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Brief Communication

Sex hormones in the risk of breast cancer: a two-sample Mendelian randomization study

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Abstract: Multiple evidence has suggested the essential role of sex hormones in the susceptibility of breast cancer. However, whether there exists a causal association and the effect direction remains controversial. To examine the causative role of hormones in the risk of breast cancer, we first estimated their genetic correlation, and then conducted two-sample and multivariable Mendelian randomization analyses using summary statistics from genome-wide association studies of major sex hormones including testosterone (N=230,454), estradiol (N=163,985) and progesterone (N=1,261), together with breast cancer (N=228,951). We further performed subtype analysis focusing on estrogen receptor (ER)+ breast cancer (N=175,475) and ER- breast cancer (N=127,442), and conducted extensive sensitivity analyses. We identified significant positive genetic correlation between testosterone level and risk of breast cancer (genetic correlation: 0.09, P=1.10E-03). Genetically determined higher total testosterone level was associated with an increased risk of breast cancer (OR: 1.11, 95% CI: 1.06-1.16, P=4.55E-06). In the subtype analysis, higher total testosterone was associated with an increased risk of ER+ breast cancer (OR: 1.18, 95% CI: 1.11-1.26, P=6.00E-08). In contrast, no association was identified between estradiol, progesterone and the risk of breast cancer. These results elucidated the causal role of major sex hormones in the risk of breast cancer, especially in ER+ breast cancer. Future development of preventive or therapeutic interventions in clinical trials could attach importance to this.

Keywords: Sex hormone, breast cancer, Mendelian randomization

Introduction

Breast cancer is the most frequent malignancy in women worldwide, mainly affecting women over the age of 50 [1]. Epidemiological studies have identified several risk factors for breast cancer such as aging, family history and lifestyles like alcohol consumption and dietary fat intake [2]. However, the identified risk factors explain only a limited amount of variance in the disease risk. Exploring novel factors influencing breast cancer could help better understand the pathogenesis of the disease, and provide care and therapeutic strategies for the patients and clinicians.

Discrepant steroid hormones between sexes and different ages might be one determinant based on evidence from previous epidemiological and clinical studies. Sex hormones, mainly including estrogens, androgens and progestogens, are molecules produced by the endocrine system that send messages to various parts of the body, and help regulate the body's processes. Multiple evidence has suggested sex hormones were involved in the etiology of breast cancer [3]. From the epidemiological perspective, the rates of breast cancer increase rapidly in the premenopausal years, while the rate of increase slows at menopause when endogenous hormone levels decline. A retrospective study among 10,786 healthy women with a follow-up of 13.5 years found that total and free testosterone levels were directly associated with increased breast cancer risk, while higher estradiol was associated with increased risk of human epidermal growth factor receptor 2 ($HER2)^-$ cancer but reduced risk of $HER2^+$ cancer [4]. Similarly, a collaborative analysis of seven prospective studies found that circulating estrogens and androgens were positively associated with the risk of breast cancer in pre-

menopausal women [5]. In contrast, other functional and epidemiological studies also reported antiproliferative effects of estrogens [6] and androgens [7, 8]. These evidence suggested the essential role of sex hormones in the pathogenesis of breast cancer. However, the observational studies might be biased by unavoidable confounding factors and small sample size, and cannot determine causation. Therefore, the causal association between sex hormones and breast cancer is still elusive.

In this context, we performed a two-sample Mendelian randomization (MR) analysis to explore the causal role of major sex hormones including estrogens measured by estradiol, androgens measured by testosterone, and progestogens in the risk of breast cancer. The MR approach is less susceptible to reverse causation or confounding factors which may distort the interpretations of conventional observational studies. We found that higher total testosterone was causally associated with higher risk of breast cancer.

Methods

Datasets

We obtained summary statistics of total testosterone levels (N=230,454) and bioavailable testosterone levels (N=188,507) in females from a previous large genome-wide association study (GWAS) based on genotype and phenotype data from the UK Biobank [9]. Testosterone was measured by "one step competitive analysis on a Beckman Coulter Unicel Dxl 800". Summary statistics of estradiol in females (N=163,985) were from another GWAS based on data from the UK Biobank [10]. Estradiol was measured by "two step competitive analysis on a Beckman Coulter Unicel Dxl 800". Summary statistics of progesterone in females (N=1,261) were from GWAS on steroid hormone levels based on individuals of European ancestry [11]. Progesterone was measured by liquid chromatography-tandem mass spectrometry. Details of the summary data from all GWAS were listed in Supplementary Table 1. Single nucleotide polymorphisms (SNP) that passed the genome-wide significance threshold (P<5E-08) were chosen as instrumental variables, which were then clumped based on the 1,000 Genomes Project linkage disequilibrium (LD) structure. Index SNPs (R^2<0.001 with any other associated SNP within 10 Mb) with the minimum *P* value were kept.

We obtained GWAS summary statistics of breast cancer in females from a genome-wide association study (N_{case}=122,977, $N_{control}$=105,974) [12]. Summary statistics of estrogen receptor (ER)-negative (ER-) (N_{case}=21,468, $N_{control}$=105,974) and ER-positive (ER+) (N_{case}=69,501, $N_{control}$=105,974) breast cancer were also from this study. Harmonization was undertaken to rule out strand mismatches and ensure alignment of SNP effect sizes.

Genetic correlation

We estimated the genetic correlation between sex hormones and breast cancer using LDSC [13] and GNOVA [14]. The LDSC method uses GWAS summary data to regress association test statistics of SNPs on their LD scores, which is defined as the sum of LD r^2 measured with all other SNPs in the reference sample. Compared with LDSC, GNOVA provides greater statistical power and higher estimation accuracy, especially when the correlation is moderate [14]. We ran LDSC and GNOVA on SNPs in both traits together with reference data derived from the 1000 Genomes Project European population using default parameters. A *P* value below 4.17E-03 (0.05/12) was considered statistically significant after the Bonferroni correction.

Mendelian randomization analysis

We hypothesized that sex hormones as a risk factor could causally influence the risk of breast cancer, and the following assumptions were satisfied: the genetic variants used as instrumental variables are associated with sex hormone levels; the genetic variants are not associated with any confounders; the genetic variants are associated with risk of breast cancer through sex hormones (namely horizontal pleiotropy should not be present) (Supplementary Figure 1).

To evaluate the causative effect of sex hormones on the risk of breast cancer, we performed a two-sample MR analysis using the random effects inverse variance weighted (IVW) method, which is most widely used in MR studies and could provide robust causal estimates under the absence of directional pleiotropy. A *P* value below 4.17E-03 (0.05/12) was considered statistically significant after the Bonferroni correction. We further verified the results using the weighted median method, which generally has greater power with a positive causal effect, particularly as the proportion

