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Article

Urinary equol is associated with bioavailable testosterone but not total testosterone in women

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Abstract: Little is known about the association between equol and bioavailable testosterone (BT) in adults. We examined the associations of urinary equol concentrations with serum total, bioavailable and free testosterone (FT), dehydroepiandrosterone sulfide (DHEAS), free androgen index (FAI) and sex hormone-binding globulin (SHBG) concentrations. This cross-sectional study included 1904 women aged 59.7 years. Urinary equol and serum sex hormone concentrations were measured. Overall, urinary equol tended to be inversely associated with bioactive forms of androgenic indices (BT, FT or FAI) but not with total testosterone (TT) or DHEAS. Urinary equol was also positively associated with SHBG. In multi-covariate-adjusted analyses stratified by menopausal status, graded and inverse associations between urinary equol and bioactive forms of androgenic indices (BT, FT and FAI) were observed in postmenopausal women (all *p*-trends <0.05), but not in premenopausal women. A significant positive association between urinary equol and SHBG was observed only in postmenopausal women. No significant associations were observed between urinary equol and TT or DHEAS in either group. A path analysis indicated that these associations of equol with androgens in postmenopausal women might be mediated by SHBG. Our findings indicated urinary equol exhibited graded and inverse associations with BT or FT but not TT in women.

Keywords: equol; bioavailable testosterone; total testosterone; sex hormone-binding globulin

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1. Introduction

Androgens have been associated with various chronic diseases, including cardiovascular diseases [1], type 2 diabetes mellitus [2] and cancer [3]. Slightly less than 50% of the total testosterone (TT) in circulation is bound tightly to sex hormone-binding globulin (SHBG) [4], while approximately 50% and 2% of TT exists in a loose albumin-bound or free state [5]. These latter states retain biological activity [6] and are classified as bioavailable testosterone (BT) [4]. Notably, BT, but not TT, plays predominant roles in the development of chronic diseases [7-9]. Therefore, it is important to explore BT as a modifiable factor in humans. Although dietary factors have been identified as important modifiable factors in the risks of chronic diseases [10], few human studies have associated dietary factors with BT levels.

Phytoestrogens, which share chemical structural similarities with estrogens [11], exert estrogenic or anti-estrogenic effects by binding to estrogen receptors (ER-α and -β) [12]. Soy isoflavones (e.g., genistein, daidzein and glycetein) are major sources of phytoestrogens in standard diets. Previous studies of the effects of isoflavones in soy protein or isolated extracts on the circulating levels of TT and FT in women have reported inverse [13] and null [14] associations. Equol, a major gut flora-dependent metabolite of daidzein [15], has a longer half-life [11] and

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stronger estrogenic/anti-estrogenic properties, compared with daidzein and genistein [12,16]. A few *in vitro* and animal studies revealed that equol exerted modulating hormone-like effects (e.g., testosterone [17-21], and SHBG [22,23]) and could alter the blood levels of androgens [24]. Accordingly, equol might affect blood androgens in humans. To date, however, few studies have verified the associations between equol and androgens in women. One cross-sectional study of 194 pregnant women reported a negative correlation between urinary equol and TT [25]. In another study of 14 premenopausal women treated with 0.15, 1.0 and 2.0 mg isoflavones/kg/day for 9 days, equol excretors had lower circulating levels of TT but higher levels of SHBG, compared with non-excretors [26]. However, two other interventional studies found no significant differences in serum TT and FT levels between equol excretors and non-excretors [27,28]. It remains uncertain whether null associations between equol and androgens, which were mainly reported in studies on Western populations, could be attributable to a limited effect size, shorter interventional period (3 days [27,28]) or lower proportion of equol excretors (20–35% [29]). No previous study has directly examined the relationship between circulating equol and BT in women, and therefore the associations between equol and androgens remain largely unclear.

In this community-based cross-sectional study, we examined the potential associations of urinary equol with BT and other androgenic indices in a sample of middle-aged and older Chinese women.

