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Biomarkers, menopausal hormone therapy and risk of venous thrombosis: The Women's Health Initiative

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Abstract

Background: Oral menopausal hormone therapy causes venous thrombosis but whether biomarkers of thrombosis risk can identify women at risk is unknown.

Methods: We completed a nested case control study in the two Women's Health Initiative hormone trials; 27 347 women aged 50-79 were randomized to hormone therapy (conjugated equine estrogen with or without medroxyprogesterone acetate) or placebo. With 4 years follow-up, biomarkers were measured using stored baseline samples prior to starting treatment, and one-year later, in 215 women who developed thrombosis and 867 controls.

Results: Overall, lower protein C and free protein S, and higher D-dimer, prothrombin fragment 1.2 and plasmin-antiplasmin complex were associated with risk of future thrombosis with odds ratios ranging from 1.9 to 3.2. Compared to women with normal biomarkers assigned to placebo, the risk of thrombosis with hormone therapy was increased among women with abnormal biomarkers, especially elevated D-dimer, elevated plasmin-antiplasmin, and low free protein S; the largest association was for D-dimer: odds ratio 6.0 (95% CI 3.6-9.8). Differences in associations by

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hormone use were not significant on the multiplicative scale. Considering a multimarker score of eight biomarkers, women with three or more abnormal biomarkers had 15.5-fold increased odds of VT (95% CI 6.8-35.1). One-year changes in biomarkers were not robustly associated with subsequent thrombosis risk.

Conclusion: Abnormal levels of biomarkers of thrombosis risk identified women at increased risk of future venous thrombosis with oral menopausal hormone therapy. Findings support the potential for clinical use of D-dimer testing in advance of hormone therapy prescription.

KEYWORDS

blood coagulation, D-dimer, menopausal hormone therapy, risk assessment, risk factor, venous thrombosis, venous thromboembolism

Essentials

- Venous thrombosis is the most common vascular complication of menopausal hormone use.
- We studied biomarkers to predict thrombosis with hormones in the Women's Health Initiative.
- Lower proteins C and S, and higher D-dimer were related to thrombosis risk.
- The 25% of women with high D-dimer had a six-times greater risk of thrombosis with hormones.

1 | INTRODUCTION

Oral menopausal hormone therapy (HT) increases the risk of venous thrombosis (VT).[1,2] As this treatment provides effective relief of menopausal symptoms and VT is the most common adverse vascular outcome of HT, knowledge of susceptibility factors might assist women and their physicians in decision-making on risks and benefits of HT use.

In perimenopausal women, the annual rate of VT is 1-2 per 1000,[3] which rises to 0.5-1% over 5 years of HT use. In the Women's Health Initiative (WHI) trials women who were older, obese, or had factor V Leiden were at higher risk of VT with HT.[1,2] For example, conjugated equine estrogens plus medroxyprogesterone acetate (E+P) doubled the risk of VT overall but in women with factor V Leiden the risk was increased 6.7-fold, predicting a cumulative incidence of 3.3-6.7% over 5 years of HT use. Other hemostatic disorders associated with VT risk might predict susceptibility for HT-related VT, as might HT-induced changes in hemostasis or inflammation factors.[4–7] We hypothesized that levels of hemostasis factors and C-reactive protein (CRP), an inflammation marker, would be associated with risk of VT with HT in the WHI trials, and that changes in some of these factors while on treatment would be associated with increased risk of VT.

We conducted a case-control study nested in the two WHI hormone trials. We measured biomarkers of thrombosis risk (factors VIIc, VIIIc, and IXc, von Willebrand factor, fibrinogen, protein C, protein S, antithrombin, prothrombin, D-dimer, and CRP) and others that are altered by HT but have no or uncertain associations with VT (plasminogen activator inhibitor-1 [PAI-1], prothrombin fragment 1.2, plasmin antiplasmin complex [PAP]). Associations of one-year HT-induced changes in some of these biomarkers with risk of VT were also studied.

2 | METHODS

The study design was a nested case control study embedded in two randomized controlled trials of hormone use versus placebo (clinicaltrials.gov identifier NCT 00000611; Women's Health Initiative).

2.1 | Subjects

Detailed descriptions and results of the WHI hormone trials, including Consolidated Standards of Reporting Trials diagrams, were previously published.[8–11] Eligible postmenopausal women aged 50-79 years were enrolled in 1993-1998. Exclusion criteria related to safety concerns with HT. Methods were approved at each site by institutional review committees, and participants provided written informed consent.