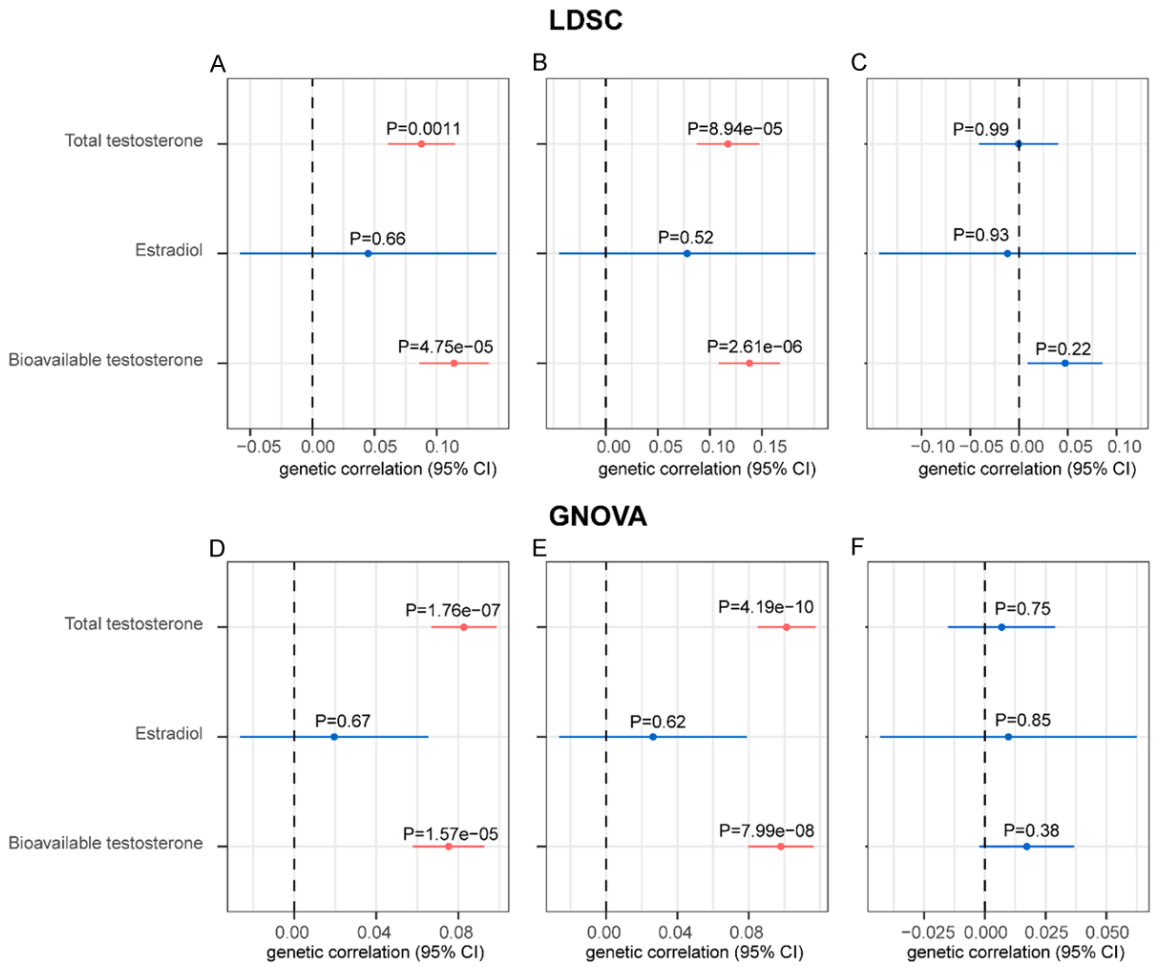

Figure 1. Genetic correlation between sex hormones and breast cancer. (A-C) Genetic correlation estimated using the LDSC method for (A) breast cancer, (B) ER+ breast cancer, and (C) ER- breast cancer. (D-F) Genetic correlation estimated using the GNOVA method for (D) breast cancer, (E) ER+ breast cancer, and (F) ER- breast cancer. Error bars indicate 95% confidence intervals. ER, Estrogen Receptor.

of invalid instrumental variables increases. Furthermore, we used the PhenoScanner v2 tool to check for variants associated with other phenotypes (P<5E-08) which might affect the risk of breast cancer independent of hormone levels [15].

In addition, we conducted comprehensive sensitivity analyses to estimate potential violations of the model assumptions in the MR analysis (Supplementary Figure 2). We conducted Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) analysis to detect outlier instrumental variables, which were removed step-by-step to reduce the effect of horizontal pleiotropy. Cochran's Q test was executed to check heterogeneity across the individual causal effects. MR-Egger regression was performed to evaluate the directional pleiotropy of instrumental variables. To evaluate the strength of each instrumental variable, we computed the F-statistic of each SNP. The statistical power was calculated using an online tool at http://cnsgenomics.com/shiny/mRnd/ [16]. The statistical analyses were conducted using the R package TwoSampleMR 0.5.5 [17].

Results

We first estimated the genetic correlation between each sex hormone and the risk of breast cancer. We detected a significant positive genetic correlation between breast cancer and bioavailable testosterone (genetic correlation: 0.11, P=4.75E-05), total testosterone (genetic correlation: 0.09, P=1.10E-03) (**Figure 1A**, **1D**). In the subtype analysis, similar results were identified between ER+ breast cancer and

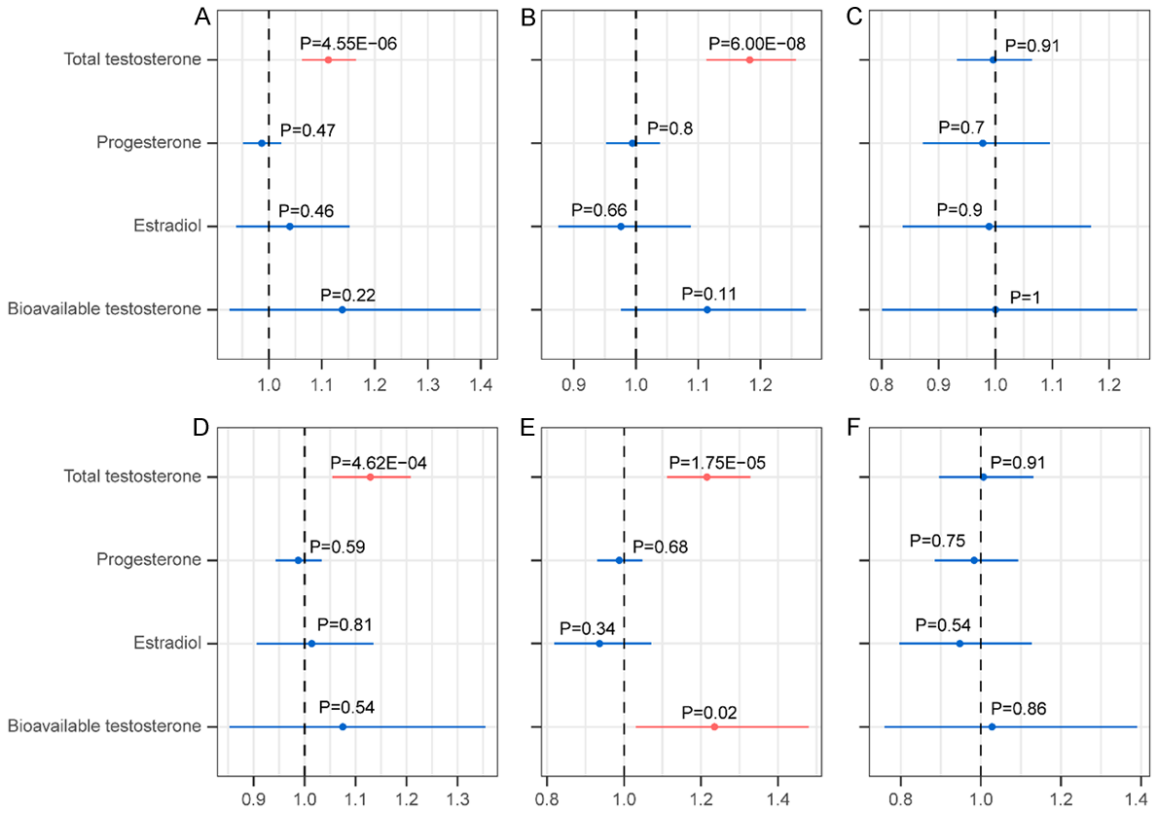

Figure 2. Forest plot showing results from the Mendelian randomization analysis. (A-C) Results from the Mendelian randomization analysis to evaluate causal role of sex hormones in (A) breast cancer, (B) ER+ breast cancer, (C) and ER- breast cancer using the inverse variance weighted method. (D-F) Results from the Mendelian randomization analysis to evaluate causal role of sex hormones in (D) breast cancer, (E) ER+ breast cancer, and (F) ER- breast cancer using the weighted median method. Estimates are per 1 standard deviation (SD) increase in the trait. ER, Estrogen Receptor.

bioavailable testosterone (genetic correlation: 0.14, P=2.61E-06), total testosterone (genetic correlation: 0.12, P=8.94E-05) (**Figure 1B**, **1E**), while for ER- breast cancer no significant association was identified (**Figure 1C**, **1F**).

We further analyzed the role of each hormone in the risk of breast cancer via the two-sample MR approach. Results showed that higher total testosterone level was associated with a higher risk of breast cancer (OR: 1.11, 95% CI: 1.06-1.16, P=4.55E-06) (**Figure 2A**). In the subtype analysis, similar association was detected between total testosterone level and ER+ breast cancer (OR: 1.18, 95% CI: 1.11-1.26, P=6.00E-08), while for ER- breast cancer no association was identified (**Figure 2B**, **2C**). Such results were further verified using the weighted median method (**Figure 2D-F**). The funnel plot displays a symmetric pattern of effect size variation around the point estimate (Supplementary Figures 3, 4, 5, 6, 7, 8, 9, 10, 11).

Furthermore, we performed extensive sensitivity analyses to validate the causal association between sex hormones and breast cancer. The Cochran's Q test did not detect the heterogeneity of effects across the instrumental variables (Supplementary Table 2). The F statistics of all the instrumental variables were above 10 (ranging from 24 to 1656), indicating the absence of weakness in the selected instrumental variables. No apparent horizontal pleiotropy was observed as the intercept of MR-Egger was not significantly deviated from zero. Meanwhile, no potential instrumental outlier was detected by the MR-PRESSO analysis. The leave-one-out results suggested that the causal effect was not driven by a single instrumental variable (Supplementary Figures 3, 4, 5, 6, 7, 8, 9, 10, 11).

