Inflammation and Hemostasis Biomarkers for Predicting Stroke in Postmenopausal Women: The Women's Health Initiative Observational Study

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Background: Inflammatory and hemostasis-related biomarkers may identify women at risk of stroke. *Methods:* Hormones and Biomarkers Predicting Stroke is a study of ischemic stroke among postmenopausal women participating in the Women's Health Initiative observational study (n = 972 case-control pairs). A Biomarker Risk Score (BRS) was derived from levels of 7 inflammatory and hemostasis-related biomarkers that appeared individually to predict risk of ischemic stroke: C-reactive protein (CRP), interleukin-6, tissue plasminogen activator, D-dimer, white blood cell count, neopterin, and homocysteine. The c index was used to evaluate discrimination. *Results:* Of all the individual biomarkers examined, CRP emerged as the only independent single predictor of ischemic stroke (adjusted odds ratio comparing Quartile_4 *v* $\text{Quartile}_1 = 1.64$, 95% confidence interval: 1.15-2.32, $P = .01$) after adjustment for other biomarkers and standard stroke risk factors. The BRS identified a gradient of increasing stroke risk with a greater number of elevated inflammatory/hemostasis biomarkers, and improved the c index significantly compared with standard stroke risk factors ($P = .02$). Among the subset of individuals who met current criteria for high-risk levels of CRP (>3.0 mg/L), the BRS defined an approximately 2-fold gradient of risk. We found no evidence for a relationship between stroke and levels of E-selectin, fibrinogen, tumor necrosis factor-α, vascular cell adhesion molecule-1, prothrombin fragment 1+2, Factor VIIC, or plasminogen activator inhibitor-1 antigen ($P > .15$). *Discussion:* The findings support the further exploration of multiple biomarker panels to develop approaches for stratifying an individual's risk of stroke. **Key Words:** Stroke—epidemiology—women.

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A number of prospective studies have reported that risk of incident cardiovascular disease (CVD) among apparently healthy individuals is associated with levels of serum pentraxins,[1-3] inflammatory cytokines,[4-7] cellular adhesion molecules,[8] and markers of coagulation and fibrinolysis activity.[9,10] In older adults, however, the results of such studies have been inconsistent, and stroke has not been as well studied as have other types of vascular events.[1,3,11-16] In light of the complex interplay of mediators that may contribute to the development of ischemic stroke, panels of several biomarkers related to inflammation, coagulation, and fibrinolysis have the potential to provide additional information about risk as compared with any single biomarker.

This investigation was designed to evaluate the ability of inflammation and hemostasis-related biomarkers to predict the risk of future acute ischemic stroke among postmenopausal women. We examined biomarkers that had been previously shown to predict risk of ischemic stroke, and others that were chosen either based on known biological mechanisms of stroke, or based on studies linking them with other vascular outcomes such as coronary events. Biomarkers included 7 circulating biomarkers of inflammation: total white blood cell count (WBC) and C-reactive protein (CRP), which are systemic markers of global inflammatory activity; pro-inflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor (TNF)-α; neopterin, a well-characterized marker of cell-mediated immunity that is synthesized by macrophages on stimulation by interferon-γ released by activated T-helper type-1 lymphocytes; and cellular adhesion molecules of the selectin (E-selectin) and Ig super-family adhesion molecule type (vascular cell adhesion molecule [VCAM]). The panel also included 6 biomarkers relating to blood coagulation, fibrinolysis, and platelets: the clotting factor Factor VII; prothrombin fragment 1+2, a marker of blood coagulation produced by cleavage of prothrombin by Factor X; tissue plasminogen activator (tPA), the main endothelial cell-derived activator of fibrinolysis; plasminogen activator inhibitor (PAI)-1, an inhibitor of fibrinolysis; D-dimer, a marker of fibrin turnover; and fibrinogen, the precursor to fibrin and mediator of platelet aggregation that also behaves as an acute-phase reactant. We also evaluated homocysteine, a marker of folate metabolism that may relate to risk of vascular events through mechanisms relating to atherogenesis, coagulation, or fibrinolysis. Multivariate approaches were used to evaluate whether stroke risk was related to a Biomarker Risk Score (BRS) based on levels of multiple inflammatory and hemostatic biomarkers.

Methods

Study Population

This investigation is part of the Hormones and Biomarkers Predicting Stroke (HaBPS) study, a case-control study of incident ischemic stroke nested within the Women's Health Initiative (WHI) observational study. A total of 93,676 postmenopausal women aged 50 to 79 years were recruited from October 1993 through December 1998. Women ineligible or not interested in the WHI clinical trial components, which examined postmenopausal hormone therapy, low-fat diet, and calcium/vitamin-D supplementation, were given an opportunity to enroll in the observational study, and others were recruited specifically for observational study participation. Institutional review board approval and informed consent were obtained.

