



## Associations of cortisol/testosterone and cortisol/sex hormone-binding globulin ratios with atherosclerosis in middle-age women



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### ABSTRACT

**Background and aims:** The cortisol/testosterone (C/T) ratio has been hypothesized to be a better predictor of atherosclerosis than cortisol alone. No study has assessed whether the C/T and C/sex hormone-binding globulin (SHBG) ratios are associated with atherosclerosis in a U.S. population sample.

**Methods:** This substudy included 367 women who had both cortisol from year 15 and testosterone and SHBG at year 16 of the Coronary Artery Risk Development in Young Adults study, an ongoing observational cohort in the United States. Of these, intima-media thickness (IMT) was available at follow-up year 20 in 339 (n = 332 with measurement at carotid bulb), and 303 were free of prevalent coronary artery calcium (CAC) at year 15. Area under the curve (AUC) of salivary cortisol was available in 302 individuals. Ratios of AUCs of cortisol to total testosterone, free testosterone, and SHBG were categorized into tertiles. Associations with CAC and IMT were assessed by regression models adjusted for age, race, body mass index, systolic blood pressure, menopause, oral contraceptive use, diabetes, alcohol, and smoking.

**Results:** Only the highest tertile of the AUC/free testosterone ratio was positively associated with carotid bulb IMT ( $\beta = 0.088$ ,  $P = 0.006$ ). This tertile was also positively associated with new onset CAC between year 15 and 25 (OR 3.45, 95% CI 1.18–10.06). Tertiles of cortisol or testosterone alone were not associated with new onset CAC.

**Conclusion:** AUC/Free testosterone ratio may be more associated with atherosclerosis in women than either indicator alone. The ratio may serve as a suitable biomarker of cortisol-linked stress.

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### 1. Introduction

The concept of chronic stress as a risk factor for atherosclerosis has been suggested in both animal [1] and human studies [2,3]. Despite many years of research, it remains difficult to precisely assess chronic stress in humans due to measurement errors on self-reported questionnaires (validity concerns) and the inability of short-term measures of stress hormones (e.g., cortisol levels) to

reflect the chronic state or trait. These problems may explain why the association between chronic stress and atherosclerosis has been inconsistent in human studies [4].

The cortisol/testosterone (C/T) ratio has been used as a chronic biomarker of stress, and has been suggested to be a better predictor of coronary heart disease than cortisol alone [4]. There are several reasons why the C/T ratio might be a plausible indicator of chronic stress. Testosterone and cortisol are derived from the same biochemical precursor [5], so if cortisol synthesis increases, there will be a corresponding decline in testosterone synthesis because it is a competitive reaction process [4–6]. Cortisol can suppress the activity of the hypothalamic–pituitary–gonadal axis [7]. Cortisol has a catabolic effect, and testosterone has anabolic effects [4].

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Therefore the C/T ratio may more accurately capture endocrine dynamics than cortisol alone.

The aim of this study was to examine the associations of cortisol alone, testosterone alone, and the C/T ratio with subclinical atherosclerosis in women. We also studied C/sex hormone-binding globulin (SHBG) ratio to provide a more complete physiologic assessment because SHBG has a physiologic role of reducing free circulating testosterone in women [8,9]. Subclinical atherosclerosis was assessed using coronary artery calcium (CAC) and carotid intima-media thickness (IMT).

## 2. Material and methods

### 2.1. The coronary artery risk development in young adults (CARDIA) study

The CARDIA study is a multicenter, longitudinal cohort study of the development of coronary artery disease risk factors in young adults. A full description of the study design is published elsewhere [10]. The full CARDIA cohort included 5115 black and white adults aged 18–30 years at the year 0 examination (1985–1986), recruited from four metropolitan areas in the United States (Birmingham, AL; Chicago, IL; Minneapolis, MN; and Oakland, CA) [11]. Within each center, the sample was designed to comprise approximately equal numbers of participants by sex, race (self-defined: black or white), age (18–24 or 25–30 years), and education ( $\leq$ high school or  $>$ high school) [11]. Follow-up examinations (follow-up rates) were conducted at years 2 (91%), 5 (86%), 7 (81%), 10 (79%), 15 (74%), 20 (72%), and 25 (72%). All examinations were approved by institutional review boards at each institution, and informed consent was obtained from each study participant [11].

### 2.2. Study population

For this sub-study, we utilized data from two CARDIA ancillary studies that measured cortisol, testosterone, and SHBG to test our hypothesis. One ancillary study was conducted in year 15 at the Oakland and Chicago sites and included salivary cortisol measurement [12]. The second ancillary study was conducted in year 16 and included sex hormones and SHBG measurements. Together, the 2 studies provided a total of 367 women who defined the sub-study population. Of these 367 women (age range 32–51, mean age 40 years old), 28 were missing all data on IMT measurements (resulting  $N = 339$ ). Additionally 5 were missing data on confounding variables, and 27 were missing some but not all IMT measures. For the CAC analysis, 320 women had CAC data from both years 15 and 25, of whom 17 already had positive CAC findings at year 15. Therefore, 303 women were included in the analysis assessing CAC incidence from year 15 to year 25. Fig. 1 illustrates the study population.

