

SHBG Gene Promoter Polymorphisms in Men Are Associated with Serum Sex Hormone-Binding Globulin, Androgen and Androgen Metabolite Levels, and Hip Bone Mineral Density

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Context: SHBG regulates free sex steroid levels, which in turn regulate skeletal homeostasis. Twin studies have demonstrated that genetic factors largely account for interindividual variation in SHBG levels. Glucuronidated androgen metabolites have been proposed as markers of androgenic activity.

Objective: Our objective was to investigate whether polymorphisms in the SHBG gene promoter [(TAAAA)_n microsatellite and rs1799941 single-nucleotide polymorphism] are associated with serum levels of SHBG, sex steroids, or bone mineral density (BMD) in men.

Design and Study Subjects: We conducted a population-based study of two cohorts of Swedish men: elderly men (MrOS Sweden; n = 3000; average age, 75.4 yr) and young adult men (GOOD study; n = 1068; average age, 18.9 yr).

Main Outcome Measures: We measured serum levels of SHBG, testosterone, estradiol, dihydrotestosterone, 5 α -androstane-3 α ,17 β -

diol glucuronides, androsterone glucuronide, and BMD determined by dual-energy x-ray absorptiometry.

Results: In both cohorts, (TAAAA)_n and rs1799941 genotypes were associated with serum levels of SHBG ($P < 0.001$), dihydrotestosterone ($P < 0.05$), and 5 α -androstane-3 α ,17 β -diol glucuronides ($P < 0.05$). In the elderly men, they were also associated with testosterone and BMD at all hip bone sites. The genotype associated with high levels of SHBG was also associated with high BMD. Interestingly, male mice overexpressing human SHBG had increased cortical bone mineral content in the femur, suggesting that elevated SHBG levels may cause increased bone mass.

Conclusions: Our findings demonstrate that polymorphisms in the SHBG promoter predict serum levels of SHBG, androgens, and glucuronidated androgen metabolites, and hip BMD in men. (*J Clin Endocrinol Metab* 91: 5029–5037, 2006)

SHBG IS SYNTHESIZED in the liver, and in the blood it transports and regulates the access of sex steroids to their target tissues (1). Serum levels of SHBG are influenced by hormonal as well as nutritional and metabolic status (2). However, twin studies have demonstrated that genetic factors account for more than 60% of interindividual variations

in serum concentrations of SHBG in men (3, 4). Furthermore, some studies have indicated that SHBG is an independent predictor of bone mineral density (BMD) (5, 6).

Recently, several genetic polymorphisms have been characterized in the human SHBG gene. In the NCBI database (May 25, 2006), there are 11 reported single-nucleotide polymorphisms (SNPs) in the SHBG gene and seven SNPs and a (TAAAA)_n microsatellite in the genomic region near the gene (including the promoter region). The promoter region is important for the regulation of gene transcription. The (TAAAA)_n repeat polymorphism, rs5030991, is located at a distance of approximately –700 bp from the transcription start site. Three recent studies in women (<350 subjects) have found an association between this polymorphism and SHBG levels (7–9). The rs1799941 A/G SNP is located within the human SHBG proximal promoter sequence, only eight base pairs from the transcription start site. One study found an association between this SNP and SHBG levels in postmenopausal women (10), and recently the same association was

First Published Online August 22, 2006

Abbreviations: aBMD, Areal BMD; ADT, androsterone; ADTG, ADT-glucuronide; BMD, bone mineral density; CV, coefficients of variation; DHT, dihydrotestosterone; 3 α -diol, 5 α -androstane-3 α ,17 β -diol; 3 α -diol-3G, 5 α -androstane-3 α ,17 β -diol-3-glucuronide; 3 α -diol-17 G, 5 α -androstane-3 α ,17 β -diol-glucuronide; DXA, dual-energy x-ray absorptiometry; E2, estradiol; FE2, free E2; FT, free testosterone; GC/MS, gas chromatography/mass spectroscopy; LC/MS/MS, liquid chromatography/tandem mass spectroscopy; pQCT, peripheral quantitative computerized tomography; sBMD, standardized BMD; SNP, single-nucleotide polymorphism; T, testosterone.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

found in a small study of men ($n = 233$) (11). Because differences in serum SHBG levels affect the free fraction of sex steroids, it is critically important to establish whether these genetic variations are associated with diseases that are linked to inappropriate sex steroid exposures.

The production rate and serum levels of testosterone (T) are also largely influenced by genetic as well as environmental factors (12). In recent years it has become clear that the biologically most active androgens, namely 5 α -dihydrotestosterone (DHT) and T, are synthesized from androgen precursors at their site of action in peripheral tissues. These androgens are subsequently metabolized and then glucuronidated before excretion (13). Only a small proportion of DHT diffuses into the circulation (14), and we have recently found that serum levels of DHT are not correlated with BMD in elderly men (own unpublished data). Glucuronidated androgen metabolites, on the other hand, are released into the circulation before excretion (15). Previous studies have suggested that the glucuronidated androgen metabolites 5 α -androstane-3 α ,17 β -diol-3-glucuronide (3 α -diol-3G) and 3 α -diol-17G and androsterone-glucuronide (ADTG) reflect the total androgenic pool in women (16). We have recently found that 3 α -diol-G is a stronger predictor of BMD and prostate volume in elderly men than the bioactive androgens T, free T (FT), and DHT (own unpublished data). Accordingly, glucuronidated androgen metabolites might be good markers of androgenic activity also in men. Serum levels of sex steroids and SHBG interact. However, it is not known whether SHBG also interacts with serum levels of androgen metabolites and thereby participates in regulating androgenic activity in androgen-responsive organs such as bone.

The aim of the present study was to investigate whether any of the two above mentioned polymorphisms within the SHBG promoter sequence are associated with serum levels of SHBG, sex steroids, or BMD in a large ($n \approx 4000$) well-characterized cohort of men, including both young adult ($n = 1068$) and elderly ($n \approx 3000$) men.