Data Collection

All women who enrolled in WHI completed visits at baseline to determine eligibility and collect data including questionnaires, physical measurements, biological specimens, and laboratory test results. During an initial visit, a physical examination was performed by trained staff using standardized procedures to obtain height and weight and seated blood pressure. Fasting blood samples were collected at study baseline by clinic staff members who followed a standardized protocol for venipuncture, centrifugation and separation of blood, freezing of specimens on site at –70°C, and shipping of specimens to the central WHI repository for long-term storage. Questionnaires elicited information on many health-related factors including medical history, health behaviors including smoking habits, and demographics. Use of prescription drugs was inventoried.

Variable Definition

Hypertension was defined as self-report of hypertension diagnosis with antihypertensive medication use, and/or systolic blood pressure greater than or equal to 140 mm Hg, and/or diastolic blood pressure greater than or equal to 90 mm Hg. Diabetes was defined as being on treatment for diabetes by self-report and/or having a fasting glucose level greater than or equal to 126 mg/dL. Body mass index (BMI) was calculated from measured weight and height as kg/m^2. A prior diagnosis of high cholesterol requiring medication was determined by self-report.

Follow-up and Outcome Ascertainment

All incident strokes, other vascular events, and deaths were identified through self-report at annual participant contacts and through third-party reports by family members and proxies. Medical records were obtained for potential strokes and other predefined health events, and adjudication was performed locally by trained physician adjudicators who assigned a diagnosis according to standard criteria. As part of the HaBPS study, all locally adjudicated strokes were then sent for central adjudication by study neurologists (D. M. R., A. E. B., J. L.). Ischemic stroke was defined as the rapid onset of a persistent neurologic

deficit attributed to an obstruction lasting more than 24 hours and without evidence for other causes. Stroke subtype was defined using Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.[17] Only stroke events that required hospitalization were considered as potential outcomes.

Case and Control Subject Identification

For the HaBPS case-control study, cases included the first 972 confirmed incident ischemic strokes occurring between study baseline and July 1, 2003. All cases not verified during central adjudication as being an ischemic stroke were excluded, including transient ischemic attacks or hemorrhagic strokes. Control subjects were matched individually to the cases according to age (2 years), race/ethnicity, date of enrollment, and follow-up time. Women with a history of myocardial infarction or stroke at baseline were excluded from both the case and control groups.

Biomarker Measurement

Stored blood specimens were sent to the WHI core laboratory for measurement of levels of plasma CRP, IL-6, TNF-α, neopterin, E-selectin, VCAM, Factor VII, prothrombin fragment 1+2, tPA, PAI-1, fibrinogen, homocysteine, fasting plasma glucose, and lipids. These laboratory tests were performed between September 2005 and March 2006, approximately 7 to 12 years since specimen collection (depending on when participants were enrolled). Baseline blood samples had been sent to a local laboratory for analysis of WBC at the time of collection.

Statistical Analyses

To compare characteristics of patients and matched control subjects, the McNemar's Chi-square test was used for categorical variables. We examined distributions of biomarkers to assess the need for normalizing transformations and identify outlying values. Because of nonnormality of several biomarkers, Wilcoxon signed ranks were used to compare levels of median biomarkers between the matched case-control pairs. Spearman correlations between all biomarkers were calculated among the control subjects. Multivariate unconditional logistic regression, with adjustment for matching factors and confounders, was used to estimate odds ratio (OR) and 95% confidence interval (CI) across quartiles of biomarkers. Quartile cut points were defined according to the distribution of biomarkers among control subjects. Significance tests were computed using two approaches: (1) fitting an ordinal variable for biomarker quartiles as a continuous variable; and (2) examining the OR comparing extreme biomarker quartiles. To examine the joint predictive value of several biomarkers, we derived a BRS by assigning one point for each biomarker value that was in the highest quartile of the control group distribution. Biomarkers that were used in computing the BRS were those that were individually associated with stroke at P_{trend} less than .15 when modeled as a trend across quartiles; this liberal significance level reflects our hypothesis that a biomarker may contribute importantly to a multiple biomarker strategy even if it does not achieve the conventional level of statistical significance in its individual association with stroke risk. We examined the association of BRS with risk of stroke using logistic regression models, both overall and in subgroups defined by CRP above and below 3.0 mg/L, which is the cutoff for defining high CRP in currently available guidelines.[18]

