

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Ding EL, Song Y, Manson JE, et al. Sex hormone-binding globulin and risk of type 2 diabetes in women and men. *N Engl J Med* 2009;361:1152-63. DOI: 10.1056/NEJMoa0804381.

SUPPLEMENT**APPENDIX 1.****A. Physician's Health Study II Details**

The PHS II is a randomized, double-blind, placebo-controlled 2x2x2x2 factorial trial of vitamin E, vitamin C, β -carotene, and a multivitamin in the prevention of CVD and cancer among 14,641 US male physicians aged 50 years and older free of baseline cancer and CVD.¹ The PHS II is an extension of the Physicians' Health Study I (PHS I),^{2,3} a 2x2 factorial trial of low-dose aspirin and β -carotene among 22,071 healthy male physicians aged 40-84 at entry in 1982. Recruitment for PHS II was completed in two phases. In the first phase, which began in July 1997, PHS I participants were invited to enroll in PHS II. In the second phase, which took place from July 1999 to July 2001, new participants were recruited from a roster of physicians provided by the American Medical Association.

B. Ascertainment of Diabetes

As described in details elsewhere,⁴⁻⁶ T2D has been diagnosed at baseline and annually during follow-up in these two ongoing cohorts. All participants are health professionals, who have been shown to provide reliable self-reported diagnostic information. Using the diagnostic criteria recommended by the ADA, all self-reported T2D cases were confirmed by a supplemental questionnaire on diabetes symptoms, results of glucose tests, and treatments. Glucose tolerance screening rates for diabetes were high, with at least 85-90% of all participants reporting a recent blood glucose screening on their annual questionnaire. Previous WHS diabetes validation via physician-led telephone interviews and supplementary questionnaires both yielded positive predictive values >91%, and confirmation of diabetes via combined supplementary questionnaire and medical records was 99%,⁴⁻⁶ supporting the validity of self-reported diabetes in our study population. Only confirmed cases were included in this study. Thus, we believe that self-reported T2D in our study has very high levels of validity.

C. Genotyping Methods

To identify common genetic variants across the *SHBG* gene that have known functional consequences, we initially surveyed all the common SNPs spanning 4 kb of the *SHBG* gene, covering at least its 10 kb 5' upstream regions. This initial assessment was based on extensive sequencing done the National Cancer Institute's cohort consortium (<http://uscnorriscancer.usc.edu/Core/DocManager/DocumentList.aspx>). We further searched the National Center for Biotechnology Information database SNP (NCBI dbSNP) for additional functional SNPs. The set of 5 putative functional SNPs with a minor allele frequency (MAF) $\geq 5\%$ in at least one ethnic group were genotyped in our samples.

All DNA samples were genotyped using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), in 384-well format. 5-nuclease assay (TaqMan) was used to distinguish gene alleles. PCR amplification was carried out on 5-20ng DNA using 1 X TaqMan universal PCR master mix (No Amp-erase UNG), 900nM forward and reverse primers, 200nM of the FAM labeled probe and 200nM of the VIC labeled probe in 5ml reaction volume. Amplification conditions on AB 7900 dual plate thermal cycle (Applied Biosystems, Foster City, CA) were as follows: 1 cycle of 95°C for 10min, followed by 50 cycles of 92°C for 15s and 60°C for 1 min. TaqMan primer and probe sequences are available upon request.

Although, the *rs6257* in intron 1, sited 17bp upstream of exon 2, is significantly associated with plasma SHBG levels, the precise mechanisms remain unknown. This intronic polymorphism may regulate SHBG expression through its location in a regulatory element or its high linkage disequilibrium (LD) with an as yet unknown functional variant located in the promoter region. *Rs6259* located within exon 8, a nonsynonymous amino acid substitution of asparagines (Asn) for aspartic acid (Asp), introduces an additional *N*-glycosylation consensus site, which may lead to reduced clearance rate of SHBG from the circulation by altering the binding of SHBG to membrane receptors or other proteins.

