Genetic Determinants of Serum Testosterone Concentrations in Men

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Genetic Determinants of Serum Testosterone Concentrations in Men


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Abstract

Testosterone concentrations in men are associated with cardiovascular morbidity, osteoporosis, and mortality and are affected by age, smoking, and obesity. Because of serum testosterone's high heritability, we performed a meta-analysis of genome-wide association data in 8,938 men from seven cohorts and followed up the genome-wide significant findings in one in silico (n = 871) and two de novo replication cohorts (n = 4,620) to identify genetic loci significantly associated with serum testosterone concentration in men. All these loci were associated with lower serum testosterone concentration defined as <300 ng/dl. Two single-nucleotide polymorphisms at the sex hormone-binding globulin (SHBG) locus (17p13-p12) were identified as independently associated with serum testosterone concentration (rs12150660, p = 1.2 x 10-47 and rs6258, p = 2.3 x 10-22). Subjects with 3 alleles of these variants had 6.5-fold higher risk of having low serum testosterone than subjects with no risk allele. The rs5934505 polymorphism near FAM9B on the X chromosome was also associated with testosterone concentrations (p = 5.6 x 10-16). The rs6258 polymorphism in exon 4 of SHBG affected SHBG’s affinity for binding testosterone and the measured free testosterone fraction (p < 0.01). Genetic variants in the SHBG locus and on the X chromosome are associated with a substantial variation in testosterone concentrations and increased risk of low testosterone. rs6258 is the first reported SHBG polymorphism, which affects testosterone binding to SHBG and the free testosterone fraction and could therefore influence the calculation of free testosterone using law-of-mass-action equation.
GWAS of Testosterone

The authors have declared that no competing interests exist.

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These authors contributed equally to this work.

These authors were joint senior authors on this work.
Introduction

Testosterone, the most important testicular androgen in men. Low serum testosterone concentrations are associated with cardiovascular morbidity, metabolic syndrome, type 2 diabetes mellitus, atherosclerosis, osteoporosis, sarcopenia, and increased mortality risk. Thus, there is growing evidence that serum testosterone is a valuable biomarker of men’s overall health status. Studies in male twins indicate that there is a strong heritability of serum testosterone. Here we perform a large-scale genome-wide association study to examine the effects of common genetic variants on serum testosterone concentrations. By examining 14,429 men, we show that genetic variants in the sex hormone-binding globulin (SHBG) locus and on the X chromosome are associated with a substantial variation in serum testosterone concentrations and increased risk of low testosterone. The reported associations may now be used in order to better understand the functional background of recently identified disease associations related to low testosterone. Importantly, we identified the first known genetic variant, which affects SHBG’s affinity for binding testosterone and the free testosterone fraction and could therefore influence the calculation of free testosterone. This finding suggests that individual-based SHBG-testosterone affinity constants are required depending on the genotype of this single-nucleotide polymorphism.

Results

Meta-analyses of genome-wide association studies for autosomal SNPs

We performed a GWAS of serum testosterone concentrations, investigating ~2.5 million SNPs in 8,938 men of Caucasian ancestry, 18 to 98 years, from seven cohorts. Genome-wide significant SNPs were found in the discovery analysis at one locus on chromosome 17 (17p13-p12) using the criteria described in the methods. The strongest association was found for rs12150660 (p = 1.9×10^{-17}), located 11.5 kb upstream of the major transcription start site of sex hormone-binding globulin (SHBG), with a minor allele frequency (MAF) of 23% (Table 1 [SNPs rs12150660 and rs6258], Figure 1A and Figures S1A, S2 and S3). Tests for independently associated SNPs with serum testosterone in this region revealed a second SNP, rs6258 (p = 4.1×10^{-15}), which represents a missense [P→L] polymorphism located in exon 4 of SHBG (Table 1 [SNPs rs12150660 and rs6258], Figure 1B) and which had a MAF of 2%. Based on HapMap release 22 (CEU), the r² between rs12150660 and rs6258 was 0.04. To validate the independence of these two SNPs, conditional meta-analysis of the discovery cohorts including both rs12150660 and rs6258 in an additive genetic linear model adjusted for covariates was calculated. Because the associations remained significant and mostly unchanged (rs12150660, p = 7.0×10^{-15}, rs6258, p = 1.6×10^{-15}), both SNPs were independently associated with serum testosterone concentrations. No additional autosomal locus fulfilled the criteria for genome-wide significance.