Subjects and Methods

Study subjects: young adult men

The Gothenburg Osteoporosis and Obesity Determinants (GOOD) study was initiated to determine environmental and genetic factors involved in the regulation of bone and fat mass. Study subjects were randomly identified using national population registers, contacted by telephone, and asked to participate in the present study. Men aged 18.9 ± 0.6 yr ($n = 1068$) from the greater Gothenburg area were included. To be included in the GOOD study, subjects had to be more than 18 and less than 20 yr of age and willing to participate in the study. Almost half (48.6%) of the study candidates agreed to participate and were enrolled (17). Seventy-two subjects (6.7%) reported regular medication use. Inhaled β -2 stimulants ($n = 19$, 1.8%) and inhaled glucocorticoids ($n = 14$, 1.3%) were the most common medications.

Study subjects: elderly men

The MrOS study is a multicenter study including elderly men in Sweden (~3000), Hong Kong (~2000), and the United States (~6000). In the present study, associations between SHBG polymorphisms, hormone levels, and BMD were investigated in the Swedish cohort. Study subjects (men aged 69–80 yr) were randomly identified using national population registers, contacted, and asked to participate. To be eligible for the study, the subjects had to be able to walk without aids and were not allowed to have bilateral hip prosthesis. There were no other ex-

clusion criteria (18), and 78.6% of study subjects reported regular medication use. In a subsample consisting of all subjects residing in the town of Malmö ($n = 994$), more detailed information on medications was available. The most common medications were aspirin (34.1%), β -blockers (27.5%), and statins (23.2%).

The studies were approved by the ethics committees at the Göteborg, Lund, and Uppsala Universities. Informed consent was obtained from all study participants.

Assessment of covariates

Height and weight were measured using standard equipment. Standardized questionnaires were used to collect information about amount of physical activity, nutritional intake, and smoking. Calcium intake was calculated using information from the questionnaires about amount of calcium-containing foods. In the MrOS study, physical activity was the subject's average daily walking distance, including both walking as a means of exercise and leisure and as a means of outdoor transportation. In the GOOD study, subjects reported amount (hours per week) of physical activity.

Assessment of BMD by dual-energy x-ray absorptiometry (DXA)

Areal BMD (aBMD; g/cm²) of the total hip, femoral neck, femoral trochanter, and lumbar spine (L₁₋₄) was assessed using the Lunar Prodigy DXA (MrOS $n \approx 2000$; GOOD $n = 1068$; GE Lunar Corp., Madison, WI) or the Hologic DXA Hologic QDR 4500/A-Delphi (MrOS $n \approx 1000$; Hologic, Whaltman, MA). The coefficients of variation (CV) for the aBMD measurements ranged from 0.5–3%, depending on the application.

To be able to use DXA measurements performed with equipment from two different manufacturers, standardized BMD (sBMD) was calculated in the MrOS Study, as previously described (19–21). To minimize further the possible confounding effect of using different DXA equipment, DXA type (Hologic or Lunar) was included as a covariate in all regression analyses with BMD as a dependent factor.

Assessment of sex hormones

Serum levels of SHBG, T, and estradiol (E2) were analyzed for all subjects, whereas serum levels of DHT, 3 α -diol-3G, 3 α -diol-17G, and ADTG (GOOD $n = 502$; MrOS $n = 631$) and urinary glucuronides of androstane-3 α ,17 β -diol (3 α -diol) and androsterone (ADT) (GOOD $n = 124$; MrOS $n = 448$) were analyzed in subsamples of the two cohorts because of high costs.

RIA. Total serum E2 was measured using an ultrasensitive RIA (Orion Diagnostics, Espoo, Finland) with a limit of detection of 5 pmol/liter, intraassay CV of 3%, and interassay CV of 6%. Total serum T was measured using RIA (Orion Diagnostics) with a limit of detection of 0.1 nmol/liter, intraassay CV of 6%, and interassay CV of 6%. Serum SHBG was measured using immunoradiometric assay (Orion Diagnostics) with a limit of detection of 1.3 nmol/liter, intraassay CV of 3%, and interassay CV of 7%. FT and free E2 (FE2) were calculated according to the method described by Vermeulen *et al.* (22) and Van den Beld *et al.* (23) taking the concentrations of total T, total E2, and SHBG into account and assuming a fixed albumin concentration of 43 g/liter.

Serum analyses using gas chromatography/mass spectroscopy (GC/MS). The validated GC/MS system (24) was used for the analysis of T (limit of detection 0.05 ng/ml, intraassay CV 2.9%, and interassay CV 3.4%) and DHT (limit of detection 0.02 ng/ml, intraassay CV 3.1%, and interassay CV 4.1%). Analytes and internal standard were detected using a HP5973 quadrupole mass spectrometer equipped with a chemical ionization source.

Serum analyses using liquid chromatography/tandem mass spectroscopy (LC/MS/MS). ADTG (limit of detection 2.00 ng/ml, intraassay CV 3.1%, and interassay CV 3.7%), 3 α -diol-3G (limit of detection 0.50 ng/ml, intraassay CV 10.3%, and interassay CV 10.7%), and 3 α -diol-17G (limit of detection 0.50 ng/ml, intraassay CV 4.6%, and interassay CV 5.3%) were analyzed by a validated LC/MS/MS method using TurboIonSpray.

Urinary analyses using GC/MS. Urinary glucuronides (3 α -diol and ADT) were determined by GC/MS. One microgram of internal standard (methyltestosterone) was added to each 2.0 ml of urine, and steroids were extracted after hydrolysis of the conjugates with β -glucuronidase as described (25) with minor modifications (26). Within- and between-assay CV were less than 7% and less than 10% for all steroids analyzed.