E. Covariates in Multivariable Model

In the primary multivariable models in women, we adjusted for matching factors, BMI (continuous), smoking (current, former, never), alcohol consumption (rarely/never, 1-3 drinks/month, 1-6 drinks/week, ≥ 1 drink/day), exercise (rarely/never, 1, 2-3, 4-6 and ≥ 7 times/week), family history of diabetes (yes, no), history of hypertension (yes, no), past HRT use (yes, no), years of oral contraceptive use (<0.5 , 0.5-2, >2 yrs), multivitamin use (current, former, never), years since menopause, and cause of menopause (natural vs. surgical, radiation, or chemotherapy). In expanded models, we adjusted for: age at menarche (<12 , 12, 13, >13), total pregnancies (0, 1-2, 3-4, ≥ 5), pregnancies lasting ≥ 6 months, age at first pregnancy of ≥ 6 months (none, <25 , ≥ 25), marital status (current, former, never married), and education (high school, associates, bachelors, masters, doctoral).

Similar analyses were carried out in men, adjusting for BMI (continuous), smoking (current, former, never), alcohol consumption (drinks: <1 /mo, 1-3/mo, 1/wk, 2-4/wk, 5-6/wk, 1/day, 2/day), exercise (0, 1, 2, 3, ≥ 4 days/wk), systolic blood pressure (continuous), current use of multivitamins, and family history of diabetes (yes, no).

Appendix 1 Table. Descriptive characteristics of Sex Hormone-Binding Globulin polymorphisms genotyped

Gene	SNP ID	Gene Region	Allele	Function	Amino Acid Substitution	Hydropathy Index [†]	Acid/Base Property
SHBG	<i>rs6257</i>	Intron 1, -17bp Exon 2	[C/T]	--	--	--	--
SHBG	<i>rs6258</i>	Exon 4	[T/C]	Non-syn	Leu for Pro	3.8, 1.6	Neutral, neutral
SHBG	<i>rs6259</i>	Exon 8	[A/G]	Non-syn	Asn for Asp	-3.5, -3.5	Neutral, acidic
SHBG	<i>rs6260</i>	Exon 1	[G/A]	Non-syn	His for Arg	-3.2, -3.5	Weak base, strong base
SHBG	<i>rs9282845</i>	Exon 1	[A/G]	Non-syn	His for Arg		

* Allele order designates [variant/ancestral] alleles

† *Hydropathy Index*⁷ describes the hydrophobic (a.k.a. lipophilic) nature of amino acids; larger values indicate greater hydrophobicity

APPENDIX 2.**Relative Prediction of Plasma SHBG and Risk of Type 2 Diabetes using C-statistics**

