

RESEARCH ARTICLE

Do sex hormones confound or mediate the effect of chronotype on breast and prostate cancer? A Mendelian randomization study

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Data Availability Statement: Summary data for GWAS of chronotype in females has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4861/> Summary data for GWAS of total testosterone in females has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4864/> Summary data for GWAS of bioavailable testosterone in females has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4869/> Summary data for GWAS of sex hormone binding globulin (SHBG) in females has been made

Abstract

Morning-preference chronotype has been found to be protective against breast and prostate cancer. Sex hormones have been implicated in relation to chronotype and the development of both cancers. This study aimed to assess whether sex hormones confound or mediate the effect of chronotype on breast and prostate cancer using a Mendelian Randomization (MR) framework. Genetic variants associated with chronotype and sex hormones (total testosterone, bioavailable testosterone, sex hormone binding globulin, and oestradiol) ($p < 5 \times 10^{-8}$) were obtained from published genome-wide association studies ($n \leq 244,207$ females and $n \leq 205,527$ males). These variants were used to investigate causal relationships with breast ($n_{\text{Cases}}/n_{\text{Controls}} = 133,384/113,789$) and prostate ($n_{\text{Cases}}/n_{\text{Controls}} = 79,148/61,106$) cancer using univariable, bidirectional and multivariable MR. In females, we found evidence for: I) Reduced risk of breast cancer per category increase in morning-preference (OR = 0.93, 95% CI: 0.88, 1.00); II) Increased risk of breast cancer per SD increase in bioavailable testosterone (OR = 1.10, 95% CI: 1.01, 1.19) and total testosterone (OR = 1.15, 95% CI: 1.07, 1.23); III) Bidirectional effects between morning-preference and both bioavailable and total testosterone (e.g. mean SD difference in bioavailable testosterone = -0.08, 95% CI: -0.12, -0.05 per category increase in morning-preference vs difference in morning-preference category = -0.04, 95% CI: -0.08, 0.00 per SD increase in bioavailable testosterone). In males, we found evidence for: I) Reduced risk of prostate cancer per category increase in morning-preference (OR = 0.90, 95% CI: 0.83, 0.97) and II) Increased risk of prostate cancer per SD increase in bioavailable testosterone (OR = 1.22, 95% CI: 1.08, 1.37). No bidirectional effects were found between morning-preference and testosterone in males. While testosterone levels were causally implicated with both chronotype and cancer, there was inconsistent evidence for testosterone as a mediator of the relationship. The protective effect of morning-preference on both breast and prostate cancer is clinically

available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4870/> Summary data for GWAS of oestradiol in females has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4872/> Summary data for GWAS of chronotype in males has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4862/> Summary data for GWAS of total testosterone in males has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4865/> Summary data for GWAS of bioavailable testosterone in males has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4868/> Summary data for GWAS of sex hormone binding globulin (SHBG) in males has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4871/> Summary data for GWAS of oestradiol in males has been made available at: <https://gwas.mrcieu.ac.uk/datasets/ieu-b-4873/> This research was conducted using the UK Biobank Resource under application number 16391. Summary data for the breast cancer GWAS used in this study is available at: <http://bcac.ccg.medschl.cam.ac.uk/bcacdata/oncoarray/oncoarray-and-combined-summary-result/gwas-summary-associations-breast-cancer-risk-2020/> Summary data for the prostate cancer GWAS used in this study is available at: http://practical.icr.ac.uk/blog/?page_id=8164.

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interesting, although it may be difficult to effectively modify chronotype. Further studies are needed to investigate other potentially modifiable intermediates.

Author summary

Although previous studies have demonstrated that morning-preference chronotype is associated with reduced breast and prostate cancer risk, the role of sex hormones in this relationship has yet to be elucidated. In this study we use genetic variants strongly associated with morning-preference chronotype, total testosterone, bioavailable testosterone, sex-hormone binding globulin and oestradiol to explore the potential of these sex hormones to act as either mediators or confounders of this relationship. The findings of this study implicate testosterone as a potential mediator of the relationship between chronotype and both prostate and breast cancer risk, although results were inconsistent between methods. As such, further studies are warranted to better understand the mechanisms underlying this relationship in order to inform the development of sleep-based intervention studies that may help to reduce breast and prostate cancer risk in high-risk populations.

Introduction

Breast and prostate cancers are hormonally related tumours with a significant burden of morbidity and mortality: an estimated 2.1 million women and 1.3 million men were diagnosed with these conditions in 2018, with age-standardised mortality rates of 13.0 and 7.6 per 100,000 respectively [1]. Despite improvements in cancer screening, treatment [2, 3] and monitoring [4, 5], they remain the most common cancers in women and men, respectively, and their incidence is increasing [6]. Improved understanding of the aetiology of these cancers is an important step towards the development of effective primary prevention strategies.

Circadian rhythms underlying the sleep-wake cycle are implicated in cancer development, particularly in breast and prostate cancers [7, 8]. Variation in sleep-wake cycles between individuals [9] can manifest as circadian preference (chronotype), with most people fitting into one of three broad categories: morning-preference; evening-preference; or no preference. Using multivariable regression, we showed that over five categories a per category increase from extreme evening preference to extreme morning-preference chronotype was associated with a reduction (OR = 0.95; 95% CI: 0.93, 0.98) in breast cancer incidence in the UK Biobank prospective cohort study (n = 151,421; 2,732 incident cases [10]), and this result was consistent when triangulated across different methods, including Mendelian randomization (MR) (see below). A prostate case-control study (1095 cases and 1388 controls) found limited evidence for a difference in chronotype between cases and controls. However, among men with an evening preference, overall risk was higher among night shift workers compared with non-shift workers (OR 1.50; 95% CI 0.85, 2.66), whereas among men with no preference chronotypes, there was no difference in risk between night shift workers and non-shift workers (OR 1.02; 95% CI: 0.72, 1.44) [11]. More recently, an MR study found morning preference to be associated with a reduced risk of prostate cancer (OR = 0.71; 95% CI: 0.54, 0.94) [12].

An individual's chronotype is largely determined by genetic variants [13], external environmental cues (e.g. light exposure [14]), and age [15]. Typically, children exhibit morning-preference, which becomes progressively later during adolescence, then as adults age there is a gradual shift back towards morning preference [16]. Generally, females reach these markers

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earlier than males, and males are more likely to retain their adolescent evening-preference into adulthood [9]. However, sex differences reduce around 50 years of age, coinciding with the onset of menopause [16]. This variability over time and by sex suggests that an individual's chronotype may, in part, be hormonally driven.

Hormones, such as cortisol, melatonin and some sex steroids, have previously been studied in relation to chronotype [17–21] and shift work, which is another circadian trait [22, 23]. In particular, evening chronotype has been associated with higher levels of testosterone in males, even after adjusting for age [20], with little evidence of an association of progesterone, dehydroepiandrosterone (DHEA) or testosterone with chronotype in females [21]. However, in cross-sectional studies such as these, it is difficult to fully account for unobserved confounding and the direction of effect between chronotype and hormone levels is difficult to resolve.

MR is an approach which uses genetic variants robustly associated with exposures to estimate potential causal effects on outcomes. It attempts to overcome some potential limitations of conventional analyses used in observational studies, including confounding and reverse causation, though has additional potential biases [24–26]. Using genetic variants related to chronotype and sex hormones, this approach may be used to investigate the bi-directional relationship between these traits and also to provide additional evidence regarding their effects on breast and prostate cancer. The statistical power and precision of MR analyses can be increased by using two-sample MR, in which summary genetic association data from independent samples representing genetic variant-exposure and genetic variant-outcome associations are combined in order to estimate causal effects [27].