Genetic polymorphism analysis

The (TAAAA)_n repeat polymorphism was successfully genotyped in 3872 individuals (2851 in the MrOS cohort, 1021 in the GOOD cohort) using PCR forward primer 5'-GAACTCGAGAGGCAGAGGCAG-CAGTGA-3' and reverse primer 5'-GTTTCTTAGAAATCACCCACTC-CCTGA-3' labeled with FAM and HEX (DNA technology) and tailed as previously described (27). Amplifications were performed using GeneAmp 9700 machines with dual 384 heads. The PCR products were separated on an ABI 3730 capillary machine using LIZ or ROX as size standard. Raw data were converted to genotype data using GeneMapper (version 3.0; Applied Biosystems, Foster City, CA). The reliability of the marker system was confirmed by repeated analysis of 48 samples and by checking for Mendelian inheritance in eight trios. Genotyping for rs1799941 was performed using FAM- and VIC-labeled PCR forward primers 5'-TCCCGGGCAACCTTTAAC-3' and PCR reverse primer 5'-CAGACAACCTCTGGGAGAAATGTGTA-3'. Amplifications were performed on a GeneAmp PCR System 9700 machine with dual 384 heads (Applied Biosystems). The 7900 HT Sequence Detection System (ABI PRISM) was used to analyze the PCR products (TaqMan). Genotyping was performed using the SDS 2.1 program (Applied Biosystems) and was successful in 3933 individuals (2875 in the MrOS cohort, 1058 in the GOOD cohort). Haplotypes were inferred using the EM-algorithm as implemented in *gc.em* (gene counting EM-algorithm) in the R-package *gap* (28). In the calculations, only haplotypes of individuals successfully genotyped for both polymorphisms were used.

Animals

Transgenic mice (*shbg4*) overexpressing a 4.3-kb region of the human *SHBG* gene have been described previously (29). In these mice, human *SHBG* transcripts are present in liver and kidney, and high levels of human *SHBG* are present in their blood. The 4-wk-old and 6-month-old male *SHBG* transgenic mice and control mice (approximately corresponding to men in late middle age) were investigated by peripheral quantitative computerized tomography (pQCT) analyses. Animal procedures were approved by the ethics committee for animal care at Göteborg University and conducted in accordance with the guidelines.

pQCT analyses of mice

Computerized tomography was performed with the STRATEC pQCT XCT operating at a resolution of 70 μ m (30). Trabecular BMD was determined *ex vivo*, with a metaphyseal pQCT scan of the distal femur. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 4% of the total length of the femur, and the trabecular bone region was defined as the inner 45% of the total cross-sectional area. Cortical bone parameters were determined *ex vivo* with a mid-diaphyseal pQCT scan of the femur (31).

Statistical analysis

Continuous variables were compared using ANOVA with Tukey's *post hoc* test. Univariate associations were examined using Pearson's correlation. Regression analyses including *SHBG* genotype as the independent predictor were used to investigate the associations between genotype and levels of *SHBG*, androgens, and androgen metabolites. Regression analyses treating the (TAAAA)_n microsatellite as a continuous variable were used to investigate the biological effects of increasing number of repeats. Regression analyses including both the (TAAAA)_n microsatellite and rs1799941 were used to investigate the independent impact of the individual polymorphisms. Regression analyses using *SHBG* genotype with or without inclusion of serum *SHBG* levels as a covariate were used to investigate whether the impact of *SHBG* genotype on levels of androgens and metabolites were mediated via affected

SHBG levels in serum. Regression analyses using height, weight, age, smoking habits, calcium intake, physical activity, DXA type, and the *SHBG* genotype as covariates were used to estimate the contribution of *SHBG* polymorphisms to sBMD. T levels were added to this model to investigate whether the results were mediated via T. All analyses of the pooled sample of both cohorts included study cohort (coded as MrOS Sweden = 1 and GOOD study = 2) and their interaction. Standardized β -values were used. Values are given as mean \pm SD. All tests were two tailed and conducted at the 5% significance level. Log transformation was used for nonnormally distributed values. In the study of mice, values are expressed as mean \pm SEM, and differences between groups were tested using Student's *t* test.

Results

Characteristics of the study subjects are shown in Table 1.

Genotyping

Genotype frequencies for the (TAAAA)_n polymorphism are shown in Fig. 1A. In both populations, the number of repeats ranged from six to 12. The 12-repeat allele was very uncommon (young adult men, *n* = 1; elderly men, *n* = 3). Genotype frequencies did not differ between the two cohorts. We first analyzed the (TAAAA)_n repeat as a continuous parameter, demonstrating a significant inverse correlation between number of (TAAAA)_n repeats (both alleles) and *SHBG* levels for both populations (*P* < 0.001). Linear regression analyses using different cut points (seven or more, eight or more, nine or more, and 10 or more; coded S/S = 1, S/L = 2, L/L = 3) and including both populations demonstrated that for cut points of seven or more (β = -0.104; *P* < 0.001), eight or more (β = -0.088; *P* < 0.001), nine or more (β =

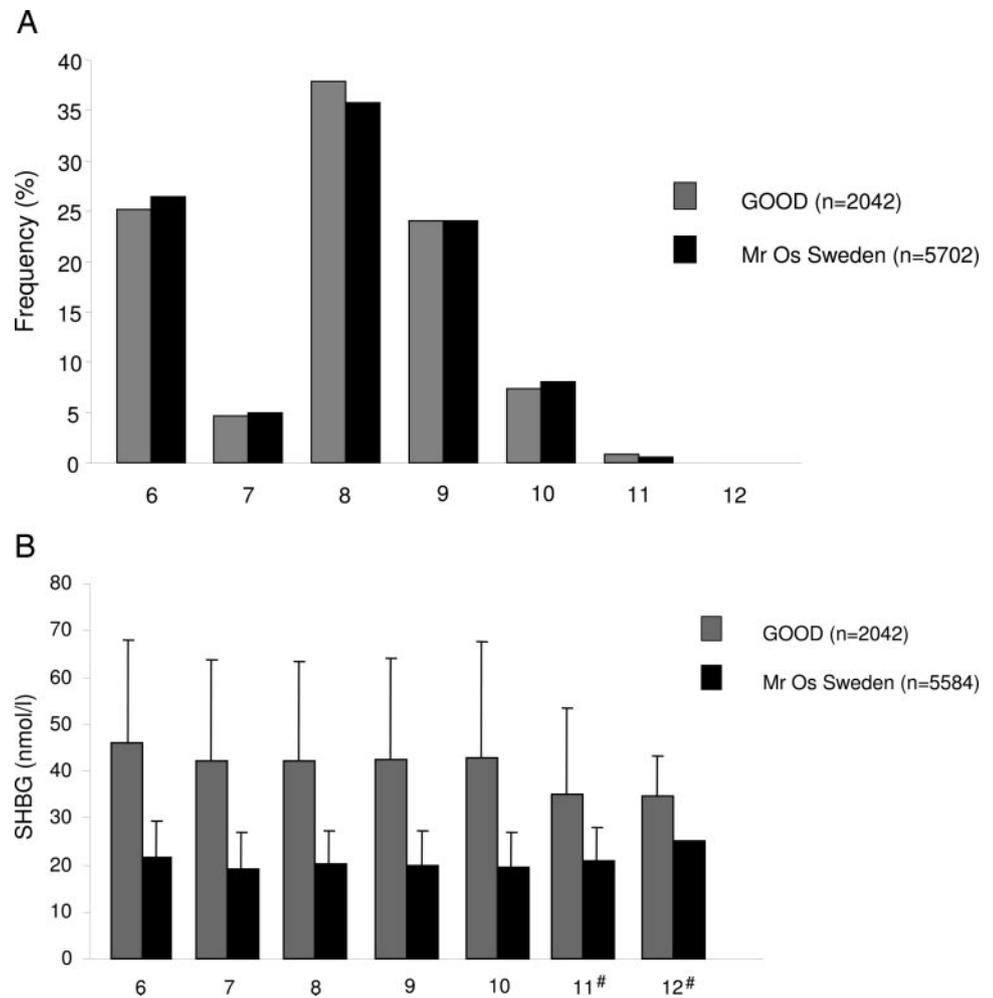
TABLE 1. Characteristics of the study subjects