The WHI hormone trials included 16 608 women with an intact uterus who were randomly assigned in double-blind fashion to receive E + P or identical placebo, and 10 739 women without a uterus who were randomized to E or placebo. Treatment included one daily tablet containing 0.625 mg conjugated equine estrogen with or without 2.5 mg medroxyprogesterone acetate or identical placebo. At baseline and one year later, blood was drawn and stored at −70 °C.

TABLE 1 Baseline characteristics by case-control status

Characteristic, mean or frequency	Venous Thrombosis Cases (n = 215)		Controls (n = 867)	
	N[a]	Geometric Mean (SD) or %	N[a]	Geometric Mean (SD) or %
HT assigned	215	67%	867	52%
Age	215	66.4 (6.6)	867	66.8 (6.7)
Race, % white	215	87%	867	83%
BMI, kg/m^2	214	31.3 (6)	862	28.6 (5.7)
Prebaseline VT, %	215	4%	867	2%
Procoagulant factors				
Prothrombin Ag, ug/ml	204	107 (18)	838	107 (18)
Factor VIII, %	214	107 (54)	863	97 (49)
Factor IX, %	213	127 (37)	858	128 (37)
von Willebrand Factor, %	212	105 (47)	862	90 (42)
Fibrinogen, g/L	214	3.02 (0.87)	864	2.98 (0.86)
D-dimer, mg/L	212	0.50 (0.36)	864	0.32 (0.27)
Fragment 1.2, nmol/L	192	1.43 (0.42)	760	1.30 (0.38)
Anticoagulant factors				
Protein C, %	146	106 (19)	611	110 (20)
Protein S total, %	146	105 (18)	609	107 (18)
Protein S free, %	145	97 (23)	605	101 (20)
Antithrombin, %	209	86 (15)	837	90 (21)
Fibrinolytic factors				
PAI-1 Ag, ng/mL	149	24.9 (18.9)	600	26.6 (17.9)
PAP, nmol/L	200	4.74 (2.20)	805	4.48 (1.76)
Inflammation factor				
C-reactive protein, mg/L	209	2.90 (2.80)	838	2.17 (2.26)

BMI, body mass index; HT, hormone therapy; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin antiplasmin complex; SD, standard deviation; VT, venous thrombosis.
[a]Sample size varied due to availability of plasma for the study.

Race/ethnicity was self-reported using a list and categorized as black or white/other. Body mass index (BMI) was measured to define overweight (BMI 25-30 kg/m^2) and obesity (BMI >30 kg/m^2).

2.2 | Events ascertainment

Participants were queried every 6 months for possible VT. Hospital discharge summaries were reviewed at each clinical center for all overnight hospitalizations except selected elective procedures. Outpatient-treated VT events were ascertained starting in 1999 by investigating self-reports of participants. Validation of potential VT events was done as previously described.[9] Validated deep vein thrombosis (DVT) was based on a physician diagnosis and positive findings on doppler or duplex ultrasound, or rarely venogram, plethysmography, isotope scan, or at autopsy. Validated pulmonary embolism (PE) was based on a discharge summary diagnosis of PE and positive findings on ventilation-perfusion lung scan, pulmonary angiogram, computed tomography, or at autopsy.

2.3 | Nested case control study

Among all trial participants, excluding baseline warfarin users, a nested case control study of biomarkers in relation to VT, stroke, and myocardial infarction occurring between randomization and February 28, 2001 was conducted. One control was selected for each case with matching on age, randomization date and prevalent vascular disease (myocardial infarction, stroke, or VT). In this study we utilized data from the 215 VT cases and all selected controls (867 total controls).