Models adjusted for the matching variables age and race/ethnicity, and were additionally adjusted for aspirin use, BMI, diabetes, systolic blood pressure, antihypertensive medication use, smoking, lipid-lowering medication use, fasting glucose, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol. Additional adjustment for history of atrial fibrillation, diastolic blood pressure, history of revascularization, or estrogen and progestin therapy did not affect the results substantially. To assess variation in associations by stroke subtype, we determined the ORs for incident stroke in subgroups of women defined by the TOAST classification. We also examined for effect modification by age, hormone use, hypertension, diabetes, smoking, race, HDL cholesterol, and LDL cholesterol. Adjusted models were based on women for whom complete data were available on all covariates of interest. To assess the ability of models to discriminate between ischemic strokes and control subjects we calculated the c index, with the use of cross-validation methods to reduce bias introduced by the use of the same population to develop and evaluate models.[19]

Results

Subject Characteristics

The follow-up time in years was, for patients with stroke, mean = 4.4, SD = 2.3, median = 4.5, and for control subjects, mean = 7.9, SD = 1.3, median = 8.0. Patients with ischemic stroke were more likely than control subjects to be current smokers, to have high BMI, and to report a history of atrial fibrillation, angina, or revascularization (Table 1). In addition, patients were more likely to have hypertension, diabetes, and use of lipid-lowering drugs and aspirin. Significant correlations among inflammatory and hemostasis biomarkers were observed, ranging as high as $r = 0.51$ ($P < .001$) for Factor VII and prothrombin fragment 1+2, and $r = 0.47$ ($P < .001$) for CRP and IL-6 (Table 2). Significant differences ($P < .05$) were present between matched patients and control subjects in median baseline levels of several of the biomarkers under study (CRP, IL-6, tPA, WBC, neopterin, E-selectin, TNF-α, and VCAM-1), whereas case-control

Table 1. *Baseline characteristics among patients with ischemic stroke and age- and race-matched control subjects*

	Control subjects (n = 972)		Patients (n = 972)		
	N	%	N	%	*P* value
Age groups (y)					
50-59	95	9.8	95	9.8	NA
60-69	392	40.3	392	40.3	
70-79	485	49.9	485	49.9	
Race/ethnicity					
American Indian/Alaskan Native	5	0.5	5	0.5	NA
Asian/Pacific Islander	21	2.2	21	2.2	
African American	80	8.2	80	8.2	
Hispanic	20	2.1	20	2.1	
Other	13	1.3	13	1.3	
White	833	85.7	833	85.7	
Smoking					
Never	526	54.6	505	52.6	<.01
Past	400	41.5	377	39.2	
Current	37	3.8	79	8.2	
Alcohol					
Nondrinker	113	11.7	118	12.2	.33
Past drinker	179	18.5	212	21.9	
<1 Drink/mo	119	12.3	115	11.9	
<1 Drink/wk	202	20.8	201	20.7	
1-6 Drinks/wk	240	24.7	199	20.5	
≥7 Drinks/wk	117	12.1	124	12.8	
Hormone use					
No current hormone use	603	62.0	588	60.5	.51
Any current hormone use	369	38.0	384	39.5	
Type of hormone among current users					
Estrogen alone	237	64.2	271	70.6	.60
Estrogen + progestin	132	35.7	113	29.4	
BMI					
<25	390	40.7	335	34.8	<.01
25-30	346	36.1	365	37.9	
>30	222	23.2	263	27.3	
History of atrial fibrillation					
No	894	94.2	858	90.2	<.01
Yes	55	5.8	93	9.8	
History of angina					
No	906	94.5	876	90.7	<.01
Yes	53	5.5	90	9.3	
History of revascularization					
No	937	98.8	913	96.0	.0001
Yes	11	1.2	38	4.0	
Diastolic blood pressure (mm Hg)					
<90	923	95.0	892	92.2	.02
≥90	49	5.0	75	7.8	
Systolic blood pressure (mm Hg)					
≤120	329	33.9	198	20.4	<.0001
120-140	384	39.5	372	38.4	
>140	259	26.7	400	41.2	
Hypertension*					
No	531	55.6	347	36.6	<.0001
Yes	424	44.4	600	63.4	
Use of hypertensive medications					
No	632	65.0	511	52.6	<.0001
Yes	340	35.0	461	47.4	

Table 1. (*Continued*)

	Control subjects (n = 972)		Patients (n = 972)		
	N	%	N	%	*P* value
Diabetes†					
No	889	91.7	805	83.3	<.0001
Yes	81	8.4	162	16.7	
Use of aspirin					
No	732	75.3	675	69.4	<.01
Yes	240	24.7	297	30.6	
High cholesterol requiring medication					
No	807	84.7	768	81.0	.05
Yes	146	15.3	180	19.0	

