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Mendelian randomization suggests non-causal associations of testosterone with cardiometabolic risk factors and mortality

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SUMMARY

Prospective studies showed that low serum testosterone concentrations are associated with various cardiometabolic risk factors and mortality. However, the causal nature of these associations is controversial. We studied 1 882 men aged 20–79 years with serum testosterone concentrations and genotyping data from the longitudinal population-based Study of Health in Pomerania. Testosterone concentrations were cross-sectionally associated with cardiometabolic risk factors, including anthropometric, lipid, blood pressure and glycaemic parameters; and prospectively with all-cause mortality (277 deaths, 14.7%) during the 10-year follow-up. To overcome problems of residual confounding, reverse causation, or regression dilution bias in the investigated testosterone-outcome associations, we used two-stage least square regression models with previously identified polymorphisms at the *SHBG* gene (rs12150660) and X chromosome (rs5934505) as multiple genetic instruments in an instrumental variable (IV) approach, also known as Mendelian randomization. In standard regression analyses, testosterone was robustly associated with a wide range of cardiometabolic risk factors. In subsequent IV analyses, no such significant associations were observed. Similarly, prospective analyses showed a consistent association of low testosterone concentrations with increased all-cause mortality risk, which was not apparent in subsequent IV analyses. The present Mendelian randomization analyses did not detect any evidence for causal associations of testosterone concentrations with cardiometabolic risk factors and mortality, suggesting that previously reported associations might largely result from residual confounding or reverse causation. Although testosterone assessment might improve risk prediction, implementation of testosterone replacement therapy requires further evidence of a direct effect on cardiometabolic outcomes from double-blinded randomized controlled trials and large-scale Mendelian randomization meta-analyses.

INTRODUCTION

Findings from prospective cohort studies accumulated evidence suggesting that low serum testosterone concentrations are associated with various cardiometabolic risk factors, including obesity (Blouin *et al.*, 2008), dyslipidaemia (Haring *et al.*, 2011b), hypertension (Torkler *et al.*, 2011), subclinical inflammation (Haring *et al.*, 2011a), metabolic syndrome (Haring *et al.*, 2009b; Kupelian *et al.*, 2006), type 2 diabetes (Vikan *et al.*, 2010) and atherosclerosis (Dörr *et al.*, 2009). In contrast, low serum testosterone concentrations are not predictive of incident 'hard' cardiovascular disease (CVD) endpoints, including myocardial infarction, angina pectoris, stroke, congestive heart failure, or peripheral vascular disease (Arnlov *et al.*, 2006; Ruige *et al.*, 2010). Furthermore, associations between low serum testosterone concentrations and mortality were reported in some, but not all prospective studies (Araujo *et al.*, 2011). Given these inconclusive findings, we previously suggested that low serum testosterone as an intermediate or surrogate marker of subclinical disease progression rather than a causal precursor (Haring *et al.*, 2010b). However, low serum testosterone concentrations are associated with a wide range of lifestyle and socioeconomic characteristics (Haring *et al.*, 2010a) and may be lowered by the

presence of subclinical CVD (Laaksonen *et al.*, 2005). Thus, there remains the possibility of confounding by unobserved covariables or reverse causality as potential explanations for the reported associations of low serum testosterone concentrations with increased CVD risk factor burden and mortality. Like other biomarker measurements, testosterone is also subject to temporal and intra-individual variability and laboratory measurement error (Haring *et al.*, 2011d). Failure to adjust for this will usually lead to regression dilution bias and therefore underestimation of the causal effect (Steyerberg, 2008).

A recent large-scale genome-wide association study among 14 429 European men revealed genetic variants associated with low testosterone concentrations (Ohlsson *et al.*, 2011). The Mendelian randomization method uses genetic data as instrumental variables to estimate treatment effects of phenotypes influenced by those genetic variants (Smith *et al.*, 2008). Given the current gap in knowledge about testosterone's upstream (causal) vs. downstream (reactive changes) role in CVD pathogenesis, Mendelian randomization provides a statistical method to overcome residual confounding, reverse causation and regression dilution bias in the observed associations between low testosterone and CVD risk and to strengthen potential causal inferences (Lawlor *et al.*, 2008). This study applied the Mendelian randomization method to investigate the possible causal effects of testosterone on cardiometabolic risk factors and mortality in a population-based sample of 1 882 men aged 20–79 years.

