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ORIGINAL ARTICLE

Cross-sectional association of endogenous steroid hormone, sex hormone-binding globulin, and precursor steroid levels with hemostatic factor levels in postmenopausal women

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Essentials

- Endogenous hormone levels' influence on hemostatic factor levels is not fully characterized.
- We tested for associations of endogenous hormone with hemostatic factor levels in postmenopause.
- Estrone levels were inversely associated with the natural anticoagulant, protein S antigen.
- Dehydroepiandrosterone sulfate levels were inversely associated with thrombin generation.

Summary. *Background:* Oral use of exogenous estrogen/progestin alters hemostatic factor levels. The influence of endogenous hormones on these levels is incompletely characterized. *Objectives:* Our study aimed to test whether, among postmenopausal women, high levels of estradiol (E2), estrone (E1), testosterone (T), dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), and androstenedione, and low levels of sex hormone-binding globulin (SHBG), are positively associated with measures of thrombin generation (TG), a normalized activated protein C sensitivity ratio (nAPCsr), and factor VII activity (FVIIc), and negatively associated with antithrombin activity (ATc) and total protein S antigen (PSAg). *Methods:* This Heart and Vascular Health study cross-sectional analysis included 131 postmenopausal women without a prior venous thrombosis who were not currently using hormone therapy. Adjusted mean differences in TG, nAPCsr, FVIIc, ATc and PSAg levels associated with differences in hormone levels were estimated using multiple linear regression. We measured E2, E1, total T, DHEAS, DHEA and androstenedione levels by mass spectrometry, SHBG levels by immunoassay, and calculated the level of free T. *Results:* One picogram per milliliter higher E1 levels were associated with 0.24% lower PSAg levels (95% Confidence Interval [CI]: −0.35, −0.12) and 1 μg mL^{-1} higher DHEAS levels were associated with 40.8 nM lower TG peak values (95% CI: −59.5, −22.2) and 140.7 nM×min lower TG endogenous thrombin potential (ETP) (95% CI: −212.1, −69.4). After multiple comparisons correction, there was no evidence for other associations. *Conclusions:* As hypothesized, higher E1 levels were associated with lower levels of the natural anticoagulant PSAg. Contrary to hypotheses, higher DHEAS levels were associated with differences in TG peak and ETP that suggest less generation of thrombin.

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Introduction

Oral contraceptive and hormone therapy (HT) use is positively associated with the risk of cardiovascular disease (CVD), including a 1.5-fold to more than three-fold greater risk of venous thrombosis (VT) [1–4]. Pathways contributing to the increase in VT risk have been demonstrated for several hemostatic measures, including endogenous thrombin potential (ETP), prothrombin fragment 1 + 2, total protein S, plasminogen activator inhibitor-1 (PAI-1), normalized activated protein C (APC) sensitivity ratio (nAPCsr), and antithrombin, levels of which are associated with oral use of these exogenous estrogens and progestins [5–8]. Furthermore, high levels of endogenous free and total testosterone (T) have been positively associated with some cardiovascular biomarkers and disease risk among midlife and postmenopausal women [9–11]. Although a substantial body of research has formed regarding the positive association between use of oral exogenous estrogens and progestins and both hemostatic factor levels and VT event risk [1–4], the relationship between endogenous steroid hormone, sex hormone-binding globulin (SHBG), and precursor steroid levels and thrombotic risk is poorly characterized.

Better characterization of the relationship between endogenous hormone and hemostatic factor levels may provide insights into the underlying risk of thromboembolic disease in postmenopausal women. Eight hemostatic biomarkers were previously measured among Heart and Vascular Health (HVH) study participants, including the four parameters of thrombin generation (TG), nAPCsr, factor VII activity (FVIIc), antithrombin activity (ATc), and total protein S antigen (PSAg). Thrombin plays a multitude of roles in the complex coagulation system, and TG is useful as a global measure of plasma's tendency to generate thrombin [12]. The TG assay produces a TG curve composed of four parameters that characterize: initiation (lag time); time to thrombin peak; peak thrombin concentration; and the individual's ETP. Greater TG peak and ETP levels have been positively associated with the risk of incident VT [13]. From the ETP-based nAPCsr, resistance to APC can be quantified, with high nAPCsr values being positively associated with VT risk [14]. The complex formed by tissue factor (TF) and FVIIc initiates the coagulation cascade; however, high FVIIc levels have not consistently been associated with an increased risk of VT [15,16]. The natural anticoagulant ATc inactivates the coagulation cascade by binding with heparin, and ATc levels have been shown to be one of the primary determinants associated negatively with thrombin formation [17]. In the presence of APC, PSAg shows anticoagulant activity, and lower PSAg levels are thought to be associated with a higher VT risk, although evidence for an association between PSAg levels and VT risk in population-based studies is lacking [18]. The levels of most of these hemostatic factors have not previously been evaluated in relation to the levels of endogenous sex hormones (estradiol [E2], estrone [E1], total T, and free T), SHBG, and sex steroid precursors (dehydroepiandrosterone sulfate [DHEAS], dehydroepiandrosterone [DHEA], and androstenedione) in postmenopausal women.