Variables	GOOD	MrOS Sweden
	<i>n</i> = 1068	<i>n</i> = 2971
Age (yr)	18.9 \pm 0.6	75.4 \pm 3.2
Height (cm)	181.4 \pm 6.8	174.7 \pm 6.5
Weight (kg)	73.8 \pm 11.9	80.7 \pm 12.1
Physical activity ^a	4.3 \pm 5.3	4.0 \pm 3.1
Smoking (%)	8.7	8.5
Calcium intake (mg/d)	1095 \pm 724	895 \pm 435
Serum by RIA	<i>n</i> = 1068	<i>n</i> = 2920
SHBG (nmol/liter)	20.4 \pm 7.4	43.2 \pm 21.9
T (ng/ml)	4.91 \pm 1.7	4.88 \pm 2.1
FT (pg/ml)	133.2 \pm 45.2	90.6 \pm 47.5
E2 (pg/ml)	19.2 \pm 8.0	26.9 \pm 11.2
FE2 (pg/ml)	0.41 \pm 0.18	0.47 \pm 0.21
Serum by LC/MS/MS or GC/MS	<i>n</i> = 502	<i>n</i> = 631
T (ng/ml)	4.94 \pm 1.56	4.46 \pm 1.81
DHT (ng/ml)	0.31 \pm 0.11	0.36 \pm 0.19
3 α -Diol-G (ng/ml)	5.66 \pm 2.64	4.10 \pm 3.07
3 α -Diol-3G (ng/ml)	1.60 \pm 0.99	1.47 \pm 1.26
3 α -Diol-17G (ng/ml)	4.06 \pm 2.06	2.63 \pm 2.13
ADTG (ng/ml)	59.7 \pm 32.4	31.3 \pm 28.1
Urine by GC/MS	<i>n</i> = 124	<i>n</i> = 448
3 α -Diol (ng/ μ mol creatinine)	5.40 \pm 3.01	2.59 \pm 1.68
ADT (ng/ μ mol creatinine)	261 \pm 100	69 \pm 42
DXA analyses	<i>n</i> = 1068	<i>n</i> = 2944
BMD total hip (g/cm ²)	1.17 \pm 0.16	0.95 \pm 0.15
BMD trochanter (g/cm ²)	0.97 \pm 0.15	0.79 \pm 0.14
BMD femur neck (g/cm ²)	1.17 \pm 0.16	0.83 \pm 0.13
BMD lumbar spine (g/cm ²)	1.24 \pm 0.15	1.14 \pm 0.20

Values are given as mean \pm SD.

^a Assessed as weekly physical activity (hours per week) in GOOD and daily walking distance (km) in MrOS.

FIG. 1. A, The distribution of *SHBG* (TAAAA)_n repeat alleles among elderly men (MrOS Sweden) and young adult men (the GOOD study), respectively; B, SHBG levels according to (TAAAA)_n repeat length among elderly men (MrOS Sweden) and young adult men (the GOOD study), respectively. Values are means \pm SD; # denotes that the results are very unreliable due to low sample number ($n < 35$).



-0.046 ; $P < 0.001$), and 10 or more repeats ($\beta = -0.033$; $P = 0.01$), the (TAAAA)_n repeat was significantly associated with SHBG levels. No interaction effects for pooling of the samples were significant. The largest discrepancy in SHBG levels between consecutive genotypes was seen when comparing the six- and seven-allele genotypes (Fig. 1B). Therefore, seven or more repeats was used as a cut point in additional analyses, and six-repeat alleles were classified as short (S) and seven or more repeat alleles were classified as long (L). Genotype frequencies were S/S 5.6%, S/L 39.1%, and L/L 55.3% among the young adult men ($n = 1021$) and S/S 6.5%, S/L 40.0%, and L/L 53.6% among the elderly men ($n = 2851$).

For the rs1799941 *SHBG* SNP, genotype frequencies were AA 4.8%, AG 37.3%, and GG 57.8% among young adult men ($n = 1058$) and AA 6.4%, AG 38.0%, and GG 55.6% among elderly men ($n = 2875$). There was a high degree of linkage between the (TAAAA)_n repeat polymorphism and the rs1799941 SNP ($D' 0.97$; $P < 0.001$). All frequency distributions were in Hardy-Weinberg equilibrium.