METHODS

Study population

The Study of Health in Pomerania (SHIP) is a population-based cohort study conducted in northeastern Germany. Details on the SHIP study design, recruitment and procedures have been published previously (Völzke *et al.*, 2011). In brief, from the total population of West Pomerania comprising 213 057 inhabitants in 1996, a two-stage stratified cluster sample of adults aged 20–79 years was drawn. The net sample (without migrated or deceased persons) comprised 6 265 eligible subjects. Only individuals with German citizenship and main residency in the study area were included. All subjects received a maximum of three written invitations. In cases of non-response, letters were followed by repeated phone calls or by home visits if contact by phone was not possible (Haring *et al.*, 2009a). After written informed consent was obtained, 4 308 participants were examined (response 68.8%) in two examination centres (Greifswald and Stralsund) between October 1997 and May 2001. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in an *a priori* approval by the local Ethics Committee of the University of Greifswald. From the 2 116 male baseline participants, we excluded men with unmeasured concentrations of serum testosterone ($N = 86$), self-reported intake of sex steroids [anatomical-therapeutical-chemical (ATC) code G03; $N = 3$], testosterone 5-α reductase inhibitors (ATC code G04CB; $N = 4$), or sex steroid antagonists (ATC code L02B; $N = 1$), missing genotyping information ($N = 102$) and covariate data ($N = 38$). The final study population comprised 1 882 men.

Measures

Computer-assisted personal interviews assessed information about socio-demographic and behavioural characteristics. Men who participated in physical training for at least 1 h a week were classified as being physically active. Mean daily alcohol consumption was calculated using beverage-specific pure ethanol volume proportions (Alte *et al.*, 2004). Smoking habits were assessed by dividing men into categories of current, former and never-smokers. After a resting period of at least 5 min, systolic and diastolic blood pressure was measured three times at the right arm of seated subjects using an oscillometric digital blood pressure monitor (HEM-705CP; Omron Corporation, Tokyo, Japan). The interval between the readings was 3 min. The mean of the second and third measurements was calculated and used for these analyses. Hypertension was defined by systolic blood pressure or diastolic blood pressure of ≥ 140 and ≥ 90 mmHg, respectively, or use of antihypertensive medication (ATC codes C02, C03, C04, C07, C08, or C09). Waist circumference (WC) was measured to the nearest 0.1 cm using an inelastic tape midway between the lower rib margin and the iliac crest in the horizontal plane, with the subject standing comfortably with weight distributed evenly on both feet.

During 17 906 person-years (median, 10.0 years; 25th, 9.3; 75th, 10.7) of follow-up, 277 men (14.7%) died, reflecting an overall crude all-cause mortality rate of 15.5 deaths per 1000 person-years. Information on vital status was collected from population registries at annual intervals from time of enrolment into the study through 15 December 2009. Subjects were censored at either death or loss to follow-up and the number of months between baseline examination and censoring was used as follow-up length (Haring *et al.*, 2011c).

For the laboratory examinations, non-fasting blood samples were taken from the cubital vein in the supine position between 07:00 AM and 06:00 AM, and prepared for immediate analysis or for storage at −80 °C for further analysis. Measurements of serum total testosterone concentrations were performed from frozen serum aliquots from December 2005 to January 2006 using competitive chemiluminescent enzyme immunoassays with an Immulite 2500 analyser (Siemens Healthcare Medical Diagnostics, Bad Nauheim, Germany). At the 3.2 nmol/L level, the inter-assay coefficient of variation was 13.2%, with a systematic deviation of +2.3%, and at the 22.5 nmol/L level, the inter-assay coefficient of variation was 8.9%, with a systematic deviation of +0.24% (Friedrich *et al.*, 2008). Serum total cholesterol and high-density lipoprotein cholesterol (HDL) concentrations were measured photometrically (Hitachi 704; Roche, Mannheim, Germany). Serum low-density lipoprotein cholesterol (LDL) was measured using applying a precipitation procedure, using dextran sulphate (Immuno, Heidelberg, Germany) on an Epos 5060 (Eppendorf, Hamburg, Germany). Serum triglyceride and glucose concentrations were determined enzymatically using reagents from Roche Diagnostics (Hitachi 717; Roche Diagnostics, Mannheim, Germany). Glycated haemoglobin (HbA1c) concentrations were determined using high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany). Plasma fibrinogen concentrations were determined according to Clauss using an Electra 1600 analyser (Instrumentation Laboratory, Barcelona, Spain) (Haring *et al.*, 2011a). All assays were performed according to the manufacturers' recommendations by skilled technical personnel. In addition, the laboratory participated in official quarterly German external proficiency testing programs.