In this cross-sectional analysis among postmenopausal women not using HT at the time of phlebotomy, we evaluated the relationship between postmenopausal endogenous steroid hormones, SHBG, and precursor steroid levels and hemostatic factor levels. Given the greater risk of VT associated with exogenous HT use, we hypothesized *a priori* that estrogen levels (E2 and E1) would be positively associated with measures of TG, nAPCsr values, and FVIIc, and negatively associated with levels of the anticoagulant factors ATc and PSAg. *A priori*, we hypothesized that there would be similar directional differences in hemostatic factors associated with higher total and free T and with lower levels of SHBG, given the relationships between high free and total T levels and cardiovascular biomarkers and disease, and the relationship between lower SHBG levels and higher levels of circulating androgens. As the adrenal and precursor steroids DHEAS, DHEA and androstenedione are converted into androgens, and, to a lesser extent, estrogens, we *a priori* hypothesized that there would be similar directional differences in hemostatic factor levels associated with higher levels of DHEAS, DHEA, and androstenedione.

Materials and methods

Setting and study design

This cross-sectional study was conducted within the HVH study, which is a population-based, case–control study set in Group Health Cooperative (GHC), an integrated healthcare system in Washington State. The HVH study was designed to evaluate risk factors for CVD, including VT, myocardial infarction, stroke, and atrial fibrillation [19–21]. The GHC Human Subjects Review Committee approved this study.

Cross-sectional study participants

The participants in this study were controls from the HVH case–control study. Control subjects were assigned an index date, which was defined as a random date in the year for which they were frequency-matched to a case. Controls had not experienced an incident VT event prior to their index date. Subjects eligible for study inclusion were females aged 18–89 years with an index date between 2003 and 2010 who had consented to blood draw, had provided a blood specimen, had not been prescribed an anticoagulant in the previous 180 days, were not currently using oral estrogen-alone or estrogen plus progesterone HT, and who were postmenopausal at the

time of blood draw (n = 3353). A woman was considered to be postmenopausal if there was a notation of the cessation of menses in the medical record or at ≥ 55 years of age. For women with a prior hysterectomy, we defined postmenopause at the start of menopausal symptoms.

Hemostatic factor levels were measured for a random subset of these postmenopausal women (n = 136), for the original purpose of an HVH study that evaluated differences in the levels of hemostatic factors associated with the use of oral conjugated equine estrogens, oral E2, and non-use of HT [8]. Women not using oral estrogen-containing HT for whom hemostatic factor levels were measured were randomly selected, with non-HT users matched to the age distribution of users of oral HT. Current and recent oral HT use at the time of phlebotomy was determined from GHC computerized pharmacy records, which included prescription fill dates, medication quantity, and dosing instructions or the anticipated days' supply, assuming 80% compliance. For this analysis, we further excluded women who were using transdermal estrogens (n = 4), or who were using oral progesterone alone (n = 1), resulting in an eligible population of 131 women.

Measures

Blood collection, processing, and storage Venous blood specimens were collected into separate tubes containing 3.2% sodium citrate and EDTA, centrifuged at 4 °C for 10 min at 1300 × g, and stored at − 80 °C within 6 h of collection.

Hemostatic factor measurements Stored tubes of citrated plasma were shipped on dry ice from Seattle, Washington to Leiden University Medical Center, Leiden, the Netherlands, for measurement of TG, nAPCsr, FVIIc, ATc, and PSAg. Prior to shipment, samples were stored for an average of 4.5 years (standard deviation [SD] 2.0). Samples were thawed once in October 2010, for these measurements of hemostatic factors.

The TG assay measured four TG parameters: lag time, time to peak, peak thrombin concentration value, and the area under the curve, which approximates ETP [17]. A fluorogenic assay (Diagnostica Stago, Asnieres, France) was used to measure these four TG parameters (coefficient of variation [CV] for ETP from normal pooled plasma: 19.8%) [8]. The ETP-based nAPCsr was estimated from the normalized ratio of the area under the TG curve without added APC to the area under the TG curve with added APC [22]. ETP in the absence of APC had been measured as part of the TG assay; ETP in the presence of APC was measured with a fluorogenic assay (Thrombinoscope TM, Synapse BV, Maastricht, the Netherlands). FVIIc and ATc were measured with the STA-R analyzer (Diagnostica Stago) (CVs: 9.2% and 3.0%, respectively). PSAg levels were measured with an ELISA (Diagnostica Stago) (CV with a commercial quality control: 4.2%) [8].

Hormone measurements Stored EDTA plasma samples were transported locally on dry ice from storage to the Department of Veterans Affairs in Seattle, where hormone levels were measured in February 2014. On average, samples had been stored for 6.8 years (SD 2.0).