2. Materials and Methods

2.1. Design and participants

This community-based cross-sectional study was based on the Guangzhou Nutrition and Health Study (GNHS). The GNHS was a prospective study designed to explore the determinants (e.g., lifestyle, dietary and biochemical indices) of common chronic diseases in Guangzhou, Guangdong province, China. The study design was described in detail previously [30]. As shown in Figure 1, 4048 community-dwelling residents who had lived in Guangzhou for at least 5 years were enrolled between 2008 and 2013. The participants had a mean age of 60.7±6.0 years. They were followed up approximately every 3 years until May 31, 2017. Of the participants, 3169 were recruited between 2008 and 2010 and 2465 completed the first follow-up visit between 2011 and 2013. A further 879 participants were recruited and completed the baseline survey between 2012 and 2013. A total of 2144 subjects were excluded for the following reasons: (1) self-reported chronic renal dysfunction (n = 4) or cancer (n = 19); (2) refusal (n = 710); (3) emigration or lost to follow-up (n = 423); (4) death or serious disease (n = 25); (5) incomplete data (n = 81); and (6) male sex (n = 882). The present study included 1904 women, of whom 1666 were postmenopausal.

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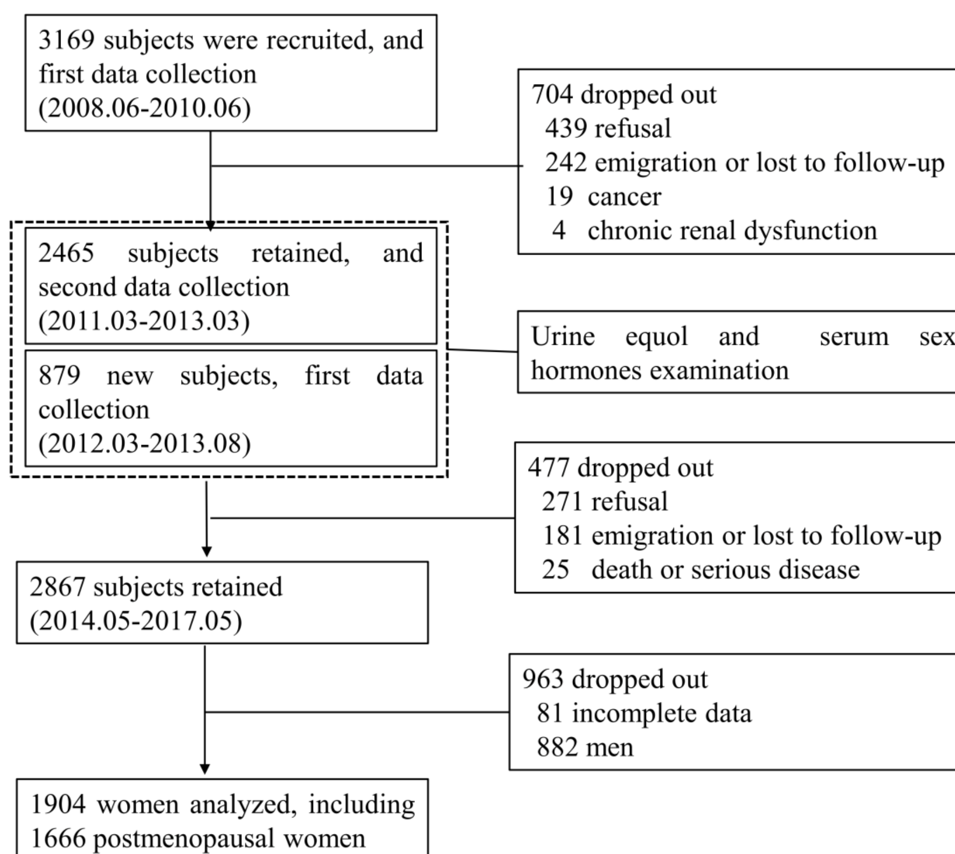

Figure 1. Flow chart of the study subjects

The GNHS was registered at ClinicalTrials.gov (ID: NCT03179657) and approved by the Ethics Committee of the School of Public Health of Sun Yat-sen University. All procedures were performed in accordance with the rules of the Declaration of Helsinki of 1975 revised in 2013. Consent was obtained from each subject after full explanation of all procedures used. Written informed consent was obtained from all the study participants.