### 2.3. Cortisol, testosterone, and ratios

The detailed cortisol measurement protocol was described elsewhere [13]. Participants were given materials and instructed regarding the collection of saliva samples at the conclusion of their year 15 follow-up CARDIA clinic visit [13]. Samples were collected from participants on a single weekday, in most cases the Monday after a Friday or Saturday clinic visit [13]. Participants were instructed not to eat, brush their teeth, or drink liquids for at least 15 min before collecting a sample [14]. They were provided six saliva sample containers to be used over the course of the day: upon awakening (“when your eyes open and you are ready to get up”), 45 min, 2.5 h, 8 h, and 12 h after awakening, and at bedtime (“right before getting into bed”) [13]. Participants were instructed

to record the time they woke up in a log and were provided alarm watches (preset to their regular wakeup time) to remind them to collect samples; they were also given a form that allowed them to easily recalculate the desired sample times if they woke up at a different time than anticipated [13]. Cortisol concentrations were determined by time-resolved immunoassays with fluorometric end point detection [13]. Intra- and interassay variabilities were each less than 12%.

Area under the curve (AUC) for cortisol, a time-adjusted measure of total cortisol exposure while awake, is thought to reflect cumulative tissue exposure to cortisol across the day; persistently high total daily output may create “wear and tear” on various body tissues, resulting in structural or functional changes that could affect disease vulnerability [15]. On the other hand, the additional use of diurnal slopes attempts to capture cortisol circadian fluctuation patterns. The slope is usually operationally defined as the line resulting from regression of cortisol values collected across the day onto hours since awakening excluding the morning awakening response [15]. A negative diurnal slope is generally considered indicative of healthy hypothalamus–pituitary–adrenal (HPA) axis function, with a flattened or positive diurnal slope suggestive of potential HPA axis dysfunction [15]. AUC and slope were chosen for the chronic cortisol indices because they have greater 12-month stability than cortisol awakening response [15]. One study reported 12-month intra-class correlation coefficients for AUC of about 0.5 and for slope of about 0.25 [15].

The AUC for cortisol was calculated as the plot of log-transformed cortisol values against collection times from the first to the last sample [13]. The AUC was computed only for those people who had data for the first sample and a minimum of 12 h between their first and last samples [13]. The cortisol slope was calculated for those who had the first sample and the sixth sample as reported previously [13]. All 367 women in the substudy had both 1st and 6th samples. The slope was estimated by separately fitting a linear regression line for each participant that predicted the log-transformed cortisol concentrations from time (hours) since awakening [13]. To minimize the impact of morning rise in cortisol on slope estimation, the second saliva sample was excluded from the slope calculation [13].

Detailed testosterone and SHBG measurement protocols are published elsewhere [16]. Briefly, SHBG, total testosterone, and free testosterone were measured in a single batch on serum specimens collected at year 16 in the CARDIA Women’s Study [16]. SHBG was determined using equilibrium dialysis on a Sephadex G-25 column [16]. This method estimates the amount of testosterone capable of being bound by SHBG [16]. Total testosterone was measured with a competitive immunoassay (Bayer Diagnostics, Tarrytown, NY) that employed direct chemiluminescent technology on the ACS:180 automated chemiluminescent system (Beckman Coulter, Brea, CA). The coefficient of variation (CV) for this assay is less than 10% for total testosterone  $>50$  ng/dL, while The College of American Pathologists document an inter-assay CV of 13.4% for 30 ng/dL samples with the Beckman Coulter system, the lowest of 16 systems surveyed [16]. Due to imprecision at the lower end of the detection limit of the assay, total testosterone levels below 10.0 ng/dL were all reported as 5 ng/dL [16]. Free testosterone which is used for estimation of free testosterone level in women [9] was calculated based on measured total testosterone and SHBG levels using the method described by Pearlman [16,17]. The analyses only included measures obtained from women who reported that they were not pregnant.

The following ratios of AUC and of slope of cortisol to total testosterone, free testosterone, and SHBG were computed: AUC/

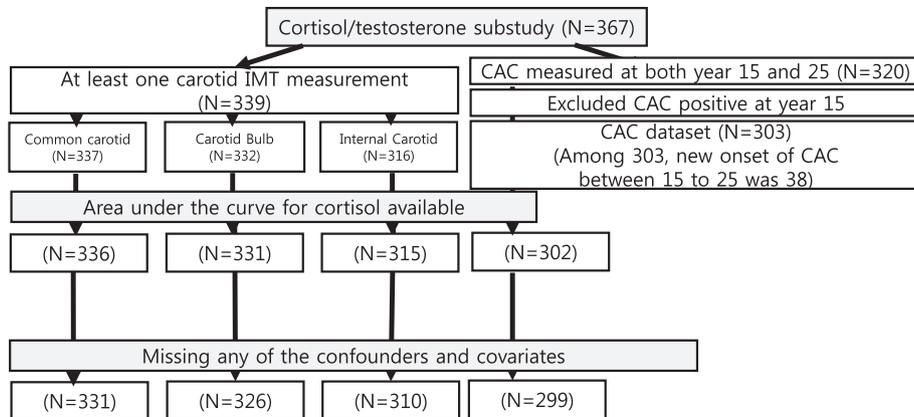


Fig. 1. Flow chart of participants for analyses in the CARDIA cortisol/testosterone substudy.

total testosterone, AUC/free testosterone, AUC/SHBG, slope/total testosterone, slope/free testosterone, and slope/SHBG.