2.4 | Laboratory analysis

Baseline and follow-up blood samples were analyzed in cases and controls using the following methods: fibrinogen (clot-rate assay, STA-R instrument, Diagnostica Stago, Parsippany, NJ, USA), factor VIII and IX activity (clotting time on mixing with factor VIII or IX deficient plasma using STA-Deficient VIII or IX;

TABLE 2 Odds ratio (95% CI) of VT by categories of baseline biomarkers[a]

	Odds Ratio[b]	(95% CI)
Procoagulant factors		
Prothrombin >P90, ug/mL	0.6	(0.4, 1.2)
Factor VIIIc > P75, %	1.3	(0.9, 1.9)
Factor IXc >P90, %	0.9	(0.5, 1.5)
von Willebrand factor >P75, %	1.3	(0.9, 1.9)
Fibrinogen >P90, mg/dL	0.7	(0.4, 1.2)
D-dimer >P75, ug/mL	2.8	(2.0, 4.0)
Fragment 1.2 > P90, nmol/L	1.9	(1.2, 3.1)
Anticoagulant factors		
Protein C <P5, %	1.8	(0.9, 3.8)
Total protein S <P5, %	1.9	(0.9, 4.1)
Free protein S <P5, %	3.2	(1.6, 6.2)
Antithrombin <P5, %	1.7	(0.9, 3.2)
Fibrinolytic factors		
PAI-1 > P90, ng/ml	0.9	(0.5, 1.7)
PAP >P90, nmol/L	2.4	(1.5, 3.8)
Inflammation factor		
C-reactive protein >P75, mg/L	1.2	(0.8, 1.7)
Number of abnormal biomarkers		
0-1	1.0	(ref)
2-3	2.9	(2.0, 4.3)
4+	7.8	(1.7, 35.1)

BMI, body mass index; CI, confidence interval; P, percentile; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin antiplasmin complex; VT, venous thrombosis.

[a]Cutoff values: prothrombin >137 ug/mL, factor VIIIc >150%, factor IXc >172%, vWF >140%, fibrinogen >4.17 g/L, D-dimer >0.54 mg/L, F1 + 2 > 1.76 nM, protein C < 84%, total protein S < 83%, free protein S < 75%, antithrombin <67%, TAFI >7.53, PAI-1 > 57.7 ng/ml, PAP >7.5 ng/ml, CRP >4.74 mg/L.

[b]Adjusted for age, race, BMI, treatment assignment, self-reported VT, and hysterectomy at screening.

STA-R instrument, Diagnostica Stago), von Willebrand factor, antithrombin and D-dimer (immunoturbidometric or colorimetric assays, Liatest von Willebrand factor, Liatest D-Di, Stachrom ATIII; STA-R instrument, Diagnostica Stago), PAI-1, PAP and prothrombin antigen (in-house immunoassays),[12,13] prothrombin fragment 1.2 (ELISA, Dade Behring, Marburg, Germany), protein C antigen, free and total protein S antigen (Asserachrom ELISA, Diagnostica Stago), CRP (nephelometry, N High Sensitivity CRP, Dade-Behring, Deerfield, IL, USA). Distributions of each biomarker were examined blind to case control status. Analytical outliers were defined based on knowledge of the biology and excluded from analysis as follows: factor VIIIc or prothrombin antigen <10%, fragment 1.2 > 7.2 nmol/L (>3 SD above the mean), PAI-1 > 70 ng/mL.[14]

2.5 | Statistical analysis

Data from both trials were combined for primary analysis. Separate analyses by trial were completed secondarily. For baseline biomarkers in cases and controls, skewed distributions were log-transformed to achieve a normal distribution and geometric means were reported. Hormone therapy use was based on intention-to-treat.

Logistic regression, adjusting for age, race, BMI, treatment assignment, pre-baseline self-reported VT, and hysterectomy status, was used to determine odds ratios of VT for abnormal levels of each biomarker compared to normal levels. Cutoff levels for most biomarkers were defined a priori based on the literature with values shown in the footnote to Table 1. For the following biomarkers, since there is no evidence on VT to suggest cutoffs for abnormal values a priori, we selected the following cutoffs: fragment 1.2 and PAI-1 > 90th percentile, and for antithrombin, protein C and free and total protein S values less than the 5th percentile. Assessments for linear association were also made using each biomarker or its log transformed distribution treated as a continuous variable. We determined the association of each woman's number of abnormal biomarkers with VT risk, including previously published data for factor V Leiden.[1,2]

The additive risk of VT with abnormal biomarkers and HT was assessed by cross-classifying women by treatment assignment and whether they had an abnormal level of each biomarker. Odds ratios were determined by logistic regression adjusted for age, race, and BMI, with women having normal levels of each biomarker and assigned to placebo comprising the reference group. Multiplicative interaction terms between HT assignment and each biomarker were also evaluated.