Using two-sample MR, recent studies have reported a 7% reduction in breast cancer risk [28] and a 9% reduction in prostate cancer risk [29] per 1 SD (25 and 30nmol/L) increase in SHBG, respectively. A 15% increase in breast cancer risk and 22% increase in prostate cancer risk per 1 SD (4nmol/L) increase in bioavailable testosterone has also been reported [29]. While MR studies for the effects of oestrogen on cancer risk in males and premenopausal females is lacking, a recent randomised control trial found that postmenopausal females undergoing oestrogen-only hormone replacement therapy (HRT) for 1–14 years experienced a 17–33% increase in breast cancer risk compared to never-users [30]. A retrospective cohort study in post-menopausal females compared oestradiol levels from highest fifth to lowest (measured by immunoassay) and found an increased risk of breast cancer (OR = 2.66; 95% CI: 1.99–3.54), with similar results reported across different oestradiol-measurement methods [31].

Regarding the effect of chronotype on breast cancer, in our previous study, we used results from both one-sample and two-sample MR analysis to support the multivariable regression result of a protective causal effect of morning chronotype on breast cancer risk (OR = 0.85; 95% CI: 0.70, 1.03 and OR = 0.88; 95% CI: 0.82, 0.93 per increased category towards morning preference, for one- and two-sample MR, respectively) [10].

The aims of this study were to use two-sample MR to explore whether: (i) the causal effect of chronotype on breast cancer risk found previously [10] remains consistent using newly available breast cancer data, including data on subtypes [32]; (ii) chronotype exerts a causal effect on prostate cancer risk; and (iii) the effect of chronotype on breast, and any effect on prostate cancer risk, is mediated or confounded by sex hormones (total testosterone, bioavailable testosterone, sex hormone binding globulin (SHBG) and oestradiol).

Methods

Overall study design strategy

Our overall strategy was to perform a series of two-sample MR analyses. First, univariable MR (uvMR) was used to estimate the effects of: chronotype on breast and prostate cancer risk (step

1, Fig 1A); and sex hormone levels on cancer risk (step 2, Fig 1B). In step 3, bidirectional MR (bdMR) was used to determine the direction of associations between sleep traits and sex hormones (Fig 1C) [25]. In step 4, multivariable MR (mvMR) was used to further explore whether the chronotype effect on breast or prostate cancer is mediated by any of the hormones (Fig 1D) [33, 34]. Only those sex hormones with a potential role in mediating the effect of chronotype on cancer risk in step 3 were used in subsequent mvMR. A potential mediating role was determined based on the following criteria: (i) evidence of a causal effect of chronotype on a specific cancer; (ii) evidence of a causal effect of chronotype on the specific sex hormone; (iii) evidence of a causal effect of the specific sex hormone on the specific cancer, and (iv) no evidence of a strong bidirectional effect. For further information regarding study design see Fig 2 and Text A in [S1 Methods](#).

Genome-wide association study populations

UK Biobank. Summary genetic data for chronotype and sex hormones were obtained from GWAS conducted in the UK Biobank (UKB). UKB identified the chronotype of each participant with the question “do you consider yourself to be” and six possible options: definitely an ‘evening’ person; more an ‘evening’ than a ‘morning’ person; neither (do not know); more a ‘morning’ person than ‘evening’ person; definitely a ‘morning’ person and prefer not to answer. Participants who chose ‘prefer not to answer’ were excluded and the remaining categories were treated as a score to give per category outcomes from definitely an ‘evening’ person to definitely a ‘morning’ person. More details on the study, the genotyping procedure, and the data sources for chronotype and the sex hormones are described in [S1 Methods](#).

While GWAS summary statistics for both chronotype and sex hormones using data from UK Biobank currently exist [29, 35], we conducted our own GWAS for chronotype and sex hormones separately in men and women, given the sex-specific nature of the cancers we were investigating. For this, we used a linear mixed model (LMM) association method to account for relatedness and population stratification, as implemented in BOLT-LMM (v2.3) [36]. More details of this approach can be found in Text B in [S1 Methods](#).

Breast Cancer Association Consortium and Prostate Cancer Association Group. For breast and prostate cancer outcomes, genome-wide association studies (GWAS) summary data were obtained from the Breast Cancer Association Consortium (BCAC) [32, 37]; and the Elucidating Loci Involved in Prostate Cancer (ELLIPSE) and Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome (PRACTICAL) consortia [38, 39] (PRACTICAL/ELLIPSE). Sample sizes for these GWAS are shown in [Table 1](#).

The GWAS of overall breast cancer included 133,384 cases and 113,789 controls from 67 studies, using data from OncoArray and iCOGS arrays which were meta-analysed [37]. Summary statistics were also obtained on 106,491 cases on whom breast cancer subtype data was available: Luminal A (oestrogen receptor positive (ER+)/progesterone receptor positive (PR+), human epidermal growth factor receptor 2 Negative (HER2-)); Luminal B (ER+/PR+/-, HER2+); Luminal B HER2 negative (ER+/PR+/-, HER2-, Ki-67 > 14%); HER2 (ER-, PR-, HER2+); and Triple Negative (ER-, PR-, HER2-), as well as 94,407 controls [32]. All participants were females of European ancestry [32, 37]. Details of genotyping and imputation for BCAC can be found in Text C in [S1 Methods](#).

The GWAS for prostate cancer included data from the PRACTICAL (46,939 cases and 27,910 controls from 52 studies) and ELLIPSE (34,379 cases, 33,164 controls from 78 studies) consortia [38, 39]. The resulting overall meta-analysis included 79,148 cases and 61,106 disease free controls. All participants included in these analyses were males of European ancestry. Details of genotyping and imputation for ELLIPSE/PRACTICAL can be found in Text D in [S1 Methods](#).

