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## ORIGINAL ARTICLE

# Venous thrombosis with oral postmenopausal hormone therapy: Roles of activated protein C resistance and tissue factor pathway inhibitor

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## Abstract

**Background:** Oral postmenopausal hormone therapy (HT) increases the risk of venous thrombosis (VT). We postulated that activated protein C (APC) resistance induced by HT is one of the mechanisms causing VT, and also assessed the role of one of the main determinants of APC resistance (i.e., tissue factor pathway inhibitor [TFPI]).

**Methods:** We performed a nested case-control study embedded within two Women's Health Initiative hormone trials. Women were randomized to hormone therapy or placebo. Biomarkers were measured at baseline and after 1 year in 217 cases and 817 controls.

**Results:** Increased APC resistance and decreased TFPI at baseline were associated with VT (odds ratio 1.20–2.06). However, women with such prothrombotic profile at baseline did not have further increased risk of VT when randomized to HT compared with placebo. Although there was no change in APC resistance or TFPI in placebo group after 1 year, HT group showed prothrombotic changes in the biomarkers (i.e., an increase in APC resistance) (mean [standard deviation] 0.39 [0.54]) and decrease in TFPI (–0.21 [0.50]: free TFPI, –0.24 [0.22]: TFPI activity –0.22 [0.20]: total TFPI). However, HT induced prothrombotic change in biomarkers did not increase risk of VT.

**Conclusion:** Women with prothrombotic levels of APC resistance and TFPI at baseline were not at increased risk of VT when randomized to HT compared with placebo. This suggests that testing for these biomarkers before starting HT is not required. HT led to prothrombotic change in these biomarkers after one year, but this did not relate to increased risk of VT.

## KEYWORDS

blood coagulation, menopausal hormone therapy, risk assessment, risk factor, venous thrombosis

## Essentials

- The mechanism by which oral postmenopausal hormone therapy (HT) increases the risk of venous thrombosis (VT) is unknown.
- A nested case-control study embedded within two Women's Health Initiative (WHI) hormone trials was performed.
- HT group showed prothrombotic changes in the biomarkers, i.e. an increase in activated protein C (APC) resistance and a decrease tissue factor pathway inhibitor (TFPI).
- HT induced prothrombotic change in biomarkers did not increase risk of VT.

## 1 | INTRODUCTION

Oral postmenopausal hormone therapy (HT) is associated with an increased risk of venous thrombosis (VT), as detailed in a Cochrane review from 2005, which included 10 randomized controlled trials (RCTs).<sup>1</sup> A Women's Health Initiative (WHI) clinical trial included in the review found an increased risk of VT (hazard ratio [HR] 1.47; 95% confidence interval [CI] 1.06–2.06) in women using combined oral HT, containing conjugated equine estrogen plus medroxyprogesterone acetate (CEE+MPA).<sup>2</sup> Only one trial used 17 $\beta$ -estradiol with norethisterone and reported an increase in VT risk.<sup>3</sup> Estrogen-only oral HT containing 17 $\beta$ -estradiol was not associated with an increased risk of VT; however, only two RCTs contained data on estradiol-only HT.<sup>4,5</sup>

The WHI published a separate trial of CEE alone in women who had undergone hysterectomy (not included in the previously mentioned Cochrane review).<sup>6,7</sup> The results from this trial showed that the use of estrogen-only oral HT increased the risk of VT (HR 1.32; 95% CI 0.99–1.75),<sup>7</sup> although not as strongly as with the use of combined oral HT evaluated in the WHI CEE+MPA trial.<sup>2</sup>

Oral HT has a prothrombotic effect on the coagulation system (i.e., an increase in procoagulant and a decrease in anticoagulant and fibrinolytic biomarkers).<sup>8,9</sup> In 2001, it was demonstrated in an RCT among women with at least one previous VT, that HT containing 17- $\beta$ -estradiol and norethisterone induced acquired activated protein C (APC) resistance and also reduced tissue factor pathway inhibitor (TFPI) and protein S.<sup>10</sup> In a study by Smith et al., CEE users were also resistant to APC and had decreased levels of protein S.<sup>11</sup> A recent study demonstrated that APC resistance induced by oral contraceptive use can at least in part be explained by effects of oral contraceptives on free protein S and TFPI.<sup>12</sup> Recently, Cushman et al.<sup>13</sup> showed using a combined score of eight biomarkers (factor V Leiden, D-dimer, F1-2, protein C, total protein S, free protein S, antithrombin, and plasmin antiplasmin complex), that women with three or more abnormal biomarkers had more than 15-fold increased risk of VT when taking HT compared with placebo. Because APC resistance is a global measurement of coagulation, this assay may be useful in identifying high-risk individuals. Furthermore, TFPI, one of the main determinants of APC resistance, has not yet been described as a VT risk factor when considered in combination with APC resistance.