Genotyping

Details on the genotyping and quality control procedures have been published previously (Ohlsson *et al.*, 2011; Suhre *et al.*, 2011). In brief, SHIP participants were exclusively Caucasian and samples were genotyped using the Affymetrix Human single nucelotide polymorphism (SNP) Array 6.0. Hybridization of genomic DNA was carried out in accordance with the manufacturer's standard recommendations. The genetic data analysis workflow was created using the Software InforSense (InforSense Ltd., Guildford, UK). Genetic data were stored using the database Caché (InterSystems, Darmstadt, Germany). Genotypes were determined using the Birdseed2 clustering algorithm. For quality control purposes, several control samples were added. On the chip level, only subjects with a genotyping rate on QC probe sets (QC call rate) of at least 86% were included. All remaining arrays had a sample call rate > 92%. The SNPs that were used as IV were imputed. Imputation of genotypes in SHIP was performed using the software IMPUTE v0.5.0 (Genetics Software Suite, University of Oxford, UK) based on HapMap II (specific SNP call rate > 0.99). Uncertainties for imputed genotypes were taken into account, by including the allele dosage of each SNP into the models.

Statistical analysis

Baseline characteristics were presented for the full sample and by genotype of the applied genetic instruments rs12150660 and rs5934505. We used one-way analysis of variance to test for significant differences between the different SNP genotypes and the outcome variables for each SNP separately.

Linear (ordinary least square, OLS) regression models, adjusted for age and multiple covariables, were used to assess cross-sectional associations of continuous serum testosterone concentrations with continuous cardiometabolic risk factor outcomes. Next, we used two-stage least square (2SLS) regression to fit IV regression models and to obtain estimates of the association between testosterone and cardiometabolic risk factors free from endogeneity. In these analyses, we used the *SHBG* SNP rs12150660 and the *FAM9B* SNP rs5934505 as multiple genetic variants modelling the allele dosage instead of categorized genotypes in the first-stage regression of the 2SLS. We tested 2SLS assumptions. First, we used the Durbin-Wu-Hausman test of endogeneity. Second, Hansen's test of overidentifying restrictions was applied in models using multiple instruments. Third, F statistics from the first-stage were used to assess the strength of the genetic instruments and values greater than 10 for all 2SLS models indicate sufficient strength to ensure the validity of the applied instruments (Cameron & Triveldi, 2008). We compared the estimates from 2SLS and OLS regression models using the Durbin-Wu-Hausman statistic (Cameron & Triveldi, 2008).

We implemented Poisson regression models with robust standard errors to assess the prospective association of low serum testosterone concentrations with mortality and incorporated a log-transform of time as an offset into the models (Hardin & Hilbe, 2007). As an IV analogue, we used a two-step approach of a Poisson regression (Cameron & Triveldi, 2008; Rassen *et al.*, 2009). The first-stage generated residuals from a logistic model of low testosterone on the instruments and covariables were included as a predictor in the second stage Poisson regression for mortality. The *p*-value for the coefficient of the included residuals provides tests for the null hypotheses of exogeneity. We checked the findings of the 2SLS and two-stage Poisson IV regression with the generalized methods of moments (Cameron & Triveldi, 2008) and found similar coefficients. Two-sided probability values < 0.05 were considered statistically significant. All statistical analyses were performed using Stata 11.1 (Stata Corporation, College Station, TX, USA).

RESULTS

Baseline characteristics of the 1 882 participants were presented for the full sample and by genotype of the applied genetic instruments rs12150660 and rs5934505 (Table 1). Variations in *SHBG* (rs12150660) and *FAM9B* (rs5934505) genotype were not associated with any of the outcome variables or potential confounding variables, but with the predictor variable testosterone. These findings met the general assumptions of an IV analysis summarized in Fig. 1. As expected, men with one or two risk alleles for rs12150660 (major allele T modelled as the 'risk' allele) had significantly higher serum testosterone concentrations compared with men with the protective minor allele (G) (Table 1).