	Control subjects (n = 972)		Patients (n = 972)		
Continuous variables	Mean	SD	Mean	SD	*P* values from paired *t* tests
BMI	27.0	5.3	27.7	5.9	<.01
Systolic blood pressure	130.1	18.0	137.2	19.4	<.001
Diastolic blood pressure	74.1	9.5	75.5	10.1	<.01
Low-density lipoprotein cholesterol	139.0	36. 7	140.8	37.4	.31
High-density lipoprotein cholesterol	59.8	16.4	57.2	16.2	<.01

Abbreviation: BMI, body mass index; NA, not applicable.
Subjects with missing values excluded from table.
Patients and control subjects were matched on age and race/ethnicity.
*Hypertension defined as on medication by self-report or systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg.
†Diabetes defined as on treatment for diabetes by self-report or fasting glucose level > 126 mg/dL.

differences in D-dimer, homocysteine, and PAI-1 antigen were of borderline statistical significance (P = .05-.10) (Table 3).

Biomarkers and Risk of Ischemic Stroke

We examined the associations of incident ischemic stroke with levels of each individual biomarker. In analyses of linear trends across biomarker quartiles, the associations for CRP (P_{trend} < .001), IL-6 (P_{trend} < .001), tPA (P_{trend} = .02), D-dimer (P_{trend} = .03), and WBC (P_{trend} = .03) met the standard P less than .05 criteria for statistical significance in models that adjusted for aspirin use, BMI, diabetes, systolic blood pressure, smoking, high cholesterol requiring medication, antihypertensive medication use, fasting glucose, and LDL and HDL cholesterol (Table 4). Biomarkers for which the trends across quartiles were of borderline significance (P = .05-.15) were neopterin (P_{trend} = .05) and homocysteine (P_{trend} = .10). Similar results were observed in analyses that compared individuals in the highest versus the lowest quartiles of biomarkers. Specifically, significant (P < .05) associations were found for quartile comparisons of CRP (adjusted OR comparing $Quartile_4$ *v* $Quartile_1$ = 1.78, 95% CI 1.32-2.39), IL-6 (OR = 1.68, 95% CI 1.25-2.26), tPA (OR = 1.42, 95% CI 1.03-1.94), D-dimer (OR = 1.52, 95% CI 1.12-2.08), and WBC (OR = 1.46, 95% CI 1.10-1.94). There was no evidence for a relationship between risk of incident ischemic stroke and levels of E-selectin, fibrinogen, TNF-α, VCAM-1, prothrombin fragment 1+2, Factor VIIC, or PAI-1 antigen, either as analyses of quartile trends or comparisons of extreme quartiles.

In models that included CRP, IL-6, tPA, D-dimer, WBC, neopterin, and homocysteine together as predictor variables, CRP retained an independent association with risk of ischemic stroke (adjusted OR comparing $Quartile_4$ *v* $Quartile_1$ = 1.64, 95% CI 1.15-2.32, P_{trend} = .01) (Table 4). The only other biomarker that achieved even a borderline level of statistical significance when multiple biomarkers were included in models was tPA (adjusted OR comparing $Quartile_4$ *v* $Quartile_1$ = 1.35, 95% CI 0.96-1.89, P_{trend} = .06). In analyses of CRP in relation to subtypes of stroke, the adjusted OR comparing $Quartile_4$ versus $Quartile_1$ was 2.27 (95% CI = 1.35, 3.84) for cardioembolic stroke (n = 186 cases), 1.35 (95% CI = 0.67, 2.70) for large-artery stroke (n = 86 cases), and 1.68 (95% CI = 1.07, 2.63) for small-vessel stroke (n = 230 cases).

BRS and Risk of Ischemic Stroke

BRS was derived based on levels of 7 biomarkers that met predefined statistical criteria (P_{trend} < .15): CRP, IL-6, tPA, D-dimer, WBC, neopterin, and homocysteine. Individuals were assigned one point for each biomarker measurement that was in the top quartile of the control distribution. In multivariate analyses, BRS was an

Table 2. *Correlations among inflammatory and hemostasis biomarkers among control study participants*

