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Genetically predicted testosterone and cardiovascular risk factors in men: a Mendelian randomization analysis in the Guangzhou Biobank Cohort Study

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Background Observationally lower testosterone is associated with an unhealthier cardiovascular (CVD) risk profile, but this association is open to confounding and reverse causality. The authors examined the association of testosterone with well-established cardiovascular disease (CVD) risk factors (blood pressure, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL)cholesterol and fasting glucose) and the Framingham score using a Mendelian randomization analysis with a separate-sample instrumental variable estimator.

Methods To minimize reverse causality, a genetic score predicting testosterone was developed in 289 young Chinese men from Hong Kong, based on three selected testosterone-related single nucleotide polymorphisms (rs10046, rs1008805 and rs1256031). Multivariable censored and linear regressions were used to examine the association of genetically predicted testosterone levels with CVD risk factors and Framingham score among 4212 older Chinese men from the Guangzhou Biobank Cohort Study.

Results Predicted testosterone was unrelated to systolic blood pressure [−0.11 mmHg, 95% confidence interval (CI) −0.70 to 0.48], diastolic blood pressure (0.04 mmHg, 95% CI −0.27 to 0.36), fasting glucose (0.02 mmol/l, 95% CI −0.02 to 0.06) or Framingham score (0.02, 95% CI −0.0001 to 0.03) but associated with higher LDL-cholesterol (0.02 mmol/l, 95% CI 0.01 to 0.04) and lower HDL-cholesterol (−0.01 mmol/l, 95% CI −0.02 to −0.001), after adjustment for potential confounders (age, education, smoking status, use of alcohol and body mass index).

Conclusions Our findings did not corroborate observed protective effects of testosterone on cardiovascular risk factors or risk of ischaemic heart disease among men, but raises the possibility that higher testosterone may be associated with an unhealthier lipid profile.

Keywords Mendelian randomization, testosterone, cardiovascular disease, risk factors, cholesterol

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity globally.[1] Observationally, serum testosterone is associated with healthier cardiovascular risk factors such as lower blood pressure,[2,3] lower low-density lipoprotein (LDL) cholesterol,[4] higher high-density lipoprotein (HDL) cholesterol[2,5] and lower fasting glucose.[6,7] Testosterone therapy is increasingly used in men aged 40+ years,[8] not only in those with hypogonadism.[9] Observational studies are open to residual confounding and reverse causality.[10] The climate of opinion is also being set by pharmaceutical companies[11] 'promoting low T'.[12] No large-scale randomized controlled trial (RCT) has assessed the long-term effect of testosterone on CVD events or mortality. The Institute of Medicine concluded in 2004 that 'there is not clear evidence of benefit for any of the health outcomes examined' from testosterone.[13] Moreover, anti-androgens treat prostate cancer, so such trials could increase prostate cancer. Meta-analyses of small RCTs among men suggest testosterone therapy increases cardiovascular-related events[14] and decreases HDL-cholesterol but has little effect on blood pressure.[15] One RCT suggests testosterone therapy improves glucose metabolism.[16]

Differences between observational and experimental evidence may reflect differences in the quality of evidence from different sources, or aetiologically important differences in the effects of exogenous and endogenous androgens. Using naturally occurring testosterone-related genetic variants in a Mendelian randomization (MR) study design provides a means of examining the causal effects of endogenous testosterone, without any potentially harmful interventions. Genetic variants determined at conception, resulting in life-long differences in endogenous exposures, are unlikely to be associated with socioeconomic position or lifestyle. To date, one MR study[10] has examined the association of testosterone with CVD risk factors and suggested no protective effects of endogenous testosterone. The study was limited by small sample size and potentially reverse causality,[17] because testosterone falls with age and ill health,[18,19] perhaps due to accumulated age-related comorbidities rather than androgen deficiency.[20] Thus genetically predicted testosterone in older men may not be a reliable indicator of lifetime exposure when CVD has a long preclinical course. Here, we examined the association of testosterone with well-established CVD risk factors and the Framingham score, using an MR analysis with a separate-sample instrumental variable (SSIV) estimator based on the association of genetic variants with testosterone in young men, when testosterone levels are at their lifetime peak, to minimize reverse causality.

