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Testosterone, dihydrotestosterone and estradiol are differentially associated with carotid intima-media thickness and the presence of carotid plaque in men with and without coronary artery disease

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Abstract. Clarifying the relationship of sex hormones to preclinical atherosclerosis could illuminate pathways by which androgens are associated with cardiovascular events and mortality. Our aim was to determine hormone profiles associated with carotid intima-media thickness (CIMT) and carotid atheroma, in men with and without known coronary artery disease (CAD). We included 492 community-based men aged 20-70 years (Group A) and 426 men with angiographically proven CAD aged <60 years (Group B). Fasting early morning sera were assayed for testosterone (T), dihydrotestosterone (DHT) and estradiol (E2) using mass spectrometry. CIMT and carotid plaque were assessed ultrasonographically. Mean (\pm SD) age was Group A: 53.8 \pm 12.6 and Group B: 49.6 \pm 5.1 years. Higher T was associated with reduced CIMT (-0.011 mm per 1-SD increase, $p=0.042$) and lower prevalence of carotid plaque (odds ratio [OR] per 1-SD increase, 0.68, $p=0.012$) in Group A, but not B. E2 was associated with increased CIMT in Group A (0.013 mm, $p=0.011$) but not B. Higher DHT and E2 were associated with reduced carotid plaque in Group B (DHT: OR=0.77, $p=0.024$; E2: OR=0.75, $p=0.008$), but not A. In community-dwelling men, higher T is associated with favourable CIMT and lower prevalence of carotid plaque, while higher E2 is associated with worse CIMT. In men with CAD, higher DHT or E2 are associated with less carotid plaque. T, DHT and E2 are differentially associated with preclinical carotid atherosclerosis in a cardiovascular phenotype-specific manner. Interventional studies are needed to examine effects of exogenous T and its metabolites DHT and E2, on atherogenesis.

Key words: Testosterone, Dihydrotestosterone, Estradiol, Intima-media thickness, Carotid atheroma

CIRCULATING TESTOSTERONE (T) is lower in older men compared with younger men, and older men have a higher incidence and prevalence of cardiovascular disease (CVD) [1, 2]. In epidemiological studies, lower T concentrations are also associated with a range of adverse health outcomes, including increased risk of CVD and all-cause mortality [3, 4]. On the contrary, benefits of T supplementation have not been reciprocated in randomised controlled trials (RCTs) which demonstrate inconsistent results [5, 6], including concerns raised over potential harm in the TOM trial [5]. Existing RCTs have not been powered for endpoints such as angina or myocardial infarction; nevertheless meta-analyses of T RCTs have shown no increase in cardiovascular adverse events [7]. Given the use of T supplementation in various clinical settings and the burden of CVD [2], it is important to clarify mechanisms by which T might modulate risk of cardiovascular events.

Carotid intima-media thickness (CIMT) is a marker of preclinical atherosclerosis and is a predictor of

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future vascular events, including stroke and myocardial infarction [8]. Carotid plaque is also a marker of atherosclerosis and represents a more advanced stage of atherogenesis [9]. Studies that have examined the relationship between T with CIMT and carotid plaque in middle-aged men have shown inconsistent results. Cross sectional studies have demonstrated an inverse association between T with CIMT and carotid plaque [10-14], while others have shown no association [15]. One longitudinal study demonstrated no association between T and progression of CIMT or plaque area, while another showed an inverse relationship [13, 16]. Furthermore, T is metabolised by 5 α -reductase into the more potent androgen receptor ligand, dihydrotestosterone (DHT), and by aromatase into estradiol (E2), a ligand for estrogen receptors [17]. However, the effect of DHT and E2 on atherosclerosis is uncertain. Observational studies examining the relationship between E2 and CIMT in middle-aged men have also yielded conflicting results [15, 18, 19], whereas an RCT of estrogen administration in men increased cardiovascular risk [20]. These studies of T and E2 consist of heterogeneous populations of men with coronary artery disease (CAD), and are limited by the use of radioimmunoassay for assay of T and E2. More accurate assay of T, and of DHT and E2 can be undertaken using mass spectrometry [21, 22]. Studies examining the association of DHT with preclinical carotid atherosclerosis are lacking.