Replication of autosomal hits

The associations of rs12150660 and rs6258 were confirmed in the three replication cohorts (in silico replication in YFS and de novo replication in MrOS Sweden and EMAS), demonstrating a combined p-value in the discovery and the replication cohorts of 1.2×10^{-41} and 2.3×10^{-22}, respectively (Table 1 [SNPs rs12150660 and rs6258]). Both SNPs showed considerable heterogeneity of results across the studies as measured by the F statistic [18]. The F values for the discovery meta-analysis using the untransformed total testosterone values were 76.7% and 81.6% for rs12150660 and rs6258, respectively. The heterogeneity was reduced to 39.3% and 75.5% for rs12150660 and rs6258, respectively, by meta-analysing the z-score based untransformed total testosterone values and to 30.9% and 78.0%, respectively, by meta-analysing the inverse-normal transformed testosterone values. For rs12150660, a substantial amount of heterogeneity could be explained by phenotypic variation among the cohorts, whereas for rs6258 one cohort (InCHIANTI) showed consistent opposite effect directions in all models used. To take into account this heterogeneity, we additionally calculated a random effects model for untransformed total testosterone values. The association for rs12150660 remained genome-wide significant in the combined discovery and replication stage meta-analysis, the association for
Concentrations of testosterone and SHBG and a fixed value for SHBG's dissociation constant were measured using plasma but the analyses after excluding KORA yielded similar results. Calculated free testosterone was calculated for all subjects with both testosterone and SHBG available by using a modified law of mass action equation. The concentrations of testosterone and SHBG and a fixed value for SHBG's dissociation constant were used in these calculations.

The role of SHBG in the observed associations

As SNP rs12150660 is located 11.5 kb upstream of SHBG and SNP rs6258 is non-synonymous and located in exon 4 of SHBG, we evaluated the influence of these polymorphisms on SHBG concentrations. Both of these polymorphisms demonstrated a significant association with SHBG concentrations in both the discovery and replication cohorts (Table 1 [SNPs rs12150660 and rs6258]). However, even after adjusting for SHBG concentrations, the associations between these two SNPs and serum testosterone concentrations were still significant (p = 9.0 \times 10^{-6} for rs12150660 and p = 4.5 \times 10^{-7} for rs6258). Free testosterone calculated using law-of-mass-action equation was not associated with either of the two polymorphisms (Table 1 [SNPs rs12150660 and rs6258]). As serum testosterone and SHBG are highly correlated (e.g., in MrOS Sweden rs<sub>p</sub> = 0.53), variations in SHBG concentration might have influenced the observed associations of serum testosterone with other non-SHBG-related loci. Therefore, we performed an additional SHBG-adjusted genome-wide meta-analysis among the discovery cohorts, wherein none of the non-SHBG-related autosomal SNPs reached genome-wide significance (Figure S1B).
The rs6258 polymorphism affects SHBG binding affinity for testosterone and the measured free testosterone fraction

As rs6258 is non-synonymous (P156L) and located in exon 4 of SHBG, we evaluated the serum SHBG steroid-binding capacity of the different rs6258 genotypes. As shown in Figure S5, serum SHBG from CT but not CC subjects had a lower steroid-binding capacity than expected from values obtained by an SHBG immunoassay (p = 0.003). Therefore, we analyzed the SHBG affinity for testosterone using Scatchard plots of SHBG in serum of men with the rs6258 genotype (Figure 3A), and revealed (Figure 3B) a higher mean dissociation constant (Kd) indicative of a lower affinity in CT (Kd = 4.5 nM) and TT (Kd = 4.9 nM) individuals than in CC individuals (Kd ≈ 2.9 nM). Recombinant SHBG corresponding to the T genotype demonstrated a higher dissociation constant (lower affinity) compared with recombinant SHBG corresponding to the C genotype (T genotype Kd 2.5 nM; C genotype Kd 1.2 nM, Figure 3C). In addition, the free testosterone fraction measured by an equilibrium dialysis method was 22% higher (p = 1.4 × 10^{-5}) in serum from CT subjects than in serum from CC subjects (Figure 3D).