Methods

Study design

A separate-sample two-stage MR analysis was used. First, a genetic score predicting serum testosterone was developed in young Chinese men from Hong Kong. Second, the association of predicted testosterone with CVD risk factors and Framingham score was examined in older Chinese men from the Guangzhou Biobank Cohort Study (see flow chart in Figure 1).

Sources of data

Two groups of men of different ages were recruited from the same genetic background, i.e. from Hong Kong and Guangzhou (the capital of Guangdong province) in China. Most residents of Hong Kong are first-, second- or third-generation migrants from Guangdong.[21] In the first stage, male students were recruited from the University of Hong Kong, restricted to those with parents and at least three grandparents born in Hong Kong or Guangdong and not taking hormone-related medication. Morning blood samples were collected for testosterone assessment and DNA extraction. Testosterone was assessed by radioimmunoassay (Roche Diagnostics GmbH, Mannheim, Germany). Self-administered questionnaires were used to collect other information, such as socioeconomic position and health status. The University of Hong Kong-Hospital Authority Hong Kong West Cluster Joint Institutional Review Board approved the study and all participants gave written, informed consent prior to participation.

In the second stage, we used a large sample of older men (50+ years) from the Guangzhou Biobank Cohort Study (GBCS). GBCS is an ongoing collaboration of Guangzhou Number 12 Hospital and the Universities of Hong Kong and Birmingham, UK.[22] Recruitment of participants was in three phases. All participants were permanent residents of Guangzhou and members of the Guangzhou Health and Happiness Association for the Respectable Elders (GHHARE), a community social and welfare association unofficially aligned with the municipal government. Membership is open to older people for a monthly fee of 4 Yuan (50 US cents). About 7% of permanent Guangzhou residents aged 50+ years are members of GHHARE, of whom 11% (about 10 000 participants) enrolled for each of phases 1, 2 and 3. Inclusion criteria were that they were capable of

