuous variables (e.g. age, BMI) and using chi-square test for categorical variables (e.g. family history of osteoporosis and the percentage of past smokers) as appropriate**.** In the G to A base change in the 5′UTR, GA and AA genotypes were combined in every analysis as there were only 8 patients with the AA genotype. The data were managed using Excel 2000 (Microsoft Corp., Redmond, WA, U.S.A.) and analyzed using Statgraphic Plus 5.0 (Manugistic, Inc., Rockville, MD, U.S.A.). Analysis of Hardy-Weinberg equilibrium was carried out using the Polymorphism and Haplotype Analysis Suite (http://krunch.med.yale.edu/hwsim/).

Results

A total of two hundred and thirteen Caucasian postmenopausal women, who were at least one year from the last menstrual period, participated in the study. Genotypes were available in 207 subjects for the G to A base change in the 5′ UTR (rs1799941) and in 199 subjects for the Asp356Asn polymorphism (rs6259). BMD data were available for all participants, SHBG and FEI levels were available in 161 subjects and 24-hour urine N-telopeptide (NTx) levels were available in 132 subjects.

For rs1799941, 60.0% (124/207) had GG, 36.2% (75/207) had GA, and 3.8% (8/207) had AA genotype, while 77.4% (154/199) had Asp/Asp, 22.6% (45/199) had Asp/Asn, and 0% (0/199) had Asn/Asn genotype for rs6259. The absence of subjects homozygous for the Asn allele reflects the rare occurrence of this genotype as has been reported in previous studies (21). The genotype frequencies calculated for both polymorphisms were in agreement with the Hardy-Weinberg equilibrium.

Simple correlation analysis (Table 1) showed significant negative correlations between SHBG with BMI and waist to hip ratio, a negative correlation between SHBG and estradiol, and a positive correlation between SHBG and 24-hour urine NTx. There were no other correlations observed between SHBG and the rest of the clinical variables analyzed.

Table 2 shows significant negative correlations between SHBG with BMD of the proximal femur. Adjusting these correlations with variables known to affect BMD (age, YSM, BMI and serum estradiol) showed reductions in the correlations observed.

The clinical features of the participants stratified according to genotypes for each polymorphism are given in Table 3. There were no differences in the baseline characteristics or potential confounding factors such as age, BMI, age at menopause, years since menopause (YSM), past history of smoking, daily calcium intake, family history of osteoporosis and physical activity score among the genotypes of both polymorphisms.

Comparing phenotypes for the G to A base substitution in the 5′UTR (rs1799941) showed no significant differences in SHBG levels between the genotypes but a trend for lower FEI among carriers of the A allele (GA+AA) (Table 4) was observed. There were no significant differences in total estradiol and NTx levels. Further analysis also showed significantly lower covariate-adjusted BMD in the intertrochanter, and a trend for lower BMD in the total femur in women with the A allele. Bone areas at all the skeletal sites evaluated were found to be comparable between the genotypes (data not shown).

Phenotype analysis for the Asp to Asn amino acid change (rs6259), showed no differences in total estradiol, SHBG, FEI and NTx levels among the genotypes (Table 4). However, significantly lower covariate-adjusted BMD at the total femur and intertrochanter, and a trend for lower BMD at the femoral neck were observed in those with the Asn allele. There were no significant differences in bone size in any of the skeletal sites analyzed (data not shown).

Controlling for genotypes in both polymorphisms, showed persistent positive correlation between urine NTx and SHBG (r=0.27, p=0.003). On the other hand, there was no significant correlation between urinary NTx with estradiol (r=0.18, p=0.047) and free estradiol levels (r=0.07, p=0.41) with or without the added effect of genotypes.

DISCUSSION

Our data suggest that polymorphisms in the SHBG gene may influence bone mineral density in the postmenopausal period. Women with the A allele for the G to A base change in the 5′UTR and the Asn allele for the Asp356Asn amino acid substitution have lower BMD in the regions of the femur. To our knowledge, this is the first study to report the association between polymorphisms of the SHBG gene on bone density in post-menopausal women.