The aim of this study was to assess the relationship between endogenous sex hormone profiles with CIMT and the prevalence of carotid plaque in two distinct cohorts of middle-aged men. The first consisted of community-dwelling men largely free of CVD, while the second consisted of men with accelerated atherosclerosis with proven CAD on angiography. We utilized liquid chromatography-tandem mass spectrometry (LC-MS) to accurately quantify of sex steroids. We tested the hypotheses that T, DHT and E2 are differentially associated with CIMT and presence of carotid plaque, and associations of sex hormones with preclinical carotid atherosclerosis would vary according to the cardiovascular phenotype of men studied.

Methods

Study population

The study population comprises two distinct cohorts of predominantly middle-aged men, the Carotid

Ultrasound Disease Assessment Study (CUDAS) and the Carotid Ultrasound in Patients with Ischaemic Heart Disease (CUPID) cohorts. The CUDAS cohort was derived from a random electoral roll survey of 2,000 people living in the metropolitan area of Perth, Western Australia, described previously [23]. A total of 1,111 adults (61% of those eligible) agreed to participate. Of these, 558 were men, aged between 20 and 70 years. Subjects who had previous carotid artery surgery were excluded. The CUDAS cohort represents a sample of community-dwelling men largely free of prior CVD history and was designated Group A. The CUPID cohort was assembled from patients undergoing coronary angiography at the Sir Charles Gairdner Hospital in Perth, Western Australia [24]. This cohort consisted of 556 adults of whom 485 were men, aged between 26 and 60 years. All were medically stable at the time of recruitment but all had a past history of angina or myocardial infarction, and angiographically confirmed CAD with at least one coronary vessel with >50% stenosis [24]. The CUPID cohort represents a cohort of men with a defined phenotype of proven coronary atherosclerosis, and was designated Group B. The University of Western Australia Human Research Ethics Committee and the Sir Charles Gairdner Hospital Research Institutional Ethics Committee approved the study and all participants provided written informed consent.

Assessment of medical comorbidities

A self-administered questionnaire was used to report the prevalence of smoking, hypertension, hyperlipidaemia, diabetes, angina pectoris, myocardial infarction, stroke, or a family history of premature onset coronary or cerebrovascular disease. Resting blood pressures and anthropometric measurements (height, weight, waist and hip measurements) were taken from all subjects in an identical manner [23, 24].

Assessment of CIMT and carotid plaque

Bilateral carotid B mode ultrasound was performed on all subjects using a 7.5-MHz annular phased-array transducer on an interspec (Apogee) CX 200 ultrasound machine. The ultrasound study was used to determine the presence of focal carotid plaque and to measure the mean common carotid artery intimal-medial wall thickness as previously described [23]. Carotid atheromatous plaque was defined as a clearly identified area of focal increased thickness of ≥ 1 mm of the intima-media layer.

Laboratory assays

Fasting blood samples were collected in the early morning in all subjects. Serum was prepared immediately after phlebotomy and stored at -80 degrees Celsius until assayed. Sera were assayed in 2013. Serum T, DHT and E2 were quantified within a single LC-MS run without derivatization, using atmospheric pressure photoionization in positive mode for androgens and negative mode for oestrogens, from 200 µL samples. Between-run imprecision was 8.6% at 5.3 nmol/L and 7.9% at 26.9 nmol/L for T, 11.3% at 1.3 nmol/L and 9.1% at 5.3 nmol/L for DHT, and 14.5% at 73 pmol/L and 9.9% at 279 pmol/L for E2. Luteinising hormone (LH) was assayed using a 2-step noncompetitive chemiluminometric immunoassay (Abbot Architect, Abbott Diagnostics, North Ryde, NSW, Australia), with between run imprecision of 5.6% at 4.8 IU/L. Sex hormone-binding globulin (SHBG) was assayed using a solid-phase, two-site enzyme immunometric assay with chemiluminescent substrate (Immulite 2000Xi; Siemens Healthcare, Bayswater, Vic., Australia), with between run imprecision of 3.4% at 39.4 nmol/L. Free T was calculated using an empirical formula, which provides closer concordance with measured free T compared to calculations based on equilibrium binding equations [25].