The aim of this study was to further unravel the role of APC resistance and TFPI in HT-induced VT. We postulated that acquired

APC resistance induced by HT is one of the mechanisms causing VT, and that the changes in procoagulants and anticoagulants induced by HT result in acquired APC resistance.

## 2 | METHODS

### 2.1 | Study design and study subjects

The study design was a nested case-control study embedded within two WHI RCTs of hormone use versus placebo (clinicaltrials.gov identifier NCT 00000611). Detailed descriptions and results of the WHI trials, including Consolidated Standards of Reporting Trials diagrams, were previously published.<sup>2,14-16</sup>

The women included in the trials were postmenopausal women between 50 and 79 years old. Recruitment took place between 1993 and 1998, at 40 US clinical centers. Exclusion criteria were related to competing risks (any medical condition associated with a predicted survival of <3 years), safety (e.g., cancer within the past 10 years), and adherence and retention concerns (e.g., alcoholism, dementia).

The protocol and consent forms were approved by the institutional review board for each participating site, and all women provided written informed consent.

The first WHI trial included 16 608 women with an intact uterus who were randomly assigned to estrogen + progestogen (E+P), as 0.625 mg of CEE with 2.5 mg of MPA, or placebo. The second WHI trial included 10 739 women without a uterus who were randomized to estrogen only (E only), as 0.625 mg of CEE, or placebo. Race/ethnicity was self-reported and weight and height measured.

The current nested case control study investigated biomarkers in relation to VT, stroke, and myocardial infarction occurring between randomization and February 28, 2001. One control was selected for each case with matching on age, body mass index (BMI), prevalent vascular disease (VT, stroke, or myocardial infarction), randomization date and hysterectomy status (i.e., the trial the women belonged to: E+P or E only trial).

The nested case control sample included 217 VT cases and all selected controls (i.e., controls matched on VT, stroke, or myocardial infarction [total 817 controls]). Of these, there were 148 cases and 482 controls from the E+P trial and 69 cases and 335 controls from the E only trial.

## 2.2 | Outcome ascertainment

Participants were contacted by telephone 6 weeks after randomization to assess symptoms and reinforce adherence. Follow-up for VT events occurred every 6 months, with annual in-clinic visits.

At each center, hospital discharges were reviewed for all overnight hospitalizations. Starting in 1999, outpatient-treated VT events were ascertained by examining self-reports of participants. VT validation<sup>15</sup> was done as following: deep vein thrombosis was validated based on a physician diagnosis and positive findings on Doppler or duplex ultrasound, or rarely venogram, plethysmography, isotope scan, or autopsy. Pulmonary embolism was validated based on a discharge summary diagnosis of pulmonary embolism and positive findings on ventilation-perfusion lung scan, pulmonary angiogram, computed tomography, or autopsy.

## 2.3 | Biomarker analysis

Blood samples were collected into tubes containing sodium citrate at baseline and 1 year later. The samples were centrifuged and plasma was stored at  $-70^{\circ}\text{C}$ . The endogenous thrombin potential-based APC resistance test (ETP-based APC resistance test) was performed at the Department of Biochemistry at the University of Maastricht, the Netherlands.<sup>17</sup> The test result is expressed as the ratio of thrombin generation without and with added APC, normalized against pooled normal plasma. In this assay, higher normalized against pooled normal plasma values indicate increasing APC resistance. TFPI was assayed at the Department of Hematology, Oslo University Hospital at Ullevål, Oslo, Norway; free and total TFPI antigen using the Asserachrom enzyme-linked immunosorbent assay (Stago, Asnieres, France) and TFPI activity by an in-house chromogenic substrate activity assay.<sup>18</sup> APC resistance and TFPI were measured at baseline and after 1 year of follow-up.

## 2.4 | Statistical analysis

The primary analysis was performed combining the two WHI trials (i.e., in the combined population of the women randomized to E+P and E only vs their respective placebo control women). However, because in the WHI trials, the risk of VT was slightly different in women using E+P compared with women using E only, additional to the overall analyses, analyses were also performed separately in both groups when there was sufficient power.