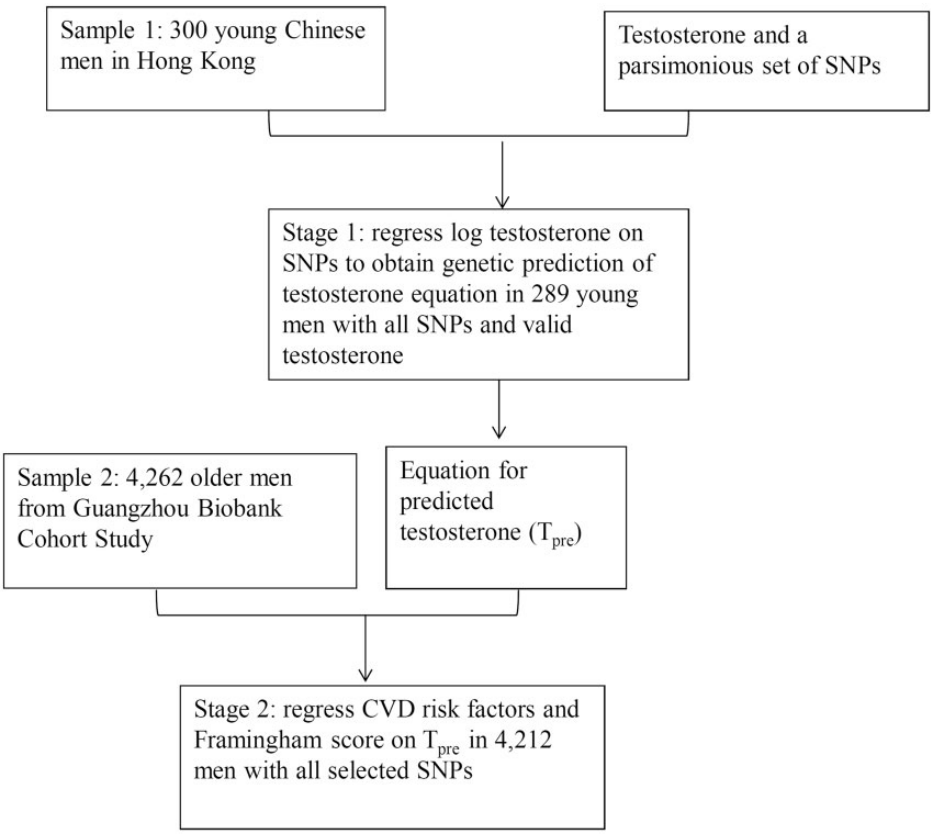

Figure 1 Flow chart of the study. A separate-sample two-stage Mendelian randomization design is used in this study. See details in study design

consenting, ambulatory and not receiving treatment which if omitted might result in immediate life-threatening risk, such as chemotherapy or radiotherapy for cancer, or dialysis for renal failure. Fasting blood samples were collected at recruitment in phase 3 and at follow-up for participants recruited in other phases. Samples were stored as whole blood or as buffy coat and sera, at -80°C[22] for all apart from a subset of phase 3 participants whose DNA was extracted from fresh blood and stored at -80°C.[23] The Guangzhou Medical Ethics Committee of the Chinese Medical Association approved the study and all participants gave written, informed consent prior to participation.

DNA extraction and analysis of single nucleotide polymorphismss

DNA was extracted using standard procedures [QIAamp DNA Blood Midi Kit (Catalog No. 51185)] for fresh blood in Hong Kong, phenol-chloroform extraction for fresh blood in GBCS and magnetic bead extraction for previously stored specimens in GBCS.[23] Candidate **single nucleotide polymorphisms** (SNPs) were selected from genes (*ESR1, ESR2* and *CYP19A1*) functionally relevant to androgens or prostate cancer,[24–27] with minor allele frequency >5% in Chinese people.[28] SNPs from *ESR1* (rs722208 and rs2175898), *CYP19A1* (rs10046 and rs1008805) and *ESR2* (rs1256030 and rs1256031) were analysed at the Centre for Genomic Sciences of the University of Hong Kong, for the Hong Kong sample, and a commercial company (Beijing Capital Bio Corporation) in Beijing, for the GBCS sample, using a Mass ARRAY system (Sequenom, San Diego, CA). For DNA quality analysis we used spectrophotometry for most of the samples and gel electrophoresis for four duplicate check controls and six randomly selected samples in each DNA sample plate. The determined sample concentration and A260/280 ratios were between 10–20 ng/μl and 1.7–2.0 ng/μl, respectively. A call rate <80% was considered failure. All the SNPs passed with a call rate >95%.

Exposure

The primary exposure was predicted testosterone estimated as the anti-log of predicted log testosterone from the genetic score established in stage 1. Testosterone, instead of log testosterone, was used as the exposure for ease of interpretation as it gave the same pattern of results as log testosterone.

Outcomes

The primary outcomes were CVD risk factors (blood pressure, LDL-cholesterol, HDL-cholesterol and fasting glucose) and the Framingham score.[29] The Framingham score overestimates absolute risk of CVD in Chinese populations[30] but provides a risk ranking. The Framingham score was calculated from age, LDL-cholesterol, HDL-cholesterol, systolic blood pressure (SBP), diastolic blood pressure (DBP), diabetes (fasting plasma glucose $\geqslant 7.0$ mmol/l, previous diagnosis or use of anti-diabetic medication) but excluded smoking to assess CVD risks reflected by biological factors which provide more mechanistic information and are assessed more precisely.

