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Ohlsson, Claes; Wallaschofski, Henri; Lunetta, Kathryn L.; Stolk, Lisette; Perry, John R. B.; Koster, Annemarie; Petersen, Ann-Kristin; Eriksson, Joel; Lehtimaki, Terho; Huhtaniemi, Ilpo T.; Hammond, Geoffrey L.; Maggio, Marcello; Coviello, Andrea D.; Ferrucci, Luigi; Heier, Margit; Hofman, Albert; Holliday, Kate L.; Jansson, John-Olov; Kahonen, Mika; Karasik, David; Karlsson, Magnus; Kiel, Douglas P.; Liu, Yongmei; Ljunggren, Osten; Lorentzon, Mattias; Lyytikainen, Leo-Pekka; Meitinger, Thomas; Mellstrom, Dan; Melzer, David; Miljkovic, Iva; Nauck, Matthias; Nilsson, Maria; Penninx, Brenda; Pye, Stephen R.; Vasan, Ramachandran S.; Reincke, Martin; Rivadeneira, Fernando; Tajar, Abdelouahid; Teumer, Alexander; Uitterlinden, Andre G.; Ulloor, Jagadish; Viikari, Jorma; Voelker, Uwe; Voelzke, Henry; Wichmann, H. Erich; Wu, Tsung-Sheng; Zhuang, Wei Vivian; Ziv, Elad; Wu, Frederick C. W.; Raitakari, Olli

Published in:
PLoS Genetics

DOI:
10.1371/journal.pgen.1002313

2011

Link to publication

Citation for published version (APA):
Ohlsson, C., Wallaschofski, H., Lunetta, K. L., Stolk, L., Perry, J. R. B., Koster, A., Petersen, A.-K., Eriksson, J., Lehtimaki, T., Huhtaniemi, I. T., Hammond, G. L., Maggio, M., Coviello, A. D., Ferrucci, L., Heier, M., Hofman, A., Holliday, K. L., Jansson, J.-O., Kahonen, M., ... Haring, R. (2011). Genetic Determinants of Serum Testosterone Concentrations in Men. *PLoS Genetics*, *7*(10). https://doi.org/10.1371/journal.pgen.1002313

Total number of authors:
59

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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 also associated with low 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, $p = 1.2\times10^{-41}$ and rs6258, $p = 2.3\times10^{-22}$). Subjects with ≥3 risk 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 ($p = 5.6\times10^{-16}$). 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.

Citation: Ohlsson C, Wallaschofski H, Lunetta KL, Stolk L, Perry JRB, et al. (2011) Genetic Determinants of Serum Testosterone Concentrations in Men. PLoS Genet 7(10): e1002313. doi:10.1371/journal.pgen.1002313

Editor: Gonçalo R. Abecasis, University of Michigan, United States of America

Received April 4, 2011; **Accepted** August 11, 2011; **Published** October 6, 2011