SHBG polymorphisms and hormone levels

Linear regression analyses demonstrated that the (TAAAA)_n genotype (coded as S/S = 1, S/L = 2, and L/L =

3) was clearly associated with serum levels of SHBG in young adults as well as in elderly men (GOOD $\beta = -0.127$, $P < 0.0001$; MrOS $\beta = -0.111$, $P < 0.0001$). Individuals with the S/S genotype had the highest serum levels of SHBG, and individuals with the L/L genotype had the lowest in both cohorts (Table 2). Because of the age-related increase in SHBG levels, serum levels of SHBG were about twice as high among the elderly men compared with the young adult men, but the percentage difference between the S/S and L/L groups was similar for both age groups (S/S 17 and 22% over L/L in young adult and elderly men, respectively). Among the elderly men, T and E2 levels were increased by 16 and 9%, respectively, in individuals with the S/S genotype compared with the L/L genotype. Levels of calculated FT and FE2 were not significantly correlated with (TAAAA)_n genotype. In a subsample of the MrOS population, T levels were also analyzed by GC/MS, and they were 28% higher in the S/S genotype than in the L/L genotype, confirming the RIA results. Levels of DHT were associated with (TAAAA)_n genotype in both cohorts (young adult $P = 0.02$; elderly $P < 0.01$). Associations between rs1799941 and both serum SHBG levels and sex steroid levels were very similar to those for the (TAAAA)_n microsatellite (Table 3).

TABLE 2. Hormone levels according to SHBG (TAAAA)_n genotype

	GOOD			MrOS Sweden			GOOD/MrOS Sweden					
	S/S (n = 57)	S/L (n = 399)	L/L (n = 565)	P1	P3	S/S (n = 184)	S/L (n = 1115)	L/L (n = 1493)	P1	P3	β	P2
Serum by RIA												
SHBG (nmol/liter)	23.0 ± 8.5 ^b	21.0 ± 7.6 ^c	19.6 ± 6.9 ^c	<0.001	<0.001	50.4 ± 24.8 ^{b,d}	44.6 ± 20.8 ^b	41.3 ± 22.0 ^d	<0.001	<0.001	-0.104	<0.001
T (ng/ml)	5.38 ± 2.01	4.99 ± 1.68	4.82 ± 1.60	0.31	0.23	5.45 ± 2.02 ^{b,c}	5.00 ± 2.08 ^a	4.70 ± 2.20 ^c	<0.001	<0.001	-0.073	<0.001
FT (ng/ml)	141.6 ± 56.6	133.9 ± 48.6	132.7 ± 48.1	0.69	0.94	93.8 ± 40.0	91.1 ± 41.8	89.6 ± 52.9	0.32	0.06	-0.022	0.15
E2 (pg/ml)	19.4 ± 9.0	19.2 ± 8.0	19.3 ± 7.9	0.68	0.66	28.7 ± 11.0 ^a	27.3 ± 11.4	26.3 ± 11.1	0.009	<0.001	-0.038	0.01
FE2 (pg/ml)	0.40 ± 0.19	0.41 ± 0.18	0.41 ± 0.18	0.35	0.36	0.48 ± 0.21	0.47 ± 0.21	0.47 ± 0.22	0.66	0.22	-0.005	0.74
Serum by MS												
Testosterone (ng/ml)	5.11 ± 1.73	5.11 ± 1.53	4.81 ± 1.53	0.10	0.69	5.45 ± 1.80	4.71 ± 1.87 ^a	4.26 ± 1.76 ^c	0.01	0.02	-0.085	0.004
DHT (ng/ml)	0.33 ± 0.13	0.33 ± 0.11 ^a	0.30 ± 0.10 ^c	0.04	0.047	0.44 ± 0.21	0.38 ± 0.20 ^a	0.34 ± 0.19 ^c	0.01	<0.001	-0.112	<0.001
3α-Diol-3G+17G (ng/ml)	5.99 ± 2.18	6.10 ± 2.84 ^b	5.34 ± 2.55 ^d	0.008	0.03	5.38 ± 8.06	4.34 ± 2.42 ^a	3.78 ± 2.45 ^c	0.01	0.02	-0.108	<0.001
3α-Diol-3G (ng/ml)	1.70 ± 0.92	1.72 ± 0.99 ^c	1.54 ± 1.01 ^c	0.04	0.30	1.82 ± 2.59	1.49 ± 1.06	1.40 ± 1.13	0.48	0.67	-0.065	0.03
3α-Diol-17G (ng/ml)	4.29 ± 1.77	4.38 ± 2.21 ^b	3.81 ± 1.98 ^d	0.02	0.03	3.56 ± 5.58	2.84 ± 1.72 ^b	2.38 ± 1.70 ^d	0.005	0.001	-0.108	<0.001
ADTG (ng/ml)	68.0 ± 33.1	61.1 ± 30.3	58.4 ± 34.3	0.10	0.16	33.3 ± 38.3	31.7 ± 27.3	29.6 ± 23.6	0.47	0.25	-0.044	0.07
Urine by GC-MS												
3α-Diol (ng/μmol creatinin)	5.90 ± 2.02	5.39 ± 3.24	5.37 ± 2.98	0.74	0.11	3.00 ± 1.88	2.86 ± 1.91 ^a	2.40 ± 1.53 ^c	0.02	0.007	-0.103	0.007
ADT (ng/μmol creatinin)	284.7 ± 87.9	261.4 ± 106.4	254.3 ± 94.8	0.76	0.15	74.2 ± 50.0	71.4 ± 40.4	66.6 ± 41.5	0.24	0.11	-0.049	0.13

Values are given as mean ± SD. P1 values are for comparison of the three genotypes (one-way ANOVA). P3 are for regression models treating the polymorphism as a continuous variable. The β-values and P2 are for (TAAAA)_n genotype in regression models including subjects from both the GOOD and the MrOS cohorts, with age (coded as young adult = 1 and elderly = 2) and (TAAAA)_n genotype (coded as S/S = 1, S/L = 2, and L/L = 3) as covariates.

^a P < 0.05 vs. L/L.

^b P < 0.01 vs. L/L.

^c P < 0.05 vs. S/L.

^d P < 0.01 vs. S/L.

SHBG polymorphisms and androgen metabolites

Serum 3α-diol-Gs levels were associated with (TAAAA)_n genotype in both cohorts ($P < 0.01$; Table 2). 3α-diol-Gs can be subdivided into 3- and 17-glucuronidated metabolites, respectively. Serum 3α-diol-17G levels were 15% higher in the S/L than in the L/L genotype in the young adult men and 19% higher in the S/L than in the L/L genotype in the elderly men (Table 2). The (TAAAA)_n genotype was significantly associated with 3α-diol-3G in young adult men but not in elderly men.

