Can Biomarkers Identify Women at Increased Stroke Risk? The Women's Health Initiative Hormone Trials

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Abbreviations: BMI, body mass index; BP, blood pressure; 95% CI, 95% confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; HT, hormone therapy; IL-6, interleukin-6; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); MI, myocardial infarction; MMP-9, matrix metalloproteinase-9; MTHFR, methylene tetrahydrofolate reductase; OR, odds ratio(s); PAI-1, plasminogen activator inhibitor-1 antigen; PAP, plasmin-antiplasmin complex; TAFI, thrombin activatable fibrinolysis inhibitor; VTE, venous thromboembolism

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ABSTRACT

Objective: The Women's Health Initiative hormone trials identified a 44% increase in ischemic stroke risk with combination estrogen plus progestin and a 39% increase with estrogen alone. We undertook a case-control biomarker study to elucidate underlying mechanisms, and to potentially identify women who would be at lower or higher risk for stroke with postmenopausal hormone therapy (HT).

Design: The hormone trials were randomized, double-blind, and placebo controlled.

Setting: The Women's Health Initiative trials were conducted at 40 clinical centers in the United States.

Participants: The trials enrolled 27,347 postmenopausal women, aged 50–79 y.

Interventions: We randomized 16,608 women with intact uterus to conjugated estrogens 0.625 mg with medroxyprogesterone acetate 2.5 mg daily or placebo, and 10,739 women with prior hysterectomy to conjugated estrogens 0.625 mg daily or placebo.

Outcome Measures: Stroke was ascertained during 5.6 y of follow-up in the estrogen plus progestin trial and 6.8 y of follow-up in the estrogen alone trial.

Results: No baseline clinical characteristics, including gene polymorphisms, identified women for whom the stroke risk from HT was higher. Paradoxically, women with higher baseline levels of some stroke-associated biomarkers had a lower risk of stroke when assigned to estrogen plus progestin compared to placebo. For example, those with higher IL-6 were not at increased stroke risk when assigned to estrogen plus progestin (odds ratio 1.28) but were when assigned to placebo (odds ratio 3.47; p for difference = 0.02). Similar findings occurred for high baseline PAP, leukocyte count, and D-dimer. However, only an interaction of D-dimer during follow-up interaction with HT and stroke was marginally significant ($p = 0.03$).

Conclusions: Biomarkers did not identify women at higher stroke risk with postmenopausal HT. Some biomarkers appeared to identify women at lower stroke risk with estrogen plus progestin, but these findings may be due to chance.

Editorial Commentary

Background: The Women's Health Initiative hormone trials originally set out to evaluate whether postmenopausal hormone therapy (HT, estrogen in the case of women who had had a hysterectomy, and estrogen plus progestin for women who had not had a hysterectomy) reduced the risk of heart attacks and strokes, as compared to placebo. The trials were stopped early, and the investigators found that both estrogen alone, as well as estrogen plus progestin increased the risk of stroke amongst women participating in the trials. As part of a secondary analysis of data from these trials, the investigators aimed to explore possible associations between various biological markers (such as variants in particular genes, and levels of particular lipids, proteins, and other markers in blood), and the risk of a woman experiencing a stroke in the trials. Specifically, they wanted to evaluate whether there was any evidence for particular markers being associated with the risk of a stroke; and then whether that risk was modified by whether a woman took HT in the trials.

What the trial shows: In this study, the researchers collected early cases of ischemic stroke in the trials (combining cases among women taking estrogen with those for women taking both estrogen and progestin), and matched these to control individuals, or women participating in the trials who did not experience a stroke. Two hundred five women who experienced a stroke were compared to 878 control individuals. The markers analyzed included those for which there was already some evidence for an association with stroke. Several clinical characteristics and some biomarkers, as measured at the start of the trial, but none of the gene variants, were linked with later risk of stroke. However, none of these clinical characteristics or gene variants specifically identified women who were at greater risk of experiencing a stroke within the HT arms of the trial. High levels of two biomarkers, IL-6 and PAP, did seem to identify women who were at lower risk of experiencing a stroke within the HT arms of the trial. This finding is interesting, because high levels of these markers had previously been suggested as being associated with a higher risk of stroke. Levels of several biomarkers changed during the trial, but for only one biomarker, D-dimer, did the change (an increase in levels) seem to predict higher risk of stroke amongst women receiving HT.

Strengths and limitations: A particular strength of this study includes the nesting of a case-control study within the Women's Health Initiative trials, in which HT or placebo was randomly assigned. This design minimizes the chance that individuals taking HT differ in their stroke risk from individuals taking placebo. However, the power of this study to detect anything other than large associations is limited; together with the limitation of multiple statistical testing, the findings here must be interpreted as hypotheses for further study and not definitive conclusions.

Contribution to the evidence: This study adds data relating to possible predictive risk markers for stroke among users of HT. The hypotheses raised here remain to be tested in further studies.
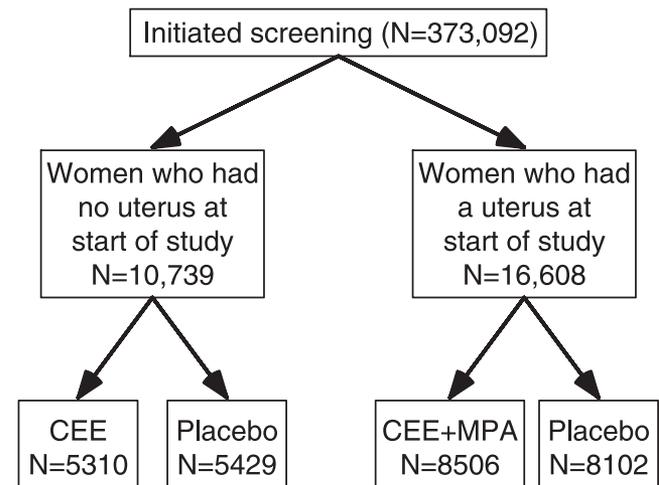
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Women's Health Initiative Hormone Trials

Figure 1. Women's Health Initiative Hormone Trials

range of genetic and baseline phenotypic biomarkers in order to formulate hypotheses about how HT increased stroke risk. We evaluated the association between baseline biomarkers and stroke, whether that association was modified by HT, whether biomarkers were influenced by HT, and whether biomarker changes influenced stroke risk.