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Funding: Framingham Heart Study (FHS): The FHS phenotype-genotype analyses for this work were supported by the National Institute of Aging (Genetics of Reproductive Life Period and Health Outcomes, R21AG032598; JM Murabito, KL Lunetta, D Karasik, DP Kiel, WV Zhuang). The Framingham Heart Study of the National Heart Lung and Blood Institute of the National Institutes of Health and Boston University School of Medicine is supported by the National Heart, Lung, and Blood Institute's Framingham Heart Study Contract No. N01-HC-25195 and its contract with Affymetrix for genotyping services (Contract No. N02-HL-6-4278). Sex hormone measurements were funded primarily by National Institute on Aging grant 1RO1AG31206 (PIs: S Bhasin and RS Vasan); additional support was provided by the Boston Claude D. Pepper Older Americans Independence Center (5P30AG031679) and a grant from the National Institute on Aging and the National Institute of Arthritis Musculoskeletal and Skin Diseases to DP Kiel (R01 AR/AG 41398). Analyses reflect intellectual input and resource development from the Framingham Heart Study investigators participating in the SNP Health Association Resource (SHARe) project. A portion of this research was conducted using the Linux Cluster for Genetic Analysis (LinGA-II), funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center. Study of Health in Pomerania (SHIP): Computing resources have been made available by the Leibniz Supercomputing Centre of the Bavarian Academy of Sciences and Humanities (HLRB project h1231). SHIP is part of the Community Medicine Research net of the University of Greifswald, Germany, which is funded by the Federal Ministry of Education and Research (grants no. 01ZZ9603, 01ZZ0103, and 01ZZ0403), the Ministry of Cultural Affairs as well as the Social Ministry of the Federal State of Mecklenburg – West Pomerania. Genome-wide data have been supported by the Federal Ministry of Education and Research (grant no. 03ZIK012) and a joint grant from Siemens Healthcare, Erlangen, Germany, and the Federal State of Mecklenburg West Pomerania. The University of Greifswald is a member of the ''Center of Knowledge Interchange'' program of the Siemens AG. This work is also part of the research project Greifswald Approach to Individualized Medicine (GANI_MED). The GANI_MED consortium is funded by the Federal Ministry of Education and Research and the Ministry of Cultural Affairs of the Federal State of Mecklenburg – West Pomerania (03IS2061A). The testosterone reagents used were sponsored by Siemens Healthcare Diagnostics, Eschborn, formerly DPC Biermann GmbH, Bad Nauheim, Germany. Novo Nordisc provided partial grant support for the determination of serum samples and data analysis. Gothenburg Osteoporosis and Obesity Determinants (GOOD) Study: Financial support was received from the Swedish Research Council (K2010-54X-09894-19-3, 2006-3832, and K2010-52X-20229-05-3), the Swedish Foundation for Strategic Research, the ALF/LUA research grant in Gothenburg, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, Petrus and Augusta Hedlunds Foundation, the Västra Götaland Foundation, the Göteborg Medical Society, the Novo Nordisk foundation, the Canadian Institutes of Health Research (MOP-15261), and the European Commission grant HEALTH-F2-2008-201865-GEFOS. We would like to acknowledge Maria Nethander at the genomics core facility at University of Gothenburg for statistical analyses. We would also like to thank Dr. Tobias A. Knoch, Luc V. de Zeeuw, Anis Abuseiris, and Rob de Graaf as well as their institutions the Erasmus Computing Grid, Rotterdam, The Netherlands, and especially the national German MediGRID and Services@MediGRID part of the German D-Grid, both funded by the German Bundesministerium fuer Forschung und Technology under grants #01 AK 803 A-H and # 01 IG 07015 G for access to their grid resources. Cooperative Research in the Region of Augsburg (KORA): The KORA research platform was initiated and financed by the Helmholtz Center Munich, German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (BMBF) and by the State of Bavaria. Part of this work was financed by the German National Genome Research Network (NGFN-2 and NGFNPlus: 01GS0823). Our research was supported within the Munich Center of Health Sciences (MC Health) as part of LMUinnovativ. This study was in part supported by a grant from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD e.V.). Health, Aging, and Body Composition (Health ABC) Study: This study was supported by National Institute on Aging contracts N01-AG-6-2101, N01-AG-6-2103, and N01-AG-6-2106. The genome-wide association study was funded by NIA grant 1R01AG032098-01A1 to Wake Forest University Health Sciences and genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the National Institutes of Health to The Johns Hopkins University, contract number HHSN268200782096C. This research was supported (in part) by the Intramural Research Program of the NIH, National Institute on Aging. Rotterdam study (RS1): The generation and management of GWAS genotype data for the Rotterdam Study is supported by the Netherlands Organisation of Scientific Research NWO Investments (nr. 175.010.2005.011, 911-03-012). This study is funded by the Research Institute for Diseases in the Elderly (014-93-015; RIDE2), the Netherlands Genomics Initiative (NGI) – Netherlands Consortium of Healthy Aging (NCHA) project nr. 050-060-810, and funding from the European Commision (HEALTH-F2-2008-201865, GEFOS; HEALTH-F2-2008-35627, TREAT-OA). The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University Rotterdam; Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Ministry of Education, Culture, and Science; the Ministry for Health, Welfare, and Sports; the European Commission (DG XII); and the Municipality of Rotterdam. We thank Pascal Arp, Mila Jhamai, Dr. Michael Moorhouse, Marijn Verkerk, and Sander Bervoets for their help in creating the GWAS database. The authors are grateful to the study participants, the staff from the Rotterdam Study, and the participating general practioners and pharmacists. We would like to thank Dr. Tobias A. Knoch, Luc V. de Zeeuw, Anis Abuseiris, and Rob de Graaf as well as their institutions the Erasmus Computing Grid, Rotterdam, The Netherlands, and especially the national German MediGRID and Services@MediGRID part of the German D-Grid, both funded by the German Bundesministerium fuer Forschung und Technology under grants #01 AK 803 A-H and # 01 IG 07015 G for access to their grid resources. Invecchiare in Chianti (InCHIANTI): The InCHIANTI study baseline (1998–2000) was supported as a ''targeted project'' (ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336); the InCHIANTI Follow-up 1 (2001–2003) was funded by the U.S. National Institute on Aging (Contracts: N.1-AG-1-1 and N.1-AG-1-2111); the InCHIANTI Follow-ups 2 and 3 studies (2004–2010) were financed by the U.S. National Institute on Aging (Contract: N01-AG-5-0002); supported in part by the Intramural research program of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland. European Male Ageing Study (EMAS): The EMAS is funded by the Commission of the European Communities Fifth Framework Programme ''Quality of Life and Management of Living Resources'' Grant QLK6-CT-2001-00258 and supported by funding from the UK Arthritis Research Campaign. The EMAS Principal Investigator is Professor Frederick Wu, MD; Dept of Endocrinology, Manchester Royal Infirmary, UK. The ''EMAS Study Group'' consists of the following people: Gyorgy Bartfai, Steven Boonen, Felipe Casanueva, Joseph D Finn, Gianni Forti, Aleksander Giwercman, Thang S Han, Kate L Holliday, Ilpo T Huhtaniemi, Krzysztof Kula, Michael EJ Lean, David M Lee, Terence W O'Neill, Neil Pendleton, Margus Punab, Stephen R Pye, Alan J Silman, Abdelouahid Tajar, Wendy Thomson, Dirk Vanderschueren, and Frederick CW Wu. The authors wish to thank the men who participated in the eight countries and the research/nursing staff in the eight centres: C Pott, Manchester, E Wouters, Leuven, M Nilsson, Malmö, M del Mar Fernandez, Santiago de Compostela,M Jedrzejowska, Lodz, H-M Tabo, Tartu, A Heredi, Szeged for their data collection and C Moseley, Manchester for data entry and project coordination. Dr. Vanderschueren is a senior clinical investigator supported by the Clinical Research Fund of the University Hospitals Leuven, Belgium. Dr. Boonen is a senior clinical investigator of the Fund for Scientific Research-Flanders, Belgium (F.W.O.–Vlaanderen). Dr. Boonen is holder of the Leuven University Chair in Metabolic Bone Diseases. The Osteoporotic Fractures in Men Study – Sweden (MrOS Sweden): Financial support was received from the Swedish Research Council (K2010-54X-09894-19-3, 2006-3832), the Swedish Foundation for Strategic Research, the ALF/LUA research grant in Gothenburg, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, Petrus and Augusta Hedlunds Foundation, the Västra Götaland Foundation, the Göteborg Medical Society, the Novo Nordisk Foundation, and the European Commission grant HEALTH-F2-2008-201865-GEFOS. The Cardiovascular Risk in Young Finns Study (YFS): YFS has been financially supported by the Academy of Finland (grant no. 117797, 121584, and 126925), the Social Insurance Institution of Finland, Turku University Foundation, Tampere and Turku University Hospital Medical Funds, Emil Aaltonen Foundation (T.L), Juho Vainio Foundation, Paavo Nurmi Foundation, the Tampere Tubeculosis Foundation, the Orion-Farmos Research Foundation, Finnish Foundation of Cardiovascular Research, and Finnish Cultural Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Author Summary