Evaluation of the association of one-year change in biomarkers with VT risk required exclusion of 83 women with VT between the two phlebotomies. We calculated change in each biomarker by subtracting the baseline from one-year values. Change was divided into quartiles with the lowest quartile including those that decreased the most and the top quartile those that increased the most. Logistic regression was used to analyze the association of quartiles of change in biomarkers, and the change values as continuous variables, with subsequent VT.

To examine change in biomarker levels in HT compared to placebo recipients, linear regression was used comparing treatment groups, adjusting for age, race, BMI, pre-baseline VT, and hysterectomy status.

3 | RESULTS

With mean follow up of 4.1 years, 215 women had VT, 69 in the E trial and 146 in the larger E+P trial. There were 359 and 508 controls selected in each trial. Among cases, 59% in the E trial and 54% in the E+P trial had DVT without PE, with the remainder having PE. There were 132 women with VT after the one-year follow up phlebotomy.

3.1 | Baseline characteristics

Table 2 shows baseline characteristics by case-control status. Few women had pre-baseline self-reported VT. Cases had higher mean BMI, higher prevalence of pre-baseline VT, and higher mean baseline levels of factor VIII, von Willebrand factor, D-dimer, fragment 1.2, and CRP than controls and slightly lower protein C, antithrombin and free protein S. These differences were similar considering the trials separately, but in the E+P trial, cases had similar free protein S levels as controls.

3.2 | Associations of biomarkers with VT

Table 1 shows the odds ratios of VT for abnormal biomarkers, adjusted for age, race, BMI, pre-baseline VT, treatment assignment, and hysterectomy status. High levels of D-dimer, fragment 1.2 and PAP, and low free protein S were significantly associated with increased risk of VT with adjusted odds ratios between 1.9 and 2.8. Four factors were only associated with risk when considered as continuous variables (all $P < .05$). Specifically, for these the adjusted odds ratios per 1 SD higher value were: factor VIII (1.2; 95% CI 1.03-1.4), von Willebrand factor (1.3; 95% CI 1.1-1.5), total protein S (0.8; 95% CI 0.7-0.98), and antithrombin (0.8; 95% CI 0.7-0.98). Considering factor V Leiden and binary terms for abnormal D-dimer, F1-2, protein C, total protein S, free S, antithrombin, PAP, women with increasing numbers of abnormal biomarkers had a higher risk of VT.

When the activation markers D-dimer, F1.2 and PAP were included together in the same model, the odds ratios of VT for each of these were 2.7 (95% CI 1.9-4.0), 1.6 (95% CI 1.0-2.6) and 2.1 (95% CI 1.3-3.5), respectively.

None of the above results differed materially comparing the two trials (data not shown).

3.3 | Joint associations of HT and abnormal biomarkers with VT

To evaluate whether abnormal biomarkers were susceptibility factors for HT-related VT, women were cross-classified by treatment assignment and whether they had an abnormal biomarker and odds ratios for VT calculated for exposed groups compared to women randomized to placebo with a normal biomarker (Table 3). In general, HT in the absence of an abnormal biomarker was associated with a 2-2.5-fold increased risk of VT, while women with abnormal biomarkers assigned to placebo had a 1.0- to 6.5-fold increased risk. Women with the combination of HT plus an abnormal biomarker had consistent elevated risks for VT (OR 2.4-6.0) with the largest odds ratio seen for the combination of HT and high D-dimer at 6.0 (95% CI 3.6-9.8). The odds ratios associated with the combination of HT plus elevated factor VIII or von Willebrand factor, or lower total protein S or antithrombin were approximately additive, while the odds ratios for the combination of HT and elevated fragment 1.2, PAP, CRP, free protein S, and low protein C were less than additive. There were no material differences between the two trials in these results (data not shown). Despite the elevated risk of HT plus abnormal biomarkers, tests for multiplicative interaction between HT assignment and each biomarker as a continuous or binary variable revealed no statistically significant multiplicative interactions (all $P > .05$).

To evaluate a multi-marker score considering eight biomarkers associated with VT risk (factor V Leiden and binary terms for abnormal D-dimer, F1-2, protein C, total protein S, free protein S, antithrombin, and PAP), women were classified as having 0-1, 2, or 3+ abnormal factors. In the figure, compared to women with 0-1 abnormal factors assigned to placebo, the odds ratio of VT with 2 or 3+ abnormal factors rose progressively such that women assigned to HT who had 3+ abnormal factors had 15.5-fold increased odds of VT (95% CI 6.8-35.1) adjusting for age, race, BMI, pre-baseline VT, and hysterectomy status.