Statistical analysis

We used an SSIV estimate from two separate samples[31] (Figure 1). In stage 1, Hardy–Weinberg equilibrium was tested for each SNP with an exact test on a contingency table of observed-vs-expected frequencies. SNPs which deviated from Hardy–Weinberg equilibrium were deleted. Linkage disequilibrium among SNPs in each gene was tested with Haploview 4.2. SNPs with D′ close to 1.0 and r^2 greater than 0.7 were considered linked. Stepwise linear regression was used to find a parsimonious set of SNPs which best predicted log testosterone, because the distribution of testosterone was skewed. Replication in 1000 bootstrapping samples was used for internal validation. The significance level was set at 0.20 to ensure that the initial inclusion criterion was not too restrictive. The F-statistic for the regression of testosterone on genetic score was obtained; F-statistic >10 suggests a reliable genetic instrument.[32]

In stage 2, selected SNPs from stage 1 were assessed in GBCS. Hardy–Weinberg equilibrium was tested as above. Estimated testosterone was calculated according to the equation from stage 1. Analysis of variance (ANOVA) was used to compare estimated testosterone by key characteristics. Multivariable linear regression was used to assess the adjusted association of estimated testosterone with the Framingham score. Censored linear regression was used for SBP, DBP, LDL-cholesterol, HDL-cholesterol and fasting glucose, because some participants were taking medication to reduce blood pressure, improve lipid profile or reduce fasting glucose. Estimated testosterone should not be confounded, but age (considered as continuous), education, smoking status, use of alcohol and body mass index (BMI) were included to achieve more precise estimates.[33] Model 1 adjusted for age. Model 2 additionally adjusted for education, smoking status and use of alcohol. Model 3 additionally adjusted for BMI, because how obesity affects testosterone is unclear. Model 4 used bootstrapping with 1000 replications for internal validation. All statistical analyses were conducted using Stata 10.1 (StataCorp LP, College Station, TX).

Results

Genetic prediction of testosterone in young Chinese men

Of the 300 young Chinese men (mean age 21.0 years), 289 (96.3%) had all six SNPs and valid testosterone. One SNP (rs2175898) deviated from Hardy-Weinberg equilibrium and was dropped. One SNP (rs1256030) in linkage disequilibrium was dropped (Supplementary Table 1, available as Supplementary data at *IJE* online). Three of the four remaining candidate SNPs were selected through stepwise regression with bootstrapping replication validation for log testosterone prediction as $-0.07 \times$ rs1008805 $+ 0.07 \times$ rs10046-0.07 $\times$ rs1256031 $+ 3.0$. Genotypes were coded 0, 1 and 2, and the number of alleles was used as continuous because the R^2 was larger than using the genotypes as categorical. The proportion of the variance explained by genetically predicted log testosterone was 4.1%, which is typical for MR studies.[23] The F-statistic was 13.3, suggesting a reliable genetic instrumental variable (IV). Testosterone was estimated as the anti-log of predicted log testosterone.

Association of genetically predictedlog-testosterone with CVD risk factors in older men

Among the 8450 men in all three phases of GBCS, DNA for SNP testing was available for 4262 men, with availability depending on the phase of recruitment and other logistical concerns, but not on CVD status. Among the 4262 men, 4212 (98.8%) had all three SNPs. In GBCS none of the three SNPs deviated from Hardy–Weinberg equilibrium (see Supplementary Table 2, available as Supplementary data at *IJE* online). Table 1 shows that, as would be expected, genetically predicted testosterone was not associated with age, education, smoking status or use of alcohol.

Table 2 shows genetically predicted testosterone was not associated with healthier CVD risk factors or lower Framingham score. Genetically predicted testosterone was positively associated with LDL-cholesterol and negatively associated with HDL-cholesterol. Most other estimates were close to the null. Testosterone was positively associated with Framingham score, but the confidence interval included the null. The results were similar with bootstrapping replication for internal validation.