Testosterone is 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.

Introduction

Testosterone, the most important testicular androgen in men, is largely bound to two plasma proteins. Most of the circulating testosterone (~50–60%) is bound with high affinity to sex hormone-binding globulin (SHBG), while a smaller fraction (40–50%) is bound loosely to albumin, and 1–3% is unbound and termed free testosterone [1]. In prospective cohort studies, low serum testosterone concentrations are associated with cardiovascular morbidity, metabolic syndrome [2,3], dyslipidemia [4], hypertension [5], type 2 diabetes mellitus [6], stroke [7], atherosclerosis [8–10], osteoporosis, sarcopenia, and increased mortality risk [11–13]. Thus, there is growing evidence that serum testosterone is a valuable biomarker of men's overall health status. Since age, body mass index (BMI), and smoking are known to affect serum testosterone concentrations [14], we used these parameters as common set of covariates in all association models. Studies in male twins indicate that there is a strong heritability of serum testosterone, with genetic factors accounting for 65% of the variation in serum testosterone [15]. However, the genetic determinants of serum testosterone and the genetic risk factors for low concentrations are poorly understood. Given the current gap in knowledge of the genetic factors that contribute to the inter-individual variability in serum testosterone concentration in men we conducted a meta-analysis of genome-wide association studies (GWAS). This two-stage meta-analysis included data from 14,429 Caucasian men from 10 independent cohorts within the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. In stage one, the discovery stage, genome-wide association data from seven cohorts were meta-analyzed (n = 8,938) and all genome-wide significant findings that fulfilled the criteria described in the methods section were followed up in the three replication cohorts: one *in silico* replication cohort (n = 871) and two replication cohorts with *de novo* genotyping (n = 4,620). All association analyses of the discovery stage were conducted both with and without additional adjustment for serum SHBG concentrations. Our primary aim was to identify genetic variants reproducibly associated with serum testosterone concentrations in men, evaluated as a continuous trait. We also assessed whether the lead single-nucleotide polymorphisms (SNPs) from the continuous trait analyses had a significant influence on the risk of having low serum testosterone, defined as <300 ng/dl [16]. This level is slightly lower than that suggested recently by Wu et al. [11 nmol/l = 317 ng/dl] as one of the clinical criteria for late onset hypogonadism [17].

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 ($p = 1.9\times10^{-17}$), 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 ($p = 4.1\times10^{-14}$), 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 r^2 between rs12150660 and rs6258 was 0.004. 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, $p = 7.0\times10^{-14}$; rs6258, $p = 1.6\times10^{-13}$), 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 I^2 statistic [18]. The I^2 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

Table 1. Meta-analyses of discovery and replication cohorts.