Estimates from multivariable OLS regression analyses presented in Table 2 showed robust associations of serum testosterone with all the considered cardiometabolic risk factors. In contrast, subsequent age- and multivariable-adjusted IV regression analyses showed no such associations, except for systolic blood pressure (Table 2). At this, Durbin-Wu-Hausman statistics yielded a *p*-value of < 0.05 indicating conflicting results for the association of testosterone and systolic blood pressure between OLS and IV analyses. *p*-values > 0.05 from Durbin-Wu-Hausman statistics for the other investigated cardiometabolic risk factors (Table 2) suggested that the observed testosterone-outcome associations from OLS regression models are subject to residual confounding, reverse causation, or regression dilution bias. The F statistics from IV regression analyses were consistently > 17, indicating sufficient strength of the applied genetic instrument.

The individual proportion of variance in serum testosterone concentrations in men explained by the individual and combined genetic instruments were as follows: *R*-squared for rs12150660: 0.016, for rs5934505: 0.004 and for the combined genetic instrument: 0.021. Estimates from IV models ranged between 0.05 and 0.21 for the first-stage and instrumental variables regression models, respectively. Finally, conventional

Figure 1 Directed acyclic graph (DAG) for the causal relationships between the genetic instrument (*SHBG* genotype), modifiable risk factor (serum total testosterone concentration), outcome (increased cardiometabolic risk factor burden) and measured or unmeasured confounding factors. The Mendelian randomization uses genetic information for instrumental variable regression modelling. As Mendelian randomization assumes that the *SHBG* genotype is only related to the risk factor (a) and unrelated to confounding factors (b), the association of serum total testosterone concentration with cardiometabolic risk factors (c) is free of measured or unmeasured confounding (d).

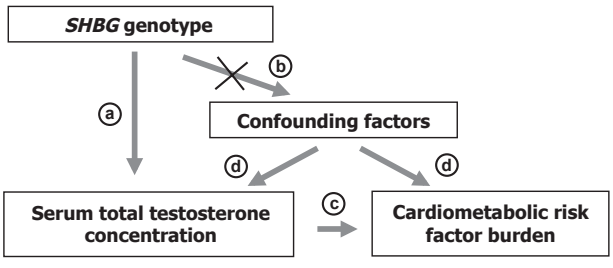

Table 1 Baseline characteristics of the study population and their association with the genetic instruments

Outcome	Baseline (N = 1,882)	rs12150660			rs5934505	
		GG (56.5%)	GT (37.8%)	TT (5.6%)	T (76.5%)	C (23.4%)
Age, years	50.8 (16.4)	51.4 (16.2)	50.1 (16.6)	48.3 (16.6)	50.7 (16.5)	50.9 (16.4)
Total testosterone (nmol/L)	16.67 (5.95)	16.1 (5.6)	17.2 (6.12)*	19.1 (6.9)*	16.5 (6.0)	17.3 (5.9)*
Sex hormone-binding globulin (nmol/L)	51.4 (25.7)	48.3 (23.4)	54.3 (24.5)*	62.1 (42.2)*	51.6 (25.9)	50.5 (24.9)
Waist circumference (cm)	95.7 (11.6)	96.0 (11.4)	95.3 (12.0)	94.8 (11.5)	95.8 (11.6)	95.4 (11.7)
Alcohol consumption (g/d)	19.7 (23.0)	18.9 (22.1)	20.5 (24.3)	22.4 (23.0)	19.3 (22.2)	21.2 (25.4)
Current smoking (%)	33.7	32.5	34.3	41.0	32.1	38.9
Low physical activity (%)	41.2	40.1	42.3	44.8	41.0	41.8
Total cholesterol (mmol/L)	5.76 (1.23)	576 (1.22)	5.73 (1.25)	5.78 (1.1)	5.8 (1.2)	5.8 (1.2)
HDL cholesterol (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.4)	1.4 (0.4)	1.3 (0.4)	1.3 (0.4)
LDL cholesterol (mmol/L)	3.6 (1.1)	3.6 (1.1)	3.6 (1.2)	3.6 (1.1)	3.6 (1.1)	3.7 (1.2)
Triglyceride (mmol/L)	2.1 (1.4)	2.1 (1.4)	2.0 (1.3)	2.0 (1.2)	2.1 (1.4)	2.1 (1.3)
Systolic BP (mmHg)	142.5 (19.3)	142.4 (19.3)	142.6 (18.8)	143.4 (22.0)	142.1 (19.0)	144.2 (20.1)
Diastolic BP (mmHg)	86.0 (11.4)	86.2 (11.3)	85.8 (11.5)	86.1 (12.3)	85.8 (11.2)	86.9 (12.2)
Glucose (mmol/L)	5.8 (1.9)	5.8 (1.9)	5.8 (1.8)	6.0 (2.4)	5.9 (2.1)	5.7 (1.5)
Haemoglobin A1c (%)	5.5 (0.9)	5.6 (1.0)	5.6 (1.00)	5.5 (0.9)	5.6 (1.0)	5.5 (0.9)
Fibrinogen (g/L)	3.0 (0.7)	3.0 (0.7)	2.9 (0.7)	2.8 (0.7)	2.9 (0.7)	3.0 (0.7)