Estrogens (E1 and E2) were measured simultaneously with liquid chromatography–tandem mass spectrometry (LC-MS/MS) (intra-assay CVs in normal male serum: 3.3% [concentration: 26.3 pg mL^{-1}] and 4.2% [concentration: 19.1 pg mL^{-1}], respectively). The lower limit of detection (LOD) for E1 was 1.96 pg mL^{-1} (0 < LOD) and for E2 was 0.98 pg mL^{-1} (2 < LOD). A separate LC-MS/MS assay was used to measure total T, DHEA, and androstenedione. Intra-assay CVs in normal male serum were 4.7% (concentration: 4.3 ng mL^{-1}), 4.8% (concentration: 7.0 ng mL^{-1}), and 5.0% (concentration: 0.93 ng mL^{-1}), respectively. The LOD for total T was 1.0 ng dL^{-1} (0 < LOD), for DHEA was 15.6 ng dL^{-1} (7 < LOD), and for androstenedione was 1.0 ng dL^{-1} (0 < LOD). DHEAS was measured with a separate LC-MS/MS assay (intra-assay CV of 3.4%; LOD of 0.04 μg mL^{-1}; 1 < LOD), as its values were greater than those of DHEA and other androgen and precursor steroid hormones. SHBG was measured with the Quantikine SHBG Immunoassay (R&D Systems, Minneapolis, MN, USA), which is a 4.5-h solid-phase ELISA (inter-assay CV: 3.3%).

In analyses, E2 (n = 2), DHEA (n = 7) and DHEAS (n = 1) values that were below the LOD were replaced with the LOD value. We calculated free T with the Mazer method [23].

Covariates Trained abstractors reviewed complete GHC medical record data available prior to the index date. Race/ethnicity, education and current smoking status at the index date were determined from self-reported data collected during a telephone interview, and were augmented with data from medical record review. Body mass index (BMI) was determined from data in the medical record, and augmented with self-reported height and weight. Risk factors for cardiovascular events were collected during medical record review, including a history of cancer, hospitalization and inpatient surgery in the 12 months prior to the index date, treated diabetes mellitus (requiring a physician diagnosis of diabetes and treatment with insulin or oral hypoglycemic agents), treated hypertension (requiring a physician diagnosis of hypertension and treatment with one or more antihypertensive medications), prevalent CVD (defined as a history of angina, claudication, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, carotid endarterectomy, or peripheral vascular disease), and the most recent systolic and diastolic blood pressure and

cholesterol measures prior to the index date. Hysterectomy and oophorectomy status and a woman's age at final menstrual period (FMP) were abstracted from the medical record. Surgical menopause was defined as a bilateral salpingo-oophorectomy prior to a woman's natural transition through menopause.

Statistical analysis

We determined the medians and interquartile ranges for hormone and hemostatic factor levels. Spearman correlation coefficients were computed among the endogenous hormone levels, among the hemostatic factor levels, and between each endogenous hormone and each hemostatic factor.

Multiple linear regression with robust standard errors was used to model the association between each individual hormone and hemostatic factor level, adjusting for potential confounders: linear age, linear BMI (kg m^{-2}), current versus never/past smoking status, treated diabetes, treated hypertension, non-white versus white race, prevalent CVD, and surgical versus natural menopause. Linear regression models were used to estimate the adjusted average difference in the hemostatic factor level associated with a 1-unit higher endogenous hormone level. To standardize comparisons within each hormone across hemostatic factors, adjusted average differences were expressed as the percentage of one hemostatic factor SD associated with a 1-SD difference in the hormone level $[([\beta \times SD_{hormone}]/SD_{hemostatic\ factor}) \times 100]$. Although not tested statistically, a visual inspection of scatterplots did not lead us to suspect that associations deviated from linearity. Scatterplots were inspected visually for possible outlying values, which were excluded from sensitivity analyses. Histograms were used to evaluate the distribution of each hormone and hemostatic factor for evidence of skewness, and all hormones and hemostatic factors other than TG peak and PSAg had evidence of skew. In sensitivity analyses, we natural log-transformed all hormones and hemostatic factors, including TG peak and PSAg, for ease of interpretation and consistency.

For each hormone, there were eight tests for association with hemostatic factors, so we defined the nominal *P*-value required for statistical significance as 0.00625 (0.05/8). To reduce the possibility of Type I error across all hormones, we set an overall threshold of statistical significance, which was a Bonferroni-corrected level of 0.00069 (0.05/72 tests).

In secondary analyses, separately for each hemostatic factor, we performed linear regression analyses with all hormone exposures and other covariates in one model. We used likelihood ratio tests to evaluate whether there was any association between hormone levels and differences in each hemostatic factor level, using a threshold for statistical significance of 0.00625 (0.05/8 hemostatic factors). All data management and analyses were conducted with STATA 13.1 [24].