SNPs rs12150660 and rs6258 (on chromosome 17 in *SHBG*) identified in GWAS for total testosterone														
	Discovery						**Replication**				**Combined**			
	A1/A2	**FREQ***	**beta**	**se**	**p**	**n**	**beta**	**se**	**p**	**n**	**beta**	**se**	**p**	**n**
Testosterone (ng/dl)														
rs12150660	T/G	0.23	26.4	3.1	1.9E-17	8938	38.8	3.6	2.3E-27	5429	31.8	2.3	1.2E-41	14367
rs6258	T/C	0.02	−74.7	9.9	4.1E-14	8938	−102.9	16.3	2.9E-10	5483	−82.3	8.5	2.3E-22	14421
SHBG (nmol/l)														
rs12150660	T/G	0.23	3.6	0.3	3.0E-42	8366	4.4	0.4	8.5E-36	5682	3.9	0.2	2.1E-75	14048
rs6258	T/C	0.02	−6.6	0.8	1.2E-15	8366	−9.5	1.3	6.7E-14	5733	−7.4	0.7	3.5E-27	14099
Testosterone (SHBG-adjusted)														
rs12150660	T/G	0.23	11.1	3.0	2.5E-04	8366	11.6	3.0	9.9E-05	5414	11.3	2.1	9.0E-08	13780
rs6258	T/C	0.02	−41.8	9.4	8.2E-06	8366	−33.2	13.8	1.6E-02	5467	−39.1	7.7	4.5E-07	13833
Calculated Free Testosterone (ng/dl)														
rs12150660	T/G	0.23	−0.1	0.1	9.6E-02	8366	0.1	0.1	1.6E-02	5414	0.0	0.0	3.9E-01	13780
rs6258	T/C	0.02	−0.2	0.2	3.2E-01	8366	−0.5	0.3	9.0E-02	5467	−0.3	0.2	6.5E-02	13833
SNP rs5934505 (on chromosome X near *FAM9B*) identified in GWAS for SHBG-adjusted total testosterone														
	Discovery						**Replication**				**Combined**			
	A1/A2	**FREQ***	**beta**	**se**	**p**	**n**	**beta**	**se**	**p**	**n**	**beta**	**se**	**p**	**n**
Testosterone (ng/dl)	C/T	0.26	14.1	3.2	1.1E-05	5067	27.2	6.0	5.4E-06	3816	17.0	2.8	1.7E-09	8883
SHBG (nmol/l)	C/T	0.26	−0.2	0.3	5.9E-01	4607	0.5	0.7	4.7E-01	4072	−0.1	0.3	8.5E-01	8679
Testosterone (SHBG-adjusted)	C/T	0.26	18.1	3.1	8.5E-09	4599	27.7	4.7	4.4E-09	3801	21.0	2.6	5.6E-16	8400
Calculated Free Testosterone (ng/dl)	C/T	0.26	0.4	0.1	4.0E-07	4607	0.6	0.1	8.7E-10	3801	0.5	0.1	6.7E-15	8408

Effects size is given per minor allele. 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. A1 = allele 1. A2 = allele 2. FREQ* = Frequency of allele 1. In the KORA cohort, testosterone was 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.

doi:10.1371/journal.pgen.1002313.t001

rs6258 reached genome-wide significance after excluding the InCHIANTI cohort (Table S3).

The genetic influence on low serum testosterone concentrations

In Table 2, the serum testosterone concentrations according to genotype are given for the three replication cohorts. As expected, mean serum testosterone concentrations were found to be lower in men with GG than in those with TT genotype for rs12150660. Similarly, men with the CT genotype for rs6258 had lower serum testosterone concentrations than those with CC genotype. The TT genotype of rs6258 was extremely rare and only found in two subjects in the replication cohorts. The two autosomal SNPs identified by GWAS had a significant influence on the risk of having low serum testosterone (serum testosterone <300 ng/dl) in both the discovery and the replication cohorts with a combined odds ratio (OR) per minor allele of 0.72 (95% CI, 0.65 – 0.79) and 2.7 (95% CI, 2.1 – 3.5) for rs12150660 and rs6258, respectively (Figure 2A). We analyzed the combined effect of the two SNPs on the risk of having low serum testosterone concentrations according to the number of combined risk alleles for rs12150660 (G) and rs6258 (T) in the three replication cohorts (MrOS Sweden, EMAS, and YFS). The risk of having low serum testosterone concentrations increased by the number of risk alleles with an OR of 1.62 (95% CI, 1.41 – 1.86) for each risk allele (Figure S4). Low serum testosterone concentrations were 6.5-times more prevalent in men with ≥3 risk alleles (30.1% prevalence of low serum testosterone) compared to men without any risk allele (4.6% prevalence of low serum testosterone; Figure 2B).

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 ($p = 9.0\times10^{-8}$ for rs12150660 and $p = 4.5\times10^{-7}$ 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 $r_s = 0.53$), 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).