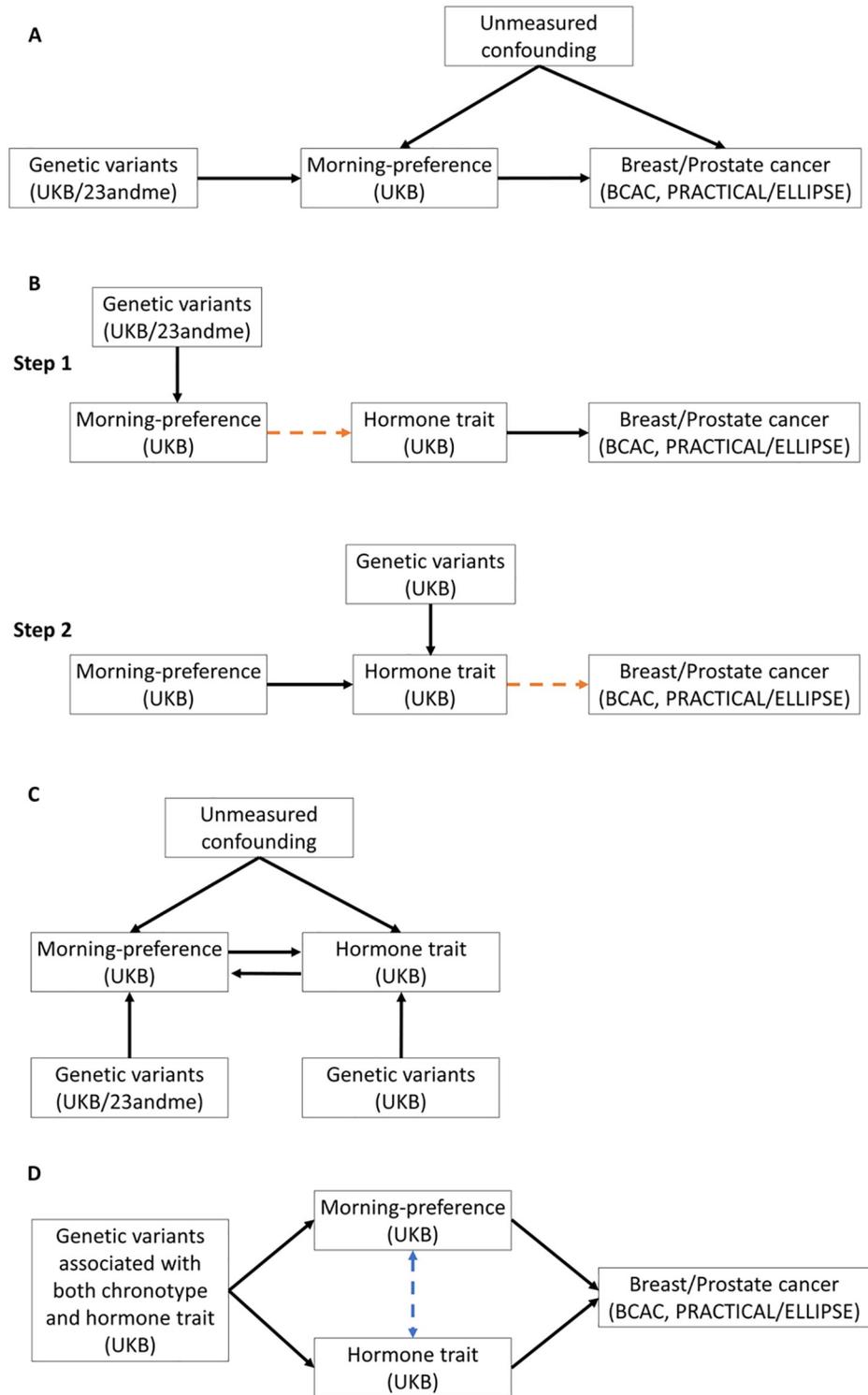


Fig 1. Schematics of MR analyses performed in this study. Participant datasets used to conduct GWAS' given in parenthesis. A) Two-sample MR to determine the effect of morning-preference on breast/prostate cancer. B) Two-sample MR to identify potential mediators. Step 1 determines causal relationships between morning-preference and hormone trait, depicted by the red dashed arrow. Step 2 determines causal relationships between the hormone trait and outcome, depicted by the red dashed arrow. C) Bidirectional MR, used to identify directionality of relationship between morning-preference and hormone traits. D) Multivariable MR to determine the direct effect of each exposure

on breast/prostate cancer risk. The blue dashed arrow in this schematic depicts adjustment of one exposure effect in the presence of another.

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Constructing genetic instruments

We next identified genetic instruments for chronotype and sex hormones, for which single nucleotide polymorphism (SNP)-exposure estimates were obtained from the sex-specific GWAS for chronotype and sex hormones in UK Biobank.

For chronotype, we identified two genetic instruments. The first comprised SNPs identified in the largest published GWAS of meta-analysed women and men combined from UKB and 23andme (N = 697,828) [35]. Of the 351 genetic variants that were genome-wide significant in that study, 341 SNPs were present in the imputed UKB-only GWAS. For these 341 SNPs we then extracted association summary results (SNP, effect allele, other allele, beta (increase in chronotype category per effect allele), SE and p-value) separately in females and males, regardless of their genome-wide significance (GWS). Those statistics were then used in our two-sample MR (irrespective of whether they reached genome-wide significance within the sex-specific GWAS). This approach maximises statistical power but might lack specificity for each sex. In the second approach we only used summary results (as above) for SNPs that reached GWAS significance ($p < 5 \times 10^{-8}$) in the sex specific GWAS within UKB. This will have less statistical

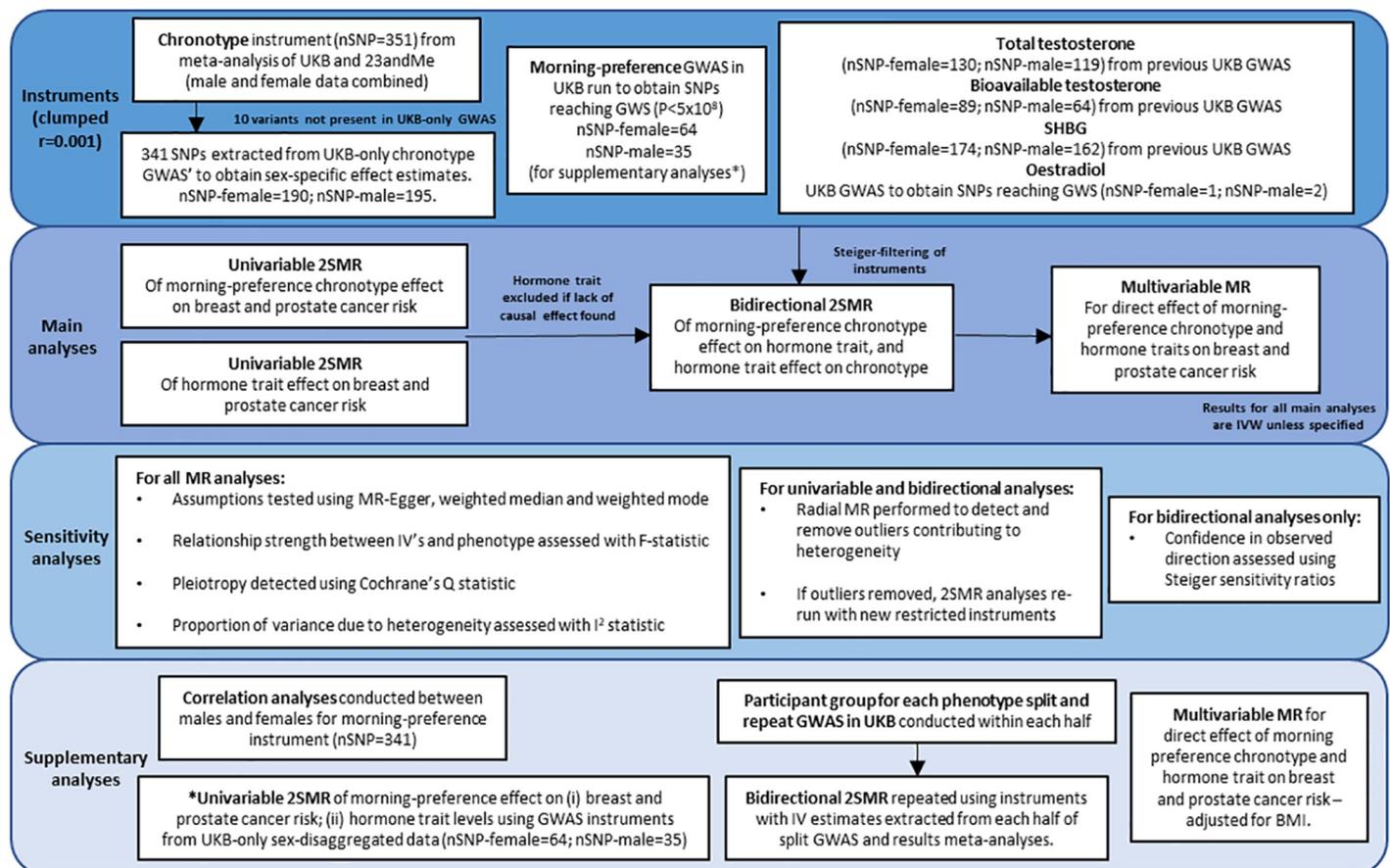


Fig 2. Overview of instruments and all main and supplementary analyses.

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Table 1. Sample sizes for exposure and outcome GWAS^s used in this study for male and female analyses. Number of SNPs (nSNP) given for clumped instruments from uvMR analyses.