	CRP	IL-6	TNF-alpha	Neopterin	E-Selection	VCAM	Factor VII	Prothrombin Fragment 1+2	tPA	PAI-1	D-dimer	Fibrinongen	Homocysteine
WBC	0.25	0.30	0.08	0.00	0.22	0.00	0.05	0.00	0.21	0.13	0.05	0.14	0.05
	<0.0001	<0.0001	0.02	0.92	<0.0001	0.96	0.14	0.99	<0.0001	<0.001	0.11	<0.0001	0.14
CRP		0.47	0.14	0.11	0.15	-0.01	0.15	0.04	0.15	0.06	0.18	0.27	0.01
		<0.0001	<0.0001	<0.001	<0.0001	0.83	<0.0001	0.38	<0.0001	0.09	<0.0001	<0.0001	0.78
IL-6			0.21	0.21	0.22	0.10	-0.01	-0.07	0.29	0.14	0.27	0.34	0.10
			<0.0001	<0.0001	<0.0001	<0.01	0.87	0.07	<0.0001	<0.0001	<0.0001	<0.0001	<0.01
TNF-alpha				0.37	0.17	0.38	0.05	0.03	0.11	0.13	0.12	0.13	0.19
				<0.0001	<0.0001	<0.0001	0.19	0.43	<0.01	<0.0001	<0.001	<0.001	<0.0001
Neopterin					0.09	0.41	-0.02	-0.02	0.14	0.05	0.21	0.11	0.30
					<0.01	<0.0001	0.48	0.70	<0.0001	0.13	<0.0001	<0.001	<0.0001
E-Selection						0.08	0.07	0.01	0.30	0.18	0.16	0.05	0.10
						0.01	0.03	0.72	<0.0001	<0.0001	<0.0001	0.13	<0.01
VCAM							-0.10	-0.02	0.08	-0.04	0.16	0.13	0.31
							0.01	0.66	0.02	0.30	<0.0001	<0.0001	<0.0001
Factor VII								0.51	-0.02	0.05	0.17	-0.26	0.02
								<0.0001	0.53	0.14	<0.0001	<0.0001	0.52
Prothrombin Fragment 1+2									-0.17	-0.04	0.17	-0.17	-0.02
									<0.0001	0.29	<0.0001	<0.0001	0.66
tPA										0.32	0.02	0.17	0.16
										<0.0001	0.57	<0.0001	<0.0001
PAI-1											0.05	0.12	0.08
											0.13	<0.001	0.02
D-Dimer												0.17	0.18
												<0.0001	<0.0001
Fibrinogen													0.07
													0.03

Data in table represent Spearman's *r* and p-value.

CRP, C-reactive protein; IL-6, interleukin-6; tPA, tissue plasminogen activator; WBC, white blood cell count; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; PAI-1, plasminogen activator inhibitor-1.

Note: We observed no significant correlation between CRP and LDL level (Spearman correlation r = -0.03, p = 0.30).

Table 3. *Inflammatory and hemostasis biomarker values among patients with ischemic stroke and age- and race-matched control subjects*

	Patients		Control subjects				Wilcoxon signed rank test	
Variable	Median	IQR	Median	Lower 25th percentile	Upper 25th percentile	IQR	No. case-control pairs	*P*-value
CRP (mg/L)	3.6	5.7	2.6	1.1	5.2	4.1	939	<.0001
IL-6 (pg/mL)	2.1	1.9	1.8	1.3	2.7	1.4	949	<.0001
tPA (mg/mL)	9.9	5.7	8.7	6.3	11.3	5.0	776	<.0001
D-dimer (ng/mL)	489.7	343.0	460.4	334.5	647.4	312.9	780	.06
WBC (cells $\times 10^3/\mu l$)	6.0	2.2	5.7	4.8	6.8	2.0	940	<.0001
Neopterin (ng/mL)	1.8	0.9	1.7	1.3	2.1	0.7	944	<.001
Homocysteine (μmol/L)	8.5	3.7	8.2	6.6	10.2	3.6	930	.06
E-selectin (ng/mL)	31.0	18.0	29.0	21.0	38.0	17.0	957	<.0001
Fibrinogen (mg/dL)	279.0	87.0	273.0	242.0	316.0	74.0	780	.43
TNF-α (pg/mL)	1.4	0.8	1.4	1.0	1.8	0.8	885	.02
VCAM-1 (ng/mL)	698.0	288.0	683.5	561.0	835.0	274.0	933	<.01
Prothrombin fragment 1+2 (nmol/L)	1.2	1.1	1.2	0.9	1.9	1.0	583	.61
Factor VII (%)	151.0	100.0	149.0	121.0	217.0	96.0	780	.65
PAI-1 (ng/mL)	26.6	32.1	23.6	14.6	43.2	28.6	773	.09

Abbreviations: CRP, C-reactive protein; IL, interleukin; IQR, interquartile range; PAI, plasminogen activator inhibitor; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; VCAM, vascular cell adhesion molecule; WBC, white blood cell count.