The biomarkers analyzed in this paper were part of a slightly larger panel of markers that were thought by members of the Women's Health Initiative laboratory working group to be associated with either stroke, venous thrombotic disease, or myocardial infarction (MI). Before analyzing the data we restricted our attention to those markers for which we felt there was evidence for an association with stroke, or for a modification of the effects on stroke in the presence of HT. Other markers in the original panel were not analyzed in relation to stroke. The major focus of this biomarker study was to understand why HT increases stroke risk.

METHODS

Details of the design, recruitment, randomization, data collection, intervention, and outcomes ascertainment procedures of the Women's Health Initiative hormone trials, including CONSORT diagrams, have been published [1,2,5–8]. Also see Figure 1.

Participants and Interventions

Between November 1993 and October 1998, postmenopausal women with prior hysterectomy ($n = 10,739$) were randomized to conjugated equine estrogens 0.625 mg/day (Premarin, Wyeth Pharmaceticals, http://www.wyeth.com) or placebo; those with an intact uterus ($n = 16,608$) were randomized to conjugated equine estrogens 0.625 mg/day with medroxyprogestrone acetate 2.5 mg/day (Prempro, Wyeth Pharmaceticals) or placebo (Figure 1). Participants provided informed consent in a form approved by local institutional review

INTRODUCTION

The Women's Health Initiative hormone trials were designed to evaluate the role of postmenopausal hormone therapy (HT) in cardiovascular risk reduction. Unexpectedly, both estrogen with progestin and estrogen alone increased stroke risk [1,2]. Evaluation of clinical characteristics and a limited number of biomarkers in the individual trials [3,4] failed to identify women at higher or lower risk for stroke with HT.

In this analysis, we pooled stroke outcomes from the Women's Health Initiative hormone trials to evaluate a broad

boards. The estrogen plus progestin arm was stopped after 5.6 y of follow-up upon recommendation of the Data and Safety Monitoring Board because of increased breast cancer risk [1]; the estrogen alone trial was stopped after 6.8 y of follow-up by the National Institutes of Health because of increased stroke risk and lack of cardioprotection [2].

Because of early adverse effects of HT on cardiovascular events in the Women's Health Initiative a nested case-control study was carried out. Participants with stroke, MI, and venous thromboembolism (VTE) as of February 2001 were matched to controls on age, randomization date, hysterectomy status, and prevalent cerebrovascular disease. For this paper we analyzed the cases of ischemic stroke, and the combined controls for each of the three outcomes to increase the power of detecting an association. In particular, this study included 205 participants who had ischemic strokes cases and 878 controls. Of the participants who had a cases stroke, 11 also experienced an MI and seven also experienced a VTE event by February 2001. Manuscripts analyzing the biomarkers discussed in this paper in relation to VTE and MI are in preparation. Participants provided informed consent using forms approved by local institutional review boards.

Outcomes

Follow-up and end-point determination. Clinical outcomes were identified by semiannual questionnaires and classified by centrally-trained local adjudicators following medical record review [8]. All locally adjudicated stroke cases, and self-reported strokes not validated by local adjudicators, were centrally-adjudicated by stroke neurologists. All adjudicators were blinded to treatment assignment. Ischemic strokes were classified according to the Trial of Org 10172 in Acute Stroke Therapy (TOAST) and Oxfordshire subtype classifications. The TOAST stroke subtypes were as follows: large artery artherosclerosis (9%), cardioembolic (12%), small vessel occlusions (29%), other etiology (5%), and unknown etiology (45%). The Oxfordshire classifications were as follows: total anterior infarct (7%), partial anterior circulation infarct (40%), lacunar infarction (37%), and posterior circulation infarct (17%).

Genetic and biomarker analysis. Blood samples were collected from all participants at baseline and 1 y. The baseline blood samples were analyzed for all 205 participants who experienced a stroke and 878 controls; the year 1 blood was analyzed for the 138 participants who experienced their stroke after the year 1 blood collection, and the 603 controls who were matched to a participant who experiences her stroke, MI, or VTE event after the year 1 blood collection.

Lipid profiles were analyzed in EDTA-treated plasma. High-density lipoprotein (HDL) was precipitated with heparin manganese (Dade-Behring, http://www.dadebehring.com). interleukin-6 (IL-6, ultra-sensitive ELISA, R&D Systems, http://www.rndsystems.com), E-selectin, and matrix metalloproteinase-9 (MMP-9) were measured at Medical Research Laboratories (http://www.mrli.ppdi.com). C-reactive protein (N-High Sensitivity CRP, Dade-Behring,), fibrinogen (clot rate assay: Diagnostica Stago, www.stago-us.com), factor VIII activity (clotting time on mixing with factor VIII deficient plasma using STA-Deficient VIII; Diagnostica Stago), von Willebrand factor activity and fibrin D-dimer (immunoturbidometric assays: Liatest von Willebrand factor, Liatest D-Di;

Diagnostica Stago), plasminogen activator inhibitor-1 antigen (PAI-1) and plasmin-antiplasmin complex (PAP, by in house immunoassay [9,10]), prothrombin fragment 1.2 (ELISA, Dade-Behring), and thrombin activatable fibrinolysis inhibitor (TAFI; immunoassay with antibodies from Affinity Biologicals, http://www.affinitybiologicals.com) were measured at the Laboratory for Clinical Biochemistry Research, University of Vermont (http://www.med.uvm.edu/lcbr/HP-DEPT.ASP?SiteAreaID=513). Complete blood counts were performed in clinics' local laboratories. Genetic polymorphisms were assayed at Wake Forest University (http://www.wfu.com); we assayed estrogen receptor 2-A1730G (NCBI SNP identification rs4986938), glycoprotein 1bα-M145T (rs6065), glycoprotein IIIa–P1 (rs5918), and the Leiden University (Factor V Leiden, thermolabile variant of MTHF, PAI-1 4G/5G). No other biomarkers were analyzed for the participants in this study.