2.2. Questionnaire interview and body measurements

Face-to-face interviews based on a structured questionnaire were conducted by trained interviewers. We collected detailed information from all participants, including socio-demographic characteristics, lifestyle factors, physical activity and menopausal status (women only). Marital status was classified as unmarried (single, divorced or widowed) or being married. Daily physical activity was estimated using a physical activity questionnaire containing 19 items, and the metabolic equivalent (MET) intensities were calculated after excluding sleeping and sitting [31]. Each participant's body hipline and waistline were measured and used to calculate the waist to hip ratio (WHR). Participants who had drunk wine ≥1 times daily for at least 6 consecutive months were defined as current drinkers. Participants who had a smoked>5 cumulative packs of cigarettes during the past year were defined as smokers.

2.3. Laboratory measurements

At the examination site, fasting venous blood samples were collected from subjects during recruitment or follow-up visits and subjected to an analysis of sex hormones. Within 2–4 h of collection, blood samples were centrifuged at 1500× g and 4ºC for 15 minutes to separate the serum. All samples were stored at -80ºC. The serum concentrations of TT, dehydroepiandrosterone sulfide (DHEAS) and SHBG were detected using chemiluminescent enzyme immunometric assay kits from Abbott Corporation (Chicago, IL, USA) and an ARCHITECT I2000 automated analyzer (Abbott Corporation). The intra-assay coefficients of variation (CV) of TT, DHEAS and SHBG were 5.90%,

4.79% and 4.64%, respectively. The FT and BT levels and free androgen index (FAI) were calculated as described by Vermeulen et al. [32].

Urinary equol levels were measured using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) methods reported by previous studies [33,34], with some modifications. Briefly, a 200-μL aliquot of urine was mixed with 3μL of β-glucuronidase (Helix pomatia; Sigma, St. Louis, MO, USA) in 300 μL of sodium acetate buffer (0.2mol/L, pH=5.5). The mixture was vortexed (10 minutes) and then incubated for 30 min at 37ºC. After a 10-min cool-down, the mixture was combined with 1mL of ethyl acetate via shaking for 30 min and centrifuged at 4000× g and 4ºC for 15 min, after which the supernatant was collected. After repeated extractions, the supernatant was dried under nitrogen and the residue was then reconstituted with 50 μL of ethyl acetate. Next, the urinary concentration of equol was analyzed using triple quad-liquid chromatography/mass spectrometry (Agilent Technologies, Santa Clara, CA, USA) with an Eclipse Plus C18 column (3.5 μm, 2.1 mm × 150 mm; Agilent Technologies). The mobile phase comprised acetonitrile (A) and a 0.1% aqueous solution of formic acid (B). The elution gradient was as follows: 45% A at 0–1 min, 45–80% A at 1–2.5 min, 80% A at 2.5–5 min and 80–45% A at 5–8 min, followed by a 6-minre-equilibration of the gradient. The flow rate was 1μL/min, and the sample injection volume was 5μL.

The urinary creatinine level was measured using a Roche-Cobas Mira Plus clinical chemistry autoanalyzer (Roche Diagnostics, Basel, Switzerland). For normalization, the equol concentrations were divided by the creatinine concentrations and were expressed as mg/mol of creatinine. The CVs of the equol and creatinine measurement methods were 8.67% and 1.20%, respectively.

2.4. Statistical analysis

The baseline characteristics of the participants were presented as means (standard deviations, SDs) or medians (interquartile ranges) for continuous variables and as frequencies (percentages) for categorical variables after stratification by menopausal status. The androgenic indices and SHBG concentrations were subjected to logarithmic transformation to achieve approximately normal distributions, and the resulting Z-scores were used in path analyses.

The subjects were categorized into tertiles according to urinary equol concentrations. Multivariate analyses of covariance (ANCOVAs) were performed to compare the mean differences in androgenic indices (TT, FT, BT, DHEAS and FAI) and SHBG levels and to test for trends according to the urinary equol tertiles. The Bonferroni test was used for pair-wise comparisons between tertiles. The percentage differences in the levels of androgenic indices (TT, BT, FT, DHEAS and FAI) and SHBG between the top and bottom urinary equol tertiles were calculated.