3.4 | Change in biomarkers

The one-year changes in biomarkers in each trial by case-control status, excluding women who had VT in that year, are shown in Supplemental Table A. In the E trial all factors changed similarly in cases and controls (all $P > .15$) except von Willebrand factor, which rose more among cases than controls (11 vs 1%, $P = .05$). In the E+P trial factor VIIIc and fragment 1.2 rose more in cases than in controls (factor VIII 10 vs. 0%, $P = .02$, fragment 1.2, 0.32 vs. 0.09 nmol/L, $P = .04$; for all other factors $P > .15$).

One-year changes in biomarkers with treatment compared to placebo were generally similar for E and E+P (Supplemental Table B). In the combined trials, fibrinogen, PAI-1 and antithrombin declined with HT compared to placebo while PAP and CRP increased and the other factors did not change.

Table 4 shows associations of quartiles of change in biomarkers with odds of VT after the second blood collection. Compared to women in the first quartile of change in each biomarker, women in the top quartile of change in prothrombin, factor VIII, von Willebrand factor, fragment 1.2, PAP, and CRP were at increased risk of subsequent VT. While the 95% confidence intervals for these odds ratios all included 1.0, PAP and CRP change in the top compared to bottom quartile were associated with a 1.9-fold increased risk. Considering the biomarkers as continuous variables, only larger increases in factor VIII were associated with subsequent VT; the odds ratio of VT for a 32% greater one-year increase of factor VIII (1 SD increment) was 1.3 (95% CI 1.1-1.6). Interpretation of results did not differ materially considering the trials separately (data not shown).

4 | DISCUSSION

4.1 | Main findings

In this study some thrombosis biomarkers were susceptibility factors for HT-associated VT, especially higher baseline D-dimer, which was associated with 6-fold increased odds of VT with HT. This risk increase is comparable to that of the combination of factor V Leiden

TABLE 3 Odds ratio (95% CI) of VT by baseline biomarkers and treatment assignment[a]

	Odds Ratio	(95% CI)
Factor VIIIc P75, %		
Normal, Placebo	1.0	(ref)
Normal, HT	1.9	(1.3, 2.7)
Elevated, Placebo	1.0	(0.5, 1.9)
Elevated, HT	2.7	(1.7, 4.5)
von Willebrand factor >P75, %		
Normal, Placebo	1.0	(ref)
Normal, HT	1.9	(1.3, 2.7)
Elevated, Placebo	1.0	(0.5, 2.1)
Elevated, HT	2.7	(1.7, 4.5)
D-dimer >P75, ug/ml		
Normal, Placebo	1.0	(ref)
Normal, HT	2.1	(1.4, 3.3)
Elevated, Placebo	2.8	(1.6, 4.9)
Elevated, HT	6.0	(3.6, 9.8)
Fragment 1.2 > P90, nmol/L		
Normal, Placebo	1.0	(ref)
Normal, HT	2.4	(1.6,3.5)
Elevated, Placebo	2.7	(1.3, 5.9)
Elevated, HT	3.9	(2.1, 7.2)
PAP >P90, nmol/L		
Normal, Placebo	1.0	(ref)
Normal, HT	2.5	(1.7, 3.6)
Elevated, Placebo	3.3	(1.6, 6.7)
Elevated, HT	4.9	(2.6, 9.3)
Protein C <P5, %		
Normal, Placebo	1.0	(ref)
Normal, HT	2.4	(1.6, 3.7)
Reduced, Placebo	3.3	(1.1, 10.1)
Reduced, HT	3.2	(1.2, 8.3)
Total protein S <P5, %		
Normal, Placebo	1.0	(ref)
Normal, HT	2.3	(1.5, 3.5)
Reduced, Placebo	2.0	(0.7, 5.9)
Reduced, HT	4.3	(1.5, 12.3)
Free protein S <P5, %		
Normal, Placebo	1.0	(ref)
Normal, HT	2.6	(1.7, 4.0)
Reduced, Placebo	6.5	(2.3, 17.8)
Reduced, HT	5.1	(2.0, 12.6)
Antithrombin <P5, %		
Normal, Placebo	1.0	(ref)
Normal, HT	2.1	(1.5, 2.9)
Reduced, Placebo	1.4	(0.4, 5.3)
Reduced, HT	3.8	(1.8, 7.9)
C-reactive protein >P75, mg/L		
Normal, Placebo	1.0	(ref)
Normal, HT	1.9	(1.3, 2.9)
Elevated, Placebo	1.0	(0.6, 1.9)
Elevated, HT	2.4	(1.5, 3.9)