X chromosome analyses

Imputed values for X chromosome-located SNPs were available for the two larger discovery cohorts (SHIP and FHS; n = 5,067).

We performed meta-analyses of imputed X chromosome SNPs for serum testosterone concentrations both with and without SHBG adjustment, revealing one genome-wide significant association for SNP rs5934505 (p = 8.5 × 10^{-9}) in the SHBG-adjusted model (Table 1 [SNP rs5934505] and Figures S1B and S3). This SNP was confirmed in two replication cohorts with de novo genotyping (MrOS Sweden p = 3.6 × 10^{-3}; EMAS p = 1.5 × 10^{-7}). Meta-analysis of discovery and replication cohorts resulted in a combined p-value of 5.6 × 10^{-16}. The rs5934505 SNP is located in a CNV-insertion area (Xp22), 145 kb upstream of the family with sequence similarity 9, member A (FAM9A) and 79 kb downstream of the family with sequence similarity 9, member B (FAM9B) (Figure 1C). In addition, rs5934505 is located 214 kb upstream of Kallmann syndrome 1 sequence (KAL1). SNP rs5934505 was associated with serum testosterone without SHBG-adjutment (combined p-value of 1.7 × 10^{-9}) and with free testosterone (combined p-value of 6.7 × 10^{-11}), but not with SHBG (Table 1 [SNP rs5934505]). The mean serum testosterone and calculated free testosterone but not SHBG concentrations were lower in men with T genotype than in those with C genotype for rs5934505 (Table 2).

Discussion

This GWAS revealed novel genetic variants that significantly affect circulating testosterone concentrations in men. The presence of three or more risk alleles for the two polymorphisms in the...
SHBG loci resulted in markedly decreased testosterone concentrations compared to men with two or less risk alleles. Importantly, one of the identified genetic variations was associated with an alteration in SHBG’s binding affinity for testosterone and the measured free testosterone fraction. In addition, we identified a locus on the X chromosome influencing serum testosterone concentrations. The genetic contribution of the polymorphisms to testosterone concentrations reported here is substantial; as a reference for comparison, the effect of these polymorphisms on testosterone concentrations in men is similar or greater than that for known risk factors such as age, smoking, and BMI [19,20].

These findings improve our understanding of the genetic factors that affect serum testosterone concentrations and contribute to the variation in testosterone concentrations in men. These polymorphisms may assist in the identification of men at risk of low serum testosterone concentrations. Our studies add to the evidence that genetic variations within known risk factors such as age, smoking, and BMI [19,20].

The SNP rs12150660 that is strongly associated with testosterone concentrations is located 11.5 kb upstream of the coding sequence for SHBG mRNA production in the liver. However, it still resides within the human SHBG locus because several other alternative exon 1 sequences are located up to ~13 kb upstream of the exon 1 sequence that encodes the secretion signal polypeptide of the SHBG precursor in the liver [22]. There are no obvious nuclear protein binding sites within the sequences spanning SNP rs12150660, and it remains to be determined whether this SNP disrupts a cis-element that directly influences SHBG transcription. We have found that rs12150660 is in strong LD (r^2 = 0.89) with another common SNP (rs1799941) in the SHBG proximal promoter that was shown to be associated with serum SHBG concentrations [23–25]. Thus, it is highly likely that only one of these polymorphisms is actually functional and therefore both SNPs represent the same signal. It should also be noted that rs1799941 is linked to the number of TAAAA repeats within an Alu sequence upstream of SHBG promoter [26] and that the rs1799941 (A allele) is linked with the presence of six TAAAA repeats in this location which has been reported to be associated with higher SHBG concentrations [27]. In addition, while there does not appear to be any putative transcriptional factor binding sites with the sequence comprising rs12150660, it remains to be determined whether rs12150660 or these other associated SNPs in