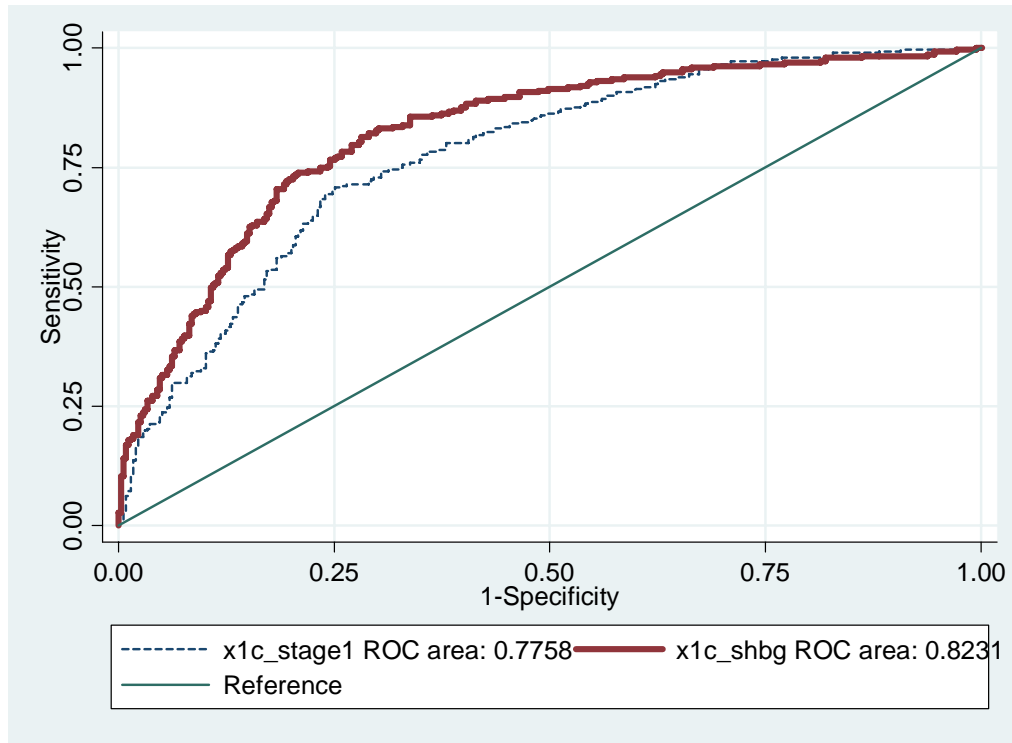
Methods To further assess the independent utility of plasma SHBG levels for clinical prediction of T2D, we modeled SHBG while simultaneously adjusting for traditional TD2 risk factors, CRP, and HbA1c, among those with normal HbA1c<6%. A C-statistic (area under an Receiver-Operator-Characteristics [ROC] curve) corresponding to a random non-informative predictor model has a value of 0.50, represented by the ROC curve as a diagonal line directly connecting coordinates (0,0) to (1,1). A C-statistic corresponding to a perfect predictor model has a value of 1.00, represented by lines connecting coordinates (0,0) to (0,1) and (0,1) to (1,1). Difference of C-statistics⁸ were used to compare models.

Results The panels of Figure S-1 show the comparative ROC curves and C-statistics of adding SHBG to various multivariable prediction models. Results indicated the addition of plasma SHBG indeed improved C-statistics in comparison to multiple base models including: 1) a base model comprised of traditional risk factors, 2) an expanded model comprised of traditional risk factors plus CRP, 3) an expanded model comprised of traditional risk factors plus HbA1c, and 4) a comprehensive model that included traditional risk factors, CRP, and HbA1c. Plasma SHBG informatively improved prediction of T2D in all models, including the comprehensive model (C-statistic 0.87 vs. 0.85, $P<0.001$), shown in Panel D.

Limitation of C-statistic Analysis Because the ROC analysis and C-statistics arise from a case-control study, rather than a full cohort, the nature of our study design does not allow us to fully derive an "absolute" C-statistics value. Rather, the C-statistic from our nested case-control design represented only the '**relative**' additive prediction that plasma SHBG contributes above other biomarkers. Secondly, our models have not included 2-hour OGTT glucose, although HbA1c are regarded a more accurate and stable metric of long-term glycemic control.