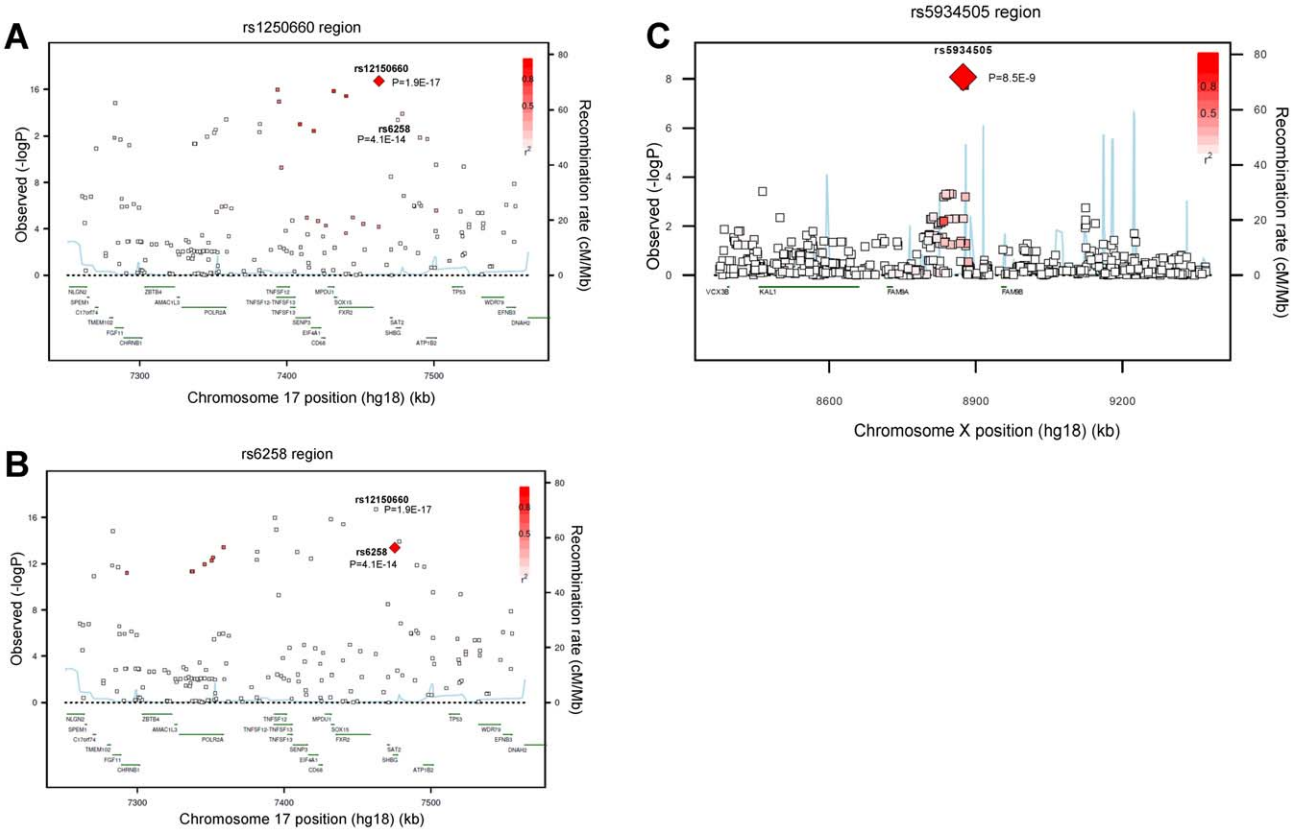

Figure 1. Regional association plots for single-nucleotide polymorphisms rs12150660, rs6258, and rs5934505. Regional association plot of the two independent signals on chromosome 17 with either (A) rs12150660 or (B) rs6258 indicated by red diamond to evaluate linkage with other single-nucleotide polymorphisms in the region. In addition, the association plot of the (C) rs5934505 signal on chromosome X is given. The r^2 is based on the CEU HapMap II samples. The blue line and right hand Y axis represent CEU HapMap II based recombination rates. (A) and (B) show the top SNPs of the inverse-variance weighted discovery stage meta-analysis of untransformed serum testosterone and (C) show the top SNP of the SHBG-adjusted serum testosterone using an imputation quality filter (observed/expected variance ratio) >0.4 at the individual cohort level during meta-analysis.
doi:10.1371/journal.pgen.1002313.g001

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 = 2.8 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 ($p = 1.4\times10^{-5}$) 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 ($p = 8.5\times10^{-9}$) in the SHBG-adjusted model (Table 1 [SNP rs5934505] and Figures S1B and S3). This SNP was confirmed in the two replication cohorts with *de novo* genotyping (MrOS Sweden $p = 3.6\times10^{-3}$; EMAS $p = 1.5\times10^{-7}$). 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-adjustment (combined p-value of 1.7×10^{-9}) and with free testosterone (combined p-value of 6.7×10^{-15}), 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

Table 2. Serum sex steroids in the three replication cohorts according to rs12150660, rs6258, and rs5934505 genotype.