BMI, body mass index; CI, confidence interval; HT, hormone therapy; P, percentile; PAP, plasmin antiplasmin complex; VT, venous thrombosis.
[a]Women were cross classified for their level of each hemostatic factor (cutoffs provided in Table 2 footnote) and treatment assignment, and each group was compared using logistic regression models to those randomized to placebo and who had normal levels of each factor. Models were adjusted for age, race, BMI, treatment assignment, self-reported VT, and hysterectomy status at screening. *P*-values for multiplicative interaction between treatment assignment and abnormal biomarkers were all >.05.

and HT previously reported in these trials.[1,2] In the presence of three or more of eight VT risk factors in combination with HT, the odds ratio of VT was substantially higher at 15.5. One-year change in biomarkers with HT was not robustly associated with subsequent VT risk, although modest associations were seen for factor VIII, PAP, and C-reactive protein. New findings regarding risk factors for VT include associations of higher levels of prothrombin fragment 1.2 and PAP with VT risk (although prothrombin fragment 1.2 has been reported in relation to VT risk in cancer patients).[15]

4.2 | Relation to other work

Venous thrombosis is a common serious vascular complication of menopausal HT and limited studies suggest the risk is higher in women who are older, obese or have factor V Leiden, prothrombin 20210A or non-O blood group.[1,2,16–19] In contrast to literature for oral contraceptives, despite many studies on effects of HT on hemostasis factors,[7] we are aware of no other prospective studies on biomarkers related to VT risk (or their changes on treatment) as predisposing factors for HT-related VT.

4.3 | Potential implications of the findings

Among the biomarkers considered here, D-dimer was most strongly related to VT. Women with D-dimer >0.54 mg/L (top quartile) had nearly a three-fold higher risk of future VT than women in the lowest quartile. While much clinical interest has focused on D-dimer in predicting recurrent VT,[20–22] our findings confirm prior publications on D-dimer and risk of first VT in men and women.[23–25] Further, women with elevated D-dimer randomized to HT were at six-fold increased risk compared to women with lower D-dimer on placebo. In the WHI trials, baseline D-dimer was also associated with increased risk of

TABLE 4 Odds ratio (95% CI) of VT after Year 1 by one-year change in biomarkers

	Odds Ratio	(95% CI)	*P* Value[a]
Prothrombin, ug/ml			.12
Q1 < −9.2)	1.0	(ref)	
Q2 (−9.2 to 1.2)	0.8	(0.4, 1.6)	
Q3 (−1.2 to 7.9)	1.3	(0.7, 2.3)	
Q4 (>7.9)	1.5	(0.9, 2.7)	
Factor VIIIc, %			.01
Q1 (<−12.9)	1.0	(ref)	
Q2 (−12.9 to 0)	0.8	(0.5, 1.5)	
Q3 (0.1 to 16)	0.9	(0.5, 1.7)	
Q4 (>16)	1.4	(0.8, 2.4)	
von Willebrand factor, %			.13
Q1 (<−16.9)	1.0	(ref)	
Q2 (−16.9 to 0)	0.8	(0.5, 1.5)	
Q3 (0.1 to 16)	1.2	(0.7, 2.1)	
Q4 (>16)	1.4	(0.8, 2.4)	
Fibrinogen, g/L			.59
Q1 (<−0.51)	1.0	(ref)	
Q2 (−0.51 to 0.14)	0.6	(0.4, 1.1)	
Q3 (−0.13 to 0.29)	0.9	(0.5, 1.5)	
Q4 (>0.30)	0.9	(0.6, 1.6)	
D-dimer, ug/ml			.32
Q1 (<−0.07)	1.0	(ref)	
Q2 (−0.07 to 0.02)	0.5	(0.3, 1.0)	
Q3 (0.03 to 0.17)	0.7	(0.4, 1.3)	
Q4 (>0.17)	1.1	(0.6, 1.8)	
Fragment 1.2, nmol/L			.27
Q1 (<−0.10)	1.0	(ref)	
Q2 (−0.10 to 0.07)	0.7	(0.4, 1.4)	
Q3 (0.08 to 0.24)	0.7	(0.3, 1.3)	
Q4 (>0.24)	1.3	(0.7, 2.3)	
Antithrombin, %			.87
Q1 (<−11.9)	1.0	(0.5, 1.8)	
Q2 (−11.9 to 4)	1.0	(0.6, 1.9)	
Q3 (−3.9 to 5.0)	0.9	(0.5, 1.6)	
Q4 (>5.0)	1.0	(ref)	
PAI-1, ng/ml			.40
Q1 (<−12.6)	1.0	(ref)	
Q2 (−12.6 to 3.9)	0.8	(0.4, 1.5)	
Q3 (−3.9 to 4.5)	0.9	(0.4, 1.7)	
Q4 (>4.5)	0.8	(0.4, 1.5)	
PAP, nmol/L			.17
Q1 (<−0.28)	1.0	(ref)	
Q2 (−0.28 to 0.44)	1.3	(0.7, 2.5)	
Q3 (0.45 to 1.19)	1.0	(0.5, 2.0)	
Q4 (>1.19)	1.9	(1.0, 3.5)	
C-reactive protein, mg/L			.49
Q1 (<−0.35)	1.0	(ref)	
Q2 (−0.35 to 0.46)	1.1	(0.6, 2.2)	
Q3 (0.47 to 2.54)	1.7	(0.9, 3.1)	
Q4 (>2.54)	1.9	(1.0, 3.4)	