Lastly, we used the PhenoScanner tool to check if the SNPs used in the MR analysis were associated with other phenotypes. As a result, several instrumental variables such as rs454-46698, rs112635299 and rs4453027 were associated with body mass index (BMI), which was suggested to affect the risk of breast cancer [18]. Therefore, we further performed multivariable MR analysis to elucidate the causal relationship between sex hormones and the risk of breast cancer adjusting potential pleiotropy due to BMI. The summary data of BMI was obtained from GWAS published by the Genetic Investigation of ANthropometric Traits (GIANT) consortium [19]. As a result, significant association was still identified between testosterone level and breast cancer in the multivariable MR analyses adjusting from BMI (Supplementary Table 3).

Discussion

In the current study, we investigated the causative role of three major hormones in the risk of breast cancer using the MR approach. The results showed that total testosterone level was positively associated with the risk of breast cancer, especially ER+ breast cancer. These findings provided a better understanding of the role of sex hormones in the risk of breast cancer, and had clinical implications.

Testosterone is a male sex hormone, mainly produced in a woman's ovaries in small amounts. Previous epidemiological studies have identified that higher testosterone level was associated with increased risk of breast cancer for women both before and after menopause [5, 20]. Similarly, another prospective cohort study found that estrogen plus testosterone therapies increased risk of invasive breast cancer compared with estrogen-only therapy [21]. These evidence suggested the close correlation between testosterone and breast cancer. Consistent with these findings, we identified that higher testosterone was associated with an increased risk of breast cancer from the genetic perspective using the MR approach. The mechanism of how testosterone increased the risk of breast cancer is still unknown. One explanation is that testosterone can be aromatized to estradiol, which increases proliferation and hence breast cancer risk. Notably, it was also reported that long term therapy with subcutaneous testosterone in women presenting with symptoms of androgen deficiency did not increase the risk of invasive breast cancer [22], and might even reduce the risk of breast cancer [23]. Therefore, moderate levels of testosterone might also be beneficial in breast cancer. However, based on the current dataset, we could not evaluate whether there exists a U-shaped effect of testosterone on the risk of breast cancer. Therefore, further studies investigating testosterone in breast cancer could pay attention to the effect of extreme levels of testosterone. In contrast, we did not identify significant association between bioavailable testosterone and breast cancer, though the effect direction was the same. Simiarly, one previous prospective study identified that total but not free testosterone was positively associatied with the risk of breast cancer [5], though such result was not consistent across studies [24]. Compared with total testosterone which has sex hormone binding globulin or albumin chemical receptors bound to it, unbound testosterone can act as receptors to any cell in the body. Therefore, the bound testosterone might play an important role in the pathogenesis of breast cancer. Nevertheless, we could not rule out the possibility that the failure to detect association might be due to the limited statistical power since the variance explained by the instrumental variables was relatively small (Supplementary Table 2). Future exploration based on summary data from GWAS with larger sample size was warranted to provide a more accurate estimate. In the subtype analysis, testosterone was associated with higher risk of ER+ breast cancer, but not ER- breast cancer. This result suggested the effect of testosterone was lower in ER- breast cancer, which does not have hormone receptors and won't be affected by endocrine treatments aimed at blocking hormones in the body.

Estradiol is a major regulator of growth for the subset of breast cancers that express the estrogen receptor. Previous prospective studies found that estrogens were positively associated with the risk of breast cancer in premenopausal women [5]. However, another cohort study also reported that estrogen was associated with lower incidence of invasive breast cancer among 10,739 postmenopausal women in a median follow-up of 11.8 years [25]. Therefore, the role of estradiol in breast cancer

was still elusive. Biologically, previous clinical findings suggested that after long-term oestrogen deprivation, adaptive changes in mammary tumor gene expression profiles render tumors paradoxically susceptible to oestrogen-induced apoptosis [26, 27]. However, as estrogen is a recognized mitogen that usually stimulates mammary cell proliferation through activation of the oestrogen receptor, too high levels of estrogen might be harmful as well. In the current study, we did not identify association between estradiol level and risk of breast cancer. However, only a few instrumental variables were available for estrodiol in the MR analysis, which limited the statistical power. Therefore, further replication with larger sample size was still necessary.

Progesterone is essential for normal breast development during puberty and in preparation for lactation and breastfeeding. A previous observational study found that estrogen plus progesterone use was associated with increased incidence of breast cancer among 41,449 postmenopausal women (HR=1.55, 95% CI=1.41-1.70, P<0.001) [28]. In the current study, we did not identify causal association between progesterone and breast cancer. This might be due to the limited effect of progesterone on breast cancer. However, we cannot exclude the possibility that we failed to detect association due to the insufficiency of current sample sizes as the effect might be relatively modest. In addition, breast cancer mainly affected women over the age of 50. Subgroup analysis on individuals of different ages might provide additional insight.

In conclusion, our results demonstrated that higher total testosterone level was associated with increased risk of breast cancer, particularly ER+ breast cancer. These findings help better understand the role of hormones in breast cancer, and will facilitate therapeutic management and drug discovery in future clinical trials.

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Disclosure of conflict of interest

None.

Abbreviations

ER, Estrogen Receptor; HER2, Human Epidermal growth factor Receptor 2; GWAS, Genome-Wide Association Study; IVW, Inverse Variance Weighted; LD, Linkage Disequilibrium; MR, Mendelian Randomization; SNP, Single Nucleotide Polymorphism.

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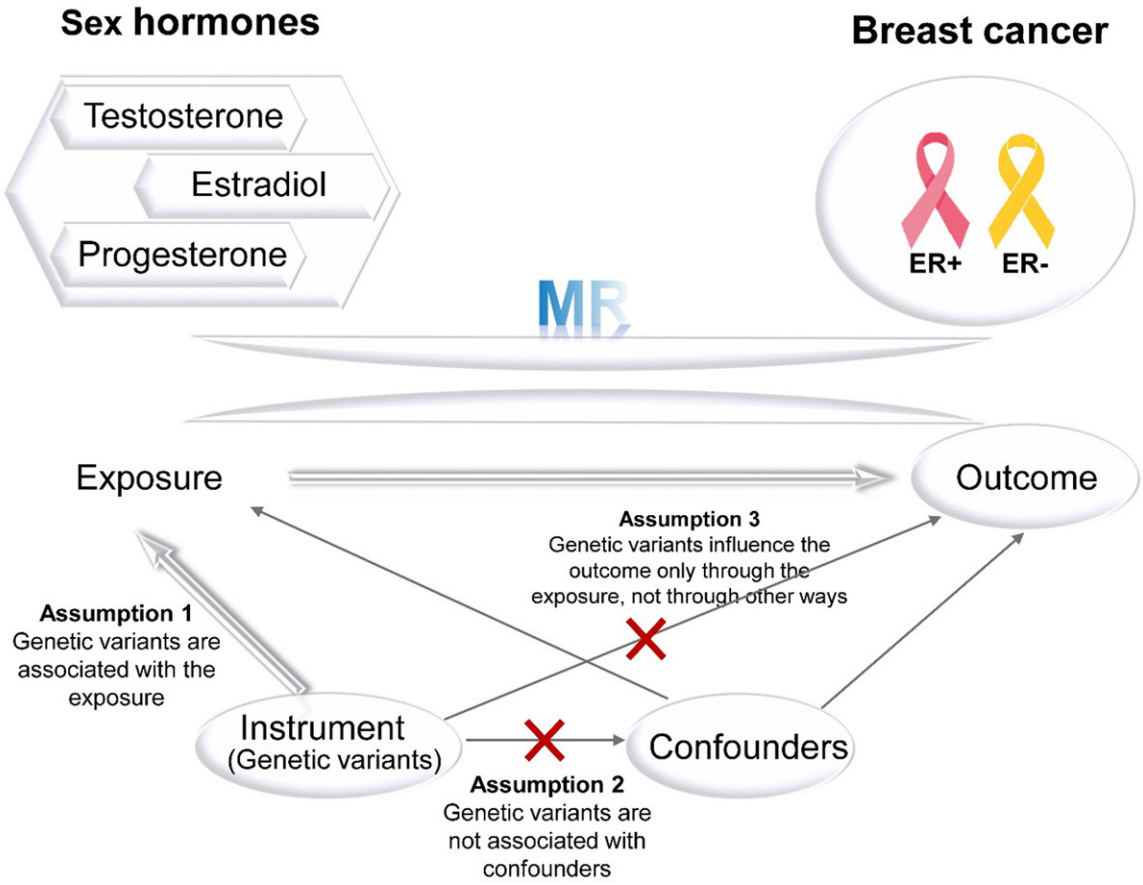
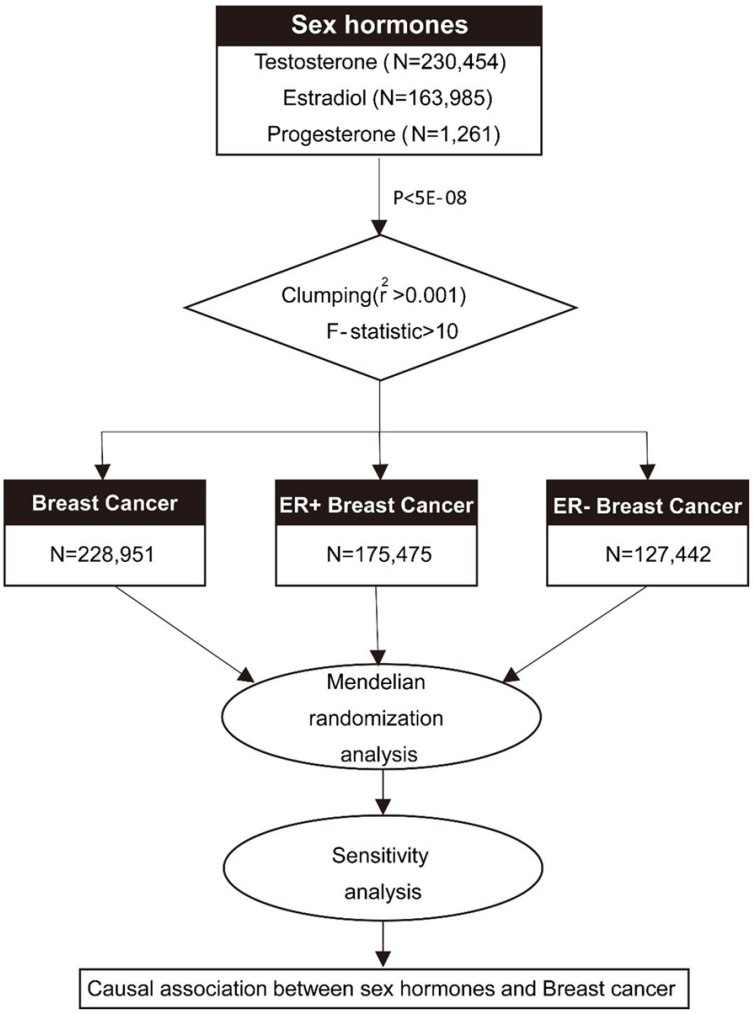
Supplementary Table 1. Summary data from all GWAS used in current study