Stratified analyses were conducted according to the menopausal status (postmenopausal vs. premenopausal). Model 1 was adjusted for age. For the overall population and premenopausal women, Model 2 was adjusted for age, WHR, alcohol consumption, smoking status, marriage, physical activity and oral estrogen use. For postmenopausal women, Model 2 was further adjusted for the years since menopause.

Path analysis was used to evaluate the mediating effects of SHBG on the associations of equol with androgenic indices (BT, FT and FAI) and was adjusted for the multi-covariates listed in Model 2. In the data analysis, bootstrapping was performed using the SPSS add-on PROCESS (Version 2.16.3) for mediation analysis, according to the method reported by Hayes [35]. Equol was entered as variable "X" (i.e., predictor), while the androgenic indices (BT, FT and FAI) were entered as "Y" variables (i.e., outcomes). SHBG was entered as variable "M" (i.e. the mediator). A two-tailed p value<0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics 21.0 for Windows (IBM, Inc., Armonk, NY, USA).

3. Results

3.1. Baseline characteristics

The baseline characteristics of the total study population and of postmenopausal and premenopausal women were listed in Table 1. The total population included 1904 women, including

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1666 postmenopausal and 238 premenopausal women. The mean ages of the total, postmenopausal and premenopausal groups were 59.7, 60.0 and 58.3 years, respectively. The majority of women in this study were married, did not smoke or drink and did not use oral estrogen supplements. Higher median urinary equol concentrations were observed in postmenopausal women than in total and premenopausal women (0.42 vs. 0.33 and 0.02 mg/mol creatinine, respectively).

Table 1. The baseline characteristics in total, postmenopausal and premenopausal women.

Characteristics	Total women (N=1904)	Postmenopausal women (n=1666)	Premenopausal women (n=238)
Age, y	59.7 ± 5.38	60.0 ± 4.79	58.3 ± 8.33
WHR	0.92 ± 0.08	0.92 ± 0.09	0.93 ± 0.07
Smoker, N (%)	9 (0.47)	7 (0.42)	2 (0.84)
Alcohol drinker, N (%)	62 (3.26)	53 (3.18)	9 (3.78)
Married, N (%)	1619 (85.03)	1420 (85.23)	199 (83.61)
Physical activity, MET/day	20.31 ± 7.20	20.43 ± 7.25	19.54 ± 6.79
Years since menopause, y	-	9.92 ± 5.38	-
Estrogen user, N (%)	222 (11.66)	207 (12.42)	15 (6.30)
Urinary creatine [a], mmol/l	5.03 (3.54, 7.32)	5.01 (3.53, 7.33)	5.20 (3.64, 7.27)
Urinary equol [a], mg/mol creatine	0.33 (0.01, 5.67)	0.42 (0.02, 6.53)	0.02 (0.01, 0.79)
Sex hormones [a]			
TT, ng/dl	1.42 (1.32, 1.51)	1.42 (1.32, 1.51)	1.41 (1.30, 1.49)
BT, ng/dl	0.90 (0.75, 1.04)	0.90 (0.76, 1.04)	0.88 (0.75, 1.03)
FT, pg/dl	0.51 (0.37, 0.65)	0.51 (0.37, 0.65)	0.49 (0.37, 0.65)
DHEAS, ug/dl	2.00 (1.84, 2.14)	2.00 (1.84, 2.14)	2.03 (1.86, 2.15)
FAI	0.21 (0.03, 0.39)	0.21 (0.03, 0.38)	0.18 (0.03, 0.39)
SHBG, nmol/l	1.75 (1.61, 1.88)	1.75 (1.61, 1.88)	1.73 (1.60, 1.89)

WHR, waist to hip ratio; TT, total testosterone; FT, free testosterone; BT, bioavailable testosterone; DHEAS, dehydroepiandrosterone sulfide; FAI, free androgen index (molar ratio between testosterone and SHBG); SHBG, sex hormone binding globulin; [a] log transformed values (median [interquartile range]).

3.2. Associations of equol with androgenic indices and SHBG

Overall, urinary equol tended to be inversely associated with the bioactive forms of androgenic indices (BT, FT or FAI), but not with TT and DHEAS, across the two models. Urinary equol also exhibited a positive association with SHBG. Among total women (Table 2), the age-adjusted mean differences between urinary equol tertiles 3 and 1 were -2.66% for BT, -5.61% for FT, -16.00% for FAI and 1.73% for SHBG. After further adjusting for the other covariates in Model 2, significant differences of -4.09% for FT and -11.82% for FAI were observed between tertiles 3 and 1 (p-trends=0.046–0.049).