A short description of the following analyses can also be found in Table S7. First, we assessed the risk of VT for prothrombotic levels of APC resistance and free, total and TFPI activity at baseline by performing logistic regression analysis. We examined nonlinear associations of log-transformed biomarker values with VT risk. There were no significant nonlinear associations, so we present only the results of statistical models using linear biomarker terms. Odds ratios (OR) and 95% CIs were calculated. In the model, we adjusted for age at

baseline, BMI at baseline, race/ethnicity, history of VT, trial randomization arm, trial randomization date, and hysterectomy status. Cutoff levels of biomarkers were defined *a priori* based on literature or if not available, based on the distribution of the total population (cases and controls). Because the controls were not selected only for the case-control analysis on VT, but also for case-control analyses on myocardial infarction and stroke, we examined the distribution of biomarkers in the total population. The distribution of the total population and controls seemed identical and hence we decided to select the total population distribution. High APC resistance was defined as values above the 75th percentile ( $>1.69$ ) value (reference group:  $\leq 75$ th percentile). Low TFPI (free  $\leq 2.29$ ) and total antigen  $\leq 4.19$ ) and TFPI activity % ( $\leq 4.43$ ) was defined as values  $\leq 10$ th percentile value (reference group  $>10$ th percentile). Robustness of our findings were assessed by repeating the analyses using more extreme cutoff values of the biomarkers (i.e.,  $>90$ th and  $>95$ th percentile for APC resistance and  $\leq 5$ th percentile for TFPI).

We assessed the joint effect of prothrombotic baseline biomarkers levels and HT use on the risk of VT. This was done by categorizing women by treatment assignment (yes/no) and levels of APC resistance ( $>75$ th percentile vs.  $\leq 75$ th percentile) and levels of TFPI ( $\leq 10$ th percentile vs  $>10$ th percentile) at baseline. Logistic regression analysis was performed after adjusting for age at baseline, BMI at baseline, race/ethnicity, history of VT, trial randomization date, and hysterectomy status. The reference group consisted of women with normal levels of each biomarker at baseline and assigned to placebo.

We further evaluated the effect of HT use on biomarker values by assessing the change in values from baseline to year 1. Change was calculated from log biomarker value:  $\log(\text{biomarker value at year 1}) - \log(\text{biomarker value at baseline})$ . In this analysis, we excluded women who reported an event before their first-year blood collection. To examine the mean change in biomarker levels in HT compared with placebo group, a two-sample t-test was performed comparing the two treatment groups.

We also assessed the association between the change in APC resistance and TFPI in both HT and placebo groups. For this, we calculated the Pearson correlation coefficient which represents the association between log-transformed biomarker values.

Next, we evaluated whether a change in biomarker levels (regardless of whether the women used HT) was associated with the risk of VT. Change was divided into tertiles as "low," "medium," and "high" categories. The low free TFPI and TFPI activity groups, indicating a prothrombotic change, consisted of all women whose free TFPI and TFPI activity levels decreased in 1 year. The change in total TFPI was dichotomized to increase (reference group) or decrease, rather than in tertiles, since the model did not converge for women belonging in the group with no or small change. The high APC resistance group, indicating a prothrombotic profile, consisted of all women whose APC resistance increased in 1 year. The medium (reference) group consisted of those women whose biomarker values did not or only moderately increased or decreased in 1 year, for APC resistance and TFPI (free TFPI and TFPI activity), respectively. This analysis was adjusted for age at baseline, BMI at baseline, race/

ethnicity, history of VT, trial randomization arm, trial randomization date, and hysterectomy status.

In the last analysis, we evaluated whether an HT-induced prothrombotic change in biomarker levels was associated with VT risk. We categorized women by treatment assignment (yes/no) and whether they had a prothrombotic change (i.e., belonging in the high group for APC resistance and in the low group for TFPI). Logistic regression analysis was used to analyze the association and adjustments were performed for age at baseline, BMI at baseline, race/ethnicity, history of VT, trial randomization arm, trial randomization date, and hysterectomy status.

### 3 | RESULTS

#### 3.1 | Baseline characteristics

Baseline characteristics by case-control status are shown in Table 1 (overall) and in Table S1 (the two trials separately).

Age was similar between cases and controls. Cases had a higher BMI compared with controls in the overall analysis: mean (standard deviation) of 31.27 (6.01) in cases and 28.49 (5.70) in controls. A higher prevalence of history of VT was seen in cases compared with controls, 4.15% and 1.96%, respectively. Cases also had higher baseline APC resistance compared with controls in the E trial (although this was not the case in the E+P trial), with a mean (standard deviation) of 4.28 (1.94) in cases and 3.29 (2.03) in controls, respectively (Table S1). Total TFPI and TFPI activity were lower in cases compared with controls, whereas free TFPI was slightly higher in cases compared with controls (Table 1).