Exposures	Female	Male	nSNP (F/M)	Study
Morning-preference	244,207	205,527	190 / 195	UKB
Total testosterone	199,569	200,159	130 / 119	
Bioavailable testosterone	180,386	184,205	89 / 64	
SHBG	214,989	185,221	174 / 162	
Oestradiol	53,391	17,134	1 / 2	
Outcomes	Cases	Controls		Study
Breast cancer (overall)	133,384	113,789	-	BCAC
Breast cancer (subtypes)	106,491	94,407	-	
Prostate cancer (overall)	79,148	61,106	-	ELLIPSE/PRACTICAL

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power and may be more prone to weak instrument bias but may be a more relevant sex-specific instrument. Following LD-clumping, to obtain independent SNP-associations for our genetic instrument (using a threshold of $R^2 = 0.001$), there were 190 and 195 SNPs for chronotype in females and males, respectively for the first approach and 54 and 32 SNPs, respectively in the second approach. Tables A and B in [S1 Data](#) provide details of the 341 SNPs identified in the published GWAS that were present in our UKB GWAS, the 190 and 195 used as genetic instruments in our first approach and the 54 and 32 used in our second approach.

Sex specific genetic instruments for total testosterone, bioavailable testosterone and SHBG were obtained from the previously published GWAS^s conducted in UKB [29]. The GWAS for total testosterone for women ($N = 230,454$) and men ($N = 194,453$) identified 254 and 231 SNP-associations respectively that were statistically independent, which LD-clumping then restricted to 130 and 119 SNPs, respectively. The GWAS for bioavailable testosterone for women ($N = 188,507$) and men ($N = 178,782$) identified 180 and 125 SNP-associations respectively that were statistically independent, which LD-clumping then restricted to 89 and 64 SNPs, respectively. The GWAS for SHBG for women ($N = 189,473$) and men ($N = 180,726$) identified 359 and 357 SNP-associations respectively that were statistically independent, which LD-clumping then restricted to 192 and 177 SNPs, respectively. Instruments for oestradiol were obtained from GWAS we undertook in UKB separately for females ($N = 53,391$) and males ($N = 17,134$), which identified 40 and 378 SNP-associations respectively that were GWS, resulting in 1 and 2 SNPs following LD-clumping for females and males, respectively. A high proportion of the oestradiol assay results had values below the limit of detection (80%) and were excluded from the GWAS [40].

The number of SNPs used to instrument chronotype and each of the sex hormone measures can be found in [Table 1](#). The summary statistics for all instruments used in this study are available in Tables A and B in [S1 Data](#).

Statistical analyses

Two-sample Mendelian randomization. For uvMR, bdMR and mvMR analysis, the TwoSampleMR package was used to combine and harmonize genetic summary data for chronotype, sex hormones and breast and prostate cancer. The inverse variance weighted (IVW) approach was used for the main analysis, whereby a causal effect is estimated from the slope of a regression line through the weighted SNP-mean exposure vs SNP-mean outcome associations (orientated to be positive) with the line constrained to have an intercept of zero. Since only 1 SNP contributed to the female oestradiol instrument, MR results were instead calculated using a Wald ratio [41].

Assessment of MR assumptions. MR analysis relies on three key assumptions: i) IVs must be robustly associated with the exposure of interest; ii) IVs must be independent of confounders of the exposure-outcome association; iii) IVs must only influence the outcome through the exposure of interest [26].

We conducted sensitivity analyses to test MR assumptions. Univariable and conditional F-statistics were calculated to assess the strength of the relationship between the IVs and phenotype, and thus to indicate potential bias in the MR estimate due to weak instruments [42–44]. To test the assumption of no unbalanced horizontal pleiotropy we conducted MR-Egger [45], weighted median [46] and weighted mode [47] MR (see Text E in *S1 Methods*). Scatter plots were used to visualise consistency between IVW, MR-Egger, median and mode effect estimates. To further test the assumption of no horizontal pleiotropy, we explored between SNP heterogeneity using Cochran's Q and leave one out analyses, as presence of heterogeneity may be due to pleiotropy of one or more SNPs [25, 48]. I^2 statistics were used to estimate the proportion of the variance between IV estimates that is due to heterogeneity [49]. Radial-MR was also conducted to identify IVs with the largest contribution to heterogeneity ($\alpha = 0.05/n\text{SNP}$) and thus identify potential outliers [50]. To assess their impact, these outliers were removed from the instrument and the data were then reanalysed. Radial-MR was also used to assess violation of both the independence and exclusion restriction assumptions. Steiger filtering was applied to instruments in the bdMR analysis of chronotype and sex hormones to reduce the likelihood of erroneous results due to pleiotropy (here where the SNP has a primary influence on the outcome rather than the exposure) [51]. Steiger sensitivity ratios (R) were calculated to ensure the likelihood that the observed direction of association was correct [52]. Since only 1 and 2 SNPs contributed to female and male oestradiol instruments, it was not possible to conduct any sensitivity analyses for oestradiol.

To accommodate additional two-sample MR assumptions, we restricted analysis to European-only individuals in all datasets and ensured sex-specific GWAS were used to meet the assumption that the exposure and outcome samples come from the same underlying population. We also performed harmonization of the direction of effects between the SNPs in the exposure and outcome GWAS. Palindromic SNPs were harmonized if they were aligned and the minor allele was <0.3 , otherwise they were excluded.

While sample overlap between UKB, BCAC and PRACTICAL/ELLIPSE is negligible, for the bdMR analysis, genetic estimates for both chronotype and sex hormones were determined in the same sample (UKB), violating the assumption of independent samples in two-sample MR. A supplementary analysis was carried out to investigate the magnitude of bias caused by overlapping samples [53]. To evaluate this, we performed split-sample MR analyses, where we randomly split UKB participants into two halves and performed additional GWAS for chronotype and the sex hormones in each of the subgroups. We used SNP-chronotype effect estimates obtained from the first subgroup (sample 1) and SNP-hormone effect estimates obtained from the second independent subgroup (sample 2), and performed IVW analysis. This method was repeated for the SNP-chronotype effect estimates obtained from sample 2 and SNP-hormone estimates from sample 1. The two MR estimates were then meta-analysed under a fixed-effects model to obtain an effect of chronotype on sex hormones. This approach was conducted separately for males and females.

Supplementary analyses. Supplementary analyses were conducted to evaluate the effects of chronotype and sex hormones on breast cancer stratified by subtype: Luminal A (ER+/PR+, HER2-); Luminal B (ER+/PR+/-, HER2+); Luminal B (ER+/PR+/-, HER2-, Ki 67 > 14%); HER2 (ER-, PR-, HER2+); and Triple Negative (ER-, PR-, HER2-). An I^2 statistic was calculated to assess variability in causal estimates between breast cancer subtypes [54].

In addition, evidence from previous research suggests that adiposity is intrinsically linked with certain sleep traits [55, 56], sex hormones [57–59], and both breast and prostate cancer

[60]. As such, supplementary mvMR analyses were conducted in which we adjusted the effect of chronotype on cancers for both body mass index (BMI) and sex hormone(s). For this, instrumental SNPs for BMI were obtained from a GIANT GWAS of males and females (N = 681,275) [61] and sex-specific association summary results for these SNPs (nSNP = 458) were obtained from female (N = 171,977) and male (N = 152,893) GIANT consortia GWAS' [62], which LD-clumping then restricted to 336 and 334 SNPs, respectively.

MR analyses used the R packages "Two SampleMR" [61] and "MVMR" [34], R version 4.0.2.

Results

Univariable MR: Effect of chronotype on breast and prostate cancer risk

The correlation (r) of the effect estimates for the 341 SNPs for chronotype in males and females was 0.60 (S1 Fig). The genetic variants (190 SNPs) contributing to the main chronotype instrument in females had a combined r^2 of 1.8% and an F-statistic of 22.75, and in males (190 SNPs) had an r^2 of 1.7% and an F-statistic of 17.18 (Table C in S1 Data). There was low to moderate heterogeneity between SNPs used within the instruments: the I^2 for MR of overall breast cancer was 41% (Qstat = 317; Qpval = 1.44×10^{-8}) and for overall prostate cancer was 26% (Qstat = 262; Qpval = 8.75×10^{-4}) (Table C in S1 Data). Weighted and unweighted I^2_{GX} [63] was also calculated for chronotype effect on both breast and prostate cancer and found to be between 50–72%. SIMEX corrections [64] were conducted to account for this and found to be consistent with MR Egger results reported in the main analyses (Table C in S1 Data).