Statistical analysis

Baseline descriptive data were shown as mean and standard deviations (SD) or percentages (%). Comparisons of means were performed using *t* tests and comparison of proportions using chi-square tests. Adjusted associations between cardiovascular risk factors (independent variable) and endogenous sex hormone levels and SHBG (dependent variable) were assessed using linear regression models. Adjusted associations between endogenous sex hormone levels and SHBG (independent variables) with CIMT were assessed using linear regression models, and their associations with presence of carotid atheromatous plaque using logistic regression models. Adjustments were made for variables that could conceivably confound the analyses, namely age, smoking, alcohol consumption, and body mass index (BMI) (comprising Model 1), and diabetes, CVD history (CUDAS only), systolic blood pressure (SBP), hypertension, lipid lowering therapy, cholesterol, high density lipoprotein (HDL)-cholesterol, (log) triglycerides (TG) and (log) C-reactive protein (CRP) (comprising Model 2). A two-tailed *p* value of <0.05, or a 95% confidence interval (CI) that did not

cross 1.0 was regarded as significant.

Results

Baseline characteristics of the study population

There were 492 community-dwelling men in Group A, and 426 men with CAD in Group B, who had hormone and carotid ultrasound results available for inclusion in this analysis. Baseline characteristics including demographic, physical, medical and biochemical data for both cohorts are shown (Table 1). Men in Group A were older, less likely to have smoked, reported lower levels of alcohol consumption, had lower BMI and lower prevalence of diabetes, compared with Group B. Men in Group B had a higher prevalence of hypertension and use of lipid-lowering medication, lower CIMT but higher prevalence of carotid plaque. Total T was not different between groups. Men in Group A had higher DHT, E2 and SHBG compared with men in Group B (Table 1). 9.6% of subjects in Group A had a history of cardiovascular disease compared to 100% in Group B.

Association of sex hormones, SHBG and LH with cardiovascular risk factors

Associations of endogenous sex hormones, SHBG and LH with conventional cardiovascular risk factors in community-dwelling men (Group A) are shown in Table 2. T and DHT were inversely associated with triglyceride (TG) concentrations (-0.43 nmol/L T per 1-SD increase in log TG, *p*=0.034; -0.07 nmol/L DHT per 1-SD increase in log TG, *p*=0.033). E2 was associated with SBP (6.28 pmol/L E2 per 1-SD increase in SBP, *p*=0.007) and log CRP (4.40 pmol/L E2 per 1-SD increase in log CRP, *p*=0.045). E2 concentrations were higher in men with hypertension compared to those without (mean difference of 13.8 pmol/L, *p*=0.016). SHBG levels were lower in men with hypertension (mean difference of -4.42 nmol/L, *p*=0.009) and those on lipid lowering medications (mean difference of -5.81 nmol/L, *p*=0.009). Lower SHBG levels were associated with higher log TG (-3.52 nmol/L per 1-SD increase in log TG, *p*<0.001), and with lower HDL (2.26 nmol/L per 1-SD increase, *p*<0.001). LH was inversely associated with cholesterol (-0.28 nmol/L per 1-SD increase, *p*=0.039).

Associations of sex hormones with CIMT

Hormone concentrations were analysed against the outcome of CIMT in both Group A and Group B using

Table 1 Baseline characteristics for 492 community-dwelling men in Group A, and 426 men with CAD in Group B.