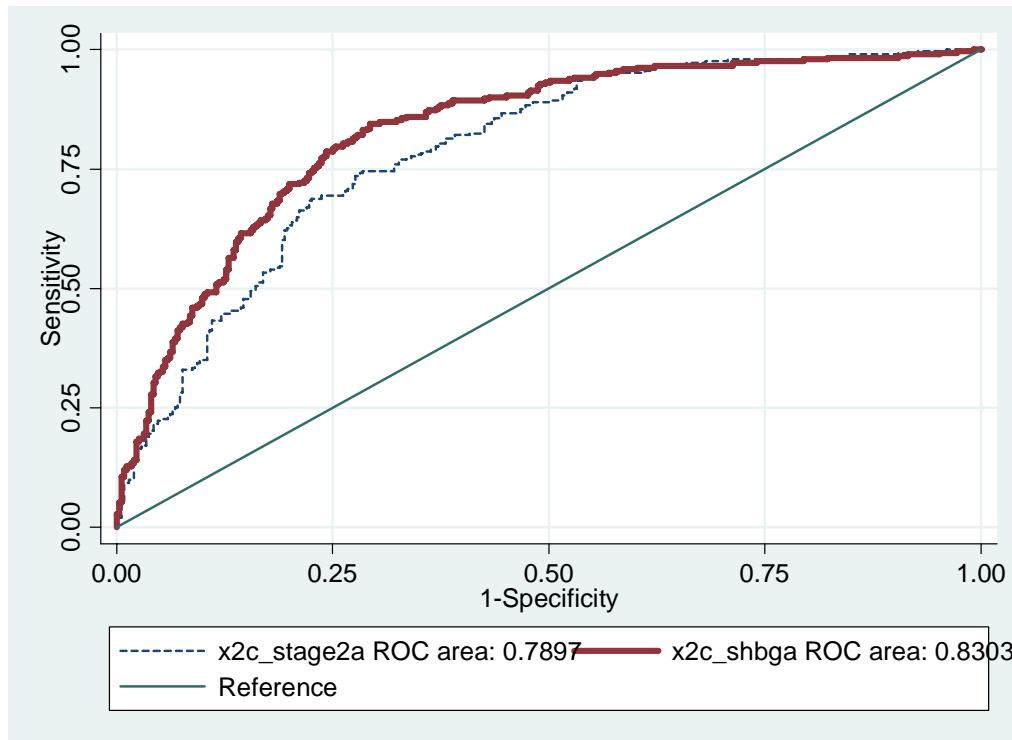
Figure S-1. C-Statistics and ROC Curves Comparing SHBG vs. Traditional Risk Factors, C-Reactive Protein, and HbA1c in Women

Panel A) [Traditional Risk Factors] + SHBG vs. [Traditional Risk Factors] alone



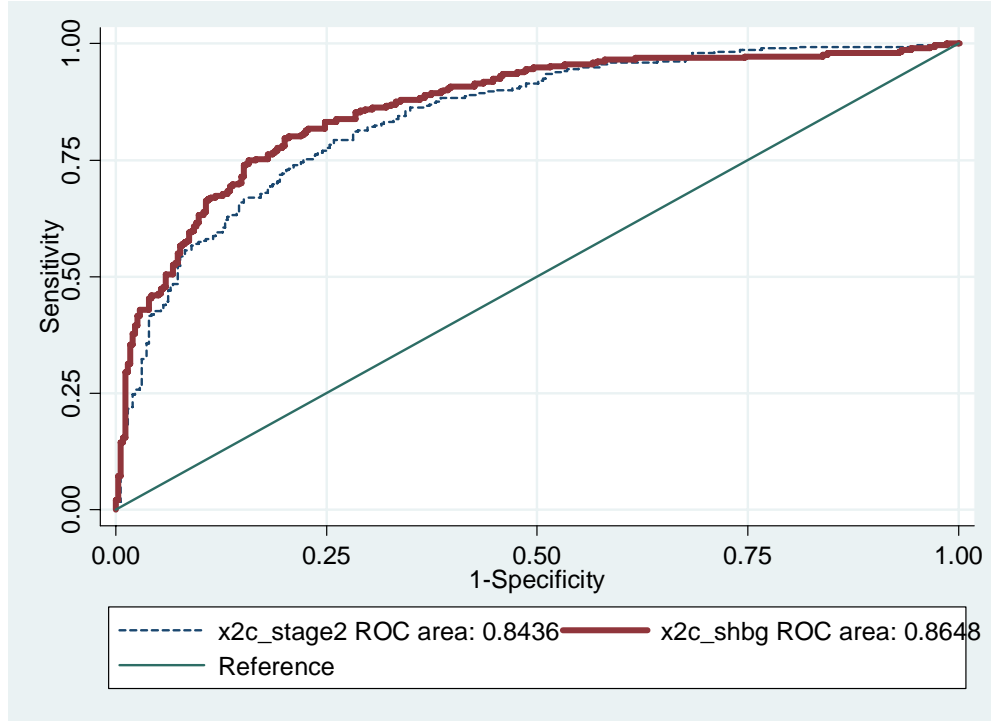
*P<0.001 for difference of ROC curves

Panel B) [Traditional Risk Factors] + CRP + SHBG vs. [Traditional Risk Factors] + CRP