Several factors have been identified to affect SHBG production. The female gender is associated with higher SHBG levels relative to males (22). Increased body weight is associated with decreased SHBG production while increasing age is associated with increased SHBG levels (23;24). Independent of age, SHBG levels have also been found to

increase 20 years after menopause (25). Since SHBG regulates the levels of free hormones, any alteration in the levels of SHBG alters the bioavailability of these hormones to target tissues. The importance of circulating SHBG in regulating bioavailable sex-steroid levels has been investigated in a variety of hormone-related disorders/diseases such as breast cancer and osteoporosis; and in other diseases also presumed to be influenced by hormonal status such as Alzheimer's Diseases, metabolic syndrome, and cardiovascular disease (26-29). Available data suggest that high SHBG is associated with increased bone loss, lower bone density and higher risk for fractures, while low SHBG is protective of bone loss (6;9;30). On the contrary, since the risk for breast cancer is opposite that of osteoporosis; women with low SHBG are at an increased risk for breast cancer while those with high SHBG are protected from the disease (31).

Findings of differences in SHBG levels resulting from polymorphisms of the gene that encodes this protein have recently been reported. These data mainly came from studies on breast cancer, polycystic ovarian syndrome, insulin resistance and hirsutism (12;14;32;33). However, very little information is available on the effect of these polymorphisms on BMD. Results from the limited studies that have examined this relationship have shown either no association or contradictory findings. A study among Swedish men showed that those with genotypes associated with high SHBG levels have paradoxically significantly higher BMD relative to those with lower SHBG (16). Meanwhile a study among postmenopausal women of Spanish ancestry, showed no significant differences in BMD despite significant differences in SHBG levels among genotypes for rs1799941 and rs6257, but found no association for rs6259 with either SHBG levels or BMD (17). On the contrary, we did not find any significant differences in the SHBG levels among genotypes of the rs179941 and rs6259 polymorphisms but significant differences in BMD in the femoral sites were noted among the variants even using a stringent p value.

The "free hormone hypothesis" stipulates that more free hormones mean more biologically active compounds (34). Thus a lower FEI in certain variants can potentially lead to lower BMD and vice-versa. In a study among women with breast cancer, Dunning et. al. reported that the G to A base change in the 5′UTR (rs1799941), and the Asp356Asn (rs6259) resulted in elevation of SHBG and lowering of estradiol to SBHG ratio (i.e. calculated free estradiol) in women with the A allele for the former and in those homozygous for the Asn allele for the latter (10). In agreement with their results, we also observed a trend for lower FEI in women with the A allele for the rs1799941 in our study. It is possible that the difference in BMD we observed among the genotypes for this polymorphism is perhaps related to the alteration in FEI.

The discordant results on BMD phenotype between our study and that of Riancho et.al., could be a result of inherent differences between the two study population under in investigation. Firstly, although both women groups are considered as whites, they differ in ethnic backgrounds; the former study was done purely on women of Spanish origin (17), while our population is combination of women from German, French, Italian, Jewish and Anglo-Saxon origin. Population stratification has been reported to cause spurious associations between a particular locus and a disease in association studies, primarily in case-control studies (35). It is possible that our results may have been influenced by population stratification given the ethnic admixture in our sample. However, an analysis of several genetic case-control studies in non-Hispanic Caucasian women in the US with cancers showed a negligible influence from population stratification, on the associations observed (36). In addition, the complimentary results in both studies, for instance the differences in SHBG levels in the Riancho study in addition to the trend in FEI levels and differences in BMD in the present study going in the same direction, favors a true rather than a spurious association between rs1799941 and BMD. Secondly, all the participants in

their study belong to an older age group, i.e. >60 years of age (17), while our subjects ranged in age from 42 to 90 years old. By influencing active hormone levels, it is possible that polymorphisms in the SHBG gene could be more relevant for bone loss experienced by younger or early postmenopausal women where residual ovarian estrogen production may still exist and becomes less important with increasing age and time lapsed from menopause. In our study, 43.5% of the women are < 60 years of age, who may at the time of enrollment are still experiencing rapid bone loss. However, the limited sample size in our study does not allow us to perform subgroup analysis of our subjects according to age.