Variable	CUDAS, Group A (n = 492)	CUPID, Group B (n = 426)	p-value
CIMT (mm)	0.73 (0.15)	0.68 (0.12)	<0.001
Carotid plaque (%)	29.3	59.6	<0.001
Total T (nmol/L)	10.8 (4.2)	11.0 (3.8)	0.490
Free T (pmol/L)	161.5 (57.0)	173.4 (54.8)	0.001
DHT (nmol/L)	1.10 (0.64)	1.01 (0.44)	0.012
E2 (pmol/L)	99.1 (43.8)	88.9 (45.4)	<0.001
SHBG (nmol/L)	38.1 (14.8)	31.9 (12.7)	<0.001
LH (IU/L)	4.42 (2.98)	4.15 (2.25)	0.122
Age (years)	53.8 (12.6)	49.6 (5.1)	<0.001
Smoking			0.001
Never (%)	38.2	26.8	
Ex (%)	45.1	54.0	
Current (%)	16.7	19.2	
Alcohol (grams per week)			<0.001
0	16.7	3.8	
0 < grams ≤ 140	64.0	76.8	
140 < grams ≤ 280	13.4	14.3	
grams > 280	5.9	5.2	
BMI (kg/m ²)	26.8 (3.6)	28.0 (3.8)	<0.001
Diabetes (%)	2.0	12.2	<0.001
CVD history (%)	9.6	100.0	<0.001
SBP (mmHg)	129.5 (16.8)	125.0 (16.1)	<0.001
Hypertension (%)	15.9	75.1	<0.001
Lipid medication (%)	8.1	66.7	<0.001
Chol (mmol/L)	5.54 (0.97)	5.16 (1.00)	<0.001
HDL (mmol/L)	1.17 (0.30)	1.08 (0.26)	<0.001
Triglycerides (mmol/L)	1.45 (0.80)	1.94 (1.40)	<0.001
log Triglycerides	0.24 (0.52)	0.52 (0.52)	<0.001
CRP (mg/L)	2.85 (3.74)	3.30 (4.93)	0.111
log CRP	0.50 (1.05)	0.67 (0.99)	0.013

Table shows the characteristics of the Carotid Ultrasound Disease Assessment Study (CUDAS, Group A) and the Carotid Ultrasound in Patients with Ischaemic Heart Disease (CUPID, Group B) cohorts. Data are presented as mean (SD) or percentage (%).

Table 2 Linear regression models examining associations of cardiovascular risk factors with testosterone (T), calculated free testosterone (cFT), dihydrotestosterone (DHT), estradiol (E2), sex hormone binding globulin (SHBG) and luteinizing hormone (LH) in the CUDAS sample of community-dwelling men (Group A).

	T (nmol/L)		cFT (pmol/L)		DHT (nmol/L)		E2 (pmol/L)		SHBG (nmol/L)		LH (IU/L)	
	Est	p-value	Est	p-value	Est	p-value	Est	p-value	Est	p-value	Est	p-value
Diabetes	-1.217	0.351	-6.565	0.715	-0.234	0.244	27.543	0.051	-8.162	0.053	1.025	0.275
CVD history	-0.340	0.597	0.225	0.980	-0.089	0.371	9.851	0.158	-1.976	0.343	0.574	0.215
Systolic BP (mmHg, SD = 16.8)	0.168	0.432	3.059	0.300	-0.010	0.761	6.277	0.007	-0.607	0.381	-0.138	0.371
Hypertension	0.039	0.942	6.511	0.371	0.026	0.746	13.795	0.016	-4.424	0.009	0.560	0.140
Lipids medication	-0.778	0.257	-2.754	0.771	-0.153	0.147	3.605	0.629	-5.805	0.009	-0.416	0.400
Chol (mmol/L, SD = 0.97)	-0.244	0.189	-2.327	0.364	-0.051	0.074	-3.541	0.079	-0.986	0.101	-0.275	0.039
HDL (mmol/L, SD = 0.30)	0.212	0.295	-0.036	0.990	0.028	0.365	2.139	0.329	2.255	<0.001	-0.072	0.619
log Triglycerides (mmol/L, SD = 0.52)	-0.428	0.034	-1.370	0.623	-0.066	0.033	-3.109	0.156	-3.515	<0.001	-0.194	0.184
log CRP (mg/L, SD = 1.05)	-0.228	0.259	-3.938	0.158	-0.020	0.521	4.397	0.045	-0.220	0.738	0.040	0.782