BMI, body mass index; CI, confidence interval; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin antiplasmin complex; Q, quartile; VT, venous thrombosis.

[a]*P* value from a logistic regression model modeling VT by continuous 1-year difference in biomarker level. All models adjusted for age, race, BMI, treatment assignment, self-reported VT, and hysterectomy status at screening.

stroke and coronary heart disease and, among a variety of biomarkers, only the change in D-dimer with HT predicted stroke risk (but not coronary risk) during follow-up.[26,27]

Considering possible clinical application of D-dimer testing, the threshold value defining VT risk in this study is similar to the threshold used to rule out acute VT with this assay (0.50 mg/L), and that which has been proposed for clinical use in determining a group at low risk of recurrent VT after completing a course of anticoagulation for first unprovoked VT.[20,21] Based on our definition of elevated D-dimer, 25% of women considering HT could be identified as having an increased risk based on D-dimer, with an estimated five-year cumulative incidence of VT of 6% with HT (assuming an annual rate without treatment and with normal D-dimer of 2 per 1000). If HT were withheld from women with elevated D-dimer, their five-year cumulative incidence of VT would be reduced to 3%. The number needed to test to prevent one VT over five years of treatment would then be 33 (1/0.03). Free protein S and PAP had similar odds ratios for VT as D-dimer in combination with HT use, but these point estimates were not precise (wide CIs) and the threshold defining abnormal values would only identify 5-10% of women at risk so the number needed to screen would be much higher. Similarly, considering a multi-marker approach (Figure 1) among women with three or more abnormal biomarkers there was an incremental increase in the odds ratio of VT to 15.5, but only 3% of non-cases had three or more abnormal biomarkers.

We are unaware of previous studies in healthy people demonstrating associations of higher levels of PAP and prothrombin fragment 1.2 with risk of future VT. In the Longitudinal Investigation of Thromboembolism Etiology, elevated PAP was not associated with VT risk.[28] Plasmin antiplasmin is formed upon plasmin generation, thus it is a marker of the fibrinolytic response to fibrin formation. Plasmin antiplasmin increases with HT.[29] Fragment 1.2 is liberated upon conversion of prothrombin to thrombin and indicates enhanced procoagulant activity. It has variably been reported to increase with HT treatment.[29] Because these factors are affected by HT and this study was by design enriched with HT users, further