**TABLE 1** Baseline characteristics of cases and controls (both E only and E+P trials)

Characteristics	Cases	Controls
Total	217	817
Age at baseline, y (mean [SD])	66.42 (6.61)	66.59 (6.74)
BMI (mean [SD])	31.27 (6.01)	28.49 (5.70)
Race/ethnicity, N (%)		
White	190 (87.56)	676 (82.74)
Black	20 (9.22)	86 (10.53)
Others	7 (3.23)	55 (6.73)
History of VT, N (%)	9 (4.15)	16 (1.96)
APC resistance (mean [SD])	3.96 (2.23)	3.97 (2.36)
Free TFPI (ng/ml) (mean [SD])	19.24 (12.53)	17.78 (10.29)
Total TFPI (ng/ml) (mean [SD])	85.54 (18.71)	89.53 (21.06)
TFPI activity % (mean [SD])	106.80 (20.75)	114.00 (25.67)

Abbreviations: APC, activated protein C; BMI, body mass index; SD, standard deviation; TFPI, tissue factor pathway inhibitor; VT, venous thrombosis

#### 3.2 | Association of biomarkers and joint association of biomarkers and HT with VT

A high APC resistance at baseline (>75th percentile vs. ≤75th percentile) was associated with an increased risk of VT, with an OR (95% CI) of 1.24 (0.83–1.84) (Table 2). Similarly, low TFPI (free and total antigen and activity) at baseline (≤10th percentile vs. >10th percentile) was associated with an increased risk. Some differences were found in the risk of VT associated with prothrombotic baseline biomarkers for the two trials separately (i.e., a high APC resistance in the E only) (OR 3.32 [95% CI 1.55–7.08]), but not in E+P trial (OR 0.86 [95% CI 0.54–1.39]) was associated with a higher risk of VT, albeit the CI were wide (Table S2). Low TFPI activity (OR 0.98 [95% CI 0.38–2.49]) at baseline was not associated with an increased risk of VT in the E only trial. Results of these analyses were in a similar direction when different cutoff values of biomarkers were used (results not shown).

Table 3 shows the joint effect between the levels of biomarkers at baseline and HT on the risk of VT. In the absence of a prothrombotic biomarker (i.e., high APC resistance or low TFPI) at baseline, HT was associated with a 2- to 2.7-fold increased risk of VT. A high APC resistance (>75th percentile vs. ≤75th percentile) in the absence of HT was not associated with an increased risk of VT, with an OR of 1.05 (0.53–2.06). For low TFPI (free, total, and activity) in the absence of HT, the OR ranged from 1 to –2.6. Women with a prothrombotic marker at baseline (high APC resistance or low TFPI) and using HT had a consistently higher risk of VT (OR 2 to –4.1) with the highest OR for total TFPI (≤10th percentile vs. >10th percentile) levels and HT: OR 4.12 (2.10–8.05). The results of these analyses for the two separate trials are reported in Table S3. The results were comparable with the results from the

**TABLE 2** Risk of venous thrombosis by categories of baseline biomarkers<sup>a</sup> (both E only and E+P trials)

Biomarker (baseline)	OR (95% CI) <sup>b</sup>
APC resistance	
>75th percentile	1.24 (0.83, 1.84)
Free TFPI	
≤10th percentile	1.30 (0.77, 2.19)
Total TFPI	
≤10th percentile	1.59 (0.96, 2.64)
TFPI activity %	
≤10th percentile	1.20 (0.73, 1.97)

Abbreviations: APC, activated protein C; CI, confidence interval; OR, odds ratio; TFPI, tissue factor pathway inhibitor

<sup>a</sup>Cutoff levels of baseline biomarkers were defined *a priori* based on literature or if not available, based on the distribution of the total population (cases and controls). APC resistance >1.69, free TFPI ≤2.29, total TFPI ≤4.19, TFPI activity % ≤4.43.

<sup>b</sup>Adjusted for age at baseline, body mass index at baseline, race/ethnicity, history of venous thrombosis, trial randomization date, trial randomization arm, and hysterectomy status.

main analyses, except for APC resistance in the E only trial, where the OR for the combination of this biomarker with HT was 12.42 (3.78–40.76), albeit the number of women in this category were

low. The combined effect between high APC resistance and HT was more than additive.