<table>
<thead>
<tr>
<th>SNP identified in GWAS for total testosterone</th>
<th>SNP identified in GWAS for SHBG-adjusted testosterone</th>
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<tbody>
<tr>
<td>rs12150660 GG</td>
<td>rs6258 GG</td>
</tr>
<tr>
<td>rs12150660 GT</td>
<td>rs6258 GT</td>
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<tr>
<td>rs12150660 TT</td>
<td>rs6258 TT</td>
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<tr>
<td>p-value</td>
<td>C</td>
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<td>EMAS Testosterone (ng/dl) (n = 1310)</td>
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<tr>
<td>454±161</td>
<td>490±172</td>
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<td>8.47±2.53</td>
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<tr>
<td>SHBG (nM) (n = 396±17.1)</td>
<td>45.2±20.4</td>
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<tr>
<td>MrOS Sweden Testosterone (ng/dl) (n = 1317)</td>
<td>(n = 844)</td>
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<td>435±170</td>
<td>475±177</td>
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<td>Calculated Free Testosterone (ng/dl)</td>
<td>7.98±3.07</td>
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<td>SHBG (nM) (n = 41.0±21.6)</td>
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<tr>
<td>YFS Testosterone (ng/dl) (n = 522)</td>
<td>(n = 329)</td>
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<td>525±182</td>
<td>549±246</td>
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<td>Calculated Free Testosterone (ng/dl)</td>
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<tr>
<td>SHBG (nM) (n = 30.0±11.7)</td>
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NA = not available. Free testosterone was calculated for all subjects with both testosterone and SHBG available by using a modified law of mass action equation. The doi:10.1371/journal.pgen.1002313.t002

GWAS of Testosterone
the SHBG gene are functionally important or simply represent proxies of SHBG and testosterone concentrations in men.

Our meta-analyses of imputed X chromosome SNPs revealed one genome-wide significant association for SNP rs5934505, located in a CNV-insertion area (Xp22), 145 kb upstream of family with sequence similarity 9, member A (FAM9A) and 79 kb downstream of family with sequence similarity 9, member B (FAM9B). Both genes, FAM9A and FAM9B, are expressed exclusively in the testis [28] and described here for the first time to be associated with total as well as free testosterone concentrations. rs5934505 is located 214 kb upstream of Kallmann syndrome 1 sequence (KAL1). Although the Kallmann syndrome, a type of hypogonadotropic hypogonadism associated with anosmia and other congenital anomalies, has been linked to mutations in the KAL1 gene on the X chromosome, only 11–14% of Caucasian patients with hypogonadotropic hypogonadism have detectable KAL1 mutations [29], reflecting the considerable genetic heterogeneity of this syndrome.
The strengths of our study include a discovery sample size of 8,938 men, which allowed us at the threshold $\alpha = 5 \times 10^{-8}$, a 90% power to detect SNPs accounting for 0.5% of the total variance in serum testosterone concentrations, and 99% power to detect SNPs accounting for 1% of the total variance. The SNPs rs12150660, rs6258, and rs5934505 explained 2.3%, 0.9%, and 0.6%, respectively, of the variance in serum testosterone concentrations when evaluated in the MrOS Sweden replication cohort. Future meta-analyses including larger samples will probably reveal additional loci associated with serum testosterone. Further research into the functional significance of these variants will be needed to enable the translation of these findings into the mechanisms of sex steroid-related diseases and strategies for risk assessment. As the causal or etiological role of these polymorphisms in the genesis of low testosterone has not been established, the reported polymorphisms associated with low serum testosterone concentration may be viewed currently as risk markers rather than causal risk factors.

In conclusion, genetic variants in the SHBG locus and on the X chromosome are associated with a substantial variation in testosterone concentrations and increased risk of low testosterone in men. Further studies are needed to determine the impact of these genetic variations on sex hormone-related disorders. rs6258 is the first reported SHBG polymorphism, which affects testosterone binding to SHBG and the free testosterone fraction and could therefore influence the calculation of free testosterone using law-of-mass-action equation.

**Methods**

**Study samples and genotyping**

The discovery stage of the GWAS included 8,938 Caucasian men of European descent drawn from seven epidemiological cohorts: the Framingham Heart Study (FHS), the Study of Health in Pomerania (SHIP), the Gothenburg Osteoporosis and Obesity Determinants (GOOD) study, the Cooperative Health Research in
the Region of Augsburg (KORA) study, the Health, Aging and Body Composition (HEALTH ABC) study, the Rotterdam Study (RS1), and the Invecchiare in Chianti (InCHIANTI) (Table S1). The replication stage consisted of 4,620 men from two epidemiological cohorts (the European Male Ageing Study [EMAS] and the Osteoporotic Fractures in Men [MrOS] Sweden study) for de novo genotyping of the top SNPs and one additional cohort (the Young Finns Study, [YFS, n = 871]) with genome-wide association data available and joining the study after stage one was finished for in silico replication (Table S2).