Results

On average, participants were 67 years of age, had experienced their FMP 19 years earlier, had a BMI in the obese range (mean BMI of 30 kg m^{-2}), and were predominantly of white race/ethnicity (93%) (Table 1). Most women had used oral estrogen HT at some point prior to phlebotomy (67%), but, on average, had not used any oral or transdermal estrogen or progestogen HT for 7.2 years previously. No study participants had a history of aromatase inhibitor prescription prior to the index date. Tables S1–S3 show unadjusted median levels of endogenous steroid hormone and hemostatic factor levels, and unadjusted Spearman correlation coefficients.

Tables 2, 3 and 4 show results from multiple linear regression models of the cross-sectional association between endogenous hormone and hemostatic factor levels, with *P*-values that are not adjusted for multiple

Table 1 Demographic and medical history characteristics of study participants

	Study participants $n = 131$
Age at blood draw (years), mean (SD)	67.1 (9.7)
Years since FMP at blood draw, mean (SD)	18.8 (10.8)*
White race/ ethnicity, no. (%)	122 (93.1)
Education > high school, no. (%)	97 (74.1)*
BMI (kg m^{-2}), mean (SD)	29.9 (7.7)
Current smoking, no. (%)	12 (9.2)
History of any CVD, no. (%)	11 (8.4)
Diabetes, no. (%)	18 (13.7)
Hypertensive, no. (%)	74 (56.5)
Systolic blood pressure (mmHg), mean (SD)	132.3 (17.3)
Total cholesterol (mg dL^{-1}), mean (SD)	208.4 (40.8)
Cancer in the 2 years prior to the index date, no. (%)	0 (0.0)
Hospitalization in the 12 months prior to the index date, no. (%)	4 (3.1)*
Inpatient surgery in the 12 months prior to the index date, no (%)	3 (2.3)*
Hysterectomy/oophorectomy, no. (%)	
No surgery	83 (63.4)
Hysterectomy-alone	20 (15.3)
Hysterectomy with BSO	22 (16.7)
Unknown	6 (94.6)
Ever estrogen HT use, no. (%)	88 (67.2)
Months since any HT use at blood draw, mean (SD)	86.4 (67.5) $n = 96$†

BMI, body mass index; BSO, bilateral salpingo-oophorectomy; CVD, cardiovascular disease; FMP, final menstrual period; HT, hormone therapy; SD, standard deviation. *Years since FMP, $n = 101$; education, $n = 129$; hospitalization, $n = 128$; inpatient surgery, $n = 128$.†Ninety-six women used any HT (estrogen or progestogen) prior to blood draw.

Table 2 Linear regression-modeled cross-sectional associations between endogenous estradiol (E2), estrone (E1) and sex hormone-binding globulin (SHBG) levels and hemostatic factor levels in postmenopausal women

		E2 (pg mL^{-1}) (n = 131)				E1 (pg mL^{-1}) (n = 131)			SHBG (nmol L^{-1}) (n = 131)		
		Hormone mean (SD)	6.5 (6.9)			27.0 (24.2)			77.7 (48.2)		
	n	Hemostatic factor mean (SD)	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†
Thrombin generation											
Peak value (nM)	131	261.1 (54.8)	0.27 (− 0.82 to 1.4)	0.62	2.9	0.031 (− 0.24 to 0.30)	0.82	1.4	0.082 (− 0.079 to 0.24)	0.32	7.2
ETP (nM×min)	131	1214.9 (268.8)	0.092 (− 5.5 to 5.7)	0.97	0.23	− 0.23 (− 1.4 to 0.97)	0.71	− 2.0	0.043 (− 0.78 to 0.86)	0.92	0.77
Lag time (min)	131	2.3 (0.63)	− 0.0053 (− 0.018 to 0.0075)	0.42	− 5.7	− 0.00076 (− 0.0041 to 0.0026)	0.66	− 2.9	− 0.00084 (− 0.0026 to 0.00088)	0.34	− 6.4
Time to peak (min)	131	4.4 (0.87)	− 0.0067 (− 0.029 to 0.015)	0.55	− 5.3	− 0.0013 (− 0.0061 to 0.0036)	0.61	− 3.5	− 0.0020 (− 0.0045 to 0.00053)	0.12	− 10.9
nAPCsr	130	1.6 (1.2)	− 0.0048 (− 0.029 to − 0.019)	0.69	− 2.8	− 0.0025 (− 0.0073 to 0.0023)	0.30	− 5.1	0.00015 (− 0.0033 to 0.0036)	0.93	0.58
FVIIc (%)	126	131.1 (36.0)	0.15 (− 0.72 to 1.02)	0.74	2.8	0.034 (− 0.22 to 0.29)	0.79	2.3	− 0.0074 (− 0.15 to 0.14)	0.92	− 0.99
ATc (%)	124	105.8 (13.9)	− 0.39 (− 0.81 to 0.029)	0.068	− 19.9	**− 0.13 (− 0.22 to − 0.039)**	**0.0052**	**− 18.9**	0.0012 (− 0.053 to 0.055)	0.96	0.36
PSAg (%)	130	109.5 (18.9)	**− 0.90 (− 1.5 to − 0.36)**	**0.0013**	**− 32.9**	***− 0.24 (− 0.35 to − 0.12)***	***0.00008***	***− 30.4***	− 0.075 (− 0.15 to 0.0018)	0.055	− 19.1