Urine levels of 3α-diol were associated with (TAAAA)_n genotype in the elderly men (S/L 19% over L/L) but not in the young adult men (Table 2). Urine levels of androsterone were not associated with (TAAAA)_n genotype. Results for regression analyses of the pooled sample of both cohorts are shown in Table 2. When pooling of the samples, none of the interaction effects was statistically significant. Associations between rs1799941 and androgen metabolites were very similar to those found for the (TAAAA)_n microsatellite (Table 3).

The relative contribution of (TAAAA)_n and rs1799941 genotype

The independent contribution of the (TAAAA)_n microsatellite and rs1799941, respectively, was investigated in regression models including both polymorphisms simultaneously. For levels of SHBG, rs1799941 but not (TAAAA)_n genotype was an independent predictor in the MrOS (rs1799941 $\beta = -0.125$, $P = 0.03$; (TAAAA)_n $\beta = -0.011$, $P = 0.84$) as well as in the GOOD cohort (rs1799941 $\beta = -0.172$, $P = 0.048$; (TAAAA)_n $\beta = 0.042$, $P = 0.63$). Haplotypes were also computed. The most common ones in the pooled sample of both cohorts were 8G (36.1%), 6A (24.4%), 9G (23.9%), 10G (7.8%), and 7G (4.7%). In the MrOS study as well as in the GOOD study, only haplotype 6A was an independent predictor of SHBG levels when all five haplotypes were included simultaneously in regression models. The adjusted r^2 value for SHBG levels using this model was 0.018 in both populations. For the rs1799941 SNP, the adjusted r^2 value was 0.018 in the elderly and 0.017 in the young adult men, indicating that compared with the SNP-based model, the haplotype-based model did not add additional information.

Role of serum SHBG as a mediator of the relationship between SHBG genotype and androgens

To investigate whether the effects of SHBG genotype on levels of androgens and metabolites were mediated via SHBG levels, serum levels of SHBG were added to regression models investigating the effects of rs1799941 on the above mentioned parameters. In the MrOS cohort, the values for T measured by RIA were $\beta = -0.082$ and $P < 0.0001$ before and $\beta = -0.056$ and $P = 0.004$ after inclusion of SHBG levels. For DHT, the values were $\beta = -0.107$ and $P = 0.009$ and $\beta = -0.092$ and $P = 0.03$, respectively. For 3α-diol-17G, the values were $\beta = -0.112$ and $P = 0.006$ and $\beta = -0.131$ and $P = 0.002$, respectively. The same pattern was seen in the GOOD cohort, except for DHT, for which the predictive role of rs1799941 was reduced from $\beta = -0.112$ and $P = 0.01$ to $\beta = -0.048$ and $P = 0.24$ after inclusion of SHBG levels. Results for (TAAAA)_n were very similar.

TABLE 3. Hormone levels according to *SHBG* rs1799941 genotype

	GOOD				MrOS Sweden				GOOD/MrOS Sweden	
	AA (n = 51)	AG (n = 395)	GG (n = 612)	<i>P</i>	AA (n = 181)	AG (n = 1069)	GG (n = 1565)	<i>P</i>	β	<i>P</i> ₂
Serum by RIA										
SHBG (ng/ml)	24.3 ± 8.8 ^b	21.2 ± 7.7 ^a	19.5 ± 7.0 ^c	<0.001	50.5 ± 25.8 ^b	44.9 ± 20.9	41.3 ± 21.8	<0.001	-0.112	<0.001
T (ng/ml)	5.29 ± 2.07	4.99 ± 1.67	4.81 ± 1.62	0.31	5.38 ± 2.03 ^b	5.05 ± 2.09 ^b	4.69 ± 2.17 ^d	<0.001	-0.074	<0.001
FT (pg/ml)	137.0 ± 60.5	133.3 ± 47.4	132.3 ± 48.9	0.69	92.4 ± 38.6	91.6 ± 42.0	89.5 ± 52.2	0.35	-0.019	0.20
E2 (pg/ml)	20.0 ± 9.5	18.9 ± 7.6	19.5 ± 8.1	0.68	28.9 ± 11.4	27.2 ± 11.2	26.4 ± 11.1	0.02	-0.033	0.03
FE2 (pg/ml)	0.41 ± 0.20	0.40 ± 0.17	0.42 ± 0.18	0.35	0.48 ± 0.22	0.47 ± 0.21	0.47 ± 0.22	0.81	0.001	0.93
Serum by MS										
T (ng/ml)	4.93 ± 1.60	5.14 ± 1.54	4.79 ± 1.56	0.10	5.40 ± 1.77	4.68 ± 1.90 ^a	4.25 ± 1.71 ^c	0.04	-0.075	0.01
DHT (ng/ml)	0.32 ± 0.12	0.33 ± 0.11 ^a	0.30 ± 0.11 ^c	0.04	0.44 ± 0.20	0.38 ± 0.21 ^a	0.34 ± 0.19 ^c	0.03	-0.103	<0.001
3 α -Diol-3G+17G (ng/ml)	6.24 ± 2.30	6.01 ± 2.76 ^b	5.35 ± 2.57 ^d	0.008	5.76 ± 8.65	4.36 ± 2.51 ^a	3.83 ± 2.41 ^c	0.04	-0.106	<0.001
3 α -Diol-3G (ng/ml)	1.78 ± 0.94	1.67 ± 0.91 ^a	1.54 ± 1.04 ^c	0.04	1.97 ± 2.78	1.49 ± 1.08	1.41 ± 1.14	0.54	-0.070	0.02
3 α -Diol-17G (ng/ml)	4.46 ± 1.94	4.33 ± 2.19 ^b	3.81 ± 1.96 ^d	0.02	3.79 ± 6.00	2.87 ± 1.76 ^b	2.42 ± 1.67 ^d	0.02	-0.103	<0.001
ADTG (ng/ml)	69.1 ± 34.4	61.3 ± 30.4	57.9 ± 33.6	0.10	34.1 ± 40.8	33.1 ± 32.2	29.6 ± 23.1	0.52	-0.050	0.05
Urine by GC/MS										
3 α -Diol (ng/ μ mol creatinine)	5.90 ± 2.02	5.27 ± 3.02	5.45 ± 3.10	0.74	3.10 ± 1.93	2.92 ± 1.92 ^a	2.40 ± 1.53 ^c	0.01	-0.111	0.004
ADT (ng/ μ mol creatinine)	284.7 ± 87.9	255.9 ± 106.8	261.0 ± 97.5	0.76	77.8 ± 21.6	72.5 ± 42.1	66.6 ± 41.2	0.23	-0.050	0.12