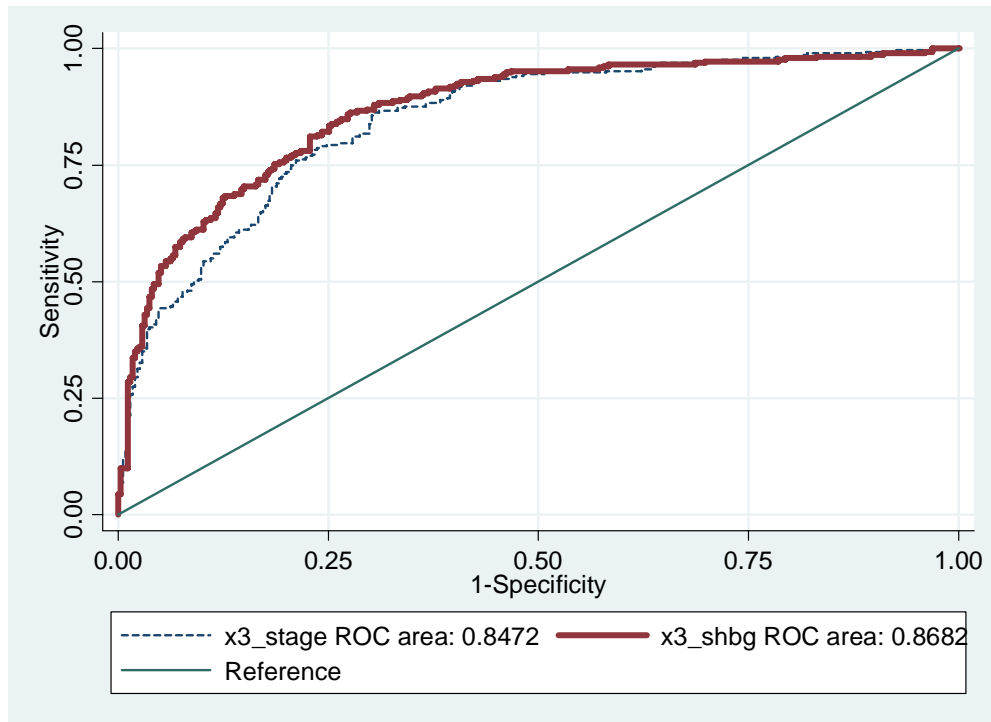
*P<0.001 for difference of ROC curves

Panel C) [Traditional Risk Factors] + HbA1c + SHBG vs. [Traditional Risk Factors] + HbA1c



*P=0.01 for difference of ROC curves

Panel D) [Traditional Risk Factors] + CRP + HbA1c + SHBG vs. [Traditional Risk Factors] + CRP + HbA1c



*P<0.001 for difference of ROC curves
(Prediction among HbA1c<6%)

APPENDIX 3.**Further Discussions of Analytical Methods, Limitations, and Interpretations of Findings****A. Potential Confounding by Adiposity**

Further evidence also suggests confounding by adiposity is unlikely. Additional adjustment for both BMI and waist circumference measures did not affect the results, which is supported by previous findings that low SHBG remained associated with glucose levels and insulin resistance independent of adiposity.⁹ Among postmenopausal women, residual confounding by adiposity is unlikely after controlling for BMI, as the association between SHBG and various compartments of adiposity, assessed via dual-energy X-ray absorptiometry (DXA), were all abolished after simple BMI adjustment.¹⁰ Moreover, we confirmed plasma SHBG's association in another independent cohort of men, among whom SHBG levels were only weakly correlated with adiposity.