Statistical Methods

All baseline marker values were log-transformed due to skewed distributions and for consistency; differences from baseline to year 1 were analyzed on the original scale. Logistic regression models were controlled for age and study (estrogen alone or estrogen plus progestin), race, BMI, waist-hip ratio, smoking, alcohol consumption, physical activity, diabetes mellitus, prevalent coronary disease (including six cases of atrial fibrillation), prevalent cerebrovascular disease, blood pressure (BP), use of antihypertension medication, aspirin, and statins at baseline. Analyses involving year 1 biomarker data only involved 138 of 205 participants who experienced their stroke after that analysis, and 603 (of 878) matched controls.

We assessed the appropriateness of using biomarkers log linearly in generalized additive models [11] using stroke as response, correcting for risk factors; linearity was rejected for CRP, TAFI, and platelets. For those markers we examined both linear and quadratic models. While we used markers linearly to assess significance (the more powerful analysis), we do not report the coefficients in the logistic regression model, but rather the more easily interpreted odds ratios (OR) comparing quartiles or quintiles. Thus, there is no one-to-one correspondence between p-values for models below 0.05 and confidence intervals for OR not containing 1.

Sensitivity analyses that excluded potential outliers were carried out; except where noted, results were unaffected. All analyses involving TAFI were repeated comparing the subjects with TAFI values above the 90th percentile (7.4 μg/ml) with those below the 25th percentile, as we a priori hypothesized that only very high TAFI levels influence stroke outcome. We only refer to this additional analysis where it differs from the analysis of TAFI on a log linear scale. As most strokes that were analyzed in this study happened early in the trials, most subjects were adhering to study medication. Of the 205 incident stroke cases in this study, only 32 out of 118 of the subjects on HT and 16 out of 87 subjects on placebo were nonadherent 6 mo before their stroke.

RESULTS

Baseline Data

Baseline characteristics are shown by case-control status (Table 1). As this study only included participants who had