Values are given as mean ± SD. *P* values are for comparison of the three genotypes (one-way ANOVA). The β -values and *P*₂ are for rs1799941 genotype in regression models including subjects from both the GOOD and the MrOS cohort, with age (coded as young adult = 1 and elderly = 2) and rs1799941 genotype (coded as A/A = 1, A/G = 2, and G/G = 3) as covariates.

^a *P* < 0.05 vs. G/G.

^b *P* < 0.01 vs. G/G.

^c *P* < 0.05 vs. A/G.

^d *P* < 0.01 vs. A/G.

SHBG polymorphisms and bone parameters

Among the elderly men, (TAAAA)_n genotype was an independent but modest predictor of sBMD in the trochanter, total hip, and femur neck when included together with height, weight, age, physical activity, smoking, and calcium intake as covariates in multiple regression models (Table 4). There was no association with spine sBMD. Results for rs1799941 were similar to those found for the (TAAAA)_n polymorphism (Table 4). Inclusion of serum levels of T as an additional covariate had no substantial impact on the results (Table 4). Among the young adult men, there were no associations between *SHBG* polymorphisms and BMD (data not shown).

SHBG transgenic mice

To further investigate the influence of the *SHBG* gene on bone parameters, we studied femurs of 6-month-old male transgenic mice expressing the human *SHBG* gene under the control of its own regulatory sequences. The pQCT measurements of the femur showed that the cortical bone mineral

content in these transgenic mice was increased by 13.5% compared with their wild-type siblings. This was because of an increased cortical thickness, whereas the cortical and trabecular volumetric BMDs were not affected (Table 5). No significant effect on skeletal parameters was seen in 4-wk-old male *SHBG* transgenic mice (data not shown).

Discussion

In this study, we show that the (TAAAA)_n polymorphism and the rs1799941 SNP in the human *SHBG* promoter are clearly associated with serum levels of SHBG in both young adult and elderly men. These *SHBG* polymorphisms were also associated with serum DHT and 3 α -diol-Gs levels in both elderly and young adult men, but this was most evident in the elderly men, in whom there was also an association between these polymorphisms and T and hip BMD.

Associations between the *SHBG* (TAAAA)_n polymorphism and SHBG levels have previously been studied only in women. These studies have been smaller in size than the present study, with the number of individuals ranging from

TABLE 4. Independent predictive role of (TAAAA)_n and rs1799941 *SHBG* genotypes for BMD

	(TAAAA) _n				rs1799941			
	β 1	<i>P</i> 1	β 2	<i>P</i> 2	β 1	<i>P</i> 1	β 2	<i>P</i> 2
Total hip (g/cm ²)	-0.048	0.007	-0.045	0.01	-0.037	0.03	-0.034	0.05
Trochanter (g/cm ²)	-0.050	0.005	-0.046	0.01	-0.037	0.04	-0.033	0.07
Femur neck (g/cm ²)	-0.046	0.01	-0.046	0.01	-0.039	0.03	-0.040	0.03
Lumbar spine (g/cm ²)	-0.029	0.11	-0.031	0.09	-0.021	0.25	-0.021	0.25

β 1 and *P*1 are for *SHBG* (TAAAA)_n genotype (coded as S/S = 1, S/L = 2, and L/L = 3) and rs1799941 genotype (coded as AA = 1, AG = 2, and GG = 3) in multiple regression models adjusted for height, weight, age, physical activity, smoking, calcium intake, and DXA type. β 2 and *P*2 are for the same analyses with the addition of serum levels of T as an additional covariate.

TABLE 5. The pQCT measurements of femur in *SHBG* transgenic and wild-type mice

	Wild type (n = 6)	<i>SHBG</i> transgenic (n = 7)	<i>P</i>
Cortical BMC (mg/mm)	1.40 ± 0.06	1.59 ± 0.06	0.03
Cortical thickness (mm)	0.23 ± 0.01	0.25 ± 0.01	0.007
Cortical vBMD (mg/mm ³)	1.28 ± 0.02	1.31 ± 0.01	0.09
Trabecular vBMD (mg/mm ³)	0.27 ± 0.03	0.27 ± 0.02	0.89

Values are given as mean ± SEM. *P* values are for Student's *t* test. BMC, Bone mineral content; vBMD, volumetric BMD.

185 (9) to 349 (7). Moreover, study subjects were affected by hirsutism (8) and/or polycystic ovary syndrome (8, 9) or had very divergent genetic backgrounds (7), whereas the cohorts investigated in the present study are population based. Concerning associations between rs1799941 and SHBG levels, we confirm the findings from the study by Low *et al.* (11) (n = 233) in our rather large cohort of elderly men (n ≈ 3000) and show that the associations are valid also in younger men as shown by the results from the GOOD study. It therefore seems likely that the polymorphisms in the human *SHBG* gene we have studied account for much of the genetic influence on SHBG levels in men, which has been described in the twin study by Ring *et al.* (4). The large sizes of the two cohorts, investigated in the present study, strengthen our data. The fact that the two polymorphisms are in linkage disequilibrium makes the very similar associations with the parameters investigated a logical finding.