Discussion

Using an MR analysis with an SSIV estimator to minimize reverse causality in Chinese men, endogenous testosterone was not associated with healthier CVD risk factors, but was associated with an unhealthier lipid profile. Our novel study provides no support for protective effects of endogenous

Table 1 Predicted testosterone by socio-demographic characteristics among 4212 men (aged 50+ years), Guangzhou Biobank Cohort Study, 2003–08

Characteristic	Number	%	Genetically predicted testosterone (nmol/l)		
			Mean	SD	ANOVA *P* value[†]
Age group, years	4212				0.94
50–54		8.1	18.05	1.10	
55–59		20.2	18.01	1.22	
60–64		25.4	18.01	1.24	
65–69		24.1	18.01	1.27	
70–74		16.5	17.99	1.19	
75–79		4.2	17.95	1.28	
⩾80		1.5	18.15	1.52	
Education	4209				0.91
Less than primary school		2.6	18.11	1.36	
Primary school		26.9	18.01	1.22	
Junior middle school		30.0	18.01	1.19	
Senior middle school		24.2	18.01	1.23	
Junior college		8.9	17.99	1.34	
College		7.4	17.96	1.24	
Smoking status	4192				0.59
Never smoker		40.8	17.99	1.22	
Ex-smoker		27.9	18.02	1.25	
Current smoker		31.3	18.03	1.22	
Use of alcohol	4212				0.49
Never		51.8	18.02	1.25	
<1/month		19.9	18.03	1.19	
<1/week		4.3	18.04	1.20	
1–4/week		6.0	18.03	1.30	
5+/week		11.8	17.90	1.19	
Ex-drinker		4.4	18.01	1.20	
Unknown		1.8	18.08	1.27	

SD, standard deviation.
[†]Predicted testosterone was not associated with socioeconomic position or lifestyle, including age, education, smoking status and use of alcohol (ANOVA *P*-value >0.05).

testosterone on CVD risk factors or Framingham score among men, although we cannot rule out the possibility of some protective effects of testosterone for specific risk factors.

Although we used MR which can mimic the randomized treatment allocation in RCT,[34] limitations exist. First, two independent samples were used and genetic association derived from one might not apply to the other. However, both samples were of ethnic Chinese from Hong Kong and Guangzhou. The Hong Kong population was largely formed by migration from Guangdong in the late 1940s and early 1950s.[35] Residents of Hong Kong and Guangzhou (the capital of Guangdong) share a recent, common ancestry. We restricted the sample of young men to those with both parents and at least three grandparents born in Hong Kong or Guangdong to ensure genetic homogeneity, reflected by the similar allele frequencies of the genetic variants in the two samples (Supplementary Tables 1 and 2, available as Supplementary data at *IJE* online). Second, lifetime testosterone cannot be assessed in older men to validate the association of testosterone with genetically predicted testosterone; neither biomaterials nor peak testosterone dating back to the 1960s are available. However, an SSIV design is valid when the phenotype of interest was either not measured or was measured with substantial error in the sample with outcome.[31] Third, this study is not totally representative, although disease prevalences were similar to those in

Table 2 Effect of genetically predicted testosterone on CVD risk factors and the Framingham Score among men (aged 50+ years), Guangzhou Biobank Cohort Study, 2003–08