Table 1. Baseline Characteristics by Case-Control Status and Hormone Trial

Category	Variable	Parameter	Estrogen Alone Trial		Estrogen Plus Progestin Trial		p-Value[a]
			Control (n = 365)	Hormone (n = 90)	Control (n = 513)	Hormones (n = 115)	
Vital statistics, mean (standard deviation)		Age at screening (y)	66.7 (6.5)	67.0 (6.7)	66.9 (6.9)	68.4 (6.2)	0.06
		BMI (kg/m^2)	29.6 (5.7)	30.1 (5.6)	27.9 (5.4)	28.3 (5.2)	0.27
		Waist-to-hip ratio	0.8 (0.1)	0.9 (0.2)	0.8 (0.1)	0.8 (0.1)	<0.001
		Systolic BP (mm Hg)	131 (17)	139 (18)	130 (18)	138 (20)	<0.001
		Diastolic BP (mm Hg)	76 (9)	78 (10)	75 (9)	76 (11)	0.04
Ethnicity, %		White	75.6	78.9	88.3	86.1	0.93
		Black	17.3	14.4	5.7	8.7	
		Other	7.1	6.7	6.0	5.2	
Risk factors		Alcohol (g/wk)	3.6 (7.9)	3.6 (7.8)	5.4 (11.5)	5.3 (12.6)	0.64
		Physical activity (MET hrs/wk)[b]	9.6 (11.7)	7.3 (8.6)	12.3 (14.7)	9.0 (9.9)	0.33
Risk factors, %	Smoking status	Never	52.0	45.5	56.1	46.5	0.07
		Past	39.3	44.3	36.2	41.2	
		Current	8.7	10.2	7.7	12.3	
	Diabetes mellitus		7.4	12.2	4.9	13.9	<0.001
	Hypertension	Never	57.8	30.0	65.5	51.0	<0.001
		Untreated	6.3	16.3	8.8	14.6	
		Treated	35.9	53.8	25.7	34.4	
	History of cardiovascular disease		19.9	28.4	11.9	14.6	0.07
	Baseline aspirin use, %		23.0	31.1	21.8	22.6	0.24
	Baseline statin use		10.7	6.7	8.2	7.8	0.37
Gene Polymorphism	Estrogen receptor 2-A1730G (rs4986938), %	CC	37.1	47.1	38.6	45.0	0.09
		CT	49.7	44.7	47.4	43.2	
		TT	13.2	8.2	14.0	11.7	
	Factor V Leiden, %	GG	96.4	95.4	95.2	93.6	0.52
		GA	3.6	4.6	4.8	6.4	
	Glycoprotein 1bα-M145T (rs6065), %	CC	75.1	77.6	85.0	81.8	0.87
		CT	22.6	20.0	13.4	17.3	
		TT	2.3	2.4	1.6	0.9	
	Integrin alpha2–807, %	CC	41.1	38.8	36.0	44.5	0.47
		CT	46.0	42.4	48.8	42.7	
		TT	12.9	18.8	15.2	12.7	
	MTHFR, %	CC	48.7	52.9	42.6	50.9	0.27
		CT	42.0	39.1	44.2	35.5	
		TT	9.2	8.0	13.2	13.6	
	PAI-1, %	4G4G	20.7	23.5	25.2	27.8	0.29
		4G5G	51.3	51.8	53.1	42.6	
		5G5G	28.0	24.7	21.8	29.6	
	Glycoprotein IIIa–P1 (rs5918), %	CC	3.4	2.4	2.7	1.8	0.28
		CT	25.2	22.6	21.2	16.5	
		TT	71.3	75.0	76.1	81.7	
Baseline biomarkers, median (IQR)	Inflammation	CRP (mg/l)	2.49 (3.56)	3.92 (4.01)	1.89 (3.48)	2.49 (4.30)	<0.001
		E-Selectin (ng/ml)	45 (27)	46 (21)	43 (26)	49 (23)	0.007
		IL-6 (pg/ml)	2.87 (2.4)	3.83 (2.48)	2.86 (2.20)	3.32 (2.20)	<0.001
		MMP-9 (ng/ml)	220 (148)	271 (192)	218 (151)	236 (165)	<0.001
	Lipids	HDL-cholesterol (mmol/l)	1.35 (0.44)	1.27 (0.40)	1.40 (0.52)	1.30 (0.44)	0.002
		HDL-2 (mmol/l)	0.36 (0.18)	0.34 (0.18)	0.36 (0.21)	0.34 (0.18)	0.19
		HDL-3 (mmol/l)	0.97 (0.31)	0.89 (0.23)	1.01 (0.34)	0.92 (0.31)	<0.001
		Lp(a) (mmol/l)	0.82 (1.21)	0.80 (1.00)	0.64 (1.00)	0.68 (1.00)	0.43
		LDL-cholesterol (mmol/l)	3.65 (1.26)	3.74 (1.01)	3.57 (1.17)	3.73 (1.30)	0.02
		Total Cholesterol (mmol/l)	5.91 (1.35)	5.91 (1.24)	5.72 (1.27)	5.85 (1.32)	0.11
		Triglyceride (mmol/l)	1.60 (1.06)	1.81 (1.36)	1.49 (0.94)	1.50 (1.04)	0.03
	Thrombosis and other blood markers	D-Dimer (ng/ml)	0.32 (0.33)	0.37 (0.50)	0.32 (0.34)	0.47 (0.38)	<0.001
		Fibrinogen (mg/l)	316 (123)	324 (86)	305 (111)	316 (126)	0.09
		Factor VIII (%)	103 (68)	122 (94)	105 (63)	111 (79)	0.04
		PAI-1 antigen (ng/ml)	44.3 (57.5)	48.4 (49.4)	35.6 (47.7)	42.5 (51.8)	0.14
		PAP (nmol/l)	4.17 (2.10)	4.24 (2.35)	4.56 (2.52)	4.49 (2.42)	0.65
		TAFI (µg/ml)	5.18 (2.60)	4.83 (1.94)	5.05 (2.42)	4.91 (2.34)	0.05
		von Willebrand factor (%)	90 (55)	106 (55)	93 (54)	91 (51)	0.02
		Prothrombin F1.2 (nmol/l)	1.29 (0.47)	1.28 (0.45)	1.29 (0.42)	1.29 (0.42)	0.90

Table 1. Continued.

Category	Variable	Parameter	Estrogen Alone Trial		Estrogen Plus Progestin Trial		p-Value[a]
			Control (n = 365)	Hormone (n = 90)	Control (n = 513)	Hormones (n = 115)	
		Leukocyte Count (10^6 cells/l)	5.8 (2.1)	6.2 (1.7)	5.8 (1.9)	6.3 (2.2)	<0.001
		Platelet count (Kcell/ml)	243 (69)	246 (64)	245 (82)	244 (65)	0.96
		Hematocrit (%)	40.5 (3.8)	40.9 (4.4)	40.4 (3.8)	41.1 (4.3)	0.61
		Homocysteine (umol/l)	8.29 (3.79)	8.48 (3.64)	8.08 (3.40)	8.61 (4.67)	0.18

[a]p-Value quantifies association of biomarker, polymorphism, or baseline characteristic with stroke from logistic regression models, controlling for treatment assignment (estrogen, estrogen-placebo, estrogen plus progestin, estrogen plus progestin-placebo).
[b]METhrs/wk, metabolic equivalent hours/week.
doi:10.1371/journal.pctr.0020028.t001

strokes before February 2001, the OR of stroke for active treatment (unadjusted OR 1.53, 95% confidence interval [CI] 1.01–2.32; adjusted for confounders OR 1.51, 95% CI 0.95–2.38) was comparable to the full duration of the trials [3,4].

Of the 14 clinical characteristics, five were associated with stroke (about one would be expected by chance); of the 23 biomarkers, 13 were associated with stroke (one or two would be expected by chance); none of the seven polymorphisms was associated with stroke. Among controls, Hardy-Weinberg equilibrium was not rejected for any of the polymorphisms. For all biomarkers we fitted a model including an interaction between biomarker and trial enrollment (estrogen alone or estrogen plus progestin versus placebo). Of 30 biomarkers and polymorphisms, only the interaction between hematocrit

Table 2. Risk of Ischemic Stroke for Baseline Biomarkers: Highest Quartile versus Lowest Quartile