	SNPs identified in GWAS for total testosterone								SNP identified in GWAS for SHBG-adjusted testosterone		
	rs12150660				rs6258				rs5934505		
	GG	GT	TT	p-value	CC	CT	TT	p-value	C	T	p-value
EMAS	(n = 1310)	(n = 833)	(n = 152)		(n = 2261)	(n = 34)			(n = 410)	(n = 1120)	
Testosterone (ng/dl)	454±161	490±172	544±181	<0.001	474±169	358±104		<0.001	495±178	473±168	0.02
Calculated Free Testosterone (ng/dl)	8.47±2.53	8.53±2.53	8.84±2.85	0.15	8.52±2.56	8.14±2.14		0.39	9.00±2.65	8.45±2.49	<0.001
SHBG (nM)	39.6±17.1	45.2±20.4	51.6±20.8	<0.001	42.6±19.0	26.8±10.6		<0.001	42.4±20.5	42.8±18.9	0.69
MrOS Sweden	(n = 1317)	(n = 844)	(n = 123)		(n = 2245)	(n = 31)			(n = 530)	(n = 1765)	
Testosterone (ng/dl)	435±170	475±177	526±171	<0.001	456±174	331±125		<0.001	473±177	448±173	0.005
Calculated Free Testosterone (ng/dl)	7.98±3.07	8.30±3.16	8.75±2.99	0.005	8.16±3.08	7.59±2.72		0.31	8.54±3.27	8.03±3.03	0.001
SHBG (nM)	41.0±21.6	45.8±22.4	49.8±23.0	<0.001	43.5±22.0	24.3±12.3		<0.001	43.7±24.1	43.1±21.5	0.51
YFS	(n = 522)	(n = 329)	(n = 51)		(n = 852)	(n = 48)	(n = 2)				
Testosterone (ng/dl)	525±182	549±246	561±158	0.063	540±209	471±157	441±75	0.065	NA		
Calculated Free Testosterone (ng/dl)	11.89±5.30	12.30±8.92	11.57±2.46	0.71	12.04±6.90	11.80±3.42	11.55±1.23	0.80	NA		
SHBG (nM)	30.0±11.7	31.3±11.9	35.2±13.1	0.007	31.2±12.0	23.0±8.1	20.5±4.0	<0.001	NA		

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 concentrations of testosterone and SHBG and a fixed value for SHBG's dissociation constant were used in these calculations.

doi:10.1371/journal.pgen.1002313.t002

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, although the clinical usefulness of these findings remains to be established. As rs12150660 and rs6258 were strongly associated with SHBG concentrations, both SNPs may at least partly affect total testosterone concentrations by modulating SHBG concentrations. Our findings that rs6258 substantially affects SHBG binding affinity and the measured free testosterone fraction raise questions about the use of a single consensus value for SHBG's dissociation constant in the law of mass action equations used to calculate free testosterone concentrations. As emphasized by the Endocrine Society's expert panel on androgen deficiency syndromes, low testosterone concentrations alone should not necessarily be viewed as evidence of androgen deficiency [16]. Whether rs593405 near the *FAM9B* and *KAL1* genes on Xp22 renders men susceptible to the increased risk of androgen deficiency remains to be determined. Further studies are required to determine the impact of these genetic variations on sex steroid-related disorders, including osteoporosis, cardiovascular diseases, prostate cancer, and male infertility [21].

Our studies add to the evidence that genetic variations within the *SHBG* gene may explain some of the inter-individual differences in SHBG concentrations. Our finding that SNP rs6258 results in the production of an *SHBG* variant with reduced affinity for testosterone provides an explanation for the association between rs6258 and low serum testosterone concentrations. This is the first described genetic variant associated with altered SHBG binding for testosterone. As rs6258 is non-synonymous (P156L), located in exon 4 of *SHBG* and associated with altered SHBG binding for testosterone and free testosterone fraction, rs6258 is likely a functional polymorphism with impact on testosterone binding to SHBG as well as testosterone bioavailability and action at target tissue level.