Phenotype	Cases	Controls	Number of SNPs	PMID
bioavailable testosterone	188,507	-	16,585,745	32042192
total testosterone	230,454	-	16,580,850	32042192
estradiol	37,461	126,524	7,870,546	34255042
progesterone	1,261	-	n.a.	31169883
breast cancer	122,977	105,974	11,792,542	29059683
ER+ breast cancer	69,501	105,974	10,643,737	29059683
ER- breast cancer	21,468	105,974	10,643,737	29059683

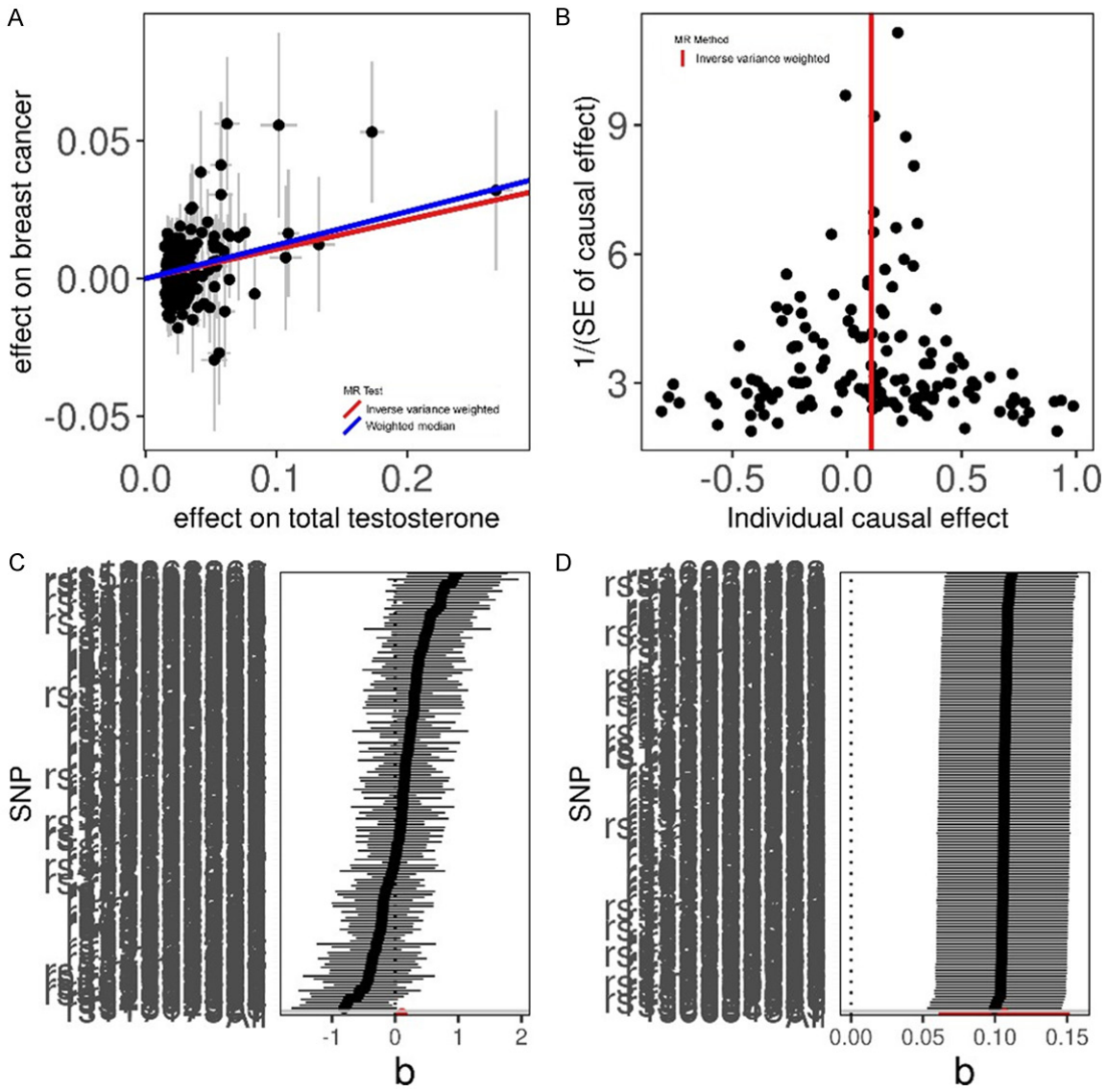
SNP, single nucleotide polymorphism; GWAS, genome-wide association study; PMID, PubMed ID; ER+, estrogen receptor positive; ER-, estrogen receptor negative; n.a., not available.