We observed a reduction in risk of breast cancer per category increase in chronotype from extreme evening preference to extreme morning preference (OR = 0.93, 95% CI:0.88, 1.00) (Fig 3A). There was also evidence of a protective effect of morning preference on prostate cancer (OR = 0.90, 95% CI:0.83, 0.97) (Fig 3B). Results from MR-Egger, weighted median and

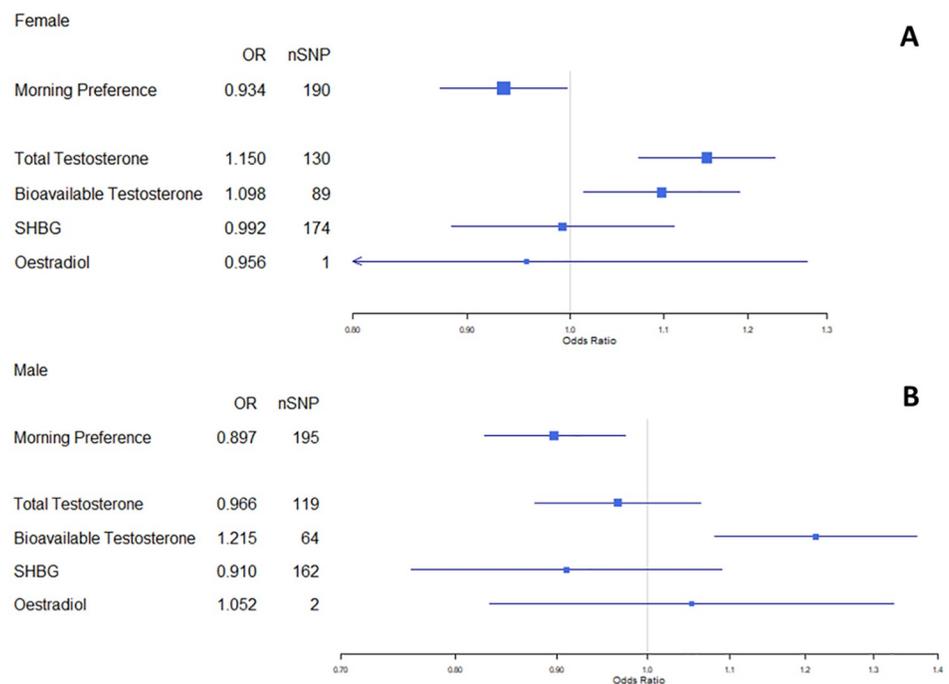


Fig 3. Forest plot of morning-preference and hormone trait effects on breast (3A) and prostate (3B) cancer risk. All estimates calculated IVW, except for oestradiol in females, which was calculated using Wald ratio analysis.

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weighted mode methods were similar to IVW estimates (Fig 3 and Table D in S1 Data). In Radial MR, three SNPs were identified as potential influencing outliers in the main IVW analyses of chronotype on breast cancer. When removed, the results were unchanged (OR = 0.94, 95% CI: 0.89, 1.00) (Table E in S1 Data). There were no outliers identified in the analysis of chronotype on prostate cancer.

The effect of chronotype on breast cancer was broadly similar between breast cancer subtypes, and heterogeneity for MR analyses between breast cancer subtypes was low ($I^2 = 26\%$) (S2 Fig).

Using UKB-only sex-specific instruments for chronotype, the results for chronotype on breast (OR = 0.92, 95% CI: 0.83, 1.01) and prostate (OR = 0.95, 95% CI: 0.81, 1.12) cancer were consistent with those in the main analysis, although less precisely estimated (Table F in S1 Data).

Univariable MR: Effect of sex hormones on breast and prostate cancer risk

The genetic variants contributing to the instruments for total testosterone, bioavailable testosterone, SHBG and oestradiol in females had a combined r^2 of 3.4%, 5.5%, 6.4% and 0.1%, respectively. F-statistics for these instruments are 44.5–83.6 (Table G in S1 Data). The genetic variants contributing to the instruments for total testosterone, bioavailable testosterone, SHBG and oestradiol in males had a combined r^2 of 4.8%, 2.5%, 10.9% and 0.4%, respectively. F-statistics for these instruments are 36.8–143.4 (Table G in S1 Data). I^2_{GX} was also calculated for sex hormone effect on both breast and prostate cancer and found to be between 45–98%. SIMEX corrections were conducted for SHBG effect on breast cancer which were found to be consistent with MR Egger results reported in the main analyses (Table G in S1 Data).

Amongst females, an increased risk of breast cancer was observed with increasing total (OR = 1.15, 95% CI: 1.07, 1.23 per SD) and bioavailable (OR = 1.10, 95% CI: 1.01, 1.19 per SD) testosterone, but not in relation to SHBG (OR = 0.99, 95% CI: 0.89, 1.11 per SD) (Fig 3A). Amongst males, an increased risk of prostate cancer was observed for bioavailable testosterone (OR = 1.22, 95% CI: 1.08, 1.37) but not in relation to total testosterone (OR = 0.97, 95% CI: 0.86, 1.07), or SHBG (OR = 0.91, 95% CI: 0.76, 1.09) (Fig 3B). The effect of oestradiol on both breast (OR = 0.96, 95% CI: 0.72, 1.27) and prostate (OR = 1.05, 95% CI: 0.83, 1.33) cancers was imprecisely estimated due to insufficient SNPs, and therefore oestradiol has been excluded from subsequent bdMR and mvMR analyses. Given the lack of evidence for a causal effect on breast or prostate cancer risk, SHBG has been excluded from all further analyses, and total testosterone has been excluded from analyses of prostate cancer.

There was moderate to high heterogeneity between SNPs used within the sex hormone instruments (excluding oestradiol): the I^2 for MR of sex hormones on overall breast cancer was 62–69% and for overall prostate cancer was 58–74% (Table G in S1 Data). However, results from MR-Egger, weighted median and weighted mode methods were similar to IVW estimates (Fig 3 and Table H in S1 Data). In Radial MR there were five, three and three SNPs identified as potential influential outliers in the main IVW analyses of total testosterone, bioavailable testosterone and SHBG on breast cancer, respectively. There were also eight, four and eight SNPs identified in the main IVW analyses of total testosterone, bioavailable testosterone and SHBG on prostate cancer, respectively. In all radial MR analyses, when outliers were removed the results were unchanged (Table I in S1 Data).

For MR of sex hormones stratified by breast cancer subtype (S3 Fig and Table J in S1 Data), the increased risk reported for total and bioavailable testosterone on overall breast cancer was consistent with all luminal-type breast cancers. However, for total testosterone on triple negative breast cancer, estimates were consistent with a reduced risk (OR = 0.91, 95% CI: 0.81, 1.03) while

there was little evidence for an effect on HER2-Enriched breast cancer (OR = 0.98, 95% CI:0.81, 1.18). Findings were similar with respect to bioavailable testosterone and breast cancer subtypes. Although little evidence was found for an effect of SHBG on overall breast cancer, subtype-specific analyses found reduced risk of luminal-A (OR = 0.90, 95% CI:0.78, 1.04) and luminal-B HER2-negative (OR = 0.90, 95% CI:0.74, 1.11) breast cancers, and increased risk of luminal-B (OR = 1.09, 95% CI:0.88, 1.35), HER2-Enriched (OR = 1.28, 95% CI:0.95, 1.71) and triple negative (OR = 1.19, 95% CI:0.99, 1.44) breast cancers, but these were imprecisely estimated.