#### 2.4. Measures of subclinical atherosclerosis (CAC, IMT)

Subclinical atherosclerosis was measured using 2 techniques (CAC, carotid IMT). First, CAC was measured at the CARDIA follow-up examinations at years 15, 20, and 25 [18]. CAC measurement was performed with an electrocardiographically gated multidetector computed tomography scanner with a standard phantom for calibration using a standardized protocol [19] with published accuracy, comparability, and reproducibility [18,20,21]. Briefly, scans were obtained, and image analysts (blinded to participant characteristics) calculated a total CAC score using a modified Agatston method with select overreading by a physician who was an expert in cardiovascular imaging [18]. The presence of CAC is defined as an Agatston score greater than 0 (CAC negative stands for a score of zero, whereas any value above zero was considered as CAC positive) [16,22] as previously suggested to predict CVD in low-risk women [16,23]. Incident CAC from year 15–25 was defined as no CAC at year 15 and newly developed CAC at year 25. We also performed analyses for associations with prevalent CAC at years 15, 20, and 25.

IMT measures of the common carotid artery (CCA), carotid bulb (CB), and internal carotid artery (ICA) were obtained at the CARDIA follow-up year 20 examination [16]. Carotid B-mode ultrasound examinations were conducted by trained sonographers at each field center employing a standard protocol using the GE Logiq 700 device (GE Medical Systems, Wauwatosa, WI). The carotid ultrasound procedures were performed in the supine position with the participant's head rotated 45° away from the side of study. Magnified longitudinal images in gray-scale of the far and near wall of the distal CCA, CB, and proximal ICA were obtained on the right and left sides. Images were read at the ultrasound central reading center (Tufts Medical Center, Boston, MA) by individuals blinded to all clinical information. The maximum IMT of each segment was defined as the mean of the maximal IMT of the near and far walls of both the left and right sides.

#### 2.5. Confounders and covariates

Demographic measures included age, sex, and race obtained at the year 15 examination by self-report. Height and weight were measured with the participant wearing light clothing with no shoes, and body mass index (BMI) was computed by dividing the weight (kg) by the height squared (m<sup>2</sup>). Seated blood pressure (BP) was measured using a random zero sphygmomanometer for the

year 15 examination [18]. After a 5-min rest in a quiet room, blood pressure was measured on the right arm at three 1-min intervals; the average of the second and third measurements was used [18]. Menopausal status was self-reported. Use of oral contraceptives (yes, no, or missing) was obtained at exam year 16. Glucose was assayed using hexokinase coupled to glucose-6 phosphate dehydrogenase. Diabetes was defined by fasting glucose  $\geq 126$  mg/dL or medication use [24]. Cigarettes smoked per day were self-reported at the year 15 examination. Alcohol intake (ml/d) was computed from self-reported frequency of consumption of beer, wine, and liquor per week at the year 15 examination [25].

#### 2.6. Statistical analysis

Data are presented as means and standard deviations or proportions. The associations of C/T and C/SHBG, categorized into tertiles, with CAC and IMT were assessed by logistic regression analysis (CAC) and multiple linear regression (IMT). Model I controlled for age and race. Model II controlled for model I variables plus body mass index (BMI), systolic blood pressure (BP), menopause, oral contraceptive usage, diabetes mellitus, alcohol consumption, and cigarette smoking. We additionally analyzed for dose-response relationship on incident CAC by trend test across tertiles (that is, by assigning the median AUC/hormone or slope/hormone value of each tertile as its value and treating this as a continuous variable in the regression model). All statistical analyses were performed using SAS version 9.4 (SAS Inc., Cary, NC, USA). A two-tailed test with  $p < 0.05$  was considered to be statistically significant.

### 3. Results

The study participants' characteristics at year 15 are shown in Table 1. Participants mean age was 40 years old (range 32–51) and 59.4% reported black race.

#### 3.1. Associations with prevalent CAC

The prevalence of CAC at year 25 was 16.6% (N = 54). The highest tertile of AUC/SHBG ratio was associated with year 25 CAC prevalence in model I (OR 2.28, 95% CI 1.05–4.95). The association was no longer significant after adjusting for age, race, menopause, oral contraceptive use, BMI, systolic BP, diabetes, alcohol drinking, and cigarette smoking (OR 1.76, 95% CI 0.70–4.42). (Data are not shown in the tables). Separate analyses of the unadjusted and adjusted associations of AUC of cortisol, slope of cortisol, total testosterone,