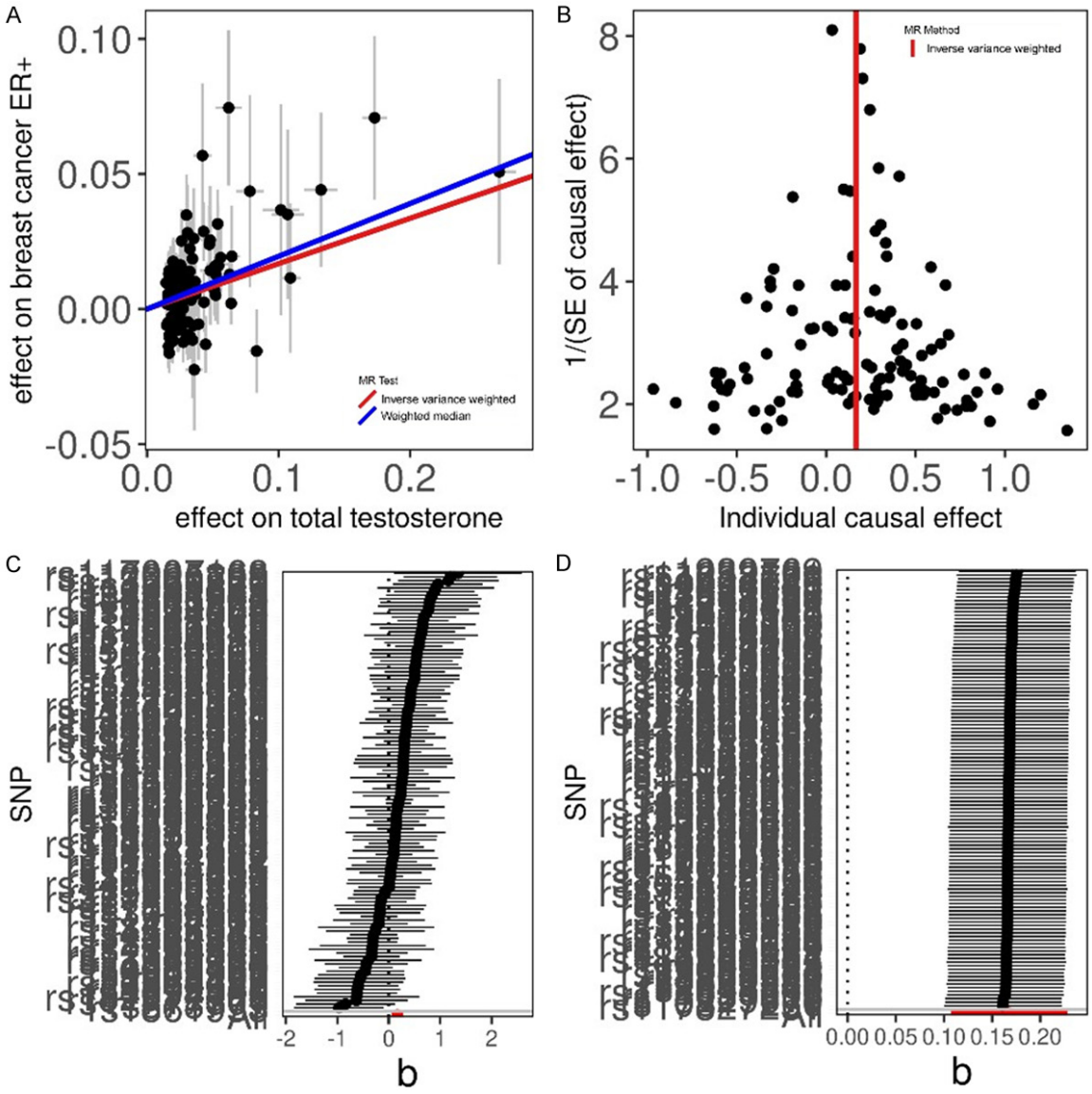
Supplementary Figure 1. Assumptions in Mendelian randomization analysis. Broken lines represent potential pleiotropic or direct causal effects between variables that would violate Mendelian randomization assumptions.



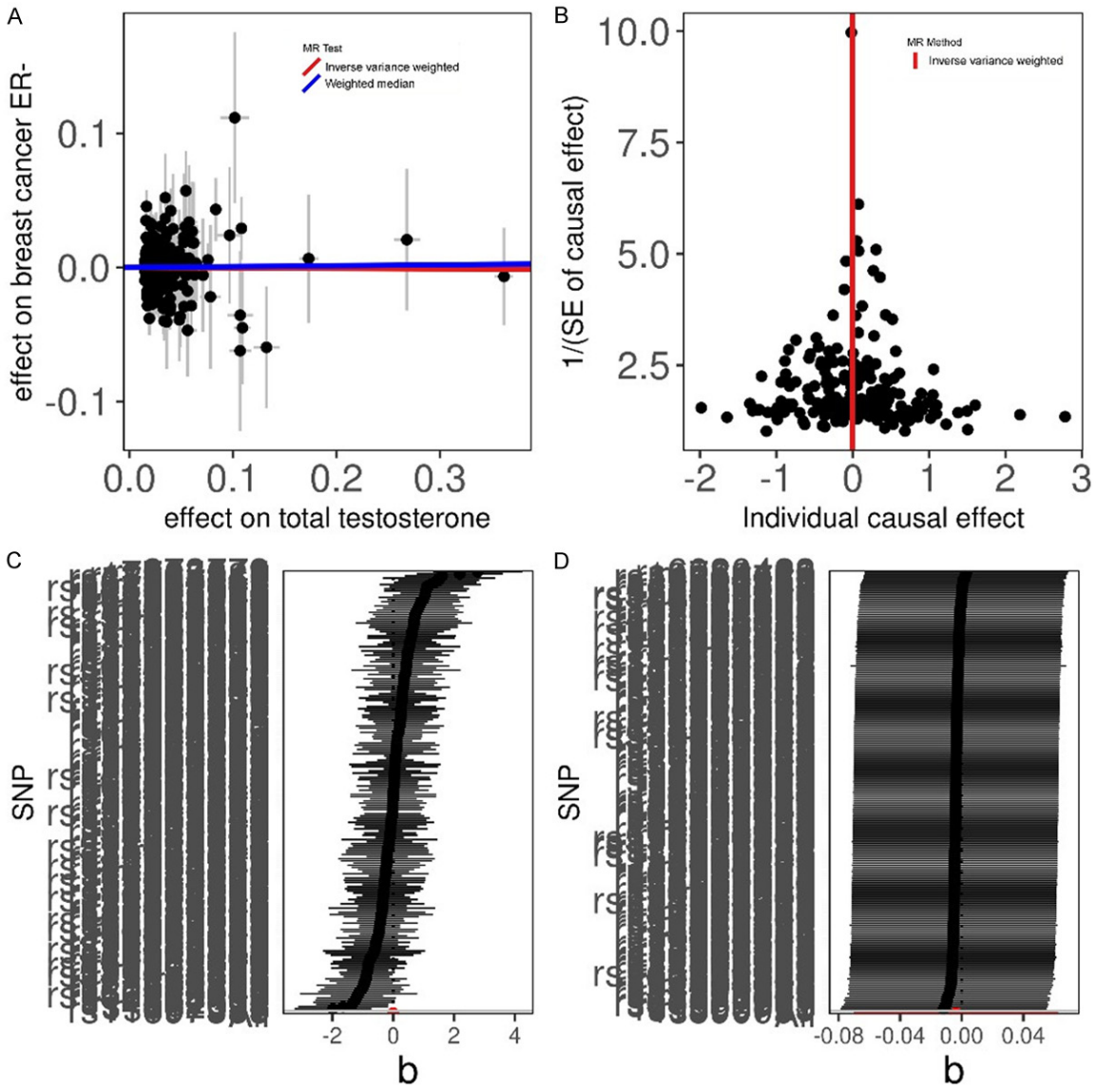
Supplementary Figure 2. Schematic analysis workflow.