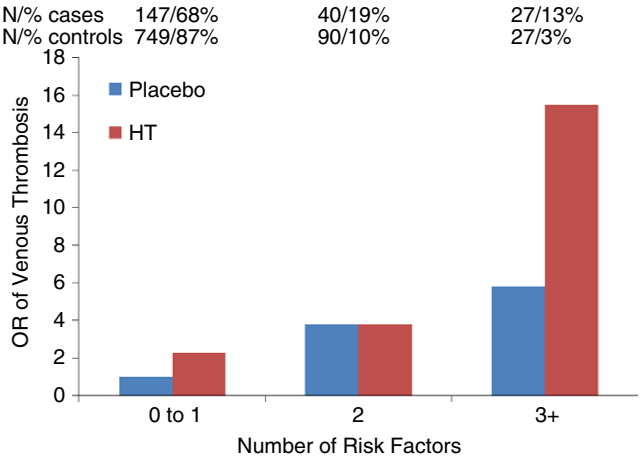

FIGURE 1 Association of Number of Risk Factors with Risk of Future Venous Thrombosis (VT), Stratified by Treatment Assignment. Combining both trials, women were cross classified by treatment assignment and their number of risk factors (including those associated with VT in Table 1 and factor V Leiden). The reference group was women with 0-1 risk factors assigned to placebo. Analyses were adjusted for age, race, BMI, pre-baseline VT, and hysterectomy status

study of their relationships with VT risk in healthy populations is indicated. Similar to another study,[30] we were unable to confirm previous findings that higher factor IX is a VT risk factor.[31,32] We also did not confirm prior conflicting reports of an association of PAI-1 with VT risk.[28,33] Lack of association of well-established VT risk factors, higher factor VIIIc and von Willebrand factor with odds of VT here was unexplained, and these were associated with stroke and coronary risk in our study.[26,27]

4.4 | Study limitations

Limitations of this study require consideration. Participants were older than women who would currently be considering starting HT. There was some nonadherence to assigned treatment in both placebo and HT groups,[11] although this was less early in the trial when most of our cases occurred. If anything, the impact of nonadherence would most likely bias our findings to the null, making our estimates of interaction of biomarkers with HT underestimates and thus conservative. We had limited power to analyze data by HT type, however most associations were similar by study. Studies suggest a lower risk of VT with estradiol or transdermal treatment than oral conjugated equine estrogens[34,35] and we could not address this. To conserve power, we did not exclude women with pre-baseline self-reported VT, but we did adjust for this. Use of ELISAs for proteins C and S would miss functional deficiencies that might have clinical relevance but would be rare. Due to concern for type I error, we did not study nonlinear associations of biomarkers with VT nor did we explore other thresholds (besides our a priori defined ones) to define abnormal values of biomarkers. Assessment of change in hemostatic factors in relation to VT risk was limited because we necessarily excluded VT cases occurring in the first year of follow-up, between the two blood collections. It would have been preferable if the second phlebotomy had been done four to six weeks after randomization to increase the opportunity to relate changes in biomarkers to VT risk. Finally, we did not measure change in protein S. Given our findings for risk of VT with HT plus low baseline protein S, this might be explored in future studies.

4.5 | Study strengths

The key strength of the study was the evaluation of participants from a rigorously conducted randomized controlled trial, eliminating selective prescribing of HT. In addition, we used baseline blood samples prior to HT use or VT for measurement of biomarkers. We are not aware of an existing or planned study with similar design that could be used for replication or which might overcome the limitations mentioned above.

5 | CONCLUSIONS

The WHI clinical trials provided a unique opportunity to examine associations of biomarkers of interest with VT, determine susceptibility factors for HT-associated VT, and determine if changes in biomarkers with HT are related to the incidence of VT. Findings here support potential for clinical use of D-dimer testing in advance of HT prescription to identify women at increased risk of VT. Further study of a multi-marker score in selected high-risk populations might be useful.

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RELATIONSHIP DISCLOSURE

None of the authors have any disclosures relevant to this paper.

AUTHOR CONTRIBUTIONS

M. Cushman: concept and design, analysis and interpretation of data, critical writing, final approval. J. C. Larson: analysis and/or interpretation of data, critical writing, final approval. F. R. Rosendaal: concept and design, analysis and/or interpretation of data, critical writing, final approval. S. R. Heckbert: analysis and/or interpretation of data, critical writing, final approval. J. D. Curb: concept and design, analysis and/or interpretation of data, critical writing. L. S. Phillips: analysis and/or interpretation of data, critical writing, final approval. A. E. Baird: analysis and/or interpretation of data, critical writing, final approval. C. B. Eaton: analysis and/or interpretation of data, critical writing, final approval. R. S. Stafford: analysis and/or interpretation of data, critical writing, final approval

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SUPPORTING INFORMATION

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