ATc, antithrombin activity; CI, confidence interval; ETP, endogenous thrombin potential; FVIIc, factor VII activity; nAPCsr, normalized activated protein C sensitivity ratio; PSAg, total protein S antigen; SD, standard deviation. Bold italic indicates associations that are significant at a multiple comparisons corrected level of $P < 0.00069$. Bold indicates associations that are significant only at a nominal level of $P < 0.00625$. *Adjusted for linear age, linear body mass index, current smoking, treated diabetes, race/ethnicity, prior cardiovascular disease, surgical menopause, and treated hypertension. †Adjusted average percent difference in 1 SD of hemostatic factor, per 1-SD difference in endogenous hormone.

Table 3 Linear regression-modeled cross-sectional associations between endogenous testosterone (T) levels and hemostatic factor levels in postmenopausal women

		Total T (ng dL^{-1}) (n = 131)				Free T (ng dL^{-1}) (n = 131)		
		Hormone mean (SD)	15.3 (10.6)			0.17 (0.12)		
	n	Hemostatic factor mean (SD)	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†
Thrombin generation								
Peak value (nM)	131	261.1 (54.8)	0.098 (− 0.73 to 0.93)	0.82	1.9	− 20.2 (− 117.5 to 77.2)	0.68	− 4.4
ETP (nM×min)	131	1214.9 (268.8)	1.5 (− 4.1 to 7.2)	0.59	6.1	120.2 (− 439.4 to 679.8)	0.67	5.3
Lag time (min)	131	2.3 (0.63)	0.00043 (− 0.0073 to 0.0081)	0.91	0.72	0.047 (− 0.53 to 0.63)	0.87	0.88
Time to peak (min)	131	4.4 (0.87)	0.0011 (− 0.012 to 0.015)	0.87	1.4	0.52 (− 0.53 to 1.6)	0.33	7.0
nAPCsr	130	1.6 (1.2)	0.0035 (− 0.014 to 0.021)	0.70	3.1	− 0.59 (− 1.7 to 1.6)	0.94	− 0.58
FVIIc (%)	126	131.1 (36.0)	− 0.21 (− 0.84 to 0.42)	0.51	− 6.2	− 3.1 (− 45.3 to 39.0)	0.88	− 1.0
ATc (%)	124	105.8 (13.9)	− 0.20 (− 0.46 to 0.048)	0.11	− 13.3	− 25.5 (− 54.5 to 3.5)	0.085	− 18.4
PSAg (%)	130	109.5 (18.9)	− 0.29 (− 0.53 to − 0.041)	0.022	− 16.2	− 18.4 (− 43.4 to 6.7)	0.15	− 11.5

ATc, antithrombin activity; CI, confidence interval; ETP, endogenous thrombin potential; FVIIc, factor VII activity; nAPCsr, normalized activated protein C sensitivity ratio; PSAg, total protein S antigen; SD, standard deviation. Bold italic indicates associations that are significant at a multiple comparisons corrected level of $P < 0.00069$. Bold indicates associations that are significant only at a nominal level of $P < 0.00625$. *Adjusted for linear age, linear body mass index, current smoking, treated diabetes, race/ethnicity, prior cardiovascular disease, surgical menopause, and treated hypertension. †Adjusted average percent difference in 1 SD of hemostatic factor, per 1-SD difference in endogenous hormone.

comparisons. For example, the adjusted average difference in TG peak value was 0.27 nM per 1 pg mL^{-1} higher E2 (95% confidence interval − 0.82 to 1.4). Expressed as a percent different in SD units of the hemostatic factor level associated with a 1-SD higher hormone level, the TG peak value was 2.9% of the TG peak value SD higher for each 1-SD higher E2 level (i.e. per 6.9 pg mL^{-1} higher E2).

At a nominal level of 0.00625 that accounted only for multiple comparisons within each hormone, higher E2 and E1 levels were associated with lower PSAg levels, and higher E1 levels were associated with lower ATc levels (Table 2). Higher DHEAS levels were associated with lower TG peak values, lower TG ETP values, and lower nAPCsr values, and higher DHEA levels were associated with lower PSAg levels (Table 4). After overall adjustment for all 72 tests, evidence for the association of E1 levels with lower PSAg levels and of DHEAS levels with lower TG peak and lower ETP persisted ($P < 0.00069$). We found no evidence of an association between SHBG, total T, free T or androstenedione levels and any hemostatic factor levels.