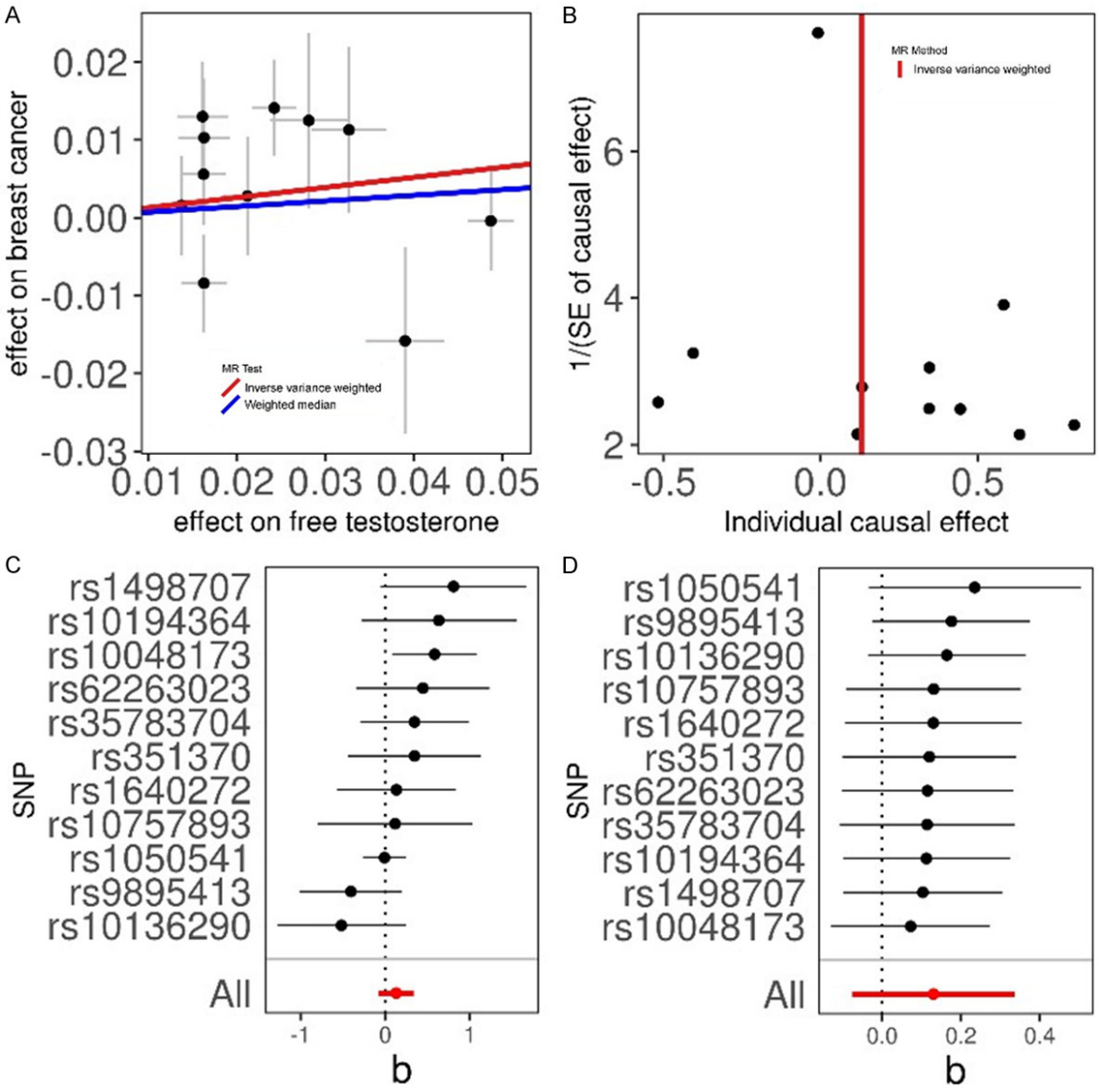
Supplementary Figure 3. Mendelian randomization analysis results for total testosterone on risk of breast cancer. A. Scatter plot of SNP effects on total testosterone and breast cancer. The 95% CI for the effect size on breast cancer is shown as vertical lines, while the 95% CI for the effect size on total testosterone is shown as horizontal lines. The slope of fitted lines represents the estimated MR effect per method. B. Funnel plot showing the estimation using the inverse of the standard error of the causal estimate with each individual SNP as a tool. The vertical line represents the estimated causal effect. C. Forest plot of the association of individual SNPs with total testosterone and breast cancer, together with pooled estimates. D. Forest plot of the results of the leave-one-out sensitivity analysis, where each SNP was iteratively removed from the instrumental variables. SNP, single nucleotide polymorphism.



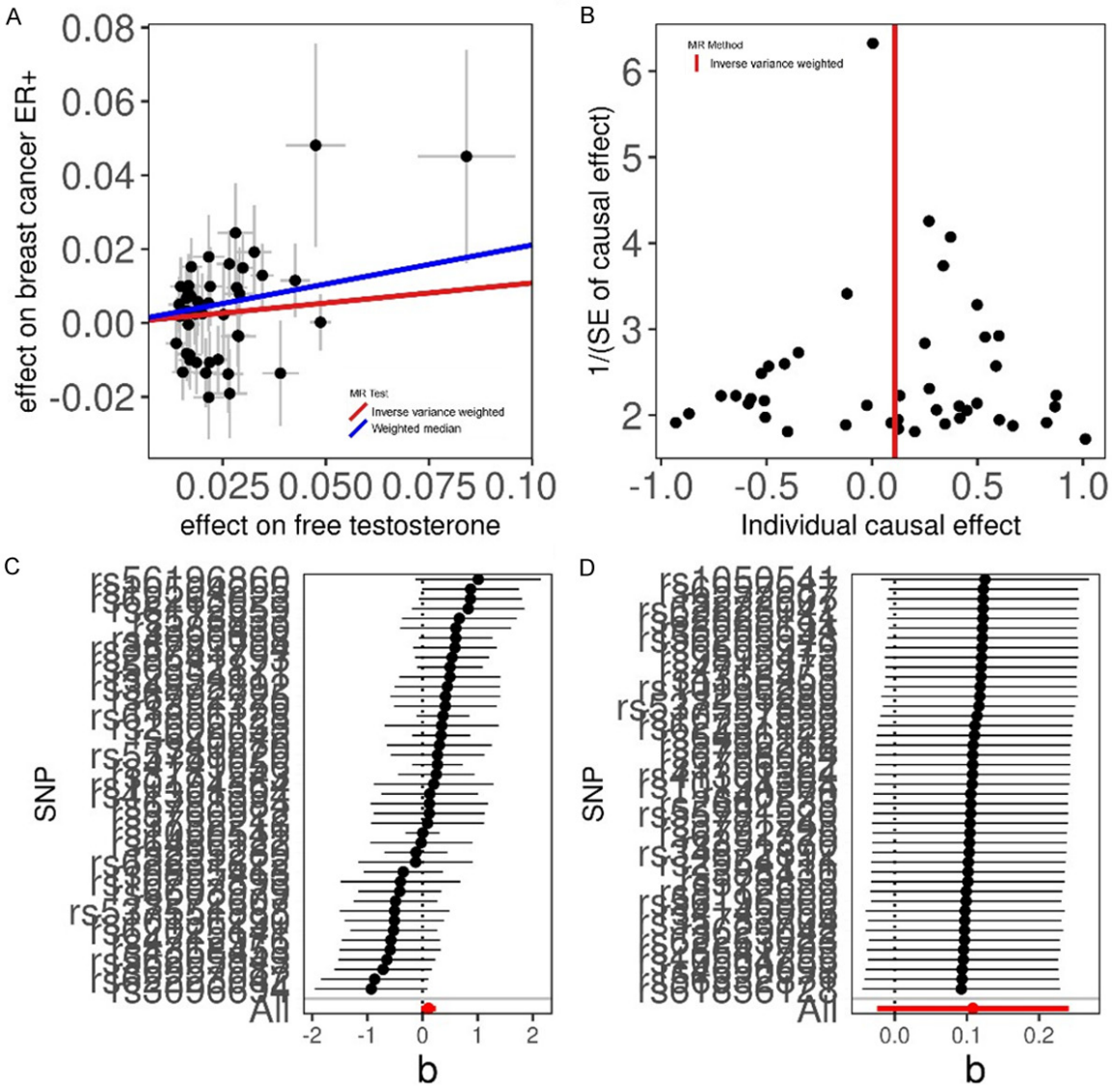
Supplementary Figure 4. Mendelian randomization analysis results for total testosterone on risk of ER+ breast cancer.