Exclusion criteria included chemical or surgical castration and/or medications affecting sex hormones such as steroid 5-alpha reductase inhibitors, and sex hormone antagonists. All studies were approved by local ethics committees and all participants provided written informed consent. Characteristics of the study samples and detailed descriptions of the participating cohorts, genotyping methods, quality control, and imputation procedures are provided in Text S1.

Genotyping and statistical analyses

Altogether, ~2.5 million SNPs, imputed using the HapMapII CEU population, were tested for association with serum testosterone in the discovery stage. Genome-wide association analyses using an additive genetic linear regression model adjusted for age, BMI, and current smoking were conducted twice within each of the discovery cohorts using serum testosterone expressed as ng/dl, as well as inverse-normal transformed serum testosterone as outcomes.

To examine the robustness of the discovery results and to reduce the risk of spurious associations due to possible testosterone measurement heterogeneity between the individual cohorts, three different types of meta-analyses were performed in the discovery stage: 1) an inverse-variance weighted fixed effect model; 2) a z-score based analysis of the untransformed serum testosterone concentrations; and 3) a z-score based meta-analysis of the inverse-normal transformed values. Model 1 was used as main analysis since it allowed the computation of effect estimates, whereas the other two analysis models were used for verification and quality control checks of the main findings. All meta-analyses were performed using METAL (www.sph.umich.edu/csg/abecasis/metal/). The random effects model of the two SHBG locus SNPs was calculated using the R-package metafor (www.r-project.org). Imputed genotypes were analyzed in all cohorts taking the genotype uncertainties into account. Genomic control was applied to each individual cohort’s results and to the discovery stage meta-analysis to correct p-values for potential effects of mild population stratification. The estimated genomic control lambda was low for both the individual cohorts (range of \( \lambda_{GC}: 1.00–1.07 \)) and the meta-analyses (range of \( \lambda_{GC}: 1.01–1.02 \)), suggesting little residual confounding due to population stratification (Figure S2).

To reduce the variance on serum testosterone induced by SHBG concentration, the GWAS included a genome-wide test for association of untransformed serum testosterone concentrations adjusted for age, BMI, current smoking, SHBG and SHBG2 concentrations, again using both an inverse-variance weighted fixed effect as main analysis and a z-score based meta-analysis for quality control purposes.

A threshold of \( p < 5 \times 10^{-8} \) was established a priori as the level for genome-wide significance in the discovery analyses [30]. SNPs that reached genome-wide significance in the inverse-variance weighed meta-analysis of untransformed serum testosterone concentrations with or without adjustment for SHBG and which had association results in at least five of the seven cohorts (for chr X: two cohorts with data available) were selected for further analyses. Notably, all autosomal SNPs that fulfilled these criteria also reached genome-wide significance in the other two types of meta-analyses. From these SNPs, all independent SNPs were taken to the replication stage.

We also assessed whether the lead SNPs from the continuous trait analyses were associated with low serum testosterone concentration (defined as <300 ng/dl [16]; this level is slightly lower than that suggested recently by Wu et al [11 mmol/1 = 317 ng/dl] [17]) by binary logistic regression including the same covariates in the model used for the main analysis and meta-analyzing the within-cohort results using inverse-variance weighted fixed-effect model. The KORA cohort was not included in the meta-analyses of low serum testosterone as testosterone was measured using plasma in this cohort.

We determined the number of low serum testosterone concentration risk alleles (0 to 4) for the two lead SNPs of the SHBG locus in each individual and assessed the risk of low serum testosterone concentrations in the three replication cohorts (MrOS Sweden, EMAS, and YFS) using a trend test. Since only two subjects in the replication cohorts had four risk alleles, individuals having three and four risk alleles were grouped into one category to obtain more reliable effect estimates during the subsequent analyses. Details of test for independence, SHBG related analysis of the top SNPs and quality control steps performed can be found in Text S1.

Sex hormone measurements

Methods for the measurement of serum testosterone and SHBG are given in Text S1. Calculated free testosterone was for all subjects with both testosterone and SHBG available (n = 13833; Table 1 and Table 2) calculated by using a modified law of mass action equation, as described by Mazer [31]. The concentrations of testosterone and SHBG and a fixed value for SHBG’s dissociation constant were used in these calculations.