**Table 1**  
Baseline characteristics in the 367 CARDIA females in the sub-study of cortisol/testosterone ratio.

Variable	
Age, mean years $\pm$ S.D.	40.23 $\pm$ 3.66
Race, black number (%)	218 (59.40%)
Menopause, number (%)	28 (7.63%)
Cortisol (AUC), nmol/hour, logged, mean $\pm$ S.D.	2.08 $\pm$ 0.51
Cortisol (Slope), nmol/hour, logged, mean $\pm$ S.D.	-0.08 $\pm$ 0.06
Total testosterone <sup>a</sup> , ng/dL mean $\pm$ S.D.	24.58 $\pm$ 19.22
Free testosterone <sup>a</sup> , ng/dL mean $\pm$ S.D.	0.19 $\pm$ 0.15
SHBG <sup>a</sup> , nmol/liter mean $\pm$ S.D.	28.13 $\pm$ 13.15
Oral contraceptive use <sup>a</sup> , number (%)	58 (15.85%)
Body mass index, kg/m <sup>2</sup> mean $\pm$ S.D.	30.10 $\pm$ 8.42
Systolic BP, mmHg mean $\pm$ S.D.	113.34 $\pm$ 15.28
Diabetes Mellitus, number (%)	24 (6.56%)
Cigarette smoking, cigarettes/day mean $\pm$ S.D.	2.06 $\pm$ 5.33
Alcohol drinking, ml/day mean $\pm$ S.D.	6.67 $\pm$ 15.47

<sup>a</sup> Results from Year 16.

free testosterone, and SHBG with CAC prevalence showed no statistically significant associations (Supplementary Table 1).

### 3.2. Associations with incident CAC

The incidence of new CAC between years 15–25 was 12.5% (N = 38). The highest tertile of the AUC/free testosterone ratio had a nonsignificant OR with incident CAC from year 15–25 in model I (Table 2, OR 2.22, 95% CI 0.89–5.51) and had a significant positive association in model II (OR 3.45, 95% CI 1.18–10.06). In model II, the AUC/free testosterone ratio showed a dose-response relationship with the second tertile being intermediate between the first and third tertiles (p for trend = 0.04). Associations of slope/total testosterone, slope/free testosterone, and slope/SHBG with incident CAC were not significant (Supplementary Table 2). Separate analyses of AUC for cortisol, slope of cortisol, total testosterone, free testosterone, and SHBG with CAC incidence showed no statistically significant associations (Supplementary Table 3).

**Table 2**  
Odds ratios for incident coronary artery calcification between year 15 and 25 and AUC of cortisol/T (or AUC of cortisol/SHBG) ratio.

Ratio	Year 15–25 incidence (N = 38)					
	Model I <sup>a</sup> (N = 302) <sup>b</sup>			Model II <sup>c</sup> (N = 299) <sup>d</sup>		
	OR	95% CI	P Trend	OR	95% CI	P trend
AUC/total testosterone						
Tertile 1	1.00	Reference	0.36	1.00	Reference	0.15
Tertile 2	1.51	(0.61–3.74)		1.24	(0.46–3.34)	
Tertile 3	1.68	(0.69–4.07)		2.01	(0.75–5.44)	
AUC/free testosterone						
Tertile 1	1.00	Reference	0.15	1.00	Reference	0.04
Tertile 2	1.82	(0.70–4.70)		2.21	(0.77–6.33)	
Tertile 3	2.22	(0.89–5.51)		3.45	(1.18–10.06)	
AUC/SHBG						
Tertile 1	1.00	Reference	0.04	1.00	Reference	0.09
Tertile 2	1.27	(0.48–3.35)		0.79	(0.26–2.41)	
Tertile 3	2.45	(0.98–6.13)		1.98	(0.69–5.66)	

Abbreviation C/T ratio; cortisol/testosterone ratio, SHBG; sex hormone binding globulin, AUC; area under the curve of six salivary cortisol.

<sup>a</sup> Adjusted for age, race.

<sup>b</sup> One person out of 303 is missing AUC.

<sup>c</sup> Adjusted for age, race, menopause, oral contraceptive use, BMI, systolic BP, diabetes, alcohol drinking, and cigarette smoking.

<sup>d</sup> 3 people were missing data on confounders or covariates.

### 3.3. Associations with carotid IMT

Table 3 and Supplementary Table 4 show the associations between IMT and C/T (or SHBG) ratio. We observed some significant associations in model I. AUC/free testosterone, which was associated with incident CAC (Table 2), was also associated with the year 20 average maximum CB in both model I ( $\beta = 0.068$ ,  $p = 0.03$ ) and model II ( $\beta = 0.088$ ,  $p < 0.01$ , Table 3). In model II, the AUC/free testosterone ratio showed a dose-response relationship with the second tertile ( $\beta = 0.059$ ,  $p = 0.05$ ) being intermediate between the first and the third tertiles. Separate analyses of AUC of cortisol, slope of cortisol, total testosterone, free testosterone, and SHBG with IMT prevalence are shown in Supplementary Table 5, and no significant results were found. The second and third tertiles of SHBG were associated with the average maximum CB. The highest free testosterone tertile was inversely associated with average maximum CB.