Data are percentages or means (standard deviation). Polymorphisms rs12150660 are located at the *SHBG* gene and rs5934505 on the X chromosome. *$p < 0.05$ p-values were calculated from one-way analysis of variance testing for significant differences between the different SNP genotypes and the outcome variables for each SNP separately.

Table 2 Cross-sectional associations between testosterone concentrations and cardiometabolic risk factors

Outcome	Model	OLS	IV	p-value
		Beta (95% CI)	Beta (95% CI)	
Waist circumference	Age-adjusted	−0.49 (−0.57; −0.41)*	−0.29 (−0.86; 0.28)	0.482
	Multiple-adjusted	−0.49 (−0.57; −0.41)*	−0.24 (−0.83; 0.36)	0.407
Total cholesterol	Age-adjusted	−0.02 (−0.03; −0.01)*	−0.01 (−0.08; 0.05)	0.891
	Multiple-adjusted	−0.02 (−0.03; −0.01)*	−0.02 (−0.09; 0.04)	0.931
High-density lipoprotein cholesterol	Age-adjusted	0.01 (0.00; 0.02)*	−0.01 (−0.02; 0.02)	0.408
	Multiple-adjusted	0.01 (0.00; 0.02)*	−0.01 (−0.03; 0.02)	0.242
Low-density lipoprotein cholesterol	Age-adjusted	−0.01 (−0.02; 0.01)	0.01 (−0.06; 0.06)	0.764
	Multiple-adjusted	−0.01 (−0.02; −0.01)*	0.01 (−0.07; 0.07)	0.768
Triglyceride	Age-adjusted	−0.06 (−0.07; −0.05)*	−0.03 (−0.10; 0.04)	0.439
	Multiple-adjusted	−0.06 (−0.07; −0.05)*	−0.05 (−0.12; 0.03)	0.646
Systolic blood pressure	Age-adjusted	−0.31 (−0.45; −0.18)*	1.14 (0.04; 2.23)*	0.004
	Multiple-adjusted	−0.29 (−0.43; −0.16)*	1.16 (0.03; 2.29)*	0.005
Diastolic blood pressure	Age-adjusted	−0.19 (−0.27; −0.10)*	0.21 (−0.43; 0.84)	0.213
	Multiple-adjusted	−0.18 (−0.26; −0.09)*	0.16 (−0.50; 0.81)	0.308
Glucose	Age-adjusted	−0.05 (−0.06; −0.03)*	−0.01 (−0.11; 0.10)	0.405
	Multiple-adjusted	−0.05 (−0.06; −0.03)*	0.02 (−0.09; 0.13)	0.243
Haemoglobin A1c	Age-adjusted	−0.02 (−0.02; −0.01)*	−0.01 (−0.07; 0.04)	0.907
	Multiple-adjusted	−0.02 (−0.03; −0.01)*	−0.01 (−0.06; 0.05)	0.672
Fibrinogen	Age-adjusted	−0.01 (−0.01; 0.01)	−0.01 (−0.04; 0.03)	0.892
	Multiple-adjusted	−0.01 (−0.01; −0.01)*	−0.01 (−0.05; 0.03)	0.936

Multiple-adjusted models included age, smoking (three categories), alcohol consumption and physical activity. p-value was calculated from Durbin-Wu-Hausman statistics to assess significant differences between ordinary least squares (OLS) & instrumental variable (IV) regression coefficients. p-values > 0.05 indicate that the observed associations from OLS regression models are subject to residual confounding, reverse causation, or regression dilution bias. The F statistics from IV regression analyses were consistently > 17, indicating sufficient strength of the applied genetic instrument. *$p < 0.05$; 95% Confidence Interval.