Free testosterone fraction

Free testosterone fraction was measured by an equilibrium dialysis method in 87 subjects with the CC genotype and 32 subjects with the CT genotype of rs6258 (Figure 3D) [32]. Detailed description of the free testosterone fraction measurements is provided in Text S1.

Sex hormone-binding globulin assays

In experiments evaluating SHBG binding capacity, serum SHBG concentrations were determined by two-site immuno-fluorometric assay (PerkinElmer Life Sciences, Turku, Finland) [33], or by a steroid-binding capacity assay [34]. For steroid-binding assays, serum samples were pre-incubated with dextran-coated charcoal (DCC) to remove endogenous steroids, prior to incubation with either \([^3H]5\alpha\text{-dihydrotestosterone (}[^3H]DHT]\); specific activity 50 Ci/mmol) or \([^3H]\text{testosterone (}[^3H]T\); specific activity 40 Ci/mmol], bound from free \([^3H]\text{steroid were separated using DCC as the separation reagent [34]. The steroid-binding properties of SHBG in diluted serum samples or tissue culture medium were determined by Scatchard analysis [34]. For the expression of SHBG protein, wild type (corresponding to the C genotype of rs6258) and rs6258 (corresponding to the T genotype of rs6258) SHBG cDNAs in the pRC/CMV expression vector were transfected into CHO cells, and G418 was used for selection of stably transfected cells. At near confluence, cells were washed with PBS and cultured in serum-free SFM4CHO medium (Thermo Scientific HyClone, Logan, UT) for four days before the SHBG-containing medium was harvested.
Supporting Information

**Figure S1** Manhattan plots giving genome-wide –log_{10} p-value according to chromosomal location for inverse-variance weighted meta-analysis of untransformed serum testosterone (A) and SHBG-adjusted serum testosterone (B) using an imputation quality filter (observed/expected variance ratio) >0.4 at the individual cohort level during meta-analysis. All seven discovery cohorts (n = 8,938) were included in the GWAS of chromosomes 1–22 while only the two largest cohorts (FHS and SHIP, n = 5,067) had GWAS data available for the X chromosome. (PDF)

**Figure S2** Quantile-quantile plot of the genome-wide association results of the inverse-variance weighted meta-analysis of untransformed serum testosterone including all SNPs (black) and after removal of the SNPs of the SHBG locus (blue). (PDF)

**Figure S3** Associations for (A) rs12150660 and (B) rs6258 with SHBG. Gray regression line r^2 = 0.866) were measured among the cohorts. (PDF)

**Figure S4** Risk of low serum testosterone concentrations (serum testosterone <300 ng/dl) according to the number of combined risk alleles for rs12150660 (G = risk allele) and rs6258 (T = risk allele) in the three replication cohorts (MrOS Sweden, EMAS, and YFS). Bars indicate 95% confidence intervals. Only two individuals in the three replication cohorts had four risk alleles and therefore individuals with three and four risk alleles were pooled into one group with ≥3 risk alleles. Two risk allele counts were used as reference, since this is the prevalent amount among the cohorts. (PDF)

**Figure S5** Subjects heterozygous for the SHBG allele containing an rs6258 SNP have lower serum SHBG steroid-binding capacity (Y-axis) when compared to the concentrations of SHBG measured by immunoassay (X-axis). Serum SHBG concentrations from 10 individuals homozygous for the wild type SHBG allele (CC, dashed regression line r^2 = 0.872) or heterozygous for the rs6258 variant SHBG allele (CT, solid regression line r^2 = 0.866) were measured by a time-resolved immunofluorometric assay[33], and a steroid-binding capacity assay using [1H]DHT as the labelled ligand.[34] (PDF)

**Table S1** Characteristics of 14,429 men from 10 cohorts included in the genome-wide association study meta-analysis. (PDF)

**Table S2** Additional genotyping information for the 10 cohorts included in the genome-wide association study meta-analysis. (PDF)

**Table S3** Meta Analysis of untransformed total testosterone using Random Effect Model. (PDF)

**Text S1** Supplemental methods. (DOC)

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Author Contributions


References


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