## 4. Discussion

In this CARDIA substudy of U.S. middle-age black and white women, we examined the associations between various C/T ratios and prospective incident CAC and carotid IMT. There were no significant associations with incident CAC or IMT for most of the C/T ratios. We found significant associations for: 1) highest tertile of AUC/free testosterone ratio with incident CAC in model II, 2) highest tertile of AUC/free testosterone ratio with IMT of carotid bulb in models I and II, 3) 2nd tertile of slope/total testosterone ratio with IMT of CCA and ICA in model I, 4) 2nd tertile of slope/free testosterone ratio with IMT of CCA in models I and II, and 5) 3rd tertile of slope/free testosterone ratio with IMT of CCA in model I. In separate analyses of cortisol and testosterone with incident CAC, we found only non-significant results.

To the best of our knowledge, there are no previous studies that have examined the relationship of the C/T ratio to measures of subclinical atherosclerosis. However if we expand the endpoint to include clinical cardiovascular disease, there was one previous population-based study that showed direct associations between the serum C/T ratio and incident cardiovascular disease. In the Caerphilly cohort study [4] (2512 men, aged 45–59 years old, with a mean follow-up of 16.5 years), a positive linear trend was seen between serum C/T ratio for incident ischemic heart disease (age-adjusted OR per z score change in ratio 1.22, 95% CI 1.07 to 1.38,  $P = 0.003$ ). However, after adjustment for confounders, their results were no longer statistically significant.

In contrast to the limited previous research on C/T ratio, many prior studies have found significant associations of SHBG with subclinical atherosclerosis [16,26]. Although SHBG plays a physiological role of reducing circulatory testosterone level in women [8,9], there were no previous studies assessing C/SHBG ratio. One of the earlier findings from the CARDIA Women's study showed an inverse association of SHBG (mean value of year 2, 10, and 16) with both CAC and IMT [16]. This previous study did not find a significant association of CAC or IMT with either total testosterone or free testosterone [16]. Our study had consistent results as the prior study with total testosterone and free testosterone, but not for SHBG. In our study, SHBG was positively associated with carotid bulb IMT; however, we did not find the association of SHBG with incident CAC. Our study also failed to show a significant association of C/SHBG with incident CAC. Possible explanations for the inconsistent results within CARDIA include differences in sample sizes between substudies (our study is 20% of previous study sample size because of within 1 year cortisol availability), differences in how CAC and SHBG were characterized (year 2, 10, 16 versus year 16), and differences in the selection of covariates included in the

**Table 3**

Beta-coefficients for the associations between intima-media thickness and AUC of cortisol/T (or AUC of cortisol/SHBG) ratio.

	Average max CCA (mm)				Average max bulb (mm)				Average max ICA (mm)			
	Model I <sup>a</sup> (N = 336)		Model II <sup>b</sup> (N = 331)		Model I <sup>a</sup> (N = 331)		Model II <sup>b</sup> (N = 326)		Model I <sup>a</sup> (N = 315)		Model II <sup>b</sup> (N = 310)	
	Beta (s.e)	p	Beta (s.e)	p	Beta (s.e)	p	Beta (s.e)	p	Beta (s.e)	p	Beta (s.e)	p
AUC/TT												
Tertile 1	Ref		Ref		Ref		Ref		Ref		Ref	
Tertile 2	−0.001 (0.015)	0.95	0.004 (0.014)	0.75	−0.038 (0.031)	0.21	−0.041 (0.030)	0.17	−0.032 (0.027)	0.24	−0.023 (0.027)	0.40
Tertile 3	−0.028 (0.015)	0.06	−0.014 (0.014)	0.33	0.020 (0.031)	0.51	0.037 (0.031)	0.24	−0.021 (0.027)	0.44	−0.007 (0.028)	0.80
AUC/FT												
Tertile 1	Ref		Ref		Ref		Ref		Ref		Ref	
Tertile 2	0.007 (0.015)	0.63	0.018 (0.014)	0.21	0.050 (0.031)	0.11	0.059 (0.031)	0.05	−0.019 (0.028)	0.49	−0.011 (0.028)	0.71
Tertile 3	−0.026 (0.015)	0.09	−0.005 (0.015)	0.72	0.068 (0.032)	0.03	0.088 (0.032)	<0.01	−0.010 (0.028)	0.72	0.010 (0.029)	0.72
AUC/SHBG												
Tertile 1	Ref		Ref		Ref		Ref		Ref		Ref	
Tertile 2	0.028 (0.015)	0.06	0.001 (0.015)	0.96	0.005 (0.031)	0.88	−0.021 (0.032)	0.50	0.042 (0.027)	0.12	0.015 (0.028)	0.61
Tertile 3	0.020 (0.015)	0.20	−0.016 (0.016)	0.30	−0.012 (0.032)	0.70	−0.046 (0.034)	0.17	0.019 (0.028)	0.50	−0.017 (0.031)	0.59

Abbreviation C/T ratio; cortisol/testosterone ratio, SHBG; sex hormone binding globulin, AUC; area under the curve of six salivary cortisol, TT: total testosterone, FT; Free testosterone, CCA; common carotid artery, Bulb; carotid bulb, ICA; internal carotid artery.

<sup>a</sup> Adjusted for age, race.

<sup>b</sup> Adjusted for age, race, menopause, oral contraceptive use, BMI, systolic BP, diabetes, alcohol drinking, and cigarette smoking.

association models.