Poisson regression models revealed a consistent association between low serum testosterone concentrations and increased risk of all-cause mortality across different cut-offs for the definition of low testosterone, whereas the subsequent IV analyses showed no such association (Fig. 2).

DISCUSSION

This is the first Mendelian randomization study to investigate whether serum testosterone concentrations in men have a direct unconfounded effect on cardiometabolic risk factors and mortality using recently identified polymorphisms as genetic instruments. On the basis of previous observational findings, low serum testosterone concentrations were recently postulated as a cardiometabolic risk factor in men (Jones, 2010; Maggio & Basaria, 2009). In contrast, the principal findings of the present study suggest that the associations of serum testosterone concentrations with cardiometabolic risk factors and mortality might largely result from imperfect adjustment for confounding or measurement error that induces spurious findings. In addition, reverse causation might have taken place where subclinical CVD influence testosterone concentrations, which in turn affects CVD risk factor burden.

As expected, the standard regression analyses confirmed the associations of serum testosterone concentrations with different

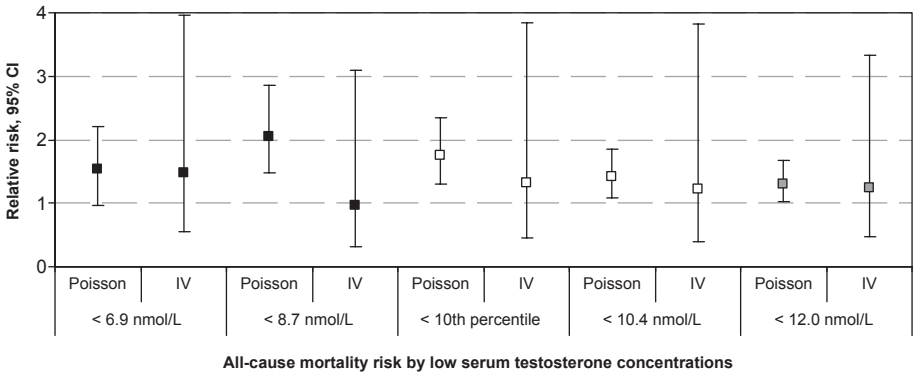

Figure 2 All-cause mortality risk for men with low serum testosterone concentrations. Multivariable conventional Poisson ('Poisson') and instrumental variable regression models ('IV') were adjusted for age, smoking, alcohol consumption, and physical activity. The number of men with low serum testosterone concentrations was as follows: < 6.9 nmol/L (N = 35), < 8.7 nmol/L (N = 99), < 10th percentile (N = 195), < 10.4 nmol/L (N = 237), and < 12.0 nmol/L (N = 405).

cardiometabolic risk factors showing inverse associations with obesity, lipid parameters, blood pressure, glucose, HbA1c and subclinical inflammation. However, when we used genetic instruments in subsequent IV analyses to represent lifetime serum testosterone concentrations free of residual confounding or reverse causation, none of these previous associations were proved to be causal. The metabolic syndrome consists of a cluster of cardiometabolic risk factors, including visceral obesity, glucose intolerance, dyslipidaemia and hypertension; which strongly predict subsequent CVD (Sattar *et al.*, 2003) and mortality (Haring *et al.*, 2010c). However, although previous prospective studies showed that low testosterone concentration precede the development of metabolic syndrome (Haring *et al.*, 2009b; Kupelian *et al.*, 2006), reverse causality remains a possibility (Laaksonen *et al.*, 2005), as it remains still unclear whether low testosterone concentrations contributes to or are a very early consequence of mechanisms finally leading to overt metabolic syndrome. Similarly, also for the reported associations of low testosterone concentrations with obesity (Blouin *et al.*, 2008), hypertension (Torkler *et al.*, 2011), or type 2 diabetes (Vikan *et al.*, 2010), the possibility exist that there is a vicious cycle between low testosterone concentration and metabolic alterations finally leading to clinical CVD and premature death.

We also found that the association between low serum testosterone concentrations and increased all-cause mortality risk is subject to residual confounding, reverse causation, or regression dilution bias. This finding is in agreement with a recent systematic review and meta-analysis concluding that the association between serum testosterone concentrations and mortality is seriously challenged by considerable between-study heterogeneity with regard to age structure, baseline testosterone concentration, length of follow-up period and day time of blood sampling (Araujo *et al.*, 2011).