As an alternative to Wilcoxon signed rank test, Mann-Whitney *U* tests were also performed and this provided similar conclusions about significant (<.05) *P* values, except for D-dimers (<.01, *v* .06 in table), VCAM-1 (.09 *v* <.01 in table), and PAI-1 (.04 *v* .09 in table).

independent predictor of ischemic stroke, after adjusting for stroke risk factors ($P < .001$) (Fig 1). Higher BRS predicted all major causal subtypes of stroke (Fig 2). In stratified analyses with tests for interaction, we found no evidence that the ORs describing the association between BRS and risk of ischemic stroke differed across subgroups of age, estrogen/progestin use, hypertension, diabetes, smoking, race, HDL cholesterol, or LDL cholesterol.

The c index for prediction of stroke was 0.633 (95% CI 0.605-0.660) for a model that included standard stroke risk factors (age, race/ethnicity, aspirin use, BMI, diabetes, systolic blood pressure, antihypertensive medication use, smoking, lipid-lowering medication use, fasting glucose, LDL cholesterol, and HDL cholesterol) but not the BRS. Addition of the BRS to the model improved the c index to 0.649 (95% CI 0.622-0.677), which was a statistically significant increase as compared with the standard stroke risk factor model ($P = .02$). A model including standard stroke risk factors and CRP alone had a c index of 0.640 (95% CI 0.613-0.668, $P = .15$ as compared with the standard stroke risk factor model). The addition of the BRS to the model containing CRP and standard stroke risk factors produced a nonsignificant increase in the c index ($P = .09$).

BRS and CRP

Additional analyses examined the association of the BRS with risk of stroke among individuals with CRP above and below the high-risk level of 3.0 mg/L. For these analyses, the reference group was defined as individuals who had CRP less than or equal to 3.0 mg/L and who had a BRS of zero (i.e., no elevated biomarkers). A gradient of increasing risk was observed across increasing numbers of elevated biomarkers, particularly among individuals with high CRP (Fig 3). Among individuals with levels of CRP greater than 3.0 mg/L, almost 40% had zero or one other elevated biomarkers (i.e., 15.2% had BRS = 1 and 23.8% had BRS = 2). Among these individuals who had high CRP but less than or equal to one other elevated biomarkers, risk of stroke was similar as compared with those who had CRP below the 3.0-mg/L threshold but who had elevated levels of one or more other biomarkers (Fig 3). In analyses of BRS as continuous variable, the adjusted OR per unit was 1.12 (95% CI 0.99-1.28) for those with CRP less than or equal to 3.0 mg/L, and the adjusted OR per unit was 1.26 (95% CI 1.13-1.40) for those with CRP greater than 3.0 mg/L (P for interaction = .53). The addition of the BRS to the model containing standard stroke risk factors significantly improved the c index both among subjects with CRP less than or equal to 3.0 mg/L ($P = .04$) and among subjects with CRP greater than 3.0 mg/L ($P = .03$). These results stratified on CRP were changed little after residual adjustment for CRP levels.

Table 4. *Adjusted analyses of the association between inflammatory and hemostasis levels with risk of incident ischemic stroke*