Bidirectional MR: Reciprocal effects between chronotype and sex hormones

The exposures used in the bdMR analyses underwent Steiger filtering. The removal of SNPs more strongly associated with the outcome were removed, resulting in greater confidence in the direction of association as determined by corresponding sensitivity ratios (Table K in S1 Data). In females, the variance (r^2) explained by the Steiger-filtered chronotype instrument was 1.9% compared to 3.9%, and 3.8% for total testosterone and bioavailable testosterone respectively (Table K in S1 Data). This is comparable to the variance explained in males, where r^2 for chronotype was 1.7%, compared with 3.0% for bioavailable testosterone (Table K in S1 Data). I^2_{GX} was also calculated for all bdMR analyses and found to be between 34–96%. SIMEX corrections were conducted for all analyses with $I^2_{GX} < 90\%$ which were found to be consistent with MR Egger results reported in the main analyses (Table K in S1 Data).

Amongst females (Fig 4A), bidirectional relationships were observed for chronotype and the testosterone measures. Morning preference was found to lower total testosterone (mean SD difference = -0.07, 95% CI:-0.10, -0.03 per category increase), and total testosterone in turn was inversely related to morning preference (difference in category = -0.06, 95% CI:-0.10, -0.03 per SD increase). Similarly, morning preference was found to lower bioavailable testosterone (mean SD difference = -0.08, 95% CI:-0.12, -0.05 per category increase), and bioavailable testosterone was inversely related to morning preference (difference in category = -0.04, 95% CI: -0.08, 0.00 per SD increase).

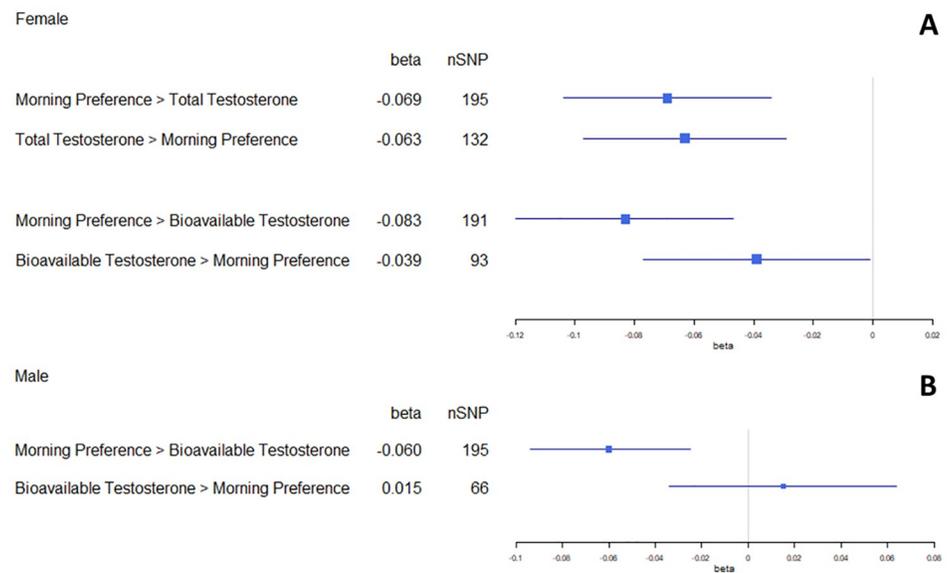


Fig 4. Forest plot of bdMR results for morning-preference and hormone traits in female (4A) and male (4B) datasets. All instruments in these analyses have been subjected to Steiger-filtering.

<https://doi.org/10.1371/journal.pgen.1009887.g004>

Amongst males (Fig 4B), morning preference was found to lower bioavailable testosterone (mean SD difference = -0.06, 95% CI:-0.09, -0.03 per category increase), but there was lack of corresponding evidence for an effect in the reverse direction (difference in mean category = 0.02, 95% CI:-0.03, -0.06 per SD increase).

Overall, the bdMR analyses suggest that the relationship between chronotype and testosterone may be bidirectional for females, but with a prevailing effect from chronotype to testosterone in males (Fig 4). Radial MR was performed for main analyses and bdMR was then repeated. The results of these analyses were consistent with the main results (Table L in S1 Data). Bidirectional MR was also repeated using split-sample MR analysis and the results were largely consistent with those reported in the main analyses (S4 Fig).

Multivariable MR: Direct effects of chronotype and sex hormones on cancer risk

Conditional F-statistics were calculated for all main mvMR analyses and varied from 7.1 to 18.4 (Table M in S1 Data). The MVMR package function `qhet_mvmmr()` was used to test for weak instruments and horizontal pleiotropy, the results of which were consistent with those found in the main mvMR analyses (Table N in S1 Data).

In females (Fig 5A), the reduced risk of breast cancer per category increase in chronotype observed with uvMR (OR = 0.93, 95% CI: 0.88, 1.00) was largely unchanged when accounting

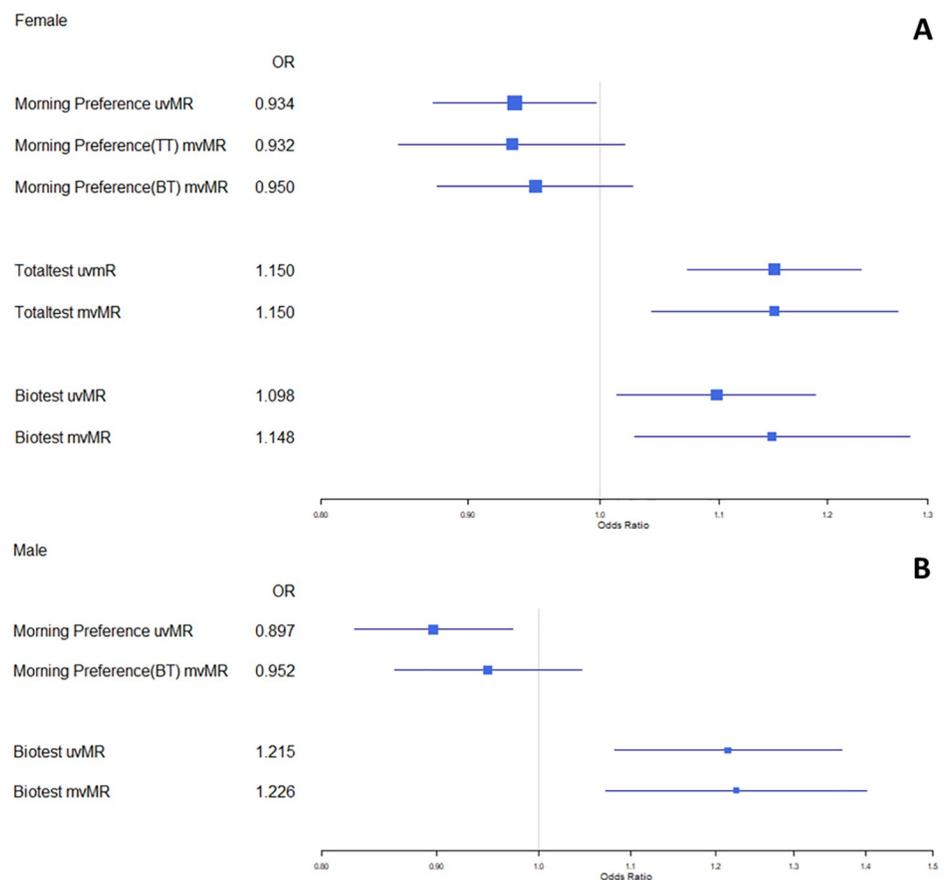


Fig 5. Forest plot of mvMR results for morning-preference and hormone traits in female (5A) and male (5B) datasets. uvMR results provided for comparison.

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for total testosterone (OR = 0.93, 95% CI: 0.85–1.02) or bioavailable testosterone (OR = 0.95, 95% CI: 0.88, 1.03). When accounting for chronotype, the increased risk of breast cancer observed per SD increase in total testosterone was consistent between uvMR (OR = 1.15, 95% CI: 1.07, 1.23) and mvMR (OR = 1.15, 95% CI: 1.04, 1.27) results. The increased risk of breast cancer observed per SD increase in bioavailable testosterone was also broadly consistent between uvMR (OR = 1.10, 95% CI: 1.01, 1.19) and mvMR (OR = 1.15, 95% CI: 1.03–1.28).