Outcome	Number	Mean (SD)	Model[a]	Beta coefficient[b]	95% CI	*P*-value
SBP (mmHg)	4200	133.24	1	−0.13	−0.74 to 0.48	0.68
	4158	(21.48)	2	−0.11	−0.72 to 0.50	0.72
	4155		3	−0.11	−0.70 to 0.48	0.72
DBP (mmHg)	4202	76.14	1	0.02	−0.30 to 0.35	0.90
	4160	(11.34)	2	0.05	−0.28 to 0.37	0.79
	4157		3	0.04	−0.27 to 0.36	0.78
LDL (mmol/l)	3956	3.07	1	0.02	0.01 to 0.04	0.006
	3917	(0.66)	2	0.02	0.01 to 0.04	0.006
	3912		3	0.02	0.01 to 0.04	0.005
	3956		4	0.02	0.01 to 0.04	0.009
HDL (mmol/l)	3959	1.52	1	−0.01	−0.02 to −0.001	0.03
	3920	(0.38)	2	−0.01	−0.02 to −0.001	0.03
	3915		3	−0.01	−0.02 to −0.001	0.03
	3959		4	−0.01	−0.02 to −0.0003	0.04
Fasting glucose (mmol/l)	3957	5.73	1	0.02	−0.02 to 0.06	0.44
	3920	(1.47)	2	0.02	−0.02 to 0.06	0.38
	3915		3	0.02	−0.02 to 0.06	0.35
Framingham score	4181	1.06	1	0.01	−0.002 to 0.03	0.09
	4138	(0.83)	2	0.02	−0.001 to 0.03	0.07
	4132		3	0.02	−0.0001 to 0.03	0.05
	4181		4	0.01	−0.003 to 0.03	0.10

SD, standard deviation; CI, confidence interval; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol.

[a]Model 1 adjusted for age; model 2 additionally adjusted for education, smoking and use of alcohol; model 3 additionally adjusted for body mass index (BMI); model 4 used bootstrapping with 1000 replications for internal validation for model 1.

[b]Beta coefficient refers to the average change in CVD risk factors (SBP, DBP, LDL, HDL and glucose) or Framingham score with each unit (nmol/l) increase in genetically predicted testosterone.

a nationally representative study.[22] Results would only be invalidated if the relation of genetic variants with testosterone or CVD risk factors differs in our sample from that in the general population, which is unlikely as the relevant genetic variants were not associated with socioeconomic position or lifestyle. Fourth, population stratification and canalization may affect MR analysis. However, the participants were restricted to genetically homogeneous Chinese men and to our knowledge no relevant canalization exists.[34] The effect of genetic variants on testosterone in early adulthood could be different from that in older adulthood, although it is unlikely.[36] Fifth, only Chinese men were recruited, which could restrict generalizability. However, the association of genetic variants with testosterone or CVD risk factors is unlikely to vary by setting or ethnicity. Sixth, the selected SNPs may affect CVD directly rather than via testosterone. However, the SNPs are from genes functionally relevant to testosterone. Seventh, genetic variants typically have small effects. MR estimates tend to be less precise (although less biased) than conventional regression estimates.[37] MR studies require large sample sizes, approximately the usual sample size for exposure on outcome divided by the R^2 (here 0.04) between instrument and exposure.[38] A sample size of around 4200 is needed for an effect size of 0.22 with 0.8 power. We used a weighted genetic score instead of one SNP as instrument, reducing variability in MR estimation.[33] However, the association of serum testosterone with CVD events is on a larger scale, so an MR approach may not generate sufficient range to test the underlying relation. Replication with a stronger instrument in a larger sample or meta-analysis of more MR analyses is needed. Eighth, correction for multiple testing could be required. However, such correction is not universally accepted[39,40] and corrections, such as

Bonferroni, may artificially increase the risk of type 2 error.[39,41] Ninth, MR estimates should be interpreted as hypothesis testing, rather than indicating the exact size of a causal effect.

Strengths of our study include the use of an SSIV estimator in an MR design to achieve an unbiased estimation of the effect of testosterone. This design, establishing a genetic prediction score for testosterone in a separate sample of young men, rather than within the sample of older men, has several advantages. First, such a design minimizes reverse causality from testosterone falling with age, ill health[18,19,42] and sub-clinical age-related comorbidities.[20] Second, testosterone at older ages may reflect ill health rather than lifelong exposure,[20] possibly inducing an underestimation of the genetic association with testosterone and inflating the MR estimate,[17] which an SSIV design avoids. Third, testosterone in early adulthood may be a better marker of lifetime exposure. The metabolic profile at the end of puberty tracks into adult life.[43] At an ecological (country) level testosterone in young men correlates with prostate cancer in older men,[44] consistent with lifetime effects of pubertal sex steroids. CVD has a long preclinical course, so early exposure may be more relevant than exposure contemporaneous with overt disease in an older adulthood. SSIV also remedies weak-instruments bias, reducing concern about using multiple polymorphisms as IVs, perhaps only weakly associated with the phenotype.[31] Moreover, any correlation of the genetic variants with unmeasured confounders in the sample with the phenotype is unlikely to be replicated in the sample with the outcome due to the different data structures.[31]