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Table 2. Mean sex hormone levels according to tertiles of urinary equol in total women.

Variables		Tertiles by urinary equol			% Diff	*p*-trend
		T1 (n=634)	T2 (n=635)	T3 (n=635)		
Equol [a], mg/mol		0.01 (0.01, 0.02)	0.32 (0.15, 0.65)	16.33 (5.61, 86.71)		
TT, ng/dl	Model 1	1.414 ± 0.006	1.428 ± 0.006	1.412 ± 0.006	-0.14	0.764
	Model 2	1.414 ± 0.006	1.428 ± 0.006	1.412 ± 0.006	-0.14	0.749
BT, ng/dl	Model 1	0.902 ± 0.009	0.909 ± 0.009	0.878 ± 0.009	-2.66	0.043
	Model 2	0.899 ± 0.008	0.908 ± 0.008	0.881 ± 0.008	-2.00	0.109
FT, pg/dl	Model 1	0.517 ± 0.008	0.524 ± 0.008	0.488 ± 0.008*¶	-5.61	0.016
	Model 2	0.513 ± 0.007	0.524 ± 0.007	0.492 ± 0.007¶	-4.09	0.049
DHEAS, ug/dl	Model 1	1.984 ± 0.010	1.964 ± 0.010	1.979 ± 0.010	-0.25	0.733
	Model 2	1.985 ± 0.010	1.964 ± 0.010	1.979 ± 0.010	-0.30	0.669
FAI	Model 1	0.225 ± 0.009	0.230 ± 0.009	0.189 ± 0.009*¶	-16.00	0.011
	Model 2	0.220 ± 0.009	0.230 ± 0.009	0.194 ± 0.009¶	-11.82	0.046
SHBG, nmol/l	Model 1	1.733 ± 0.008	1.738 ± 0.008	1.763 ± 0.008*	1.73	0.010
	Model 2	1.739 ± 0.007	1.738 ± 0.007	1.757 ± 0.007	1.04	0.074

Abbreviations were shown in Table 1 plus T: tertile; Model 1 was adjusted for age; Model 2 was adjusted for age, WHR, alcohol consumption, smoking status, marriage, physical activity and oral estrogen use; [a] Values were median (interquartile range); %Diff: Percentage difference of sex hormone levels between T3 and T1=(T3–T1)/T1×100%; * $p<0.05$ compared with T1; ¶$p<0.05$ compared with T2.

In analyses stratified by menopausal status, graded and inverse associations between urinary equol and the bioactive forms of androgenic indices (BT, FT and FAI) were observed in postmenopausal women according to both Model 1 and Model 2 (all *p*-trends <0.05) (Table 3), but were not observed in premenopausal women (Table 4). Similarly, a significant positive association between urinary equol and SHBG was observed only in postmenopausal women. No significant associations were observed with TT and DHEAS in either group.

Table 3. Mean sex hormone levels according to tertiles of urinary equol in postmenopausal women.

Variables		Tertiles by urinary equol			% Diff	*p*-trend
		T1 (n=555)	T2 (n=556)	T3 (n=555)		
Equol [a], mg/mol		0.01 (0.01, 0.02)	0.41 (0.20, 0.82)	17.75 (6.47, 95.62)		
TT, ng/dl	Model 1	1.420 ± 0.006	1.424 ± 0.006	1.416 ± 0.006	-0.28	0.637
	Model 2	1.420 ± 0.006	1.424 ± 0.006	1.416 ± 0.006	-0.28	0.675
BT, ng/dl	Model 1	0.909 ± 0.008	0.903 ± 0.008	0.878 ± 0.008	-3.41	0.017
	Model 2	0.906 ± 0.008	0.903 ± 0.008	0.881 ± 0.008	-2.76	0.039
FT, pg/dl	Model 1	0.519 ± 0.008	0.519 ± 0.008	0.491 ± 0.008	-5.39	0.021
	Model 2	0.516 ± 0.008	0.519 ± 0.008	0.494 ± 0.008	-4.26	0.048
DHEAS, ug/dl	Model 1	1.987 ± 0.011	1.958 ± 0.011	1.976 ± 0.011	-0.55	0.479
	Model 2	1.986 ± 0.011	1.959 ± 0.011	1.975 ± 0.011	-0.55	0.471
FAI	Model 1	0.228 ± 0.011	0.225 ± 0.011	0.189 ± 0.011*	-17.11	0.010
	Model 2	0.224 ± 0.010	0.224 ± 0.010	0.195 ± 0.010	-12.95	0.030
SHBG, nmol/l	Model 1	1.734 ± 0.008	1.739 ± 0.008	1.766 ± 0.008*	1.85	0.008
	Model 2	1.737 ± 0.008	1.740 ± 0.008	1.761 ± 0.008	1.38	0.031