A visual inspection of unadjusted scatterplots for hormone and hemostatic factors with associations significant at the multiple comparisons corrected level of $P < 0.00069$ (Fig. S1) and at the nominal level of $P < 0.00625$ (Fig. S2) suggested possible outlying observations in the evaluations of E2 with PSAg (n = 2), of E1 with PSAg (n = 2), of E1 with ATc (n = 2), of DHEAS with TG peak (n = 1), of DHEAS with ETP (n = 1), and of DHEAS with nAPCsr (n = 2). In sensitivity analyses that excluded possible outliers, adjusted average differences in hemostatic factor levels were similar to those estimated in primary analyses (E2 with PSAg, $\beta = -0.93$, $P = 0.055$; E1 with PSAg, $\beta = -0.31$, $P = 0.033$; E1 with ATc, $\beta = -0.28$, $P = 0.007$; DHEAS with TG peak, $\beta = -41.0$, $P = 0.000032$; DHEAS with ETP, $\beta = -140.4$, $P = 0.00019$; and DHEAS with nAPCsr, $\beta = -0.62$, $P = 0.003$).

After the natural log transformation of hormone and hemostatic factor levels, at a nominal level of statistical significance of $P < 0.00625$, higher levels of ln(DHEAS) and ln(DHEA) were associated with lower ln(TG peak) values, and higher levels of ln(E2) and ln(E1) were associated with lower levels of ln(ATc) (Tables S4–S6). Other associations were no longer nominally statistically significant.

In secondary analyses that included all hormone levels and covariates in one model per hemostatic factor level, there was evidence of a significant association between differences in hormone levels and differences in PSAg levels (likelihood ratio test, $P = 0.0020$). Likelihood ratio tests suggested no evidence of associations between levels of other hemostatic factors and differences in hormone levels when all hormones were included simultaneously as predictors.

Discussion

In this cross-sectional study among postmenopausal women, after accounting for multiple comparisons, higher E1 levels were associated with lower PSAg levels, and higher DHEAS levels were associated with lower values of two TG measures. The inverse association between E1 levels and PSAg levels was in the direction associated with greater VT risk, as hypothesized. However, the inverse association between DHEAS levels and both TG peak and ETP suggested differences in TG measures associated with lower VT risk, which is the opposite of what we hypothesized. When we accounted only for multiple comparisons made within each hormone, there was also evidence that higher E2 and DHEA levels were associated with lower PSAg levels, higher E1 levels were associated with lower ATc levels, and higher DHEAS levels were associated with lower nAPCsr values.

E2, E1, and SHBG

There was some evidence that higher E2 and E1 levels may be associated with lower PSAg levels, and that higher E1 levels may be associated with lower ATc levels. After accounting for all comparisons, evidence for an association between higher E1 levels and lower PSAg levels persisted. It has been suggested that lower levels of the natural anticoagulant PSAg may be associated with a greater risk of incident VT in studies of thrombophilic families, but evidence for this association in population-based settings is lacking [18].

Other studies have reported mixed findings relating estrogens and SHBG levels to hemostatic factors, but these studies have included women of different ages and menopausal stages, and have included primarily different hemostatic factors than were included in our study [25,26]. In the Study of Women's Health Across the Nation (SWAN), which included women aged 42–52 years at baseline, E2 levels were not associated with FVIIc levels [27], in agreement with our study, but lower SHBG levels were associated with higher FVIIc levels [10]. Although measures of PAI-1, tissue-type plasminogen activator (t-PA) and fibrinogen were not available in the HVH study, estrogen levels have been positively associated with fibrinogen levels [25,26], and negatively associated with PAI-1 and t-PA levels [27], and SHBG levels have been positively associated with PAI-1, t-PA and FVIIc levels [10] in other studies of women in midlife and postmenopause.

Total T and free T

We found no significant evidence that total and calculated free T levels were associated with hemostatic factor levels. SWAN investigators also reported no evidence of an association between total T and FVIIc levels, but they reported a positive association between total T levels and PAI-1 and t-PA levels [10].

DHEAS, DHEA, and androstenedione

We found some evidence that higher DHEAS levels may be associated with lower TG peak, ETP and nAPCsr levels, and that higher DHEA levels may also be associated with lower PSAg levels. After adjustment for all comparisons made, the association between higher DHEAS levels and lower TG peak and ETP levels persisted. Associations between DHEAS and DHEA levels and measures of TG and nAPCsr were in the direction opposite of that hypothesized, suggesting a possibly lower risk of VT.

The relationships between measures of TG and nAPCsr and the levels of these adrenal steroids have not been evaluated in other populations of postmenopausal women, and additional study is warranted. Other studies of midlife and postmenopausal women have reported that higher DHEAS levels are associated with higher PAI-1 [10], t-PA [10] and fibrinogen levels [10,25].