Our study is inconsistent with observational studies where cross-sectionally higher endogenous testosterone is associated with a healthier CVD risk profile[2–5,7] and prospectively with lower risk of CVD mortality.[45,46] These studies cannot assess whether testosterone is negatively associated with CVD mortality and risk factors as a cause, a symptom of disease or a marker of an underlying process causing both lower testosterone and CVD: hence the need to test the hypotheses generated by these observational studies in study designs capable of ascertaining causality.

Our study is consistent with most, but not all, previous evidence from similar study designs and RCTs. The previous MR study[10] also found that higher genetically predicted testosterone increased blood pressure, glucose, LDL-cholesterol and reduced HDL-cholesterol, although the wide confidence intervals included the null for all except SBP. Using a different genetic instrument, calibrated in young men, and a larger sample size, we obtained more precise estimates for lipids. Pooling both MR studies in a meta-analysis (Supplementary Table 3, available as Supplementary data at *IJE* online), genetically predicted testosterone was clearly associated with higher LDL-cholesterol (0.019 mmol/l per nmol/l testosterone, 95% CI 0.005 to 0.034) and lower HDL-cholesterol (−0.01 mmol/l, 95% CI −0.019 to −0.001), but not with blood pressure or fasting glucose. Our findings for HDL-cholesterol and blood pressure are consistent with the available meta-analysis of RCTs.[15] Our findings for HDL-cholesterol are also consistent with the changes that take place in boys at puberty under the influence of testosterone.[47] Conversely, a small meta-analysis of RCTs found testosterone improved exercise capacity in stable chronic heart failure,[48] but this may be due to testosterone enhancing the capacity to ignore discomfort rather than better cardiovascular function. A larger meta-analysis found testosterone increased cardiovascular-related events.[14]

Given that lower estrogen among men than women does not explain men's higher rate of CVD, this study adds weight to the alternative hypothesis that higher androgens among men than women is the explanation. Focus should perhaps be shifted from whether treatment of andropause is justified to the safety of testosterone for CVD.[49] Potential mechanisms include testosterone-increasing factors related to CVD such as thromboxane.[14,50] Moreover, a recent meta-analysis[51] has reported a small reduction of testosterone by statins, the most effective lipid-modulating treatment for CVD, but the clinical significance and contribution to statins' action are unclear.

Conclusion

This study did not corroborate observed protective effects of serum testosterone on CVD risk factors or risk of ischaemic heart disease among men. Instead, it supported accumulating evidence that androgens may be a modifiable causal factor underlying men's greater vulnerability to CVD, with corresponding implications for developing new effective treatment and prevention strategies. Testosterone therapy needs to be used very cautiously considering the potential detrimental effect on LDL- and HDL-cholesterol. Replication in a larger sample is required.

Supplementary Data

Supplementary data are available at *IJE* online.

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Conflict of interest: None declared.

KEY MESSAGES

- Androgen replacement therapy is increasingly used but the potential cardiovascular risk is unclear.
- A Mendelian randomization study design provides a means of examining the causal effects of endogenous testosterone on cardiovascular risk factors, without any potentially harmful interventions.
- Our separate-sample Mendelian randomization analysis did not corroborate observed protective effects of testosterone on cardiovascular risk factors or risk of ischaemic heart disease among men.
- Testosterone needs to be used cautiously considering the potential detrimental effect on LDL- and HDL-cholesterol.

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