		Adjusted for known stroke risk factors					Adjusted for known stroke risk factors and other biomarkers				
		Quartile categories					Quartile categories (740 cases, 775 controls)				
		1	2	3	4	p-trend*	1	2	3	4	p-trend*
C-Reactive Protein	OR	1	1.18	1.24	1.78	<0.001	1	1.19	1.04	1.64.	0.01
cases/controls: 874/892	95% CI	(reference)	(0.88, 1.59)	(0.92, 1.67)	(1.32, 2.39)		(reference)	(0.86, 1.63)	(0.75, 1.45)	(1.15, 2.32)	
Interleukin 6	OR	1	1.26	1.15	1.68	<0.001	1	1.07	0.91	1.22	0.43
892/912	95% CI	(reference)	(0.94, 1.68)	(0.86, 1.55)	(1.25, 2.26)		(reference)	(0.77, 1.48)	(0.64, 1.28)	(0.85, 1.75)	
Tissue Plasminogen Activator	OR	1	0.90	1.00	1.42	0.02	1	0.93	1.04	1.35	0.06
789/820	95% CI	(reference)	(0.66, 1.22)	(0.73, 1.36)	(1.03, 1.94)		(reference)	(0.68, 1.29)	(0.75, 1.44)	(0.96, 1.89)	
D-Dimer	OR	1	1.5	1.33	1.52	0.03	1	1.43	1.20	1.30	0.19
791/821	95% CI	(reference)	(1.11, 2.03)	(0.97, 1.81)	(1.12, 2.08)		(reference)	(1.04, 1.97)	(0.86, 1.68)	(0.92, 1.83)	
White Blood Cell Count	OR	1	1.21	1.03	1.46	0.03	1	1.20	0.90	1.20	0.44
885/904	95% CI	(reference)	(0.91, 1.60)	(0.77, 1.37)	(1.10, 1.94)		(reference)	(0.88, 1.64)	(0.65, 1.24)	(0.87, 1.67)	
Neopterin	OR	1	0.79	0.98	1.24	0.05	1	0.73	0.92	1.10	0.30
878/893	95% CI	(reference)	(0.59, 1.05)	(0.74, 1.30)	(0.94, 1.63)		(reference)	(0.53, 1.00)	(0.67, 1.26)	(0.79,1.52)	
Homocysteine	OR	1	1.15	1.23	1.26	0.10	1	1.13	1.15	1.11	0.60
872/886	95% CI	(reference)	(0.86, 1.52)	(0.93, 1.64)	(0.95, 1.68)		(reference)	(0.83, 1.55)	(0.84, 1.59)	(0.80, 1.54)	
E-Selectin	OR	1	0.93	1.00	1.15	0.30	Not included in model				
894/910	95% CI	(reference)	(0.70, 1.22)	(0.76, 1.31)	(0.86, 1.53)						
Fibrinogen	OR	1	0.75	0.89	0.91	0.72	Not included in model				
791/821	95% CI	(reference)	(0.56, 1.01)	(0.67, 1.18)	(0.67, 1.22)						
TNF-alpha	OR	1	0.98	1.03	1.16	0.27	Not included in model				
851/865	95% CI	(reference)	(0.74, 1.30)	(0.78, 1.37)	(0.87, 1.54)						
VCAM-1	OR	1	0.97	1.00	1.00	0.96	Not included in model				
875/891	95% CI	(reference)	(0.73, 1.28)	(0.76,1.32)	(0.75, 1.32)						
Prothrombin Fragment 1 + 2	OR	1	0.90	1.18	1.04	0.57	Not included in model				
580/601	95% CI	(reference)	(0.64, 1.27)	(0.84, 1.65)	(0.74, 1.47)						
Factor VIIC	OR	1	0.77	0.91	0.91	0.75	Not included in model				
791/821	95% CI	(reference)	(0.58, 1.03)	(0.68, 1.21)	(0.68, 1.21)						
PAI-1 Antigen	OR	1	0.80	1.03	1.00	0.60	Not included in model				
785/820	95% CI	(reference)	(0.59, 1.08)	(0.77, 1.38)	(0.74, 1.35)						

Adjusted for aspirin use, BMI, diabetes, systolic blood pressure, smoking, high cholesterol requiring pills, anti-hypertensive medication use, fasting glucose, low density lipoprotein and high density lipoprotein.

*Test of trend across quartiles of biomakers were conducted by assigning a numerical value for each quartile (1, 2, 3, 4) and fitting this continuous variable in the model.

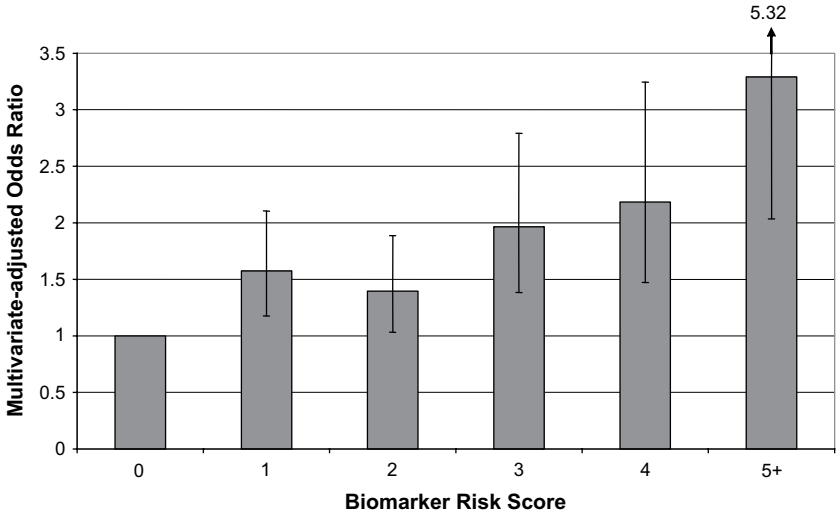

Figure 1. *Multivariate-adjusted analyses of BRS as predictor of incident ischemic stroke.* Y axis, *Multivariate-adjusted OR.* X axis, *BRS. BRS defined as number of biomarkers that were above top 25% of distribution among controls, from among: CRP, IL-6, tPA, D-dimer, WBC, neopterin, and homocysteine. See Table 3 for upper quartile cut points. N (%) among controls was 243 (25%) for BRS = 0, 263 (27.1%) for BRS = 1, 232 (23.9%) for BRS = 2, 115 (11.8%) for BRS = 3, 79 (8.1%) for BRS = 4, and 40 (4.1%) for BRS ≥ 5. N (%) among cases was 141 (14.5%) for BRS = 0, 238 (24.5%) for BRS = 1, 217 (22.3%) for BRS = 2, 160 (16.5%) for BRS = 3, 119 (12.2%) for BRS = 4, and 97 (10.0%) for BRS ≥ 5.*