B. Principles, Assumptions, and Methods in Mendelian Randomization (MR) Analysis

According to the Mendelian Law of random assortment, genetic variants should be distributed independently and randomly with respect to other genetic variants. Given some strong assumptions (no LD, population stratification, gene-gene, gene-environmental interactions¹¹, the random assortment of alleles from parents to offspring at the time of gamete formation should also lead to population distributions of genetic variants that are generally independent of behavioral and environmental factors. Therefore, the MR approach incorporating information on both the genotype (G)-intermediate phenotype (X) association and genotype – disease (Y) association into one analytical framework may allow for an unbiased estimate of the intermediate phenotype-disease association (Figure 1). Herein, we further describe the key variables in our MR application discussed in the main text, including T2D status (Y), plasma SHBG levels (X), genotype (G), and unknown confounder (U). For the i th participants in the source cohort, let y_i represent their binary disease status (T2D), p_i represent their probability of having the disease, x_i represent the level of the

biological phenotype (log of SHBG) and g_i represent their genotype (rs6259 and rs6257 variant allele), which is coded 0 and 1 to indicate the existence of the relevant risk allele.

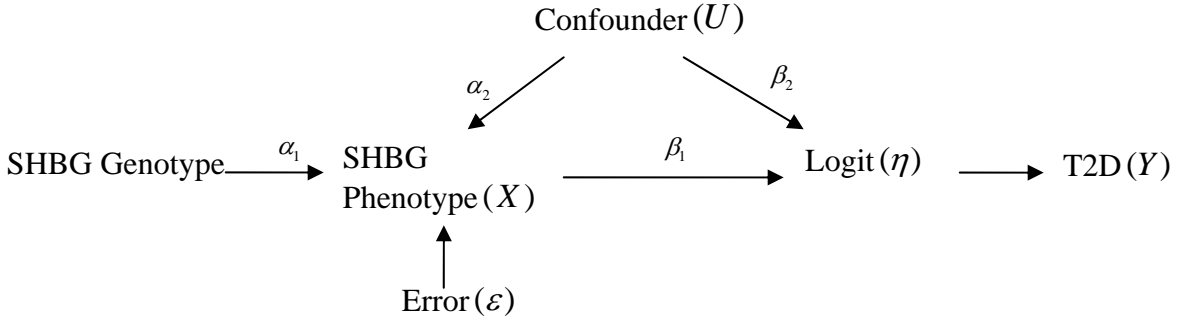


Figure 1

The regression model of SHBG phenotype on genotype is given by:

$$x_i = \alpha_0 + \alpha_1 g_i + \alpha_2 u_i + \varepsilon_i, \text{ with } \varepsilon_i \sim N(0, \sigma_\varepsilon^2) \quad (1)$$

where the error term ε_i is independent of g_i and u_i ;

also, the regression model of T2D on SHBG phenotype is given by:

$$y_i \sim \text{Bern}(p_i), \text{ with } \eta_i = \log\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_1 x_i + \beta_2 u_i \quad (2)$$

In the paradigm of Mendelian randomization, an instrumental variable is defined as a variable that satisfies the following three criteria: 1) the genotype G should be robustly associated with intermediate phenotype X ; 2) the genotype G is independent of the confounding factors U ; and 3) the outcome Y is independent of the genotype G given the intermediate phenotype X . These three main assumptions are sufficient to test the null hypothesis that intermediate phenotype X is not associated with disease outcome Y . If these assumptions were satisfied in selecting G , for example, the coefficient estimate of β_1 for the association between plasma SHBG levels (X) and risk of T2D (Y) would be unbiased by the confounder (U) in MR analysis.