The relationship between endogenous DHEAS and DHEA levels and cardiovascular biomarkers and events, including VT, is unclear. We hypothesized that higher levels of these precursor steroids would be associated with differences in hemostatic factor levels associated with greater thrombotic risk, owing to their downstream metabolism to endogenous estrogens and androgens. However, given that DHEAS and DHEA levels decrease with age, other investigators have hypothesized that the increased risk of CVD with advancing age may be associated with declining levels of serum DHEAS and DHEA [28], in contrast to our study's original hypothesis.

Biological plausibility

We found statistically significant evidence that higher E1 levels are associated with lower PSAg levels, and that higher DHEAS levels are associated with less TG. Estrogens and androgens, and the adrenal steroids, DHEAS and DHEA, may plausibly interact with functional androgen and estrogen receptors on vascular endothelial and smooth muscle cells [29,30]. Activated endothelial cells may express TF, which, in conjunction with activated FVII, initiates the extrinsic pathway of the coagulation cascade [31].

It is unclear whether any association between endogenous hormone and hemostatic factor levels would translate into an association with clinical cardiovascular endpoints. In a recent study that included postmenopausal women, there was no evidence that endogenous levels of E2 and total T measured by immunoassay were associated with VT risk [32], but the investigators did not evaluate VT risk in relation to E1, SHBG or adrenal precursor steroid levels.

Table 4 Linear regression-modeled cross-sectional associations between precursor steroid levels and hemostatic factor levels in postmenopausal women

		DHEAS (μg mL^{-1}) (n = 131)				DHEA (ng dL^{-1}) (n = 131)			Androstenedione (ng dL^{-1}) (n = 131)		
		Hormone mean (SD)	0.52 (0.46)			110.2 (82.5)			35.8 (19.1)		
	n	Hemostatic factor mean (SD)	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†	Adjusted average difference (95% CI)*	P-value	Adjusted % difference in SD†
Thrombin generation											
Peak value (nM)	131	261.1 (54.8)	***− 40.8 (− 59.5 to − 22.2)***	***0.000031***	***− 34.2***	− 0.17 (− 0.31 to − 0.034)	0.015	− 25.3	− 0.29 (− 0.88 to 0.30)	0.34	− 10.1
ETP (nM×min)	131	1214.9 (268.8)	***− 140.7 (− 212.1 to − 69.4)***	***0.00016***	***− 24.0***	− 0.66 (− 1.2 to − 0.12)	0.018	− 20.3	− 1.03 (− 3.5 to 1.4)	0.41	− 7.3
Lag time (min)	131	2.3 (0.63)	− 0.067 (− 0.23 to 0.10)	0.43	− 4.8	− 0.00017 (− 0.0013 to 0.00091)	0.75	− 0.56	0.0022 (− 0.0033 to 0.0077)	0.43	6.6
Time to peak (min)	131	4.4 (0.87)	0.23 (− 0.084 to 0.53)	0.15	11.9	0.00091 (− 0.0011 to 0.0029)	0.38	8.0	0.0048 (− 0.0031 to 0.013)	0.23	10.5
nAPCsr	130	1.6 (1.2)	**− 0.89 (− 1.4 to − 0.34)**	**0.0018**	**− 34.0**	− 0.0034 (− 0.0064 to − 0.00047)	0.024	− 21.8	− 0.0064 (− 0.017 to 0.0041)	0.23	− 10.3
FVIIc (%)	126	131.1 (36.0)	− 1.15 (− 10.8 to 8.5)	0.81	− 1.5	− 0.078 (− 0.14 to − 0.018)	0.011	− 18.6	− 0.043 (− 0.40 to 0.31)	0.81	− 2.3
ATc (%)	124	105.8 (13.9)	0.53 (− 4.7 to 5.8)	0.84	1.5	0.0028 (− 0.031 to 0.037)	0.87	2.6	0.0057 (− 0.14 to 0.14)	0.94	0.66
PSAg (%)	130	109.5 (18.9)	− 6.9 (− 14.0 to 0.12)	0.054	− 16.9	**− 0.065 (− 0.11 to − 0.021)**	**0.004**	**− 27.2**	− 0.14 (− 0.34 to 0.063)	0.17	− 14.2

ATc, antithrombin activity; CI, confidence interval; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; ETP, endogenous thrombin potential; FVIIc, factor VII activity; nAPCsr, normalized activated protein C sensitivity ratio; PSAg, total protein S antigen; SD, standard deviation. Bold italic indicates associations that are significant at a multiple comparisons corrected level of $P < 0.00069$. Bold indicates associations that are significant only at a nominal level of $P < 0.00625$. *Adjusted for linear age, linear body mass index, current smoking, treated diabetes, race/ethnicity, prior cardiovascular disease, surgical menopause, and treated hypertension. †Adjusted average percent difference in 1 SD of hemostatic factor, per 1-SD difference in endogenous hormone.