In breast cancer studies, the Asn allele for the Asp356Asn polymorphism in exon 8 has been shown to be protective from breast cancer (14). Carriers of the Asn allele were found to have increased SHBG level, and this biochemical alteration hypothesized to result in lower free estradiol, and consequently a lower risk for breast cancer. On the other hand, a study on multiethnic women showed no difference in SHBG levels among the genotypes of the Asp356Asn polymorphism (21). Similar to their finding, we did not find a significant increase in SHBG level among the Asn carriers. In contrast to the explanation proposed for the rs1799941 polymorphism, this polymorphism may alter the BMD findings of our patients through a different mechanism. Because this polymorphism occurs in the coding region of the gene and resulted in an amino acid change, it is likely that the difference in BMD between carriers of the Asn allele compared to those without the allele could be secondary to the variation in functional activity of the SHBG protein and not in the amount of protein produced. It is possible that certain variants may have lower affinity to estradiol resulting in actually higher free hormones than the level reflected by the calculated free estradiol index (i.e. calculated from SHBG and total estradiol). Thus, despite the apparent absence of biochemical alteration in SHBG and free estradiol levels, a difference in BMD in the proximal femur was observed among the variants. Another potential explanation for our observation is the likelihood of linkage between rs6259 and rs1799941 as suggested by Riancho et. al. (17), with women carrying the Asn allele for the rs6259 polymorphism expressing the same BMD phenotype as the carriers of the A allele for the rs1799941. On the other hand, a recent report indicated the existence of a receptor-mediated (megalin receptor) endocytosis of the SHBG-hormone complex in target tissues to promote action (34;37). Whether this newly recognized pathway of hormone action has any role in influencing BMD in variants for the SHBG gene remains undetermined and needs further studies.

Although urinary NTx positively correlated with SHBG levels and negatively correlated with BMD in the femoral sites, there were no significant differences in urinary NTx found among the genotypes. The failure to find an association between BMD and urinary NTx among the genotypes could be due to the wide range in age and menopausal status of our subjects. Future studies with longitudinal follow-up of bone turnover markers and serial BMD measurements in young premenopausal women and women in the early menopausal age may clarify whether low BMD seen in some variants is secondary to inadequate peak bone mass or accelerated bone loss at menopause.

In summary, our data suggest that polymorphisms of the sex hormone binding globulin (SHBG) gene are associated with differences in BMD in postmenopausal women. By altering free estradiol levels, polymorphism in the 5′UTR (G to A base change) may have resulted in significant differences in BMD. The other polymorphism, resulting in an amino acid change at codon 356 from Asp to Asn, was associated with differences in BMD without any changes in SHBG or free estradiol levels. The absence of any biochemical alteration associated with this polymorphism is not surprising given its location. It is likely that the differences in BMD phenotype associated with this polymorphism arise from subtle alteration in the functional activity among the variants rather than the level of protein

expression itself. This is the first study investigating the role of SHBG gene polymorphisms in post-menopausal BMD, once again confirming the probability of another genetic contribution from the genes regulating sex hormones in skeletal health. In conjunction with other genetic polymorphisms affecting bone health, polymorphisms in the SHBG gene may help in identifying individuals at risk for low bone density who may represent the best candidate for osteoporosis prevention strategies.

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Table 1

Simple correlation analysis between serum SHBG with clinical, and biochemical features

	Serum SHBG	
Clinical and biochemical features	**r**	**p**
Age	0.14	0.08
BMI	−0.44	<0.001*
YSM	0.08	0.33
Waist to hip ratio	−0.38	<0.001*
Serum estradiol	−0.25	0.001*
Urine NTx	0.27	0.002*

* significant p value <0.00625

Table 2

Simple and adjusted correlation analysis between serum SHBG with bone mineral density (BMD).