Thus, by substituting equation (1) into equation (2), we have

$$\eta_i = (\beta_0 + \beta_1 \alpha_0) + \alpha_1 \beta_1 g_i + (\alpha_2 \beta_1 + \beta_2) u_i + \beta_1 \varepsilon_i \quad (3)$$

From equation (3) and (1), consistent estimates of $\alpha_1\beta_1$ and α_1 can be obtained by using logistic regression of Y on G and linear regression of X on G, respectively. Thus, the potential causal effect parameter β_1 can be consistently estimated by taking the ratio of the two estimates.

Aside from the three main assumptions discussed in the paper, specific germline variants serving as MR instruments should not be in strong LDs with other functional variants and there should be no population stratification in the source population of interest. Perhaps, it would be helpful to think of MR analysis as analogous to a randomized control trial (RCT) of a hypothetical treatment using recombinant SHBG vs. placebo — where there is a degree of non-adherence to the use of recombinant SHBG, non-compliance to protocol, drop-out, drop-in, and necessary compensatory/rescue drugs used in placebo/control groups that mimic canalization and incomplete penetrance.

There are some uncertainties regarding the use of instrumental variables for binary disease outcomes, although recent studies provide some empirical evidence in support of the robustness of applying MR analysis^{12, 13} with some indication that logistic regression may yield estimates that are somewhat conservative even when the linearity assumption was violated¹². While MR analysis does require several important assumptions that need to be carefully evaluated prior to making causal inference, some of these assumptions cannot be empirically verified and requires subject-matter knowledge (e.g. pleiotropy/heterogeneity of effects and canalization/the buffering effects of germline variants by developmental compensation via other biologic pathways).¹¹ Many common genetic variants are often weakly related to an intermediate phenotype, which can also be influenced by inadequate sample size, measurement errors, model misspecification, population stratification, and potential gene-gene or gene-environmental interactions. To specifically test the suitability of *SHBG* variants (rs6259 and rs6257) as MR instruments, we herein evaluate each of these potential limitations.

First, the *SHBG* genotypes were robustly associated with plasma levels of SHBG with no significant effect modification, suggesting that the genetic effects are likely mediated through SHBG as the intermediate phenotypic product (thus satisfying the assumption of no pleiotropy). The strength of the instrument is determined by the absolute magnitude of relation with SHBG

phenotype. Indeed, as shown in Table 4, each allele altered plasma SHBG levels by 10% for each SNP, and the two independent alleles combined to effect 20% differences in plasma SHBG (Figure 1). Moreover, the F-statistic of the set of *SHBG* alleles was 12.8 (i.e., greater than the standard convention of 10 for evaluating sufficiently strong instruments in MR analysis).

Second, the frequencies of known covariates (potential confounding factors) were quite evenly distributed between *SHBG* SNP carriers and noncarriers (see table below), indicating no evidence for any known confounding factors that could bias the association between SHBG levels and T2D. Furthermore, although population stratification (a.k.a. confounding by ethnicity) is possible, it is usually minimized in a relatively homogeneous population. However, it is unlikely that false positive results from population stratification would explain our findings because our study participants were drawn from two well-characterized cohorts comprised of homogeneous populations of female health professionals of whom ~98% were Caucasian, and male physicians of whom 100% were Caucasian.

Third, we observed a strong relationship between plasma SHBG levels and T2D risk. Also, the associations between the SNPs appear to exert additive effects on both plasma SHBG and T2D risk. Because LD between *rs6257* and *rs6259* was not strong ($r^2=0.13$), these two SNPs could at least be considered two independent proxies for genetic instruments reliably associated with circulating SHBG levels. Figure 1 shows that the effects of *rs6257* and *rs6259* were each independent and additive, jointly contributing to ~57% lower risk of T2D. As discussed in the main in the paper, the independent genetic effects on SHBG expression through different regulatory mechanisms have been revealed by functional studies in animals and humans (*rs6257* is a promoter SNP and *rs6259* is a specific coding SNP not influencing protein structure but nevertheless affecting circulating SHBG levels). Thus, we submit that our MR estimates may represent the average effect of lifetime exposure differences exerted by the *SHBG* genotypes (up until the time of the measurement of plasma SHBG levels) in SHBG-dependent causal pathways leading to T2D. Because of measurement error, however, a single measure of SHBG levels may not be sufficient in capturing lifetime SHBG exposure. Notably, as genetic variants in SHBG exert their gene product effects across a lifetime, MR estimates may represent the effects of SHBG exposure differences

across an individual's lifetime—a measure of average lifetime exposure, which incorporates various mitigating, compensating, and attenuating patterns and lifestyle-related changes over a lifetime.