**TABLE 3** Risk of venous thrombosis by categories of baseline biomarkers<sup>a</sup> and treatment assignment (both E only and E+P trials)

Biomarker	HT	Cases	Controls	OR (95% CI) <sup>b</sup>
		217	817	
APC resistance (>75th percentile)				
No	No	45 (23.9)	221 (37.5)	Ref
Yes	No	16 (8.5)	68 (11.5)	1.05 (0.53, 2.06)
No	Yes	91 (48.4)	227 (38.5)	2.13 (1.40, 3.25)
Yes	Yes	36 (19.2)	74 (12.5)	2.89 (1.69, 4.95)
Free TFPI (≤10th percentile)				
No	No	56 (26.5)	296 (45.0)	Ref
Yes	No	13 (6.2)	32 (4.9)	2.61 (1.25, 5.46)
No	Yes	130 (61.6)	292 (44.4)	2.72 (1.88, 3.95)
Yes	Yes	12 (5.7)	38 (5.8)	2.01 (0.94, 4.30)
Total TFPI (≤10th percentile)				
No	No	61 (28.9)	299 (45.9)	Ref
Yes	No	8 (3.8)	29 (4.5)	1.27 (0.54, 2.97)
No	Yes	121 (57.3)	296 (45.4)	2.26 (1.57, 3.25)
Yes	Yes	21 (10.0)	28 (4.3)	4.12 (2.10, 8.05)
TFPI Activity % (≤10th percentile)				
No	No	57 (27.0)	295 (45.0)	Ref
Yes	No	12 (5.7)	33 (5.0)	1.64 (0.78, 3.44)
No	Yes	127 (60.2)	294 (44.8)	2.52 (1.74, 3.64)
Yes	Yes	15 (7.1)	34 (5.2)	2.38 (1.18, 4.78)

Abbreviations: APC, activated protein C; CI, confidence interval; HT, hormone therapy; OR, odds ratio; TFPI, tissue factor pathway inhibitor.

<sup>a</sup>Cutoff levels of biomarkers were defined a priori based on literature or if not available, based on the distribution of the total population (cases and controls). APC resistance >1.69, free TFPI ≤2.29, total TFPI ≤4.19, TFPI activity % ≤4.43.

<sup>b</sup>Adjusted for age at baseline, body mass index at baseline, race/ethnicity, history of venous thrombosis, trial randomization date, and hysterectomy status.

**TABLE 4** Effect of hormone therapy on the change<sup>a</sup> in biomarker values from baseline to year 1 (both E only and E+P trials)

Biomarker Change	Placebo Mean (SD)	Treatment Mean (SD)	95% CI
APC resistance	−0.08 (0.62)	0.39 (0.54)	−0.59 to −0.36
TFPI activity (%)	0.00 (0.15)	−0.24 (0.22)	0.21–0.28
Free TFPI	−0.01 (0.40)	−0.21 (0.50)	0.12–0.27
Total TFPI	−0.01 (0.14)	−0.22 (0.20)	0.18–0.24

Abbreviations: APC, activated protein C; CI, confidence interval; SD, standard deviation; TFPI, tissue factor pathway inhibitor.

<sup>a</sup>Change in biomarker values was calculated from log biomarker value: log(biomarker value at year 1) − log(biomarker value at baseline).

### 3.3 | Change in biomarkers

Table 4 shows the effect of HT use on biomarker levels by assessing the change in levels from baseline to year 1.

Women in the placebo groups showed no change in biomarker levels, whereas women in HT group showed changes in biomarkers levels at year 1 follow-up compared with baseline. These changes were in the expected direction (i.e., an increase in APC resistance) (mean [standard deviation]: 0.39 [0.54]), whereas free TFPI (−0.21 [0.50]) total TFPI (−0.22 [0.20]), and TFPI activity (−0.24 [0.22]) levels were decreased at year 1 follow-up compared with baseline. The results from the two separate trials were similar compared with the results from the main analysis (Table S4).

A negative correlation was found between the change in APC resistance and change in TFPI and this was more pronounced in HT versus the placebo groups (Table 5). The correlation coefficient between the change in APC resistance and the change in TFPI in HT group was as following: −0.18 (95% CI −0.30 to −0.05) for free TFPI, −0.21 (−0.33 to −0.08) for total TFPI, and −0.25 (−0.36 to −0.12) for TFPI activity. In the placebo group, these correlations were −0.10 (−0.24 to 0.04) for free TFPI, 0.01 (−0.13 to 0.15) for total TFPI, and −0.11 (−0.25 to 0.03) for TFPI activity. The results from the two separate trials were similar compared with the results from the main analysis (Table S5).

Table 6 depicts the VT risk for the 1-year change in biomarker levels (regardless of using HT).