Category	Biomarker	OR[a] (95% CI)	p-Value[b]
Inflammation	CRP	1.72 (0.97–3.05)	0.03
	E-selectin	1.27 (0.74–2.19)	0.25
	IL-6	2.26 (1.30–3.91)	0.02
	MMP-9	1.58 (0.97–2.58)	0.004
Lipids	HDL-cholesterol	0.73 (0.44–1.22)	0.14
	HDL-3	0.57 (0.34–0.97)	0.02
	LDL-cholesterol	1.46 (0.88–2.41)	0.03
	Triglyceride	1.54 (0.91–2.59)	0.52
Thrombosis and other blood markers	D-dimer	2.53 (1.50–4.27)	<0.001
	Fibrinogen	1.07 (0.72–1.71)	0.53
	Factor VIII	1.62 (1.00–2.62)	0.10
	TAFI	0.69 (0.42–1.15)	0.09
	TAFI[c]	0.24 (0.09–0.59)	0.003
	von Willebrand factor	1.50 (0.91–2.47)	0.10
	Leukocyte count	1.48 (0.88–2.48)	0.13
Polymorphism	Estrogen receptor 2-A1730G (rs4986938)		0.26
	TT versus CC	0.69 (0.39–1.22)	
	CT versus CC	0.74 (0.51–1.07)	

Only results that were statistically significant in this analysis or had a p-value < 0.10 in Table 1 are shown.
[a]OR is the comparison of the fourth quartile versus the first quartile, and is based on a logistic regression model controlled for age, trial, race, BMI, waist-hip ratio, smoking, alcohol consumption, physical activity, diabetes, history of cardiovascular disease, systolic and diastolic BP, use of antihypertension medication, aspirin, and statin use.
[b]p-Value for biomarkers based on a logistic regression model using a 1 degree-of-freedom test for biomarkers (log scale) and a 2 degree-of-freedom test for polymorphism. Covariate adjustment same as above.
[c]Comparison of the 90th percentile versus the 1st quartile.
doi:10.1371/journal.pctr.0020028.t002

and trial was marginally significant (p = 0.07). As one or two significant results would be expected by chance, this suggests that there were no interaction effects between the trial (estrogen plus progestin or estrogen alone) and the biomarkers. This result, together with the fact that the overall effects of HT on stroke were similar in both trials, suggests that it was appropriate to combine the estrogen plus progestin trial and the estrogen alone trial for analyses of the effects of these biomarkers on stroke.

Outcomes and Estimation

Risk of ischemic stroke for biomarkers. For each baseline biomarker the stroke risk was compared in multivariate analyses (Table 2). For continuous biomarkers we used log-linear models, and for the polymorphisms we used additive genetic models. As continuous variables, IL-6 (p = 0.02), CRP (p = 0.03), MMP-9 (p = 0.004), D-dimer (p < 0.001), LDL (p = 0.03), von Willebrand factor (p = 0.10), and factor VIII activity (p = 0.10) were positively associated with stroke, whereas HDL-3 was inversely associated (p = 0.02) with stroke. Of the 30 biomarkers analyzed, seven had a statistically significant association with stroke at the level of p = 0.05; one or two markers would be expected to be significant by chance. While TAFI was not strongly associated with stroke when analyzed as a continuous variable (p = 0.09), the OR of stroke for TAFI above the 90th percentile compared to the lowest quartile was 0.24 (95% CI 0.09–0.59; p = 0.003). Several other biomarkers showed less significant associations.

Risk of ischemic stroke with baseline biomarkers by treatment assignment. Of the 30 biomarkers studied, the p-value for interaction between biomarker level and treatment assignment in the pooled cohorts nearly reached significance for IL-6 (p = 0.06) and was significant for PAP (p = 0.02) (Table 3). As one or two statistically significant results are expected by chance, these results may be due to chance. For these markers, as well as D-dimer and the leukocyte count, the pattern for the estrogen plus progestin trial suggested a smaller OR associated with elevated biomarkers for the active arm than the placebo arm of the trial; no such pattern was observed in the estrogen alone trial. A post-hoc analysis restricted to the estrogen plus progestin trial yielded the following p-values for an interaction between baseline biomarker and HT with stroke in the estrogen plus progestin trial: IL-6 (p = 0.02), PAP (p = 0.002), D-dimer (p = 0.02), and leukocyte count (p = 0.04), suggesting that individuals with high levels of these biomarkers at baseline have lower stroke