In a recent systematic review and meta-analysis of 51 testosterone trials, testosterone therapy showed no significant effects on all-cause mortality, prostatic outcomes, cardiovascular events, or cardiovascular risk factors (Fernandez-Balsells *et al.*, 2010). In addition, a recent testosterone replacement trial among 209 community-dwelling men, 65 years of age or older, and with limitations in mobility and a high prevalence of chronic disease was discontinued after the application of a testosterone gel was associated with adverse cardiovascular events (Basaria *et al.*, 2010). Although this finding provides some new caution about testosterone therapy, its generalizability is limited by an elderly study population, higher cardiovascular risk burden in the intervention group, and a sample composition designed to assess frailty as the primary endpoint. However, results from existing interventional trials provide only limited evidence for causal mechanisms between exogenous testosterone, cardiometabolic risk factorsand clinical CVD. Thus, more rigorously conducted double-blinded randomized controlled trials in metabolically healthy men are strongly needed.

Strengths and limitations

The estimates derived from Mendelian randomization analyses reported herein should not be biassed or confounded, although some potential limitations of this study must be mentioned. First, the *SHBG* SNP rs12150660, presently applied as a genetic instrument, was previously related to circulating sex hormone-binding globulin (SHBG) concentrations (Ding *et al.*, 2009; Perry *et al.*, 2010). To maintain testosterone concentrations at appropriate levels, the primary function of SHBG is to bind and transport steroids in the blood to access target tissues and to determine their bioavailable fraction. In serum, most of the circulating testosterone (50–60%) is bound to SHBG, whereas a smaller fraction (40–50%) loosely bound to albumin, leaving only 1–3% to circulate as 'free' testosterone not bound to protein (Kaufman & Vermeulen, 2005). To assess the robustness of our findings independent of a potential SHBG cross-regulation, we also included SNP rs5934505 near *FAM9B* on the X chromosome (exclusively associated with serum testosterone concentrations) into our Mendelian randomization analysis. Furthermore, there were still residual associations of the *SHBG* SNP on testosterone concentrations after removing linear and quadratic effects of SHBG via adjustment (Ohlsson *et al.*, 2011), suggesting outcome effects via TT independently of SHBG. Thus, we modelled the best currently known and available instrumental variables for serum TT concentrations in Caucasian men from the general population. However, although Mendelian randomization is suggested as a novel method to overcome some of the inherent limitations of observational research, herein, present findings are still based on observational data including its unique biases and limited causal inferences (Rothman & Greenland, 2005) – longing for clinical randomized controlled trials to provide further insights (Cook & Romashkan, 2011).

Second, our IV analysis based on a comparably small sample size and therefore yielded larger confidence intervals than the estimates from standard OLS or Poisson regression model However, this is a likely finding in Mendelian randomization analyses based on small samples (Bochud *et al.*, 2009; Kivimaki *et al.*, 2008), making considerably larger sample sizes necessary to generate more precise estimates (Pierce *et al.*, 2011). For

comparison, another Mendelian randomization analysis of the causal role of serum SHBG as a risk factor for type 2 diabetes was conducted in a sample of only 320 men from the Physicians Health Study II (Ding *et al.*, 2009). However, future research using Mendelian randomization meta-analysis of pooled samples is strongly suggested to improve the power of Mendelian randomization applications (Bochud & Rousson, 2011) and to further investigate the causative nature of testosterone in subclinical and clinical CVD. And finally, the applied instruments (SNPs) may not be applicable or useful in a different ethnic context and alternative instruments may need to be sought in non-Caucasian study populations.

In conclusion, this study provided further insights into the causal role of testosterone concentrations in men with CVD and mortality. The triangulation of the associations between genetic instruments, serum testosterone and cardiometabolic risk factors suggests a non-causal effect of serum testosterone on cardiometabolic risk burden and mortality. This result adds to previous findings suggesting low serum testosterone concentrations in men as a secondary marker of subclinical disease progression rather than a causal risk factor (Sartorius *et al.*, 2012). Thus, establishing statistically significant associations is necessary, but not sufficient to prove causality (Smith *et al.*, 2008). To date, assessment of serum testosterone concentrations improves our ability to predict risk and provides a marker of good health and overall well-being in men. However, before issuing any firm clinical recommendations, additional insights into the causal role of testosterone as a CVD risk factor require further research from double-blinded randomized controlled trials, as well as large-scale Mendelian randomization meta-analyses.

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