Limitations and strengths

Due to the cross-sectional nature of this study, it cannot be determined whether differences in hormone levels may alter hemostatic factor levels, or vice versa. Our study's relatively small sample size limits power, and additional studies in larger samples are warranted. Plasma samples were stored prior to the measurement of hemostatic factor and endogenous hormone levels. The effect of long-term sample storage on hemostatic factor levels is unclear [8], but many hemostatic factor measurements appear to be relatively stable in plasma samples stored for up to 2 years [33]. Although the rank order of endogenous steroid hormone levels, including E2, total T, and free T, appears to be relatively stable over at least 3 years of storage [34], long-term sample storage appeared to decrease SHBG levels in the Baltimore Longitudinal Study of Aging in men [35]. Although the women eligible for this study were not using HT at the time of phlebotomy, and no participants had used HT in in the 2 months prior to blood draw, women who had previously used HT were included; we did not evaluate differences by ever versus never HT use, owing to our study's relatively small sample size. Furthermore, our study predominantly included women in late postmenopause; therefore, it was underpowered to evaluate the possibility of an interaction by early versus late postmenopausal status.

Our primary analyses included all observations. In sensitivity analyses that excluded women with possible outlying hormone values, estimated adjusted average differences in hemostatic factor levels were similar. As expected, associations and *P*-values were slightly diminished, with two of the three primary findings remaining significant at a level of $P < 0.00069$ (DHEAS with TG peak, and DHEAS with ETP). Although normality of the exposure and outcome variables is not required to fit a valid linear regression model [36], in small samples, the largest observations of explanatory variables with skewed distributions can have a large influence. To reduce this influence, we conducted sensitivity analyses using natural log-transformed hormone and hemostatic factor levels to determine impact on the primary analyses. The results from natural log-transformed analyses suggested similar directional differences in hemostatic factor levels as in primary analyses, but several associations that had been statistically significant in primary analyses became non-significant, and two associations (E2 with ATc, and DHEA with TG peak) became significant at a level of $P < 0.00625$. Although there was some difference in the significance of associations, it is unclear whether the transformed data better represent associations. Given that few published studies have evaluated the associations between hormone levels and hemostatic factor levels in postmenopausal women, it will be of interest to determine whether associations identified as significant in primary and sensitivity analyses are present in other study populations.

A strength of our study was the inclusion of measures of TG, which is a global marker for thrombotic risk. Another strength is that our study utilized sensitive LC-MS/MS methods of hormone measurement, which enabled us to capture generally low levels of endogenous hormones present in postmenopausal women.

Conclusions

In this study of postmenopausal women not currently using HT, we found statistically significant evidence that higher E1 levels were associated with lower levels of the natural anticoagulant, PSAg. We also found that higher DHEAS levels were associated with less TG, a directional difference that is generally associated with less thrombotic risk. When we corrected only for comparisons made within hormones, there was some suggestion that higher E2 and DHEA levels were associated with lower PSAg levels, that higher E1 levels were associated with lower ATc levels, and that higher DHEAS levels were associated with lower nAPCsr values. Replication efforts in a larger study would strengthen our understanding of the potentially complex relationship between endogenous hormone levels and thrombotic risk.

Addendum

L. B. Harrington, B. McKnight, S. R. Heckbert, N. F. Woods, A. Z. LaCroix, B. M. Psaty, and N. L. Smith contributed to the study concept and design. L. B. Harrington, B. T. Marck, S. R. Heckbert, B. M. Psaty, F. R. Rosendaal, A. M. Matsumoto, and N. L. Smith designed the collection of data. L. B. Harrington performed statistical analyses. All authors contributed to the interpretation of data, provided substantial scientific contributions to the revisions of the manuscript, and approved the final version of the manuscript.

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Disclosure of Conflict of Interests

B. M. Psaty serves on the Data Safety Monitoring Board for a clinical trial of a device funded by the manufacturer (2011 LifeCor), and serves on the steering committee of the Yale Open Data Access Project funded by Medtronic. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Correlations between endogenous hormone levels.
Table S2. Spearman correlations between hemostatic factor levels.
Table S3. Spearman correlations between endogenous hormone levels and hemostatic factor levels.
Table S4. Linear regression-modeled cross-sectional associations between natural log-transformed endogenous estradiol, estrone and sex hormone-binding globulin levels and natural log-transformed hemostatic factor levels in postmenopausal women.
Table S5. Linear regression-modeled cross-sectional associations between natural log-transformed endogenous testosterone levels and natural log-transformed hemostatic factor levels in postmenopausal women.
Table S6. Linear regression-modeled cross-sectional associations between natural log-transformed precursor steroid levels and natural log-transformed hemostatic factor levels in postmenopausal women.
Fig. S1. Scatterplots depicting the unadjusted relationships between hormone levels and hemostatic factor levels that, in adjusted linear regression models, reached a level of statistical significance of $P < 0.00069$.
Fig. S2. Scatterplots depicting the unadjusted relationships between hormone levels and hemostatic factor levels that, in adjusted linear regression models, reached a level of statistical significance of $P < 0.00625$ but did not reach statistical significance at a level of $P = 0.00069$.

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