Table 3. Ischemic Stroke Risk with Baseline Biomarker by Treatment Assignment

Category	Biomarker	Estrogen Alone		Estrogen plus Progestin		Interaction p-Value[b]
		Estrogen (46/187[a])	Placebo (44/178[a])	Estrogen plus Progestin (72/268[a])	Placebo (43/245[a])	
Inflammation	C-reactive protein	1.77 (0.62–5.00)	1.26 (0.39–4.07)	1.47 (0.60–3.60)	2.58 (0.72–9.21)	0.56
	E-selectin	0.53 (0.18–1.56)	0.82 (0.25–2.64)	1.76 (0.71–4.41)	2.90 (0.84–10.03)	0.15
	Interleukin-6	2.55 (0.81–8.07)	2.98 (1.00–8.84)	1.28 (0.53–3.12)	3.47 (1.00–12.06)	0.06
	MMP-9	4.18 (1.35–12.99)	1.88 (1.35–2.99)	0.87 (0.39–1.96)	1.38 (0.52–3.70)	0.89
Lipids	LDL-cholesterol	1.87 (0.60–5.88)	0.72 (0.25–2.09)	1.76 (0.75–4.15)	1.72 (0.62–4.76)	0.14
	HDL-cholesterol	0.77 (0.25–2.14)	0.48 (0.16–1.43)	0.91 (0.41–2.03)	0.61 (0.21–1.81)	0.28
	HDL-3	0.42 (0.12–1.42)	0.24 (0.07–0.81)	0.86 (0.38–1.93)	0.64 (0.21–1.94)	0.24
	Triglyceride	2.05 (0.68–6.17)	2.43 (0.76–7.78)	0.86 (0.38–1.97)	2.09 (0.65–6.74)	0.28
Thrombosis and other blood markers	D-dimer	2.59 (0.87–7.71)	2.92 (1.02–8.39)	1.40 (0.60–3.24)	6.33 (1.67–24.01)	0.13
	Fibrinogen	1.08 (0.37–3.15)	1.21 (0.42–3.47)	0.87 (0.38–1.96)	1.34 (0.52–3.45)	0.89
	Factor VIII	3.11 (1.18–8.15)	1.62 (0.60–4.40)	0.92 (0.39–2.15)	1.53 (0.60–3.91)	0.96
	PAP	1.73 (0.61–4.92)	1.55 (0.53–4.57)	0.32 (0.13–0.79)	7.09 (1.48–34.03)	0.02
	TAFI	0.63 (0.19–2.06)	0.37 (0.12–1.12)	0.84 (0.36–2.00)	1.04 (0.38–2.84)	0.88
	von Willebrand factor	1.79 (0.67–4.78)	1.58 (0.54–4.59)	1.23 (0.53–2.87)	1.63 (0.54–4.91)	0.47
	Leukocyte count	2.78 (0.81–9.51)	1.94 (0.54–6.88)	0.70 (0.32–1.55)	3.09 (0.94–10.14)	0.33
Polymorphism	Estrogen receptor 2-A1730G (rs4986938)					0.43
	TT versus CC	0.39 (0.10–1.49)	0.76 (0.21–2.72)	0.65 (0.25–1.66)	1.18 (0.38–3.66)	
	CT versus CC	0.52 (0.24–1.13)	0.67 (0.30–1.48)	0.70 (0.37–1.31)	1.29 (0.59–2.80)	

Values are OR (95% CI) comparing top to bottom quartile of each biomarker, and were derived from a logistic regression model controlling for age, trial, race, BMI, waist-hip ratio, smoking, alcohol consumption, physical activity, diabetes, history of cardiovascular disease (including history of AF), systolic and diastolic BP, use of antihypertension medication, aspirin, and statin. Only results that were statistically significant in this analysis or had a p-value ≤ 0.10 in Table 1 are shown.
[a]Strokes/controls.
[b]p-Value for the interaction of active treatment/placebo × biomarker or polymorphism, based on a 1 degree-of-freedom test for biomarkers (log-scale) and 2 degrees-of-freedom for polymorphism.
doi:10.1371/journal.pctr.0020028.t003

risk with estrogen plus progestin than women with lower levels of these biomarkers.

We did not find differences in biomarker by treatment assignment between stroke subtypes. In particular, for these results we found no significant differences between ischemic strokes being classified as large artery atherosclerosis or cardioembolism and those classified as small vessel occlusion (unpublished data).

Biomarker change from baseline to year 1. One-year changes in biomarkers are shown in Table 4. Several inflammatory ($p < 0.001$ for CRP, E-selectin, and MMP-9) and thrombotic biomarkers ($p < 0.001$ for PAI-1 and PAP) were altered by HT, as were lipids (all $p < 0.001$). When we removed five extreme changes in D-dimer, the p-value for that marker changed from 0.02 to 0.005. No other outliers had a substantial effect. Overall, 14 of the 20 biomarkers had significant changes from baseline to year 1, far more than the one that would be expected by chance.

Risk of ischemic stroke by change in biomarker level from baseline to year 1. One-year changes from baseline values were associated with stroke risk in multivariate analysis for only one biomarker: von Willebrand factor (lowest quintile of change OR 0.66, 95% CI 0.35–1.23; middle quintile, including no change, referent; highest quintile OR 0.95, 95% CI 0.51–1.74; $p = 0.04$).

Changes in biomarkers and the association of HT with stroke. Of the 20 biomarkers measured at baseline and 1 y, only the change in D-dimer demonstrated an interaction with randomization assignment ($p = 0.03$, Table 5). For women whose D-dimer increased, the risk of stroke was higher with HT (estrogen plus progestin and estrogen alone combined,

OR 1.38 for the top quintile of change) while for women whose D-dimer decreased, the risk of stroke was lower (OR 0.49 for the bottom quintile).

We examined whether changes in biomarker level could be considered an intermediate outcome on the pathway from HT to ischemic stroke, in that inclusion of a biomarker change in a regression model including treatment assignment would significantly reduce the association of HT with ischemic stroke [12]. This was not the case for any marker.

DISCUSSION

Interpretation

This nested case-control study was undertaken to elucidate mechanisms underlying the increased stroke risk with HT in the Women's Health Initiative clinical trials, and to attempt to identify women at lower or higher risk for treatment-related stroke. Baseline levels of several biomarkers representing inflammation and coagulation activity, but not the genotypes that were studied here, were associated with increased stroke risk. The finding that women with higher IL-6, PAP, D-dimer, and leukocyte counts at baseline were at lower risk for stroke if assigned to estrogen plus progestin than placebo, but not estrogen alone, is counterintuitive and is likely due to a noncausal association since both hormone treatment groups had similar stroke rates. When one-year changes in biomarkers were examined, only women with treatment-related increases in D-dimer had increased stroke risk with HT. The effect of other risk factors (such as BP, hypertension, and smoking) in the complete Women's Health Initiative cohort has been previously reported [3,4].