	Simple correlations		Adjusted Correlations	
Bone mineral density (g/cm^2)	**r**	**p**	**r**	***P**
Spine	−0.20	0.01	−0.03	0.68
Total Femur	−0.40	<0.001*	−0.20	0.01
Femoral Neck	−0.35	<0.001*	−0.16	0.04
Trochanter	−0.32	<0.001*	−0.12	0.12
Intertrochanter	−0.39	<0.001*	−0.17	0.03

* p adjusted for YSM, age, BMI and estradiol levels, *significant P value <0.00625

Table 3

Clinical features of the study population stratified according to genotypes for polymorphisms of the SHBG gene.

Clinical features	G to A substitution in 5′UTR N=207 (rs1799941)			(Asp356Asn) N=199 (rs6259)		
	GG (N=124)	GA+AA (N=83)	P	Asp (N=154)	Asp+Asn (N=45)	P
Age (years)	64.2 ± 0.6	63.6 ± 0.85	0.62	64.2 ± 0.6	64.7 ± 1.07	0.93
BMI (kg/m^2)	27.8 ± 0.38	27.66 ± 0.51	0.79	27.9 ± 0.45	26.9 ± 0.82	0.32
Waist to hip ratio	0.851 ± 0.008	0.843 ± 0.10	0.52	0.82 ± 0.01	0.81 ± 0.03	0.68
YSM (years since menopause)	14.45 ± 0.97	11.73 ± 1.27	0.09	13.83 ± 0.90	14.42 ± 1.67	0.76
History of smoking Percent past smokers Total past smoking (pack-years)	 48.48 8.73 ± 1.32	 41.05% 6.94 ± 1.75	 0.33 0.41	 47.06% 8.41 ± 1.32	 42.86% 5.73 ± 2.45	 0.39 0.34
Average daily calcium intake (mg/day)	1096.74 ±55.2	1073.9 ± 72.7	0.80	1174.7 ± 54.5	1248.5 ± 98.7	0.51
Alcohol intake (oz-Eq/ week)	1.90 ± 2.06	6.62 ± 2.69	0.16	4.14 ± 1.84	1.97 ± 3.32	0.56
Positive family history of osteoporosis (%)	43.48%	47.92%	0.94	42.79%	51.61%	0.43
Activity score	2.32 ± 0.49	2.34 ± 0.63	0.77	2.35 ± 0.04	2.26 ± 0.07	0.35

Table 4

Biochemical and BMD data in the genotypes of the G to A in the 5′ UTR polymorphism of the SHBG gene

Clinical data	**G to A at 5′UTR (rs1799941) N=207**			**Asp356Asn (rs6259) N=199**		
	GG	**GA+AA**	**P**	**Asp**	**Asp+Asn**	**P**
BMD (g/cm^2)	(N=124)	(N=83)		(N=150)	(N=45)	
Spine	0.952 ± 0.01	0.939 ± 0.02	0.51	0.959 ± 0.01	0.912 ± 0.02	0.05
Total femur	0.867± 0.01	0.831 ± 0.01	0.02	0.869± 0.01	0.814 ± 0.02	0.004*
Femoral Neck	0.713± 0.01	0.694 ± 0.01	0.19	0.718± 0.01	0.678 ± 0.01	0.03
Trochanter	0.652 ± 0.01	0.641 ± 0.01	0.39	0.655 ± 0.01	0.624 ± 0.01	0.05
Intertrochanteric	1.048 ± 0.01	0.980 ± 0.02	0.001*	1.044 ± 0.01	0.966 ± 0.02	0.002*
Biochemical (N=161)	(N=99)	(N=62)		(N=123)	(N=38)	
Estradiol (pmol)	53.24±1.78	49.32±2.36	0.18	51.49±1.62	53.58±2.95	0.54
SHBG (nmol)	133.7±7.4	143.9±9.3	0.4	138.9±6.1	144.9±11.0	0.77
FEI (pmol/nmol)	0.543±0.04	0.411±0.05	0.04	-0.91±0.05	-0.96±0.09	0.62
Urine NTx	96.02±7.56	87.81±10.52	0.53	91.23±7.24	108.19±12.82	0.25

BMD comparisons by analysis of covariance (ANCOVA) adjusted for age, body mass index (BMI), years since menopause (YSM), average daily calcium intake; and FEI comparison adjusted for YSM.

* significant *P* value <0.00625