Associations are adjusted for age, smoking, alcohol consumption and BMI. Table shows estimated difference in mean hormone level compared with reference category for categorical risk factors or for an increase of one standard deviation (SD) in continuous risk factors, and the associated *p*-value.

linear regression models as shown in Table 3. In the fully-adjusted models, higher total and free T concentrations were associated with lower CIMT in Group A (-0.011 mm and -0.013 mm per 1-SD increase in total and free T, $p=0.042$ and 0.014 respectively), but not in Group B. Higher E2 was associated with increased CIMT in Group A (0.013 mm per 1-SD increase in E2, $p=0.011$) but not in Group B. DHT, SHBG and LH

were not associated with CIMT in either group.

Associations of sex hormones with carotid plaque

Associations between hormone levels and the presence of carotid plaque were analysed using logistic regression models as shown in Table 4. Higher total and free T were associated with decreased odds of carotid plaque in Group A (fully-adjusted odds ratio

Table 3 Linear regression models examining associations of T, DHT, E2, SHBG and LH with carotid intima-media thickness (CIMT) in community-dwelling men (CUDAS, Group A) and in men with coronary artery disease (CUPID, Group B).

	CUDAS (Group A)			CUPID (Group B)		
	Estimate	95% CI	<i>p</i> -value	Estimate	95% CI	<i>p</i> -value
Total T						
Model 1	-0.011	(-0.021,-0.001)	0.039	0.004	(-0.008,0.016)	0.539
Model 2	-0.011	(-0.021,0.000)	0.042	0.004	(-0.008,0.016)	0.531
Free T						
Model 1	-0.012	(-0.023,-0.002)	0.018	0.007	(-0.004,0.019)	0.212
Model 2	-0.013	(-0.023,-0.003)	0.014	0.007	(-0.005,0.019)	0.237
DHT						
Model 1	-0.005	(-0.016,0.005)	0.324	-0.010	(-0.022,0.002)	0.095
Model 2	-0.004	(-0.014,0.007)	0.474	-0.009	(-0.021,0.002)	0.121
E2						
Model 1	0.013	(0.003,0.023)	0.009	-0.003	(-0.015,0.008)	0.577
Model 2	0.013	(0.003,0.023)	0.011	-0.003	(-0.015,0.008)	0.567
SHBG						
Model 1	-0.002	(-0.014,0.009)	0.673	-0.007	(-0.019,0.005)	0.246
Model 2	0.000	(-0.011,0.012)	0.961	-0.006	(-0.019,0.006)	0.298
LH						
Model 1	-0.005	(-0.015,0.005)	0.332	0.002	(-0.010,0.013)	0.774
Model 2	-0.004	(-0.014,0.006)	0.449	-0.001	(-0.013,0.011)	0.890

Table shows the estimate of the change in CIMT in mm, per increase of one SD in the hormonal variable, its 95% confidence interval (CI) and associated *p*-value. Model 1: Adjusted for age, smoking, alcohol consumption and BMI. Model 2: Adjusted for age, smoking, alcohol consumption, BMI, diabetes, CVD history (CUDAS only), systolic BP, hypertension, lipid medication, cholesterol, HDL, (log) triglycerides and (log) CRP.

Table 4 Logistic regression models examining associations of T, DHT, E2, SHBG and LH with carotid atheroma in community-dwelling men (CUDAS, Group A) and in men with coronary artery disease (CUPID, Group B).