The relatively small number of strokes limited our power to

Table 4. Changes in Biomarkers from Baseline to Year 1

Category	Biomarker	Estrogen Alone Trial, Median (IQR)		Estrogen Plus Progestin Trial, Median (IQR)		p-Value[a]
		Estrogen (27/133)[b]	Placebo (33/121)[b]	Estrogen Plus Progestin (54/195)[b]	Placebo (24/154)[b]	
Inflammation	CRP (mg/l)	2.87 (5.10)	0.11 (2.35)	1.68 (6.33)	0.64 (4.95)	<0.001
	E-selectin (ng/ml)	−7.2 (13.0)	−0.5 (9.5)	−8.4 (11.2)	0.2 (9.1)	<0.001
	IL-6 (pg/ml)	0.29 (1.62)	0.06 (4.11)	0.49 (4.84)	0.05 (2.57)	0.13
	MMP-9 (ng/ml)	29 (154)	−23 (148)	52 (146)	9 (178)	<0.001
Lipids	Total cholesterol (mmol/l)	−0.40 (0.80)	−0.02 (0.77)	−0.42 (0.63)	−0.05 (0.79)	<0.001
	LDL-cholesterol (mmol/l)	−0.74 (0.74)	−0.00 (0.77)	−0.57 (0.59)	−0.05 (0.73)	<0.001
	HDL-cholesterol (mmol/l)	0.19 (0.25)	0.00 (0.21)	0.12 (0.20)	0.01 (0.19)	<0.001
	HDL-2 (mmol/l)	0.07 (0.12)	−0.01 (0.12)	0.05 (0.10)	−0.00 (0.09)	<0.001
	HDL-3 (mmol/l)	0.12 (0.16)	0.00 (0.13)	0.06 (0.14)	0.01 (0.13)	<0.001
	Lp(a) (mmol/l)	−0.18 (0.32)	−0.07 (0.45)	−0.17 (0.33)	−0.00 (0.35)	<0.001
	Triglycerides (mmol/l)	0.30 (0.88)	−0.02 (0.71)	0.08 (0.66)	−0.00 (0.52)	<0.001
Thrombosis and other blood markers	D-dimer (ng/ml)	0.15 (0.71)	−0.01 (0.34)	0.20 (0.69)	0.08 (0.86)	0.03
	Fibrinogen (mg/dl)	−15.3 (70.2)	−1.10 (71.5)	−21.3 (68.7)	−0.5 (68.4)	0.007
	Factor VIII (%)	0.1 (33.2)	0.7 (30.5)	0.6 (33.3)	1.1 (32.7)	0.69
	PAI-1 antigen (ng/ml)	−12.2 (42.7)	2.5 (43.0)	−3.8 (50.2)	−2.0 (50.9)	0.001
	PAP (nmol/l)	1.05 (1.70)	0.15 (1.59)	0.83 (2.08)	0.07 (1.94)	<0.001
	TAFI (µg/ml)	0.25 (0.93)	−0.06 (1.41)	−0.01 (1.12)	−0.02 (0.88)	0.09
	von Willebrand factor (%)	−0.3 (33.9)	2.8 (43.6)	−2.0 (37.0)	2.5 (37.7)	0.08
	Prothrombin F1.2 (nmol/l)	0.50 (3.13)	−0.07 (2.53)	−0.32 (0.78)	0.34 (2.56)	0.06
	Homocysteine (µmol/l)	−0.63 (2.69)	−0.11 (2.18)	−0.44 (2.11)	−0.14 (2.44)	0.07

[a]p-Value from a paired t-test (per participant) of change in biomarker controlling for the same variables as in Table 2. For each participant the biomarker value at year 1 was compared with the value at baseline for the same participant. Platelet count, hematocrit, and leukocyte count were not measured at year 1.
[b]Strokes/controls.
doi:10.1371/journal.pctr.0020028.t004

definitely detect associations for any lipid parameter. Although low-density lipoprotein (LDL)–cholesterol appears to be a weak risk factor for ischemic stroke in women [13,14], there was evidence in Women's Health Initiative of a trend toward lower stroke risk in women with a reduction in LDL–

cholesterol of 0.52–0.73 mmol/l on HT. HDL–cholesterol is more strongly correlated with stroke risk in epidemiologic studies, with protection at levels above 1.45 mmol/l in women aged 45–64 y, and above 1.04 mmol/l in women over age 65 y [13,14]. In this analysis, there was some evidence of a trend

Table 5. Ischemic Stroke Risk by Treatment Assignment Modified by Change in Biomarker, Baseline to Year 1

Category	Biomarker	First Quintile of Change		Fifth Quintile of Change		Interaction p-Value[a]
		Change Value	OR (95% CI)	Change Value	OR (95% CI)	
Inflammation	CRP (mg/l)	<−0.6	0.83 (0.32–2.13)	>3.1	1.28 (0.36–4.61)	0.56
	E-selectin (ng/ml)	<−11.0	1.67 (0.46–6.14)	>3.0	0.59 (0.21–1.68)	0.26
	IL-6 (pg/ml)	<−0.9	1.12 (0.46–2.74)	>1.0	0.68 (0.28–1.63)	0.69
	MMP-9 (ng/ml)	<−70	1.59 (0.63–4.02)	>107	0.88 (0.34–2.30)	0.76
Lipids	Total cholesterol (mmol/l)	<−0.8	1.65 (0.60–4.50)	>0.3	0.85 (0.31–2.38)	0.68
	LDL-cholesterol (mmol/l)	<−0.9	0.77 (0.26–2.31)	>0.2	0.56 (0.14–2.26)	0.68
	HDL-cholesterol (mmol/l)	<−0.1	1.99 (0.70–5.65)	>0.3	0.77 (0.23–2.64)	0.13
	HDL-2 (mmol/l)	<−0.1	2.35 (0.71–7.73)	>0.4	0.44 (0.15–1.30)	0.30
	HDL-3 (mmol/l)	<−0.1	2.88 (0.99–8.44)	>0.2	1.41 (0.40–4.98)	0.20
	Lp(a) (mmol/l)	<−0.3	1.74 (0.43–7.02)	>0.1	0.95 (0.39–2.33)	0.92
	Triglycerides (mmol/l)	<−0.3	0.65 (0.26–1.63)	>0.5	0.61 (0.22–1.66)	0.30
Thrombosis and other blood markers	D-dimer (µg/ml)	<−0.1	0.49 (0.19–1.28)	>0.3	1.38 (0.48–3.92)	0.03
	Fibrinogen (mg/dl)	<−6.1	1.51 (0.54–4.21)	>42	0.94 (0.41–2.17)	0.50
	Factor VIII (%)	<−18	1.38 (0.57–3.34)	>21	1.29 (0.52–3.16)	0.88
	PAI-1 antigen (ng/ml)	<−24.4	1.58 (0.54–4.63)	>14.3	1.11 (0.45–2.74)	0.93
	PAP (nmol/l)	<−0.5	0.66 (0.22–1.95)	>1.6	2.37 (0.60–9.31)	0.46
	TAFI (µg/ml)	<−0.4	1.13 (0.42–3.03)	>0.6	0.88 (0.34–2.23)	0.57
	von Willebrand factor (%)	<−22	0.45 (0.17–1.15)	>0.2	2.04 (0.83–5.02)	0.23
	Prothrombin F1.2 (nmol/l)	<−0.2	1.21 (0.49–3.00)	>0.4	0.99 (0.34–2.85)	0.95
	Homocysteine (µmol/l)	<−1.7	1.28 (0.47–3.48)	>1.1	2.10 (0.82–5.36)	0.52