The present study has several strengths. This is the first study assessing the C/T ratio (and C/SHBG ratio) in women, and it is also the first to demonstrate an association between the C/T ratio and subclinical atherosclerosis using data from a carefully conducted epidemiologic U.S. study. We analyzed both prospective IMT and incident CAC, which are separate but complementary measures of subclinical atherosclerosis. Notably, we performed six salivary cortisol measurements in a single day rather than one measure of serum cortisol. Since cortisol exhibits a marked diurnal pattern, multiple measurements as reported here provide a more complete assessment of cortisol secretion.

It is worth considering why C/T ratio may improve prediction compared to either measure alone. Testosterone and cortisol are derived from the same biochemical precursor [5], and secretion of cortisol and testosterone are inversely related [4]: If cortisol synthesis increases, testosterone synthesis should decline because it is a competitive reaction [4–6]. Recent studies have demonstrated additional inter-relationships of cortisol and testosterone [7]. In the presence of cortisol elevation due to chronic stress via hypothalamic-pituitary-adrenal axis [27], cortisol affects testosterone in multiple ways [7] (Fig. 2). Cortisol suppresses the activity of hypothalamic-pituitary-gonadal axis, cortisol inhibits the action of testosterone on target tissue, and cortisol down regulates androgen receptors [7]. Accordingly, chronic stress leads to prolonged suppression of testosterone function [27]. Therefore the C/T ratio may capture more accurately endocrine dynamics than cortisol or testosterone alone. The connection between the C/T ratio and atherosclerosis is plausible through insulin resistance [4] and inflammation [28]. Cortisol is associated with an adverse effect (catabolic) on insulin resistance and cardiovascular risk factors (e.g. lipids), and testosterone is associated with favorable (anabolic) effects [4]. Also, it is possible that stress itself increases the proliferation of primitive hematopoietic progenitors, giving rise to higher levels of disease-promoting inflammatory leukocytes [28]. In a study of atherosclerosis-prone Apolipoprotein E (−/−) mice, chronic stress accelerated hematopoiesis and promoted plaque

features associated with lesions that cause myocardial infarction and stroke in humans [28].

We can only speculate as to why only the AUC of cortisol was significantly associated with both of the atherosclerosis measures (CAC and IMT) whereas slope of cortisol was not significant for both. The AUC is thought to reflect cumulative tissue exposure to cortisol across the day; persistently high total daily output may create “wear and tear” in various body tissues, resulting in structural or functional changes that could affect disease vulnerability [15]. On the other hand, diurnal slope attempts to capture a characteristic of the cortisol circadian pattern [15]. Abnormal patterns of cortisol slope (flat slope) can occur for a number of reasons. In some people, cortisol does not return to baseline levels as one would expect over the course of the day (high level). In other individuals, there may be a failure to exhibit the usual cortisol awakening response and thus cortisol in these people is low throughout the day. These opposite patterns may have the same cortisol slope but very different cortisol AUC. It is also worth considering why only the AUC/free testosterone ratio was significant whereas AUC/total testosterone and AUC/SHBG were not significant. We used total and free testosterone measurements for calculating C/T ratios. Total testosterone measures all circulating forms of testosterone, whereas free testosterone measures only the unbound form of testosterone. Although they are both measurements of testosterone, physiologic functions are different. Free testosterone is the biologically active form; therefore the AUC/free testosterone ratio possibly represents the most physiologically appropriate measure. It is also possible that cortisol/testosterone regulation is due to mechanisms other than SHBG binding in women [8,9] (Fig. 2) such as downregulation of the androgen receptor or inhibition of the action of T on target tissue [7]. Therefore, this may account for the observation here that AUC/SHBG did not show significant results.

Several limitations should be considered. First, the sample size is small due to combining two ancillary studies. Second, we used year 15 cortisol and year 16 testosterone and SHBG. According to a previously reported study of cortisol stability [15,29], the 12-month

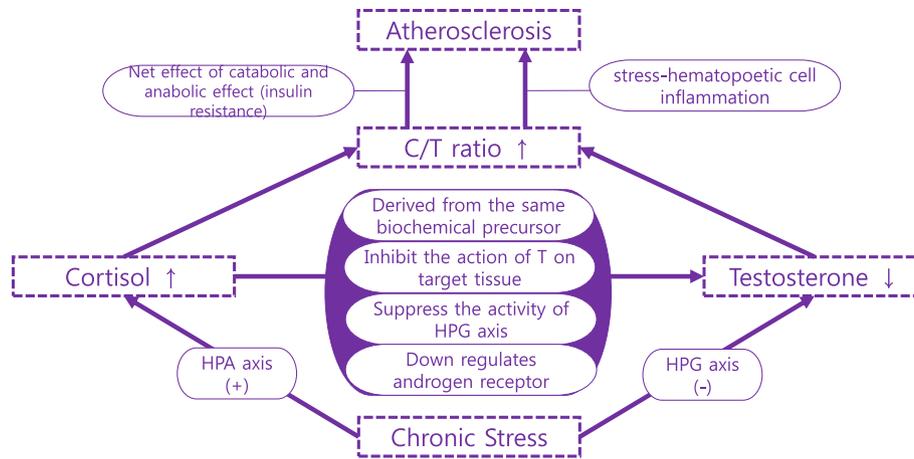


Fig. 2. Possible mechanisms associated with cortisol/testosterone ratio.

intraclass correlation coefficient is about 0.5 for AUC [15], and about 0.25 for the slope [15], so our inability to utilize concurrent results may have weakened the associations. Third, the majority of women (92%) were premenopausal with low total testosterone and free testosterone levels. Fourth, we adjusted for a large numbers of confounders and covariates given the size of our study population. These limitations may also have weakened the associations. However, the significant results may show genuine relationships considering biological mechanisms.