One may speculate that the polymorphic sequences in the human *SHBG* promoter influence its transcriptional activity and thereby the rate of SHBG production. This has been suggested by Hogeveen *et al.* (32), who found a lower transcriptional activity of human *SHBG* promoter sequence containing the (TAAA)₆ repeat allele when compared with alleles of longer length in human HepG2 hepatoblastoma cells. Their studies examined the human *SHBG* promoter in the context of a reporter gene, and it is not entirely surprising that the activities are not in line with the findings in the present study or other studies that have found higher serum SHBG levels in individuals with short alleles (7–9). This is because such reporter gene studies examine the activities of promoters of naked DNA and do not take into consideration the contributions of higher chromatin structure on transcriptional activity or the possible long-range contributions of other regulatory sequences. They do, however, help to define the presence and location of regulatory sequences that might function in an *in vivo* context. According to our multiple regression analyses, the rs1799941 SNP, but not the (TAAA)_n genotype, is an independent predictor of SHBG levels. Haplotype-based methods did not add additional information. However, the present study is an association study, and additional functional studies are required to clarify the details regarding the genetic regulation of SHBG transcription.

Genotypes with high levels of SHBG had clearly increased levels of T among the elderly men. Our regression analyses, including SHBG as a covariate, indicate that the associations between *SHBG* polymorphisms and androgens/androgen metabolites were largely independent of affected serum SHBG levels. One could speculate that assay variations in

SHBG and or T analyses or diurnal fluctuations in serum levels of SHBG and androgens might blur the picture, but the associations between *SHBG* polymorphisms and T could also be mediated via other mechanisms unknown to us. In recent years, however, data have accumulated indicating that to a large extent, sex hormones exerting effects in the peripheral tissues are synthesized and metabolized at their site of action (15). Therefore, measurements of T and calculations of FT give limited information about the hormonal milieu in the steroid-responsive tissues. Serum levels of DHT were associated with *SHBG* polymorphisms in both cohorts, and this could be an indication of an effect by the polymorphisms on the local androgenic milieu in the peripheral tissues.

Recent development of the LC/MS/MS technique enables measurement of the glucuronidated androgen metabolites 3 α -diol-G and ADTG, which are considered as the final step of the androgenic signal (16, 33). We have recently found that 3 α -diol-G is a stronger predictor of BMD in elderly men than bioactive androgens including T, FT, and DHT, supporting the notion that these metabolites are good indicators of the androgen pool in males (own unpublished data). Interestingly, 3 α -diol-G was associated with *SHBG* genotypes in both cohorts. This finding, in addition to the findings of associations with T and DHT, lends further support to the hypothesis that *SHBG* polymorphisms influence androgen levels both in the circulation and in the peripheral target tissues.

Interestingly, *SHBG* genotypes were modest but clearly significant independent predictors of BMD at all bone sites investigated in the hip but not in the lumbar spine of elderly men. The genotypes with the highest levels of T, DHT, and glucuronidated androgen metabolites had the highest hip BMD, but the impact on BMD does not seem to be mediated by serum levels of T as indicated by the fact that inclusion of T levels in the regression models had no substantial impact on the association between *SHBG* genotypes and BMD. We have recently demonstrated that 3 α -diol-G androgen metabolites are strong positive predictors of BMD in elderly men (own unpublished data). Thus, one may speculate that the *SHBG* polymorphisms result in affected local androgenic activity in bone and as a consequence an affected BMD in elderly men. The *SHBG* genotypes associated with high levels of SHBG were also associated with high BMD. Interestingly, we also found that male mice overexpressing *SHBG* had increased cortical bone mineral content in the femur, supporting the notion that elevated SHBG levels actually may cause increased bone mass. In contrast, the trabecular BMD was unaffected in the *SHBG* transgenic mice, which is in line with the present finding that the *SHBG* polymorphisms were not associated with the spine BMD, *i.e.* the bone site with the highest trabecular bone content. However, in contrast to men, mice do not normally express SHBG, which should be kept in mind when the data are interpreted. It should also be noted that the age of the 6-month-old mice corresponds to middle-aged rather than to elderly men. However, we find it noteworthy that genetically derived high SHBG levels seem to be associated with increased bone mass in both species. The associations between *SHBG* polymorphisms and hip BMDs in the elderly but not in the young adult men indicate that these polymorphisms are of impor-

tance for age-dependent bone loss but not for the attainment of peak BMD.

A recent challenging publication demonstrated that megalin, an endocytic receptor in reproductive tissues, acts as a pathway for cellular uptake of biologically active androgens and estrogens bound to SHBG (34). This finding might indicate that increased SHBG levels via cell-specific endocytosis could enhance local sex steroid action. These recent provocative results might indicate that elevated SHBG levels, such as in SHBG transgenic mice, could result in increased sex steroid levels and action in osteoblasts, resulting in increased BMD, as seen in the present study. However, we think that this possible mechanism for the stimulatory role of SHBG on BMD should be regarded as rather speculative.

In the MrOS cohort, two different DXA machines were used. To minimize the confounding effect of using two different DXA machines, we used calculated sBMD as a means of cross-calibrating between different DXA machines, a procedure that has been evaluated in several previous studies (19–21). Furthermore, DXA type (Hologic or Lunar) was included as a covariate in all regression analyses with BMD as a dependent factor. Nevertheless, we cannot fully exclude that the use of two different DXA machines had an impact on our results in the MrOS study.

In conclusion, our study shows that polymorphisms in the human SHBG gene promoter are associated with levels of SHBG, androgens, and glucuronidated androgen metabolites in elderly as well as in young adult Swedish men. These polymorphisms are also associated with hip BMD in elderly men. Additional studies are needed to investigate the biological importance of these SHBG polymorphisms.

Acknowledgments

We thank Anette Hansevi, Anna-Lena Jirestedt, Maud Petterson, Lotta Uggla, and Magid Fallahi for excellent technical assistance.

Received March 29, 2006. Accepted August 14, 2006.

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This work was supported by the Swedish Research Council (Grants 04250 and 04496), the Swedish Foundation for Strategic Research, European Commission Grant QLK4-CT-2002-02528, the ALF/LUA (Avtal om Läkarutbildning och Forskning/Läkarutbildningsavtalet) research grant in Göteborg, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, Petrus and Augusta Hedlunds Foundation, the Novo Nordisk Foundation, The Göteborg Medical Society, and World Anti-Doping Agency.

Disclosure summary: D.M. has consulted for and received lecture fees from MSD, Lilly, Novartis, and Aventis. All other authors have nothing to declare.

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