	CUDAS (Group A)			CUPID (Group B)		
	Odds ratio	95% CI	<i>p</i> -value	Odds ratio	95% CI	<i>p</i> -value
Total T						
Model 1	0.685	(0.515,0.911)	0.009	1.021	(0.826,1.261)	0.851
Model 2	0.684	(0.508,0.921)	0.012	1.002	(0.800,1.254)	0.989
Free T						
Model 1	0.682	(0.507,0.918)	0.012	1.091	(0.879,1.355)	0.429
Model 2	0.657	(0.486,0.888)	0.006	1.074	(0.858,1.344)	0.535
DHT						
Model 1	0.875	(0.673,1.138)	0.319	0.771	(0.619,0.960)	0.020
Model 2	0.912	(0.710,1.172)	0.473	0.767	(0.610,0.965)	0.024
E2						
Model 1	0.975	(0.778,1.223)	0.829	0.776	(0.631,0.954)	0.016
Model 2	0.925	(0.728,1.175)	0.522	0.749	(0.605,0.929)	0.008
SHBG						
Model 1	0.768	(0.589,1.002)	0.051	0.859	(0.696,1.061)	0.160
Model 2	0.854	(0.645,1.130)	0.269	0.842	(0.670,1.058)	0.139
LH						
Model 1	0.927	(0.745,1.153)	0.496	0.969	(0.788,1.191)	0.766
Model 2	0.915	(0.733,1.143)	0.434	0.922	(0.735,1.156)	0.482

Table shows odds ratio for prevalent carotid plaque per increase of one SD in the hormonal variable, its 95% confidence interval (CI) and associated *p*-value. Model 1: Adjusted for age, smoking, alcohol consumption and BMI. Model 2: Adjusted for age, smoking, alcohol consumption, BMI, diabetes, CVD history (CUDAS/Group1 only), systolic BP, hypertension, lipid medication, cholesterol, HDL, (log) triglycerides and (log) CRP.

[OR] per 1-SD increase, $OR=0.68$, $p=0.012$ and $OR=0.66$, $p=0.006$ for total and free T respectively), but not in Group B. Higher DHT was associated with reduced odds of carotid plaque in Group B ($OR=0.77$, $p=0.024$) but not in Group A. Higher E2 was also associated with reduced odds of carotid plaque in Group B ($OR=0.75$, $p=0.008$) but not in Group A. Neither SHBG nor LH was associated with the presence of carotid plaque in either group.

Discussion

In community-dwelling men, there were moderate associations of higher T with lower TG levels, higher SHBG with a more favourable cardiovascular risk factor profile, and higher E2 with increased SBP and CRP. Higher T was associated with reduced CIMT and carotid plaque prevalence in community-dwelling men, while higher E2 was associated with increased CIMT in the same population. Interestingly, these associations were robust to adjustment for conventional cardiovascular risk factors and were not present in men with premature CAD. On the contrary, higher DHT and E2 were independently associated with reduced prevalence of carotid plaque in men with CAD. Despite the association of SHBG with cardiovascular risk factors in community-dwelling men, neither SHBG nor LH was associated with CIMT or carotid plaque in either group of men. These findings support a potential role for sex hormones in the pathogenesis of carotid atherosclerosis, independently of conventional cardiovascular risk factors.

Associations between sex hormones and preclinical atherosclerosis were markedly different based on cardiovascular phenotype in our study. Our results differ from several previously published studies. In the Tromsø and the Study of Health in Pomerania (SHIP) cohorts, there was no association between T and CIMT, and an inverse association between T and carotid plaque [13, 14]. An earlier analysis of the Tromsø cohort reported an inverse association between T and CIMT; however this association was no longer present following adjustment for BMI [11]. The Tromsø cohort was derived from community-dwelling men with a prevalence of CVD that was slightly higher than our community-dwelling group (17.5% vs 9.6%) [11]. The SHIP cohort was derived from a population characterised by a relatively high prevalence of cardiovascular risk factors, although the proportion of men with

CVD was not reported [14]. Testosterone was quantified using immunoassay in one population [13] and the other did not report the assay used [14]. Another study in middle-aged men also showed contrasting results, with no association seen between T with CIMT or carotid plaque [15]. That study had 17% of men with CVD and quantified hormone levels using immunoassay [15].