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

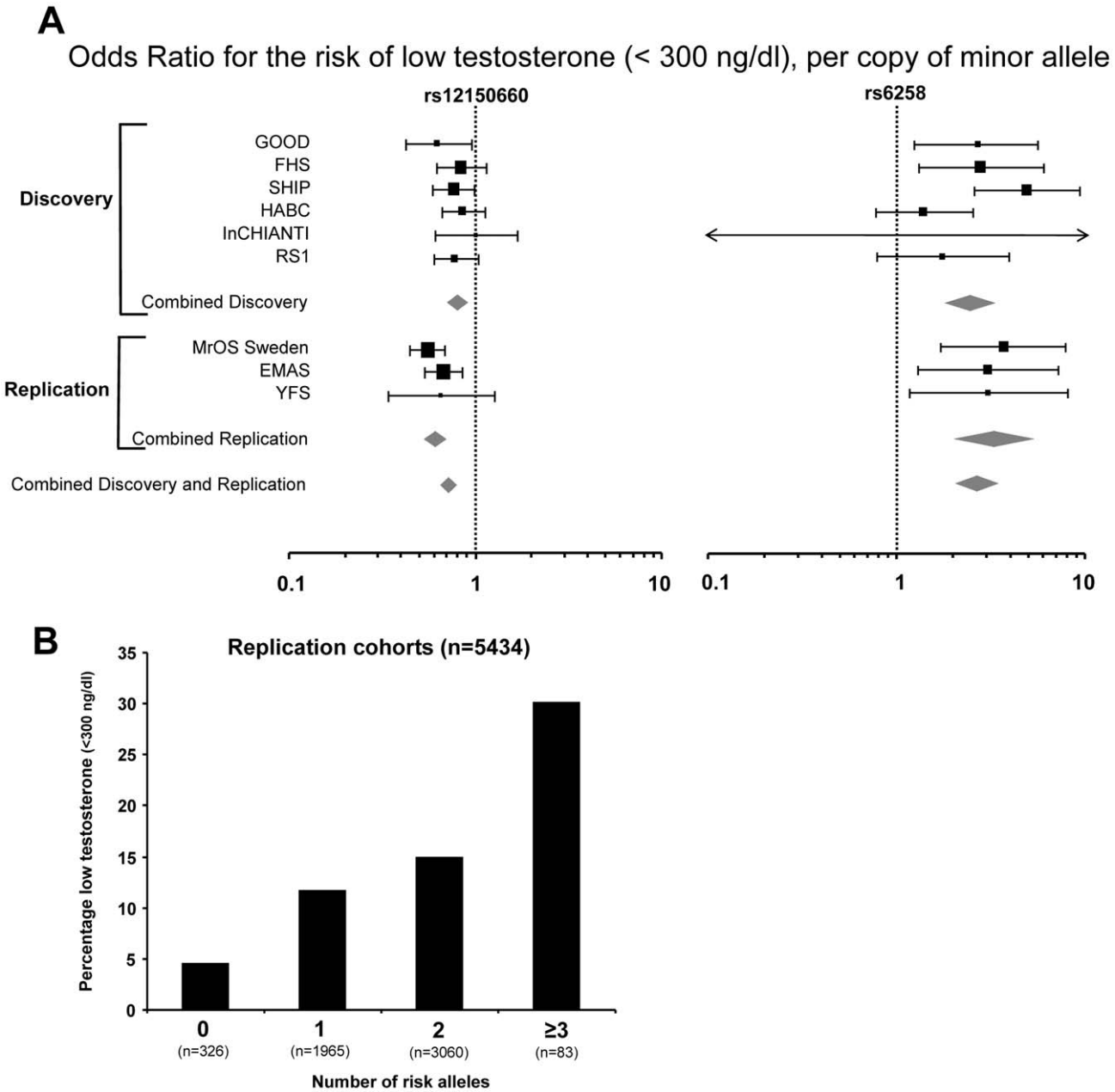

Figure 2. The genetic influence on low serum testosterone concentrations. (A) Odds ratio (OR) for risk of low serum testosterone concentrations (serum testosterone <300 ng/dl), per copy of minor allele. Summary estimates of the OR and their 95% confidence intervals (CI) are given. The size of the data markers is proportional to the weight (inverse of the variance) of each study. Combined discovery (n = 8,030, low serum testosterone 13%; KORA was not included as testosterone was analyzed in plasma rather than in serum, combined replication (n = 5,504, low serum testosterone 13%), and combined discovery and replication (n = 13,534, low serum testosterone 13%). (B) Percentage of men with low serum testosterone concentrations (serum testosterone <300 ng/dl), according to the number of combined risk alleles for rs12150660 (G) and rs6258 (T) in the three replication cohorts (MrOS Sweden, EMAS, and YFS). 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.
doi:10.1371/journal.pgen.1002313.g002

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.