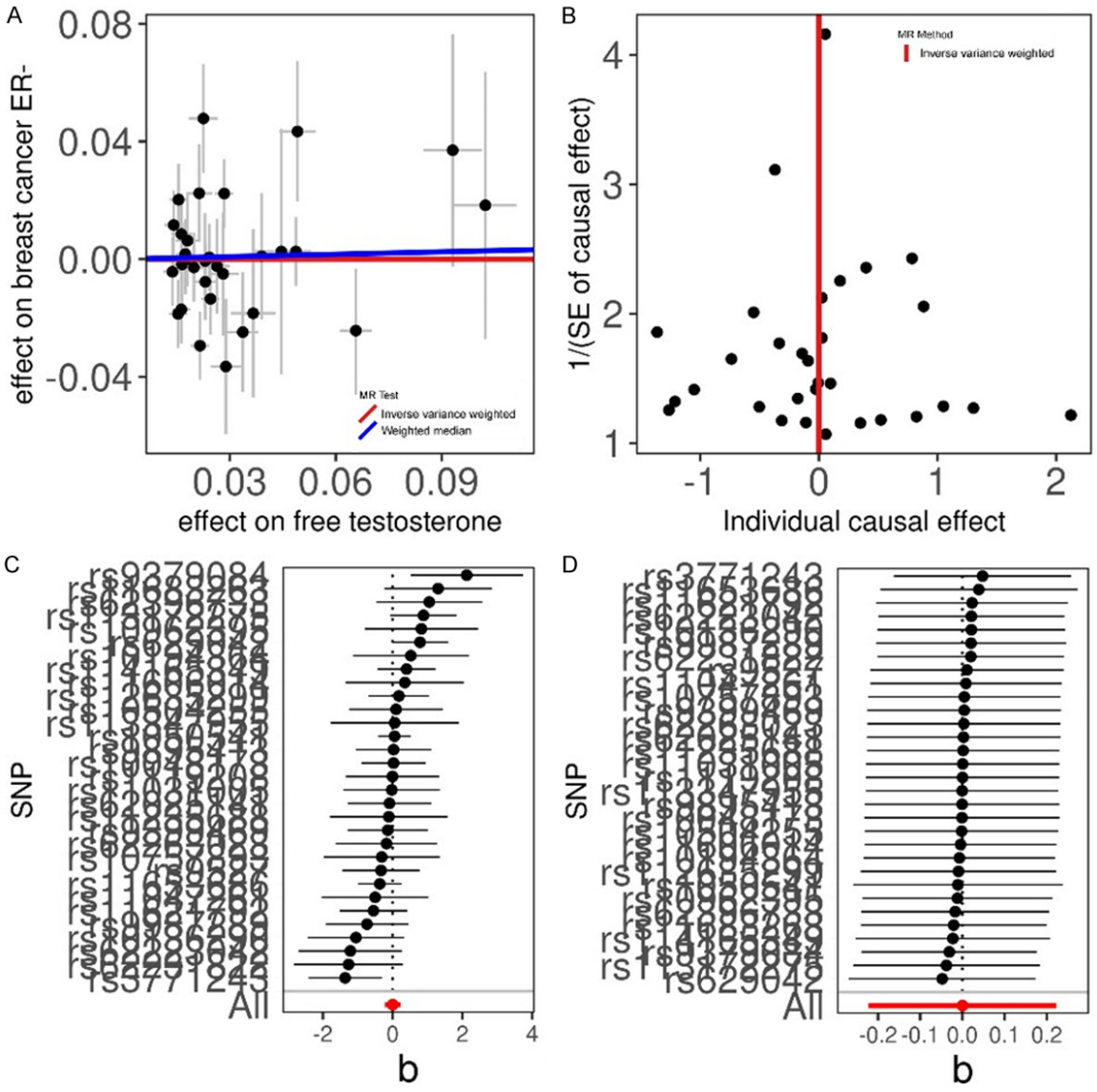
Supplementary Figure 5. Mendelian randomization analysis results for total testosterone on risk of ER- breast cancer.



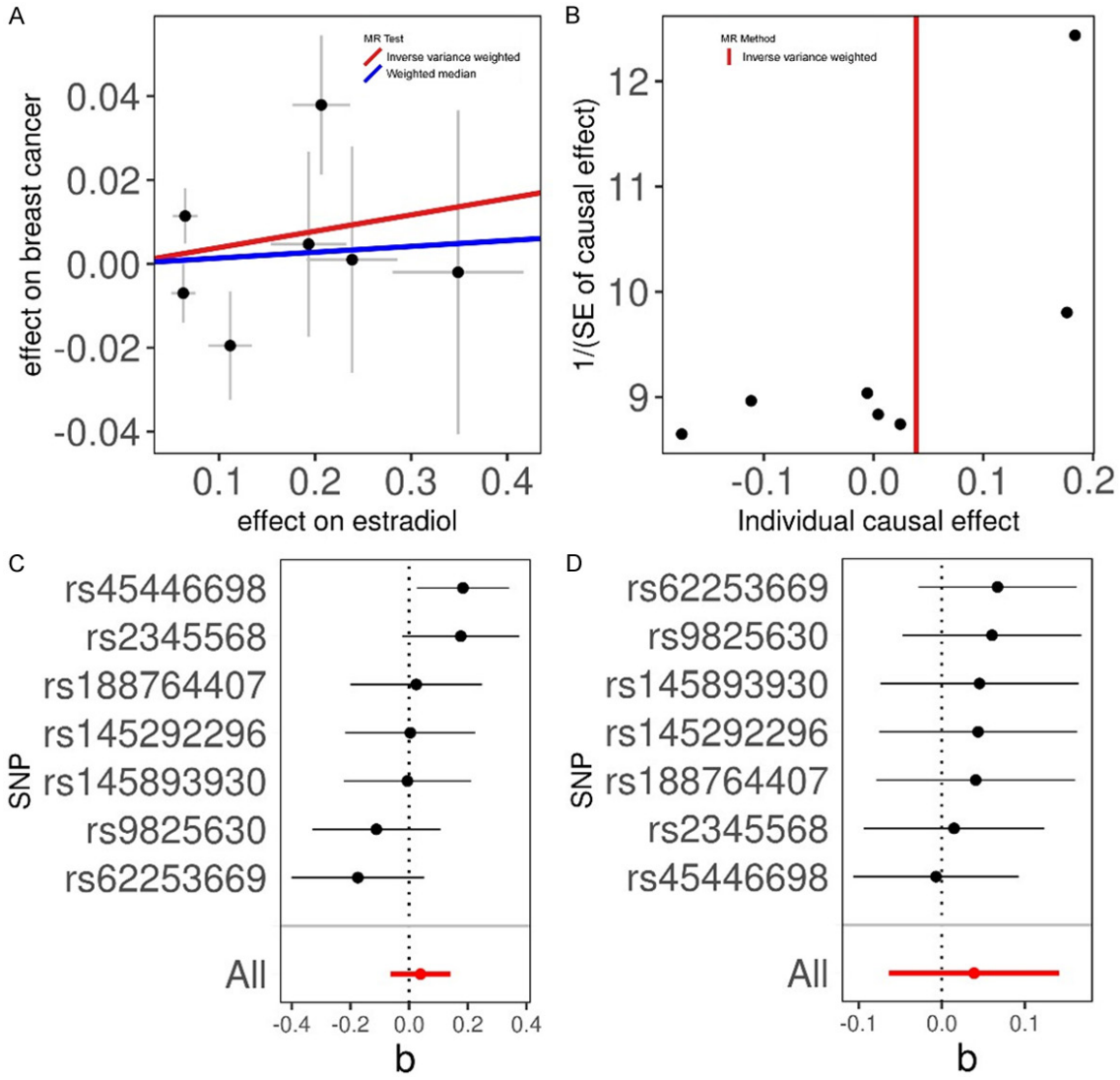
Supplementary Figure 6. Mendelian randomization analysis results for free testosterone on risk of breast cancer.



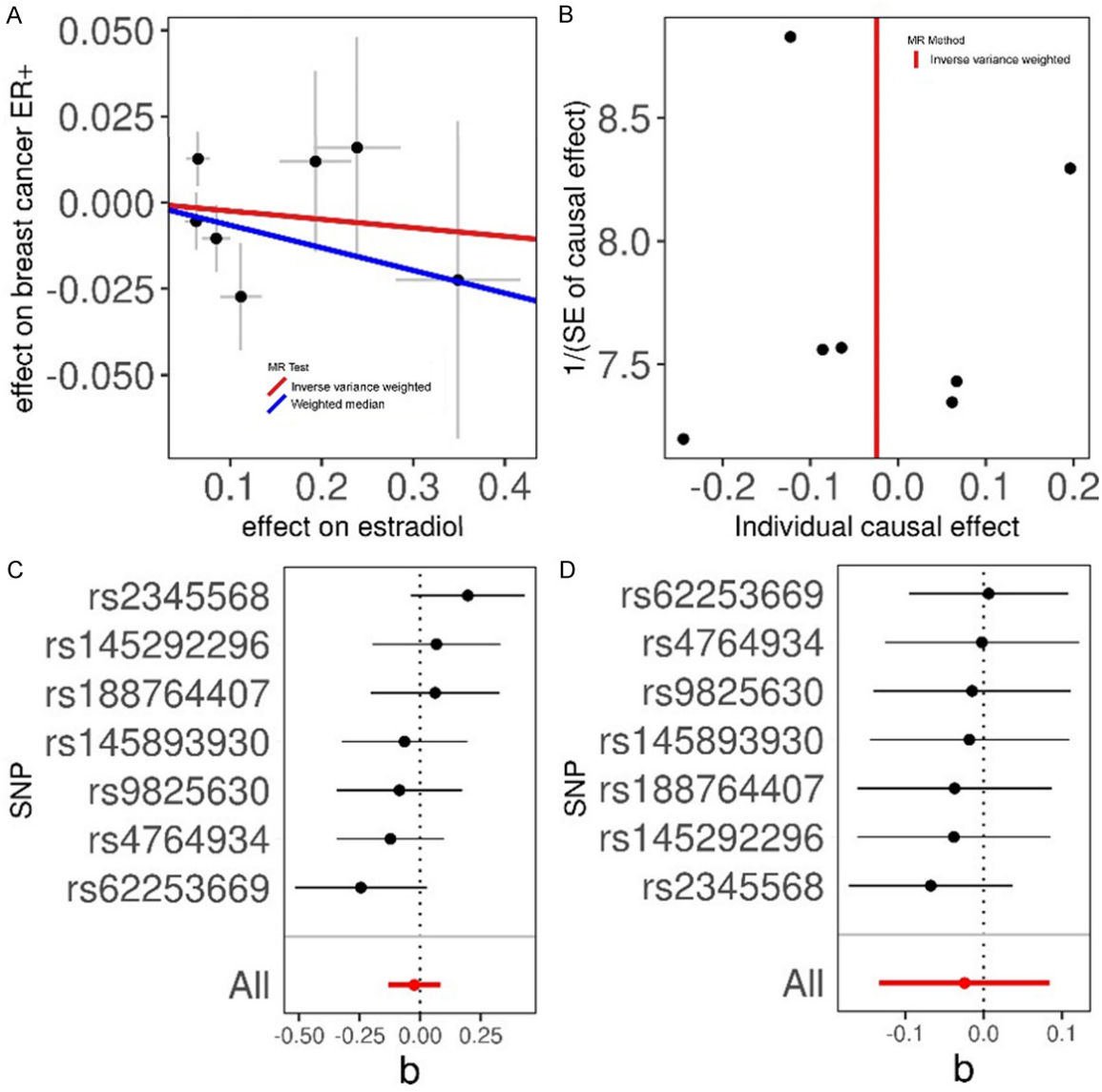
Supplementary Figure 7. Mendelian randomization analysis results for free testosterone on risk of ER+ breast cancer.