Our findings in community-dwelling men are consistent with several smaller studies of middle-aged men with symptomatic low T concentrations [10], men with Type 2 diabetes [12, 26], in overweight and obese glucose tolerant young men [27], and in healthy middle-aged men [28]. These studies showed an inverse association between T [10, 12], free T [26, 27] and bioavailable T [28] with CIMT. Of these studies, two did not report on the proportion of men with CVD [26, 28] while the rest excluded men with CVD [10, 12, 27]. In contrast to our findings, a study by van den Beld *et al* in older men reported inverse associations of T with CIMT which were comparable for men with or without CVD [29]. Our findings indicate that both total and free T are associated with favourable CIMT and reduced odds of carotid plaque in predominantly middle-aged community-dwelling men without a prior history of CVD. However, these associations between T and preclinical carotid atherosclerosis are not present in men with established premature CAD. Given the heterogeneity of previously studied cohorts, our results using LC-MS in two distinct and carefully phenotyped cohorts of men of comparable age may explain previous inconsistencies in the literature [10-15, 26-28].

With regards to the association of higher T with lower TG in community-dwelling men (Group A), this may be mediated *via* the relationship between higher T and more favourable body composition [30, 31]. High TG has been associated with reduced HDL, increased small dense LDL, production of proinflammatory cytokines, endothelial dysfunction, and thereby with increased cardiovascular risk [32]. Of note, in our multivariate model, the association of higher T with reduced CIMT in community-dwelling men was independent of conventional cardiovascular risk factors including (log) TG. This would suggest that the association of higher T with lower CIMT in this group of men is mediated by factors additional to TG.

Favourable associations between T and preclinical carotid atherosclerosis in our community-dwelling group is supported by experimental studies of T dem-

onstrating vasodilatory effects of T on rabbit coronary arteries and aorta [33]. It is possible that the influence of T on CIMT is relatively modest such that in men with advanced atherosclerosis who have stronger atherogenic risk factors, the impact of T is less. This may explain the lack of association of T and CIMT in men with premature CAD. However, we also found that higher DHT and E2 were associated with decreased prevalence of carotid plaque in men with premature CAD. Mechanistic studies of human aorta comparing advanced to early atherosclerotic lesions demonstrated that advanced lesions had decreased expression of mRNA coding for androgen receptor (AR) and increased expression of mRNA coding for estrogen sulfotransferase which catalyses the inactivation of estrogen [34]. In an animal model of apolipoprotein E-null mice, there were differential effects of T and DHT on estrogen receptor α (ER α) expression in the aortic sinus, with T decreasing and DHT increasing expression of ER α [35]. In this context, our observation that higher DHT and E2 were associated with decreased prevalence of carotid plaque in men with premature CAD is intriguing. In addition, we postulate that in men with premature CAD, DHT and E2 are better biomarkers for CVD risk, possibly reflecting altered tissue sensitivity to androgens *versus* estrogens.

Our findings for E2 are in contrast to prior studies of middle-aged men which report no association [10, 11, 15], or an inverse association between E2 and CIMT [19]. Another study consisting of healthy middle-aged men reported a positive association between E2 and CIMT progression [18]. Similarly, we found a positive cross-sectional association of E2 with CIMT in community-dwelling men even after accounting for the associations of E2 with SBP and CRP. These findings are supported by an observational study showing elevated risk of stroke with high levels of E2 [36]. Previous studies have mostly relied on E2 measured using immunoassay [11, 15, 18, 19], which has been shown to be unreliable for measurement of E2 in men [22]. Furthermore, studies assessing the association between E2 and carotid plaque in men with CAD are lacking. Our results based on E2 assayed using LC-MS, indicate an adverse association of E2 with CIMT in community-dwelling men, but a favourable association of E2 with reduced carotid plaque in men with CAD. This reflects findings in experimental studies associating estrogen with both anti- and pro-atherosclerotic effects, possibly modulated by estrogen levels, recep-

tor subtypes and cellular targets [37]. Therefore, further studies are needed to clarify whether E2 differentially modulates cardiovascular risk in men with either early or more advanced degrees of atherosclerosis.