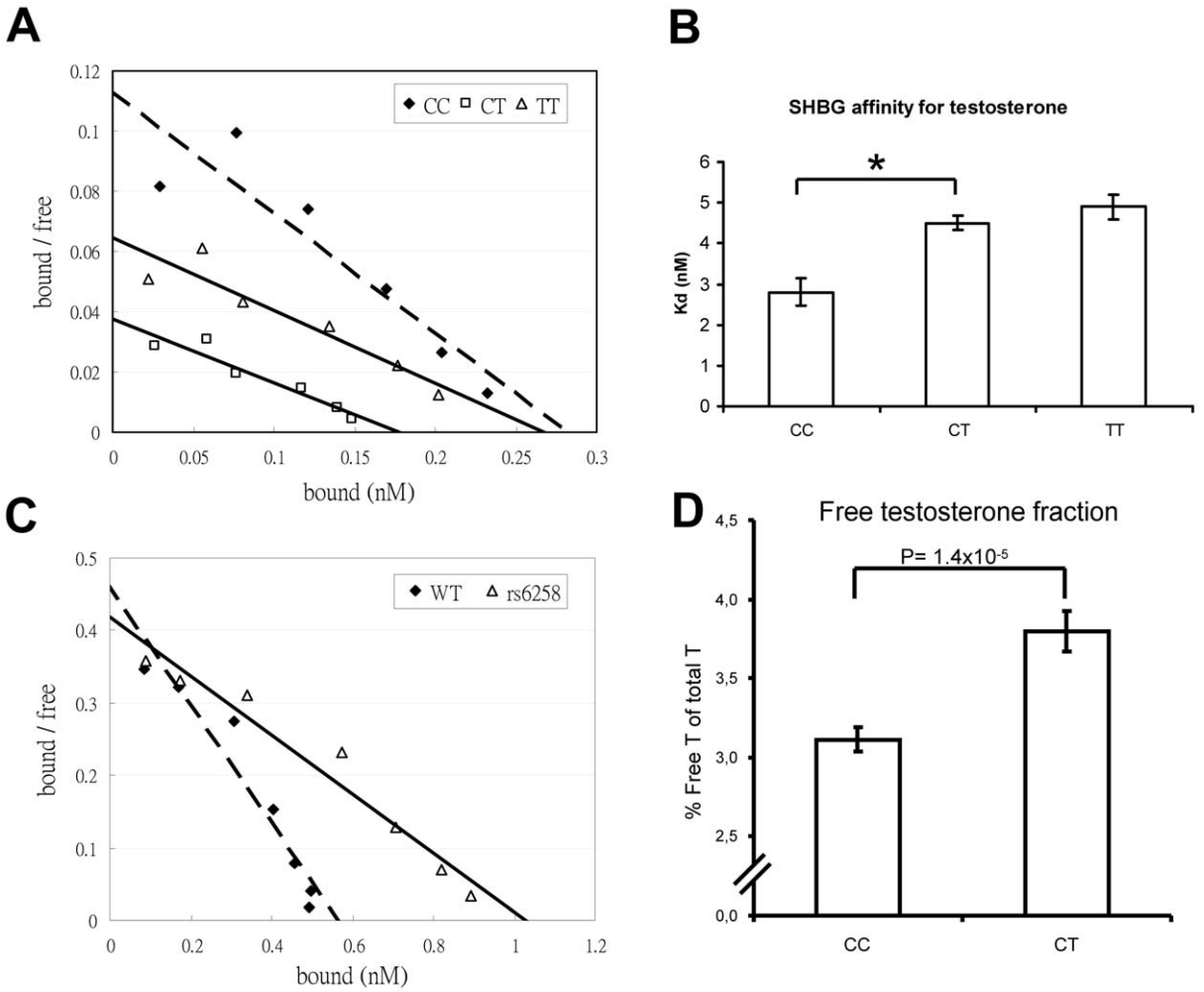

Figure 3. SHBG affinity for testosterone. (A and B) Scatchard plots of SHBG binding affinity for testosterone in serum samples according to rs6258 genotype. (A) Representative Scatchard plots of serum SHBG binding to [^{3}H]testosterone. Serum from individuals homozygous for the wild-type SHBG allele (CC dashed line) or the rs6258 SNP (TT, solid line), or heterozygous for these alleles (CT, solid line). (B) Dissociation constant (Kd) of serum SHBG according to rs6258 genotype (CC, n = 4 subjects; CT, n = 4 subjects; TT [rare variant] n = 1 and the variation for the TT subject is derived from three separate analyses). (*) p = 0.001. Values are means ± SEM. (C) Representative Scatchard plots of recombinant SHBG binding to [^{3}H]testosterone. Recombinant wild type (= WT, C genotype; dashed line) or rs6258 (T genotype; solid line) SHBG expressed by CHO cells was diluted 1:10 and subjected to Scatchard analysis, as in panel A. (D) Free testosterone fraction in serum measured by an equilibrium dialysis method according to rs6258 genotype (CC, n = 87 subjects; CT, n = 32 subjects). Values are means ± SEM.
doi:10.1371/journal.pgen.1002313.g003

The strengths of our study include a discovery sample size of 8,938 men, which allowed us at the threshold $\alpha = 5\times10^{-8}$,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 λ_{GC}: 1.00–1.07) and the meta-analyses (range of λ_{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 $SHBG^2$ 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 $p<5\times10^{-8}$ 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 weighted 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 nmol/l = 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 immunofluorometric 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 [^{3}H]5α-dihydrotestosterone ([^{3}H]DHT; specific activity 50 Ci/mmol) or [^{3}H]testosterone (specific activity 40 Ci/mmol), bound from free [^{3}H]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 testosterone and for (C) rs5934505 with SHBG-adjusted testosterone. Effects sizes are given per minor allele. Beta estimates and their 95% confidence intervals are given. The size of the data markers is proportional to the weight (inverse of the variance) of each study.
(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 most 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 [^{3}H]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)

Acknowledgments

We are indebted to the participants and staff of all the studies for their important contributions. Full individual study Acknowledgments are listed in Text S1.

Author Contributions

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