Women who had a prothrombotic change in their APC resistance levels after 1 year (i.e., who had an increase in APC resistance [high group]) were not at increased risk of VT (OR 1.01 [0.54–1.89]) compared with women who had no or little change (medium group). For TFPI, only a change in TFPI activity (i.e., women with a decrease in this biomarker [low/decrease group]) had an increased risk of VT compared with the group which showed no or little change (medium group). The results from the two separate trials were similar compared with the results from the main analysis (Table S6).

Table 7 shows the risk of VT associated with HT induced prothrombotic change in biomarker levels.



Biomarker Change	Placebo Coefficient Estimate (95% CI)	Treatment Coefficient Estimate (95% CI)
TFPI activity (%)	-0.11 (-0.25, 0.03)	-0.25 (-0.36 to -0.12)
Free TFPI	-0.10 (-0.24, 0.04)	-0.18 (-0.30 to -0.05)
Total TFPI	0.01 (-0.13, 0.15)	-0.21 (-0.33 to -0.08)

TABLE 5 Correlation between the change<sup>a</sup> in APC resistance and TFPI (both E only and E+P trials)

Abbreviations: CI, confidence interval; TFPI, Tissue factor pathway inhibitor.

<sup>a</sup>Change was calculated from log biomarker value: log(biomarker value at year 1) – log(biomarker value at baseline).

TABLE 6 Risk of venous thrombosis by tertiles<sup>a</sup> of biomarker change (both E only and E+P trials)

Biomarker Change (Tertiles)	Cases	Controls	OR (95% CI) <sup>b</sup>
APC resistance			
Low (-0.46)	30 (36.1)	113 (32.6)	1.36 (0.72–2.58)
Medium (0.15)	26 (31.3)	118 (34.0)	Ref
High (0.81)	27 (32.5)	116 (33.4)	1.01 (0.54–1.89)
Free TFPI			
Low (-0.59)	35 (31.0)	148 (33.8)	1.06 (0.61–1.85)
Medium (-0.11)	32 (28.3)	152 (34.7)	Ref
High (0.34)	46 (40.7)	138 (31.5)	1.46 (0.86–2.49)
Total TFPI			
Increase (0.11)	36 (31.9)	120 (27.5)	Ref
Decrease (-0.21)	77 (68.1)	316 (72.5)	0.69 (0.41–1.13)
TFPI activity (%)			
Low (-0.38)	43 (38.4)	141 (32.1)	1.30 (0.73–2.29)
Medium (-0.11)	30 (26.8)	153 (34.9)	Ref
High (0.09)	39 (34.8)	145 (33.0)	1.67 (0.94–2.98)

Abbreviations: APC, activated protein C resistance; CI, confidence interval; OR, odds ratio; TFPI, tissue factor pathway inhibitor.

<sup>a</sup>Biomarker change was assessed by assessing the difference in levels between measurement after 1 year and baseline. The biomarker change was subsequently divided into tertiles of this biomarker to define medium (no or little change), low (decrease in change), and high (increase in change) categories. For total TFPI, the categories were binary (i.e., increase and decrease in change over 1 year follow-up).

<sup>b</sup>Adjusted for age at baseline, body mass index at baseline, race/ethnicity, history of venous thrombosis, trial randomization date, trial randomization arms, and hysterectomy status.

Women not using HT, but who did show a prothrombotic change in their APC resistance levels from baseline to 1 year follow-up (i.e., who had an increase in APC resistance [high group]) had an OR (95% CI) of 2.31 (0.68–7.80). For women not using HT who showed a prothrombotic change in their TFPI levels (i.e., who had a decrease in TFPI [low/decrease group]) from baseline to 1 year follow-up, had an OR ranging from 0.9 to 3.6. The OR for the combined effect of HT and a prothrombotic change in biomarkers was associated with an increased risk of VT, ranging from 1 to 2.5. The combined effect was approximately additive for all the biomarkers and HT. Because of low power, the analyses could not be performed for the two trials separately.

## 4 | DISCUSSION

In this study, we assessed the role of APC resistance and TFPI in HT-induced VT. High baseline levels of APC resistance and low baseline levels of TFPI were associated with an increased risk of VT. However, women with such prothrombotic levels at baseline did not have a further increase in the risk of VT after they started using HT. HT led to a prothrombotic change in biomarkers (i.e., an increase in APC resistance and a decrease in TFPI levels after 1 year of use). However, our study did not confirm the hypothesis that HT induced prothrombotic change in the biomarkers increases the risk of VT.