Abbreviations were shown in Table 1 plus T: tertile; Model 1 was adjusted for age; Model 2 was adjusted for age, WHR, alcohol consumption, smoking status, marriage, physical activity, oral estrogen use and years since menopause; [a] Values were median (interquartile range); %Diff: Percentage difference of sex hormone levels between T3 and T1 = (T3–T1)/T1×100%; * $p<0.05$ compared with T1.

Table 4. Mean sex hormone levels according to tertiles of urinary equol in premenopausal women.

Variables		Tertiles by urinary equol			% Diff	*p*-trend
		T1 (n=79)	T2 (n=79)	T3 (n=80)		
Equol [a], mg/mol		0.01 (0.01, 0.02)	0.03 (0.03, 0.06)	6.27 (0.76, 31.83)		
TT, ng/dl	Model 1	1.395 ± 0.016	1.426 ± 0.017	1.394 ± 0.017	-0.07	0.964
	Model 2	1.388 ± 0.016	1.431 ± 0.017	1.396 ± 0.017	0.58	0.743
BT, ng/dl	Model 1	0.884 ± 0.024	0.905 ± 0.027	0.889 ± 0.026	0.57	0.888
	Model 2	0.865 ± 0.021	0.921 ± 0.023	0.896 ± 0.022	3.58	0.330
FT, pg/dl	Model 1	0.517 ± 0.024	0.523 ± 0.026	0.491 ± 0.025	-5.03	0.457
	Model 2	0.498 ± 0.021	0.539 ± 0.023	0.497 ± 0.022	-0.20	0.982
DHEAS, ug/dl	Model 1	1.975 ± 0.027	1.988 ± 0.029	2.009 ± 0.028	1.72	0.379
	Model 2	1.962 ± 0.027	1.995 ± 0.029	2.017 ± 0.028	2.80	0.160
FAI	Model 1	0.225 ± 0.029	0.227 ± 0.032	0.199 ± 0.031	-11.56	0.544
	Model 2	0.202 ± 0.025	0.247 ± 0.027	0.207 ± 0.026	2.48	0.883
SHBG, nmol/l	Model 1	1.727 ± 0.023	1.739 ± 0.025	1.735 ± 0.024	0.46	0.798
	Model 2	1.744 ± 0.019	1.724 ± 0.021	1.730 ± 0.020	-0.80	0.629

Abbreviations were shown in Table 1 plus T: tertile; Model 1 was adjusted for age; Model 2 was adjusted for age, WHR, alcohol consumption, smoking status, marriage, physical activity and oral estrogen use; [a] Values were median (interquartile range); %Diff: Percentage difference of sex hormone levels between T3 and T1 = (T3–T1)/T1×100%.

3.3. Path analysis

A path analysis revealed that the above-mentioned associations of equol with androgenic indices in postmenopausal women might be mediated by SHBG. An analysis with adjustments for multiple covariates demonstrated that urinary equol might directly influence SHBG (Figure 2). The SHBG concentration correlated positively with a higher urinary equol concentration (all r=0.053, $p<0.05$) but inversely with the androgenic indices ($r_{BT} = -0.615$, $r_{FT} = -0.624$ and $r_{FAI} = -0.758$, all $p<0.001$).