In conclusion, the AUC/free testosterone ratio may be a better indicator of subclinical atherosclerosis than either cortisol or testosterone alone, and the highest tertile of AUC/free testosterone ratio may be positively associated with subclinical atherosclerosis. If this finding is replicated in other studies, the AUC/free testosterone ratio may be a useful measure of cortisol linked chronic stress in population studies. In addition, our findings suggest that this measure of chronic stress is associated with some measures of subclinical atherosclerosis in young women.

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### Disclosure

None.

### Abstract presentation

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2016.03.028>.

### References

- [1] J.R. Kaplan, S.B. Manuck, T.B. Clarkson, F.M. Lusso, D.M. Taub, Social status, environment, and atherosclerosis in cynomolgus monkeys, *Arteriosclerosis (Dallas, Tex)* 2 (1982) 359–368.
- [2] S. Cohen, D. Janicki-Deverts, G.E. Miller, Psychological stress and disease, *JAMA* 298 (2007) 1685–1687.
- [3] H. Iso, C. Date, A. Yamamoto, H. Toyoshima, N. Tanabe, S. Kikuchi, T. Kondo, Y. Watanabe, Y. Wada, T. Ishibashi, H. Suzuki, A. Koizumi, Y. Inaba, A. Tamakoshi, Y. Ohno, Perceived mental stress and mortality from cardiovascular disease among Japanese men and women: the Japan Collaborative Cohort Study for Evaluation of Cancer Risk Sponsored by Monbusho (JACC Study), *Circulation* 106 (2002) 1229–1236.
- [4] G.D. Smith, Y. Ben-Shlomo, A. Beswick, J. Yarnell, S. Lightman, P. Elwood, Cortisol, testosterone, and coronary heart disease: prospective evidence from the Caerphilly study, *Circulation* 112 (2005) 332–340.
- [5] W. Boron, *Medical Physiology: a Cellular and Molecular Approach*, Elsevier/Saunders, 2003.
- [6] X.F. Li, J.C. Mitchell, S. Wood, C.W. Coen, S.L. Lightman, K.T. O'Byrne, The effect of oestradiol and progesterone on hypoglycaemic stress-induced suppression of pulsatile luteinizing hormone release and on corticotropin-releasing hormone mRNA expression in the rat, *J. Neuroendocrinol.* 15 (2003) 468–476.
- [7] P.H. Mehta, R.A. Josephs, Testosterone and cortisol jointly regulate dominance: evidence for a dual-hormone hypothesis, *Horm. Behav.* 58 (2010) 898–906.
- [8] Shlomo Melmed, Kenneth S. Polonsky, P. Reed Larsen, H.M. Kronenberg, *Williams Textbook of Endocrinology*, twelfth ed., Saunders, 2012.
- [9] W. Rosner, R.J. Auchus, R. Azziz, P.M. Sluss, H. Raff, Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement, *J. Clin. Endocrinol. Metab.* 92 (2007) 405–413.
- [10] G.D. Friedman, G.R. Cutter, R.P. Donahue, G.H. Hughes, S.B. Hulley, D.R. Jacobs Jr., K. Liu, P.J. Savage, CARDIA: study design, recruitment, and some characteristics of the examined subjects, *J. Clin. Epidemiol.* 41 (1988) 1105–1116.
- [11] L.L. Yan, K. Liu, K.A. Matthews, M.L. Daviglius, T.F. Ferguson, C.I. Kiefe, Psychosocial factors and risk of hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) study, *JAMA* 290 (2003) 2138–2148.
- [12] P. Ouyang, D. Vaidya, A. Dobs, S.H. Golden, M. Szklo, S.R. Heckbert, P. Kopp, S.M. Gapstur, Sex hormone levels and subclinical atherosclerosis in postmenopausal women: the Multi-Ethnic Study of Atherosclerosis,