Women with prothrombotic levels of biomarkers at baseline were not at further increased risk of VT when randomized to HT versus placebo. This may suggest that these women are already at a certain threshold, and that administration of HT does not further increase their risk of VT. This finding may also suggest that testing for these biomarkers (APC resistance and TFPI) is not required when starting HT to identify women at an increased risk of VT.

Another main determinant of the ETP-based APC test is protein S.<sup>19</sup> The WHI study by Cushman et al.<sup>13</sup> showed that women with prothrombotic levels of both free and total protein S at baseline had higher risk of VT when they started using HT, with an OR (95% CI) of 5.1 (2.0–12.6) for free protein S and 4.3 (1.5–12.3) for total protein S, respectively. However, for both the combined effects between HT and prothrombotic biomarker levels at baseline, the OR for the combined effect was approximately additive. Although testing for APC resistance or TFPI (and protein S) does not seem beneficial, the study by Cushman et al.<sup>13</sup> supported the potential clinical use of D-dimer testing before starting HT because higher baseline D-dimer was associated with a 6-fold increased risk of VT in women starting HT. Furthermore, with a multimarker score of eight biomarkers (factor V Leiden, D-dimer, F1-2, protein C, total protein S, free protein S, antithrombin, and plasmin antiplasmin complex), women with 3+ abnormal factors at baseline had a 15.5-fold increased risk of VT when starting HT, albeit the CI was 6.8–35.1 was wide.

A previous study showed that HT causes APC resistance, in both carriers and noncarriers of factor V Leiden.<sup>10</sup> As mentioned, free protein S and free TFPI are both important parameters for the acquired APC resistance. APC resistance is measured by APC sensitivity tests.<sup>17,20,21</sup> These tests quantify the effects of APC on the activated partial thromboplastin time and the ETP, respectively. The ETP-based test is also highly dependent on oral contraceptive (OC)

**TABLE 7** Risk of venous thrombosis by tertiles<sup>a</sup> of biomarker change and treatment assignment (both E only and E+P trials)

Biomarker Change (Tertiles)	HT	Cases	Controls	OR (95% CI) <sup>b</sup>
<b>APC resistance</b>				
Medium (0.15)	No	6 (7.2)	57 (16.4)	Ref
High (0.81)	No	7 (8.4)	29 (8.4)	2.31 (0.68–7.80)
Medium (0.15)	Yes	20 (24.1)	61 (17.6)	3.11 (1.13–8.58)
High (0.81)	Yes	20 (24.1)	87 (25.1)	2.29 (0.84–6.24)
<b>Free TFPI</b>				
Medium (–0.11)	No	13 (11.5)	78 (17.8)	Ref
Low (–0.59)	No	7 (6.2)	40 (9.1)	0.94 (0.34–2.61)
Medium (–0.11)	Yes	19 (16.8)	74 (16.9)	1.41 (0.64–3.11)
Low (–0.59)	Yes	28 (24.8)	108 (24.7)	1.61 (0.77–3.35)
<b>Total TFPI</b>				
Increase (0.11)	No	22 (19.5)	91 (20.9)	Ref
Decrease (–0.21)	No	21 (18.6)	113 (25.9)	0.76 (0.38–1.51)
Increase (0.11)	Yes	14 (12.4)	29 (6.7)	2.05 (0.90–4.66)
Decrease (–0.21)	Yes	56 (49.6)	203 (46.6)	1.25 (0.70–2.21)
<b>TFPI activity (%)</b>				
Medium (–0.11)	No	10 (8.9)	76 (17.3)	Ref
Low (–0.38)	No	5 (4.5)	11 (2.5)	3.57 (0.92–13.88)
Medium (–0.11)	Yes	20 (17.9)	77 (17.5)	2.36 (1.01–5.52)
Low (–0.38)	Yes	38 (33.9)	130 (29.6)	2.54 (1.17–5.53)

Abbreviations: APC, activated protein C resistance; CI, confidence interval; HT, hormone therapy; OR, odds ratio; TFPI, tissue factor pathway inhibitor.

<sup>a</sup>Biomarker change was assessed by assessing the difference in levels between measurement after 1 year and baseline. The biomarker change was subsequently divided into tertiles of this biomarker to define medium (no or little change), low (decrease in change), and high (increase in change) categories. For total TFPI, the categories were binary (i.e., increase and decrease in change over 1 year follow-up).