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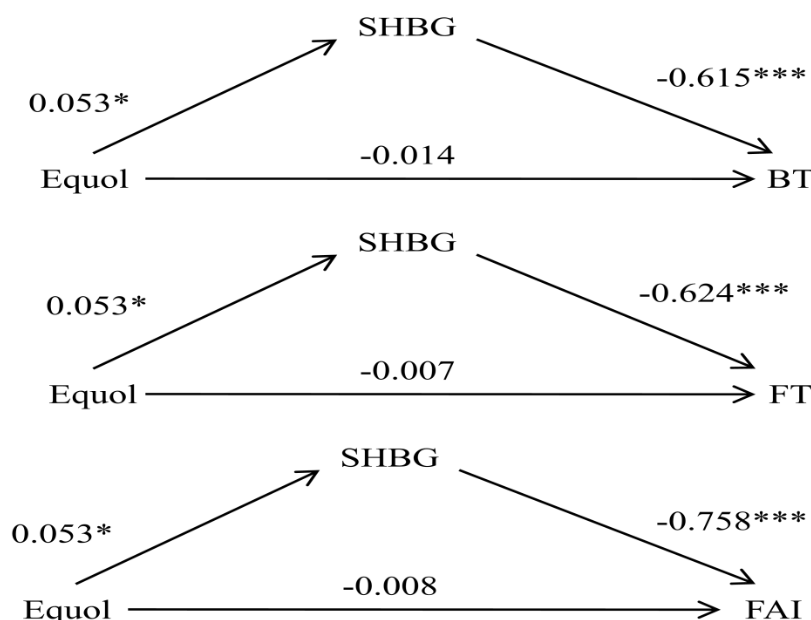

Figure 2. Path analyses of the associations of equol and the potential mediator (SHBG) with androgenic indices (BT, FT and FAI) in postmenopausal women. FT, free testosterone; BT, bioavailable testosterone; FAI, free androgen index; All variables are presented as the Z-scores of log-transformed values; Age, WHR, alcohol consumption, smoking status, marriage, physical activity, oral estrogen use and years since menopause were adjusted in the analysis; * p<0.05, *** p<0.001.

4. Discussion

In this community-based cross-sectional study of women, we observed graded and inverse associations of urinary equol with concentrations of circulating BT or FT, but not TT. The inverse associations appeared to be mediated (at least in part) by SHBG. To the best of the authors' knowledge, this study was the first to examine the associations between urinary equol and bioactive androgens in a sample of women before and after stratification by menopausal status. Our results suggested that equol might influence the blood levels of androgens, and particularly the bioactive forms.

One meta-analysis of 36 interventional studies reported the null effects of soy protein and soy isoflavones on the concentrations of TT, SHBG or BT in men [36]. Although another study reported similar null effects of soy protein and/or isoflavone interventions on estrogen and SHBG levels in both pre- and postmenopausal women, isoflavones reduced the levels of follicle-stimulating hormone and luteinizing hormone (secondary outcomes) in premenopausal women [37]. However, the effects of isoflavones on the circulating androgen levels in women remained largely unknown. It also remains uncertain whether these null effects of soy protein and/or isoflavones on androgens and estrogens could be attributable to a small effect size, short intervention period (generally 4–26 weeks [36,37]) or lower proportion of equol excretors (20–35% [29]) in Western populations.

Equol has a longer half-life and higher apparent bioavailability than its precursor daidzein [38,39] and exhibits superior estrogenic properties relative to other isoflavone metabolites [16]. Accordingly, interest in the health benefits of equol is increasing. However, few studies have examined the associations of circulating equol with androgens or SHBG. In women, three previous studies examined differences in the blood TT or FT levels between equol excretors and non-excretors [26-28]. In one cross-over trial of 14 premenopausal women who received 0.15, 1.0 and 2.0 mg of isoflavones/kg/day for 9 days, separated by approximate washout intervals of 3 weeks, Duncan et al. observed lower concentrations of TT in equol excretors than in non-excretors (mean values: 0.70–0.73 vs. 1.04–1.05nmol/L) [26]. However, no significant differences in serum TT and FT levels were observed between equol excretors and non-excretors in a study of 199 premenopausal women

challenged with a single soy bar per day for 3 days [27] or a study of 152 women challenged with 93 mg of daidzein per day [28]. A cross-sectional study of 194 pregnant women reported a negative correlation between maternal urinary equol and TT levels ($r=-0.16$, $p<0.05$) [25]. However, previous studies demonstrated that bioactive T, rather than TT, played a predominant role in the development of chronic diseases [7-9].