Our data illuminate a potential role for DHT to influence risk of atherosclerosis, as higher DHT was associated with reduced prevalence of carotid plaque in men with CAD. This may reflect tissue actions of DHT, with experimental studies demonstrating attenuation of development of atherosclerosis with DHT exposure [38]. We have previously reported that higher DHT is associated with reduced mortality from ischaemic heart disease in older men [39], thus our current findings in younger men may be important for the consideration of future interventional studies. Of note, our results reinforce the importance of T, DHT and E2 rather than SHBG or LH, for risk stratification, particularly when LC-MS is available for the assay of these sex steroids.

The different association of sex hormones with CIMT compared with carotid plaque in each group is of interest. The underlying pathological processes of CIMT and carotid plaque may be distinct rather than representing a continuum of disease [9]. This may help to explain differences in associations seen in each group based on the use of CIMT or carotid plaque as an outcome. As expected, the prevalence of carotid plaque was higher in men with CAD (Group B) compared to community-dwelling men (Group A).

Strengths of our study include the availability of two reasonably large but distinct cohorts of men: one based on a random sample of community-dwelling men and the other men with angiographically confirmed CAD. Both cohorts were carefully phenotyped allowing systematic adjustment for potential confounders in the analyses. We assayed T, DHT and E2 using LC-MS, allowing the examination of these hormones with regard to CIMT and carotid atheroma. We acknowledge several important limitations of our study. This was an observational study with a cross-sectional analysis, which limits our ability to infer causality. Our results were analysed based on the single ultrasound measurement of CIMT and carotid atheroma, although we have expertise with the performance and interpretation of these measures [23]. Similarly, blood sampling for hormones was conducted at a single time point and we do not have serial measurements of hormone concentrations.

Our cohorts were derived from the urban population of men in Perth, Western Australia, thus further work

would be needed to before extrapolating our findings to men of differing geographic or racial backgrounds, and we cannot comment on any corresponding associations in women. Additionally, our results in men with premature onset of disease (aged 60 years or less) might not apply to older men with CAD.

Our results have implications for future research and translation into the clinical arena. Firstly, we have clarified that associations between T, DHT and E2 with CIMT and carotid plaque differ according to the presence or absence of premature CAD in middle-aged men. Secondly, we show that assessment of all three sex hormones may be more informative than T alone, especially in men with pre-existing CAD. Therefore, risk stratification for atherosclerosis should encompass accurate assay of all three sex steroids, and could differ according to whether CAD was absent or present. In a study by Basaria *et al*, older men with established comorbidities given T supplementation exhibited an excess of cardiovascular events [5]. However, in a study by Srinivas-Shankar *et al*, older men who were frail or intermediate frail were given T supplementation which improved physical performance without a signal for adverse cardiovascular events [6]. Thus, there is ongoing debate over potential risks of T supplementation in older men with pre-existing CVD. Our results suggest that there may be a role for earlier intervention in men before atherosclerosis becomes established. Further mechanistic and interventional studies are needed to examine the effects of exogenous T and its metabolites DHT and E2, on cardiovascular risk in middle-aged men with varying degrees of atherosclerosis.

Conclusions

T, DHT and E2 are differentially associated with preclinical carotid atherosclerosis in a cardiovascu-

lar phenotype-specific manner. Higher T is associated with more favourable CIMT and lower prevalence of carotid plaque in community-dwelling men, whilst higher DHT and E2 are associated with lower prevalence of carotid plaque in men with pre-existing CAD. Interventional studies are required to further clarify the effects of T, and its metabolites DHT and E2, on mechanisms of atherogenesis and disease progression in men with both favourable and adverse cardiovascular phenotypes.

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Disclosures

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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