<sup>b</sup>Adjusted for age at baseline, body mass index at baseline, race/ethnicity, history of venous thrombosis, trial randomization date, and hysterectomy status.

use.<sup>19</sup> Furthermore, users of third-generation OCs (i.e., OCs considered high risk) were shown to be more resistant to APC and had lower levels of TFPI and protein S compared with users of the low-risk OCs (i.e., second-generation OCs).<sup>12</sup>

In this study, similar to prior research in OC users, HT use led to higher APC resistance and lower TFPI compared with placebo. Furthermore, there was a negative correlation between the change in APC resistance and the change in TFPI after 1 year of HT use. We did not investigate the reason for the decrease in TFPI levels in HT users. The decrease in free TFPI induced by OC use seems to be caused by changes in synthesis, clearance, or lipoprotein profile.<sup>22,23</sup> This may also be true for the observed HT-induced reduction in TFPI. Unfortunately, protein S was not measured at 1 year follow-up in the WHI trials; therefore, we were unable to assess the changes in this biomarker because of HT use.

HT-induced prothrombotic change in the studied biomarkers was not associated with an increased risk of VT. One explanation for this may be that the change in biomarkers caused by HT was not large enough to influence VT risk. Our results are consistent with a

previous study, which concluded that prothrombotic changes in APC resistance and TFPI caused by HT are not sufficient to explain the increased risk of VT caused by HT.<sup>10</sup> The combined effect of increased APC resistance and HT on VT risk showed in Table 7 suggests that these two risk factors are independent of each other. Thus, the VT risk associated with HT use may not be caused by increased APC resistance. The same independent effect is suggested by the results for TFPI activity.

Because of the complexity of the coagulation cascade, and the interplay of many coagulation factors, HT-induced increase in VT might be due to other biomarkers that were not measured in this or other studies. In a prior WHI study, change in factor VIII (FVIII) was associated with higher subsequent VT risk with HT.<sup>13</sup> FVIII levels might influence the ETP-based APC resistance test.<sup>19</sup> This study showed only a small increase in APC resistance for the highest quartile of FVIII because of high tissue factor concentrations present in the ETP test. Lowering the tissue factor concentration will make thrombin generation more dependent on the concentration of the components of the tenase complex (FIX, FVIII).



FXa may also be important in HT-mediated VT, particularly because TFPI levels were reduced in women using HT in this study. Decreased TFPI leads to increased FXa.

FXa protects FVa from inactivation by APC by selectively blocking cleavage at Arg506,<sup>24</sup> therefore inducing APC resistance. Because the ETP-based APC sensitivity test is based on the extrinsic pathway, other coagulation factors involved in this pathway need to be explored.

The strengths of this study are that the data come from well-designed RCTs in which there was not selective prescribing of HT. Also, baseline blood samples before HT use or VT measurement were available.

The limitations of this study are that the mean age was higher than of women who would consider starting HT in the contemporary era. The majority of the women in this study were White. Therefore, our results may not be generalizable to women of the other race/ethnic groups. The mean follow-up after randomization in the E+P trial was 5.2 years; in the E only trial, it was 8.5 years. There was some nonadherence to assigned treatment in both placebo and HT groups, 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. Fourth, we had limited power to analyze the data per trial type; however, most of the associations were similar to the overall analysis. Also, we did not assess the associations for the different types of HT, such as those containing estradiol or administered transdermally. Studies have suggested that VT risk may be lower for these types of HT compared with CEE.<sup>25,26</sup> Women with a history of VT were not excluded, but we adjusted for this variable to conserve power. Biomarkers were measured at baseline and at 1 year follow-up, and therefore the assessment of change in biomarkers in relation to VT risk was limited. Preferably, biomarkers would have been measured every month or every 3 months, to increase the opportunity to relate the changes in biomarkers to VT risk. Also, the risk of VT in HT users changes over time (i.e., it is increased in the first 3–12 months after starting and decreases afterwards).<sup>27</sup> Women with VT in the first year's follow-up were excluded from analysis of change, so any relationships would be underestimated. Last, protein S was not measured at 1 year follow-up, and therefore changes protein S from HT and risk of VT could not be assessed. This should be explored in future studies.

## 5 | CONCLUSION

In this study, women with prothrombotic levels of APC resistance and TFPI at baseline did not have a further increase in the risk of VT with HT use. This suggests that testing for these biomarkers before starting HT is not required. HT led to a prothrombotic change in these biomarkers, but this change was not associated with an increased risk of VT. Future studies should explore other biomarkers, such as FX, which may be involved in HT-related increased risk of VT.

## AUTHOR CONTRIBUTIONS

D. Khialani and A. van Hylckama Vlieg drafted the manuscript. S. Vasan performed the statistical analyses of the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. M. Cushman, A. E. A. Dahm, P. M. Sandset, and J. Rossouw critically revised the manuscript for important intellectual content.

## CONFLICT OF INTEREST

The authors report no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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