In males (Fig 5B), the reduced risk of prostate cancer per category increase in chronotype observed with uvMR (OR = 0.90, 95% CI: 0.83, 0.97) was broadly consistent when accounting for bioavailable testosterone (OR = 0.95, 95% CI: 0.86, 1.05). Bioavailable testosterone also remained largely consistent between uvMR (OR = 1.22, 95% CI: 1.08, 1.37) and mvMR results (OR = 1.23, 95% CI: 1.08, 1.40) when accounting for chronotype.

Supplementary mvMR analyses were conducted to adjust for the effects of BMI (Table O in S1 Data). In females, (S5 Fig) the reduced risk of breast cancer per category increase in chronotype observed for uvMR was directionally consistent when adjusting for bioavailable testosterone and BMI (OR = 0.96, 95% CI: 0.79, 1.17), and for total testosterone and BMI (OR = 0.91, 95% CI: 0.73, 1.14). However, in all cases the results were imprecisely estimated. In males (S6 Fig), the direct effects obtained for chronotype and bioavailable testosterone were largely consistent with those found for uvMR.

Discussion

This study has assessed the evidence for the role of sex hormones in mediating or confounding the effect of chronotype on risk of breast and prostate cancer using a series of MR analyses. Overall, we found evidence for a protective effect of genetically predicted tendency towards morning preference on both breast and prostate cancer risk. There was evidence that morning preference reduces both total and bioavailable testosterone levels in males and females. We also found evidence to support higher testosterone increasing the risk of both breast and prostate cancer risk. The effect of testosterone on prostate cancer was more prominent when assessing bioavailable rather than total testosterone. While findings from univariable and bidirectional MR analyses indicated that testosterone may lie on the causal pathway between chronotype and cancer risk, there was evidence for a bidirectional association between chronotype and testosterone in females, suggesting that these sex hormones may have the capacity to both confound and mediate the chronotype effect on breast cancer risk. However, the effects of chronotype remained largely unchanged when accounting for testosterone in multivariable MR suggesting that any mediating (or confounding) effect is likely to be minimal. The effect of oestradiol on breast and prostate cancer was very imprecisely estimated and may have been influenced by weak instrument bias as we only had one SNP to instrument for oestradiol in females, and two SNPs in males. Having so few SNPs meant that we could not explore potential bias due to unbalanced horizontal pleiotropy and therefore we did not explore this sex hormone further.

The effect of morning-preference on breast cancer risk is consistent, although more conservative, than our previously reported two-sample MR findings (current OR = 0.93, 95% CI: 0.88, 1.00 vs previous OR 0.88, 95% CI: 0.82, 0.93 per category increase in chronotype) [10]. This may be attributed to differences in the genetic instruments used in the two analyses, with a more stringent threshold for obtaining independent SNP-associations in the current analysis ($LD r^2 = 0.001$ vs 0.01) and the larger sample size of the breast cancer GWAS [32] ($n = 247,173$ vs $n = 228,951$). The chronotype effect on prostate cancer risk (OR = 0.90, 95% CI: 0.83, 0.97) was more pronounced in the current study than previously reported results obtained from observational multivariable logistic regression analyses, where there was a lack of association

between morning and no preference (OR = 0.98, 95% CI:0.79, 1.21) and a slightly higher risk for evening vs no preference (OR = 1.10, 95% CI:0.82, 1.46), although confidence intervals are overlapping [11]. Our results were also consistent with the recent MR study of morning preference effect on prostate cancer risk (OR = 0.71; 95% CI: 0.54, 0.94), although morning preference was scaled differently so effect estimates are not directly comparable [12].

Our findings of increased testosterone increasing breast and prostate cancer risk are comparable to a previously published study [29] which also utilised MR to estimate effects of total (OR = 1.14, 95% CI:1.08, 1.20) and bioavailable testosterone (OR = 1.15, 95% CI: 1.05, 1.25) on breast cancer, and an effect of bioavailable testosterone on prostate cancer risk (OR = 1.23, 95% CI: 1.13, 1.33), consistent with those reported in this study. Similarly, our findings that increasing SHBG reduces both breast and prostate cancer were consistent with previously reported MR results [28, 29].

That morning-preference chronotype was associated with lower testosterone levels in males is in-keeping with previous cross-sectional studies [20, 21]. However, the finding that morning preference chronotype lowers testosterone levels in females is in contrast to previous null findings [21]. This is likely due to the very small number of female participants (N = 47) as many were excluded for testosterone levels below the limit of sensitivity. Furthermore, both prior studies inferred from association analyses that chronotype is dependent on testosterone levels, whereas bidirectional MR analyses in the currently study enabled us to orient the causal direction of this association, suggesting that testosterone levels are dependent on chronotype in males, and that there is a bidirectional relationship between chronotype with testosterone in females. A bidirectional relationship between these traits in females indicates that these hormones may serve as both confounders and mediators of the chronotype effect on breast cancer. However, results of the mvMR analyses did not support evidence for total testosterone or bioavailable testosterone acting as a mediator in males or females, since the effect of chronotype on breast and prostate cancer was found to be largely independent of these sex hormones, and vice versa.

In line with the findings regarding the role of chronotype in breast cancer, network-based GWAS studies conducted to identify genetic determinants of breast cancer survival have reported circadian enrichment [65], but only in oestrogen-receptor (ER) negative breast cancers. However, in our previous study, the effect of chronotype on breast cancer risk (OR = 0.88, 95% CI:0.82, 0.93) was largely unchanged when stratified into ER-positive (OR = 0.86, 95% CI:0.80, 0.92) or ER-negative breast cancer (OR = 0.88, 95% CI:0.80, 0.97) [10]. We have extended this to also investigate the effect of chronotype on five subtypes of breast cancer: three luminal-type cancers which are ER-positive, and HER2-enriched and triple negative cancers which are ER-negative. While these results are largely consistent with our findings for a chronotype effect on all breast cancer subtypes (S2 Fig), there was some indication for an increased risk of luminal B HER2-negative breast cancer in relation to morning preference, although this was imprecisely estimated.

The effect of both testosterone and SHBG on ER-stratified breast cancer has been previously studied [29], in which both total (OR = 1.19, 95% CI:1.12, 1.26) and bioavailable testosterone (OR = 1.23, 95% CI:1.12, 1.34) were associated with increased risk of ER-positive breast cancers. This is consistent with our subtype-specific analyses for luminal-type breast cancers (S3A and S3B Fig). In the previous study, there was limited evidence for an association for either total (OR = 0.99, 95% CI:0.93, 1.06) or bioavailable testosterone (OR = 0.94, 95% CI:0.83, 1.07) on ER-negative breast cancer. While this is consistent with our finding for total testosterone and HER2-enriched breast cancer risk (OR = 0.98, 95% CI:0.81, 1.18), there was an indication that bioavailable testosterone reduced risk of HER2-enriched breast cancer (OR = 0.86, 95% CI:0.69, 1.07) although this was imprecisely estimated. Similarly, a reduction

in risk of triple negative breast cancer was found for both total (OR = 0.91, 95% CI:0.81, 1.03) and bioavailable testosterone (OR = 0.89, 95% CI:0.77, 1.04). The previous study also reported reduced risk of ER-positive (OR = 0.91, 95% CI: 0.82, 1.01) and increased risk of ER-negative (OR = 1.14, 95% CI: 1.01, 1.28) breast cancer per SD increase in SHBG. These are consistent with findings from our present study, except for luminal-B HER2-negative breast cancer (ER-positive), for which we found increased risk per SD increase in SHBG, although imprecisely estimated (S3C Fig). The modal age group for all breast cancer subtypes was >60 (Table P in S1 Data), suggesting that the subtype effects observed are unlikely to be related to age at diagnosis.