Finally, available biological evidence does not allow for any direct testing of the assumption that no significant biological network buffering the effects of *SHBG* variants, although gene knockout of *SHBG* have found SHBG attenuation lead to early fetal terminal and severe gonadal malformation in mice¹⁴—indicating that SHBG may play a key role in embryonic development. However, it remains difficult to evaluate the assumptions of no canalization and no gene-gene/gene-environment interactions which would require further experimental and intervention work. In this regard, the robustness and strength of the relations between plasma SHBG levels and T2D risk using both conventional multivariable method and the MR analysis provides some assurance to our findings.

Appendix 3 Table. Characteristics by *SHBG* Polymorphisms among controls in women*

Characteristics (median, or %)	<i>rs6257</i>			<i>rs6259</i>		
	TT	CT and CC	P value	GG	AG and AA	P value
Age	60.5	60.7	0.56	60.0	62.2	0.07
Body Mass Index	24.8	25.4	0.88	24.9	24.8	0.80
Alcohol Intake (g/day)	0.86	0.43	0.15	0.86	0.86	0.29
Current smoking (%)	12	24	0.06	15	14	0.97
Physical activity (% ≥once/wk)	41	34	0.56	42	31	0.26
Past menopausal hormone use (%)	29	23	0.28	26	36	0.08
Pregnancies, ≥5 (%)	34	40	0.74	34	37	0.95
Cause of menopause (% natural)	69	63	0.38	70	60	0.12
History of hypertension (%)	32	23	0.13	30	33	0.54
Family history of diabetes (%)	24	34	0.10	29	16	0.02 [†]

* Among controls not missing genotyping data

[†] Due to genetic inheritance, there is an expected automatic correlation between shared genotypes and family history; this does not bias the results, while in fact, it supports the role of this SNP in diabetes risk.

Further Discussion

In addition to the strong predictive findings from MR analysis, the observed plasma SHBG levels-T2D risk was also expected by the magnitude of genotype-plasma SHBG association seen in our data (Figure 1). This clear and distinctive concordance between not just direction of genotype-plasma-T2D associations but also relative magnitudes of each genotype with plasma SHBG and T2D risk further supports the dose-response nature of the potentially causal results.

Our SHBG findings may help explain some intriguing divergent effects of transdermal versus oral estrogen previously reported in the literature. Notably, the contrast between transdermal estradiol in elevating plasma glucose and oral estrogen in lowering glucose has been observed in two randomized trials (overall P-value=0.03 for interaction)¹⁵. It may be that transdermal estradiol unfavorably affecting SHBG levels¹⁶⁻¹⁸ whereas oral estrogen therapy favorably increases SHBG.¹⁶⁻²⁰ Recent studies indicate that SHBG-bound hormones may also be important for biological action,^{14, 21} which may explain why postmenopausal oral estrogen therapy increases SHBG levels,^{17, 22} and subsequently lowering T2D risk^{23, 24} Testosterone therapy has recognized adverse effects on adiposity and glucose control in women,²⁵ and thus, higher SHBG level had been thought to be favorable via sequestering adverse androgens in women. However, this old concept is not necessarily supported by our SHBG findings in men, as androgens likely lower glucose in men.²⁵ Taken together, our prospective data coupled with evidence from clinical observations and experiments support the biological importance of the interplay between SHBG and sex hormones in glucose homeostasis.

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