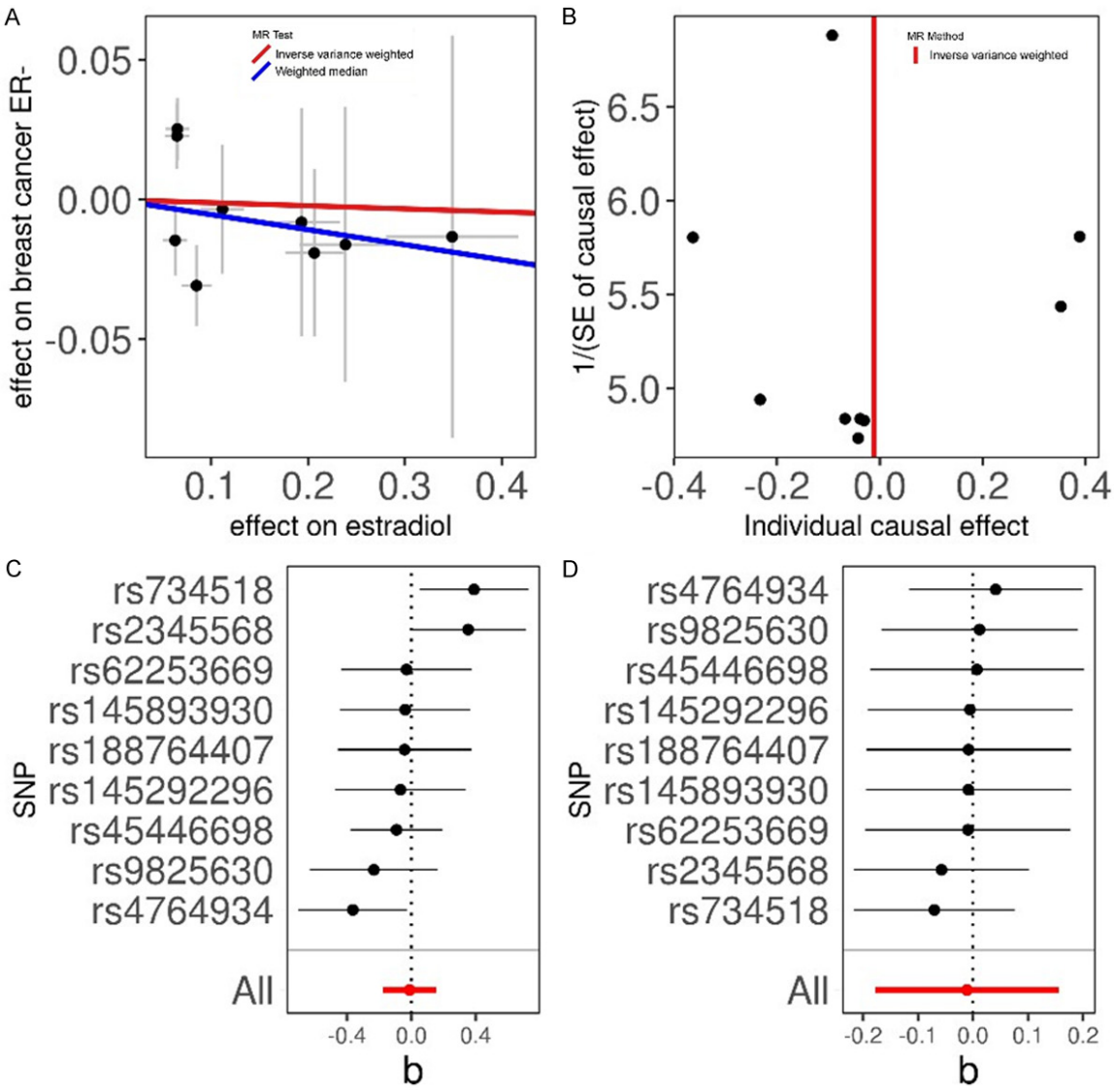
Supplementary Figure 8. Mendelian randomization analysis results for free testosterone on risk of ER- breast cancer.



Supplementary Figure 9. Mendelian randomization analysis results for estradiol on risk of breast cancer.



Supplementary Figure 10. Mendelian randomization analysis results for estradiol on risk of ER+ breast cancer.



Supplementary Figure 11. Mendelian randomization analysis results for estradiol on risk of ER- breast cancer.

Supplementary Table 2. Heterogeneity and horizontal pleiotropy analyses between hormone traits and breast cancer

hormone trait	Heterogeneity			Horizontal pleiotropy			MR-PRESSO	
	IVW Q	IVW Q df	IVW P	Egger intercept	SE	*P* value	*P* value	Beta
breast cancer as outcome								
bioavailable								
testosterone	14.89	10	0.14	7.41E-03	6.15E-03	0.26	0.12	0.17
total testosterone	178.32	149	0.05	-2.17E-03	1.46E-03	0.14	0.05	0.05
oestradiol	10.56	6	0.10	-3.23E-03	1.14E-02	0.79	0.09	0.34
progesterone	1.46	3	0.69	-6.11E-03	2.23E-02	0.81	0.71	0.07
ER+ breast cancer as outcome								
bioavailable								
testosterone	55.72	42	0.08	-4.69E-03	4.37E-03	0.29	0.09	0.11
total testosterone	143.26	119	0.06	-3.01E-03	1.92E-03	0.12	0.05	0.06
oestradiol	7.79	6	0.25	2.40E-03	1.22E-02	0.85	0.25	0.39
progesterone	0.88	3	0.83	-4.80E-03	2.66E-02	0.87	0.84	0.08
ER- breast cancer as outcome								
bioavailable								
testosterone	39.62	30	0.11	-2.99E-03	6.70E-03	0.66	0.12	0.18
total testosterone	240.04	204	0.04	-7.80E-04	2.06E-03	0.71	0.05	0.08
oestradiol	15.12	8	0.06	1.73E-02	1.69E-02	0.34	0.04	0.45
progesterone	8.69	3	0.03	3.80E-03	8.52E-02	0.97	0.05	0.12

IVW, Inverse variance weighted; Q, Cochran's Q test estimate; df, Cochran's Q test degrees of freedom; SE, standard error. Beta denotes the effect sizes can be detected with the power of 0.8 given the sample size, proportion of cases and variance explained by the instrumental variables.

Supplementary Table 3. Mendelian randomization estimates between sex hormone level and risk of breast cancer adjusting for body mass index

outcome	exposure	beta	SE	*P* value
Breast cancer	Bioavailable testosterone	0.13	0.06	0.02
	Total testosterone	0.13	0.03	1.24E-05
	Estradiol	0.05	0.50	0.93
ER+ breast cancer	Bioavailable testosterone	0.19	0.06	8.23E-04
	Total testosterone	0.17	0.03	1.65E-07
	Estradiol	0.18	0.60	0.76
ER- breast cancer	Bioavailable testosterone	-0.06	0.08	0.41
	Total testosterone	-0.02	0.4	0.61
	Estradiol	-0.49	0.61	0.42

ER+, estrogen receptor positive; ER-, estrogen receptor negative.