Strengths & limitations

A key strength of this study is the use of MR to appraise the causal effect of chronotype on both sex and prostate cancer, as well as additional MR analyses to assess the role of sex hormones underlying these effects. Furthermore, the sensitivity analyses conducted in tandem to the main analyses served to test the assumptions of MR and provide evidence for the robustness of the results. The genetic summary data used for both breast and prostate cancer in this study were obtained from the largest available GWAS available to date and for breast cancer enabled the stratification of breast cancer outcomes into five distinct subtypes, thus improving the clinical relevance of these findings.

While we were careful to use genetic variants from sex-specific GWAS when evaluating the sex-hormones in relation to breast and prostate cancer, the SNPs used to instrument chronotype in the main analyses were extracted from a sex-combined GWAS including data from UKB and 23andMe [35]. We used male and female effect estimates for the SNPs derived from UK Biobank in the MR analyses, although the correlation in effect estimates of the SNPs between males and females was only moderate (0.60), suggesting some heterogeneity in the genetic contribution to chronotype between sexes. Given this, we also derived sex-specific instruments and compared results of MR using the combined- and sex-specific chronotype instruments, with the effect estimates obtained being largely consistent with respect to both breast and prostate cancer.

While we conducted a series of sensitivity analyses to assess MR and two-sample MR assumptions, we did not directly test the assumption of independence (IVs must be independent of confounders of the exposure-outcome association). However, germline genetic variants used in MR should not plausibly be influenced by confounding factors, and as such, concerns about violation of the second MR assumption usually focus on confounding by population stratification or as a result of selection bias [66]. Steps were taken to minimise population stratification in the GWAS performed although this assumption is difficult to test, particularly in a two-sample setting. Other assumptions for causal effect estimation also exist, such as the causal estimates being homogeneous in the population [67], which we were also not able to explicitly assess.

In terms of two-sample MR specific assumptions, the use of overlapping sample populations between exposures and outcomes may also introduce bias [53]. For analyses where exposures were generated from UKB data and outcomes from BCAC or ELLIPSE/PRACTICAL there was no known overlap between participants. However, for bidirectional MR analyses, both exposure and outcomes were from UKB and as such had a 100% overlap. This has been addressed with the use of split-sample MR analysis. Comparison between whole- and split-sample analyses served as an indicator of the extent to which the main analyses are influenced by this bias. Overall, the results were consistent between these analyses, suggesting that the influence of bias from using overlapping datasets is minimal. Data from UKB may also be

influenced by selection bias, however this is a more general issue underlying all UKB data due to low participation rates (5.5% of those invited). Specific to our analyses, recruitment clinics had appointments from early morning to the evening, meaning that selection on chronotype may be minimal and to our knowledge circulating levels of sex hormones are unlikely to be importantly influenced by socioeconomic position (participants recruited to UK Biobank tended to be from more affluent areas and more educated than those who were not) and related characteristics. Thus, our results may not be importantly biased by selection into UKB.

Another potential concern is the overlap in the UKB sample used for both conducting the discovery GWAS for chronotype and sex hormones and for obtaining the effect estimates for the genetic instruments in MR analysis, given the potential for Winner's curse bias. For chronotype, this was addressed previously [10] whereby sensitivity analyses using only the SNPs that replicated in 23andMe were found to give similar results. However, in the absence of a large independent genetic study with hormone measures, this was not possible for the hormone instruments used in this study.

Further work

The analyses presented here demonstrate inconsistent evidence for the role of testosterone as a mediator for the effect of chronotype on cancer, therefore further investigation is required. For example, to improve the robustness of the findings in this study, it would be interesting to investigate the effect of morning-preference on breast cancer subtypes using more objective measures of chronotype, such as L5 timing [68]. We believe further work investigating testosterone in relation to other sleep traits (e.g. sleep duration and insomnia) would also be valuable in understanding the nuanced relationship between sleep and cancer risk. Due to the lack of SNPs reaching genome-wide significance, we were unable to sufficiently explore the mediating effect of oestradiol in this study. As such, should a large-scale genetic study of oestradiol data become available, further investigation is warranted. While our study focused on the putative role of sex hormones in the link between chronotype and cancer risk, other hormones such as cortisol and melatonin, have been strongly linked with circadian traits and may lay on the causal pathway to breast cancer. MR analysis using genetic instruments for these hormones is therefore required.

Conclusion

In conclusion, this study has extended previous findings regarding the protective effect of chronotype on breast cancer and has found evidence to support recent findings that morning-preference also reduces prostate cancer risk in men. While testosterone levels were found to be closely linked with both chronotype and cancer risk, there was inconsistent evidence for the role of testosterone in mediating the effect of morning preference chronotype on both breast and prostate cancer. Findings regarding the potential protective effect of chronotype on both breast and prostate cancer risk are clinically interesting although this may not serve as a direct target for intervention, since it is difficult to modify someone's morning/evening preference. However, a personalised breast cancer screening that considers chronotype as a causal risk factor may help to improve prevention in the future. Given this, further studies are needed to investigate the mechanisms underlying this effect and to identify potential modifiable intermediates.

Supporting information

S1 Methods. Text A: Study design. Text B: Genotyping, Imputation and association analysis (UKB). Text C: Genotyping and Imputation (BCAC). Text D: Genotyping and Imputation

(ELLIPSE/PRACTICAL). Text E: Assessment of MR Assumptions (PDF)

S1 Data. Table A: Table of Female exposures used in main MR analyses. Table B: Table of Male exposures used in main MR analyses. Table C: Table of heterogeneity and instrument strength for morning-preference chronotype instruments. Table D: Table of results for morning-preference chronotype effect on breast and prostate cancer. Table E: Table of results for Radial MR analyses for morning-preference chronotype on breast cancer risk. Table F: Table of results for morning-preference chronotype effect on breast and prostate cancer using sex-specific instruments. Table G: Table of heterogeneity and instrument strength for hormone trait instruments. Table H: Table of results for hormone trait effect on breast and prostate cancer. Table I: Table of results for Radial MR analyses for hormone trait effect on breast and prostate cancer risk. Table J: Table of results for hormone trait effect on breast cancer subtypes. Table K: Sensitivity tests for bidirectional MR analyses. Table L: Table of results for Radial MR analyses for morning-preference chronotype and hormone trait bidirectional MR. Table M: Table of results for mvMR analyses. Table N: Table of results for qhet_mvMR analyses. Table O: Table of results for trivariate mvMR including BMI. Table P: Table of results for age at diagnosis for each of the breast cancer subtypes. (XLSX)

S1 Fig. Scatter plot of genetic correlation between male and female effect estimates for morning preference chronotype instrument. (TIF)

S2 Fig. Heterogeneity analysis for MR of morning-preference chronotype on breast cancer subtypes. Study numbers = 1. Luminal A (ER+/PR+, HER2-); 2. Luminal B (ER+/PR+/-, HER2+); 3. Luminal B (ER+/PR+/-, HER2-, Ki 67 > 14%); 4. HER2 (ER-, PR-, HER2+); and 5. Triple Negative (ER-, PR-, HER2-). (TIF)

S3 Fig. Forest plot of uvMR results for A) total testosterone, B) bioavailable testosterone and C) SHBG effects on breast cancer subtypes. (TIF)

S4 Fig. Forest plot of split sample bdMR results for morning preference and sex hormones in female (A) and male (B) datasets. All instruments in these analyses have been subjected to Steiger-filtering. (TIF)

S5 Fig. Forest plot of trivariate (adjusted for morning preference/sex hormone and BMI) mvMR results in females. uvMR results provided for comparison. (TIF)

S6 Fig. Forest plot of trivariate (adjusted for morning preference/sex hormone and BMI) mvMR results in males. uvMR results provided for comparison. (TIF)

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