This study was the first to examine the associations of urinary equol with bioactive androgens in total, postmenopausal and premenopausal women. Notably, we observed inverse associations of equol with BT or FT, but not TT. Our results suggest that this highly bioactive metabolite of daidzein might be a determinant of circulating BT and FT concentrations in postmenopausal women. The inconsistent results of our and previous studies may be attributable to several factors. Interestingly, the inverse associations were more likely to be observed in Asian populations ([25] and this study) than in Western populations [26-28], in studies with a longer intervention period [26] (vs. short periods [27,28]) or with BT (vs. TT in this study). Given the limited available evidence, further longitudinal studies, and particularly randomized controlled trials (RCTs), are needed to confirm the effects of equol.

Several biological mechanisms might explain the observed association between equol and BT in postmenopausal women. One *in vitro* study showed that equol decreased the mRNA expression of CYP11A1, a key enzyme in the conversion of cholesterol to pregnenolone. As pregnenolone is the steroid precursor of testosterone, the downregulation of CYP11A1 reduced the production of testosterone in an *in vitro* ovarian follicle culture system [17]. Additionally, equol directly diminished the ability of antral follicles to produce testosterone by increasing the ratio of Bax to Bcl2 mRNA in these follicles and thus dysregulating the apoptotic pathway [17]. Equol also inhibited the secretion of testosterone from antral follicles by reducing the levels of estrogens, thus inhibiting the production of cyclic adenosine monophosphate [40,41].

This study of the testosterone status in women expanded beyond the traditional biomarkers to include SHBG. Notably, we observed a positive association between the levels of urinary equol and SHBG. Both *in vitro* and *in vivo* evidence from previous studies demonstrated that equol could increase SHBG production in HepG2 liver carcinoma cells [22,23,26]. This increased production was attributed to upregulated SHBG mRNA expression in response to elevated triiodothyronine levels [42]. Ultimately, the levels of bound-testosterone and BT may increase and decrease, respectively, in response to elevated SHBG levels. Our path analysis also identified a mediating role of SHBG on the associations of equol with various androgenic indices (BT, FT and FAI) in postmenopausal women.

This study had several strengths. To the best of our knowledge, this was the first study to evaluate the associations of urinary equol with serum BT levels in women. Previous studies indicated that BT, but not TT, was associated with the development of chronic diseases such as non-alcoholic fatty liver disease [7], osteoporosis [9] and atrial fibrillation [8]. Our results might elucidate the health effects of equol on chronic diseases via BT and may support interventions intended to modulate the levels of BT and SHBG. Next, the relatively large study size permitted us to detect potential associations. Third, the urinary equol concentrations of participants consuming normal diets would more closely represent habitual equol exposure than the concentrations in subjects challenged with isoflavones. Fourth, the observation of similar results for different bioavailable androgenic indices reduced the probability of falsely significant results.

However, some limitations of our study should be considered. First, we did not examine causality because of the cross-sectional study design. However, equol reaches a steady-state in healthy adults (terminal elimination half-life: 7–8 h), and this state is mainly influenced by dietary isoflavones rather than sex hormones. Moreover, populations with equol synthesis ability were relatively steady [29]. Therefore, cause-and-effect correlations were less likely to be inverted. Second, we collected only first-morning urine samples, which could not accurately reflect the long-term equol concentrations in the body. However, a previous study reported that urinary equol levels in the body remained relatively stable over a period of approximately 12 months [43]. Third, the androgenic indices and SHBG levels were measured only once in our study. Day-to-day fluctuations in these levels might attenuate the underlying associations. Finally, FT and BT were calculated in this study, rather than directly detected. However, previous studies confirmed the existence of

strong correlations between the calculated and measured values of FT (r= 0.97) [32] and BT (r= 0.88) [44].

5. Conclusions

Generally, our study of community-dwelling women identified graded and inverse associations of the urinary equol concentrations with the concentrations of BT or FT, but not TT. Further longitudinal studies, and particularly RCTs, are warranted to validate these results and overcome the limitations of cross-sectional studies.

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