- Atherosclerosis* 204 (2009) 255–261.
- [13] K. Matthews, J. Schwartz, S. Cohen, T. Seeman, Diurnal cortisol decline is related to coronary calcification: CARDIA study, *Psychosom. Med.* 68 (2006) 657–661.
- [14] S. Cohen, J.E. Schwartz, E. Epel, C. Kirschbaum, S. Sidney, T. Seeman, Socio-economic status, race, and diurnal cortisol decline in the Coronary Artery Risk Development in Young Adults (CARDIA) Study, *Psychosom. Med.* 68 (2006) 41–50.
- [15] K.M. Ross, M.L. Murphy, E.K. Adam, E. Chen, G.E. Miller, How stable are diurnal cortisol activity indices in healthy individuals? evidence from three multi-wave studies, *Psychoneuroendocrinology* 39 (2014) 184–193.
- [16] R. Calderon-Margalit, S.M. Schwartz, M.F. Wellons, C.E. Lewis, M.L. Daviglius, P.J. Schreiner, O.D. Williams, B. Sternfeld, J.J. Carr, D.H. O'Leary, S. Sidney, Y. Friedlander, D.S. Siscovick, Prospective association of serum androgens and sex hormone-binding globulin with subclinical cardiovascular disease in young adult women: the "Coronary Artery Risk Development in Young Adults" women's study, *J. Clin. Endocrinol. Metab.* 95 (2010) 4424–4431.
- [17] J.K. Bower, P.J. Schreiner, B. Sternfeld, C.E. Lewis, Black-White differences in hysterectomy prevalence: the CARDIA study, *Am. J. Public Health* 99 (2009) 300–307.
- [18] N.B. Allen, J. Siddique, J.T. Wilkins, C. Shay, C.E. Lewis, D.C. Goff, D.R. Jacobs Jr., K. Liu, D. Lloyd-Jones, Blood pressure trajectories in early adulthood and subclinical atherosclerosis in middle age, *JAMA* 311 (2014) 490–497.
- [19] J.J. Carr, J.C. Nelson, N.D. Wong, M. McNitt-Gray, Y. Arad, D.R. Jacobs Jr., S. Sidney, D.E. Bild, O.D. Williams, R.C. Detrano, Calcified coronary artery plaque measurement with cardiac CT in population-based studies: standardized protocol of Multi-Ethnic Study of Atherosclerosis (MESA) and Coronary Artery Risk Development in Young Adults (CARDIA) study, *Radiology* 234 (2005) 35–43.
- [20] J.J. Carr, J.R. Crouse 3rd, D.C. Goff Jr., R.B. D'Agostino Jr., N.P. Peterson, G.L. Burke, Evaluation of subsecond gated helical CT for quantification of coronary artery calcium and comparison with electron beam CT, *AJR Am. J. Roentgenol.* 174 (2000) 915–921.
- [21] R.C. Detrano, M. Anderson, J. Nelson, N.D. Wong, J.J. Carr, M. McNitt-Gray, D.E. Bild, Coronary calcium measurements: effect of CT scanner type and calcium measure on rescan reproducibility—MESA study, *Radiology* 236 (2005) 477–484.
- [22] D.E. Bild, R. Detrano, D. Peterson, A. Guerci, K. Liu, E. Shahar, P. Ouyang, S. Jackson, M.F. Saad, Ethnic differences in coronary calcification: the Multi-Ethnic Study of Atherosclerosis (MESA), *Circulation* 111 (2005) 1313–1320.
- [23] S.G. Lakoski, P. Greenland, N.D. Wong, P.J. Schreiner, D.M. Herrington, R.A. Kronmal, K. Liu, R.S. Blumenthal, Coronary artery calcium scores and risk for cardiovascular events in women classified as "low risk" based on Framingham risk score: the multi-ethnic study of atherosclerosis (MESA), *Arch. Intern. Med.* 167 (2007) 2437–2442.
- [24] C.D. Lee, D.R. Jacobs Jr., P.J. Schreiner, C. Iribarren, A. Hankinson, Abdominal obesity and coronary artery calcification in young adults: the Coronary Artery Risk Development in Young Adults (CARDIA) Study, *Am. J. Clin. Nutr.* 86 (2007) 48–54.
- [25] A.R. Dyer, G.R. Cutter, K.Q. Liu, M.A. Armstrong, G.D. Friedman, G.H. Hughes, J.J. Dolce, J. Raczynski, G. Burke, T. Manolio, Alcohol intake and blood pressure in young adults: the CARDIA study, *J. Clin. Epidemiol.* 43 (1990) 1–13.
- [26] S.H. Golden, B.N. Sanchez, M. Wu, S. Champaneri, A.V. Diez Roux, T. Seeman, G.S. Wand, Relationship between the cortisol awakening response and other features of the diurnal cortisol rhythm: the Multi-Ethnic Study of Atherosclerosis, *Psychoneuroendocrinology* 38 (2013) 2720–2728.
- [27] I. Kyrou, C. Tsigos, Chronic stress, visceral obesity and gonadal dysfunction, *Hormones (Athens, Greece)* 7 (2008) 287–293.
- [28] T. Heidt, H.B. Sager, G. Courties, P. Dutta, Y. Iwamoto, A. Zaltsman, C. von Zur Muhlen, C. Bode, G.L. Fricchione, J. Denninger, C.P. Lin, C. Vinegoni, P. Libby, F.K. Swirski, R. Weissleder, M. Nahrendorf, Chronic variable stress activates hematopoietic stem cells, *Nat. Med.* 20 (2014) 754–758.
- [29] X. Wang, B.N. Sanchez, S.H. Golden, S. Shrager, C. Kirschbaum, A.S. Karlamangla, T.E. Seeman, A.V. Roux, Stability and predictors of change in salivary cortisol measures over six years: MESA, *Psychoneuroendocrinology* 49 (2014) 310–320.