[a]p-Value for interaction of active treatment/placebo × biomarker change is based on a 1 degree-of-freedom test for change in biomarker.
doi:10.1371/journal.pctr.0020028.t005

toward a lower risk of stroke in women with a greater than 0.16 mmol/l (12%) increase in HDL–cholesterol on HT. Baseline HDL-3, but not HDL-2, appeared to be protective against stroke, perhaps explaining the lack of significance for HDL–cholesterol overall.

Inflammation promotes atherogenic events, including stroke [15,16]. IL-6 is an important regulator of the *CRP* gene [17]. HT with estrogen alone or estrogen plus progestin increases CRP to a similar extent [18]. The rise in CRP with estrogen plus progestin, but not estrogen alone, appears to be IL-6 mediated [19]. These observations suggest that estrogen plus progestin and estrogen alone have differing proinflammatory effects, consistent with the significant interaction between baseline levels of IL-6 and stroke risk that we identified with estrogen plus progestin, but not estrogen alone.

It has been hypothesized that hemostatic activation underlies increased vascular risk with hormones, and it has been previously reported that hormones raise D-dimer levels [20]. Most associations of hemostatic markers and stroke were carried out during acute ischemic stroke, which itself affects the hemostatic system. A prospective study in patients with prior transient ischemic attack or asymptomatic carotid bruits identified prothrombin fragment 1.2, but not PAP or D-dimer, as an independent predictor of subsequent cardiovascular events [21]. In contrast, D-dimer levels independently predicted ischemic stroke in healthy men, whereas prothrombin fragment 1.2 did not [22]. Our findings of higher D-dimer as a risk marker for stroke, and that increases in D-dimer with HT might identify women at risk of stroke, support a role for hemostatic activation in the development of stroke.

Generalizability

The strengths of this analysis are the ability to prospectively assess the interaction between randomly assigned HT and baseline biomarker levels and changes in biomarker levels. Some limitations should be considered. There were a relatively small number of strokes, confining us to detecting relatively large interactions of treatment with biomarkers. Because we analyzed a large number of biomarkers, some of the results may be due to chance. We studied clinical trial participants who may be different from the general population, so that the results might not be generalizable. A standardized diagnostic approach to stroke was not followed, and this may have led to misclassification of stroke types. Information on stroke subtype was limited. The fact that stroke risk was increased similarly with estrogen alone and estrogen plus progestin, whereas baseline levels of four biomarkers (IL-6, PAP, D-dimer, leukocyte count) appeared to predict a protective association from estrogen plus progestin, raises concerns about the plausibility of these findings. Regarding the findings related to change in D-dimer and stroke susceptibility of women with HT, in view of the number of markers, one interaction might be expected by chance.

Overall Summary

In this nested case-control study, no biomarker convincingly predicted the stroke risk seen with HT, although some hypotheses were raised. A genome-wide scan is in progress to search for underlying mechanisms, and a proteome study has been proposed to characterize hormone-induced proteins.

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Author Contributions

MC and AEB designed the study. CK, JGR, AKA, AEB, and LHK analyzed the data. JH, JGR, KCJ, and BR enrolled patients. All authors contributed to writing the paper. CK, MC, JH, JGR, JLK, KCJ, SAAB, and BR collected data or performed experiments for the study. SAAB oversaw the recruitment, data collection, and retention of women participating in the Seattle Clinical Center of the Women's Health Initiative. BR contributed patients from the Hawaii Center.

Funding

Competing Interests

MC participated in three meetings as a consultant for Pharmacia, Novartis, and Merck on topics related to hormone therapies or selective estrogen receptor modulators in 2001 and 2003. JGR has received to date: Grants from Abbott, Andrx Labs, Astra-Zeneca, Atherogenics, Bristol-Myers Squibb, GlaxoSmithKline, Hoffman La Roche, Merck, Pfizer, Procter & Gamble, Sankyo, Schering-Plough, Takeda, and Wyeth Ayerst; Speaker honoraria for education programs from Bristol-Myers Squibb, Merck, and Pfizer; Honoraria from Reliant; Consultant/Advisory Board for Bristol -Myers Squibb, Merck, Pfizer, Proliant, Wellmark, and American Emu Association.

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