Discussion

A multiple biomarker index (BRS) derived from levels of 7 biomarkers of inflammation and hemostasis (CRP, IL-6, tPA, D-dimer, WBC, neopterin, and homocysteine) defined a gradient of ischemic stroke risk across this population of 50- to 79-year-old postmenopausal women. Discrimination between patients with stroke and control subjects (c index) was significantly improved with addition of the BRS to standard stroke risk factors including diabetes, hypertension, and smoking.

In this study, CRP was the only single biomarker that remained associated with stroke after adjustment for standard stroke risk factors and other inflammation and hemostasis-related biomarkers. CRP is a well-established vascular risk factor,[18] although the risk associated with elevated CRP may be weaker than previously believed.[14]

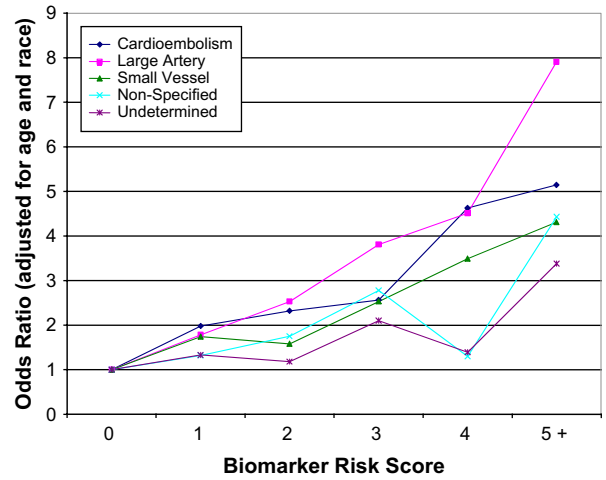

Figure 2. *Age- and race-adjusted analyses of BRS as predictor of incident ischemic stroke, by stroke subtype.* Y axis, *Multivariate-adjusted OR.* X axis, *BRS. BRS was defined as number of biomarkers that were above top 25% of distribution among controls, from among: CRP, IL-6, tPA, D-dimer, WBC, neopterin, and homocysteine. See Table 3 for upper quartile cut points.*

Moreover, fewer studies of CRP have examined ischemic stroke than have examined coronary disease, and the importance of CRP may be less in older adults than in middle-aged populations. Data from the Women's Health Study (mean age 53.7 years) suggested that CRP was more strongly associated with risk of ischemic stroke than with risk of coronary events (for stroke, adjusted hazard ratio comparing CRP Tertile 3 *v* Tertile 1 = 2.76, 95% CI 1.51-5.05; for coronary disease, adjusted hazard ratio comparing CRP Tertile 3 *v* Tertile 1 = 1.66, 95% CI 1.17-2.34).[15] In the current cohort (50-79 years old, median age 69 years), results were consistent with this finding but the association was weaker and appeared to be confined to the upper quartile (adjusted OR comparing CRP Quartile$_4$ *v* Quartile$_1$ = 1.64, 95% CI 1.15-2.32). Other studies have produced conflicting evidence on the association of CRP with ischemic stroke in older adults. In the Cardiovascular Health Study (CHS) cohort, elevated levels of CRP did not predict risk of stroke among men and women 65 years and older who were free of prior angina, myocardial infarction, or stroke; in CHS, an association between CRP and stroke was only observed among those older adults who had an increased burden of subclinical atherosclerosis as indicated by carotid artery wall thickness.[1,3] The Health, Aging and Body Composition Study found that IL-6 and TNF-α, but not CRP, were associated with stroke, heart failure, and coronary disease among 70- to 79-year-old adults.[12] In the Rotterdam (≥55 years old)[16] and Copenhagen (50-89 years old)[13] cohorts, elevated CRP had no significant association with risk of stroke after adjustment for standard stroke risk factors. Notable strengths of the current study, shared by some but not all prior investigations, included large sample size, with more than 900 incident strokes and a comparably sized control population, and neurologist review of medical records to confirm stroke events.

Current clinical guidelines have endorsed measurement of CRP level as an adjunct to standard CVD risk

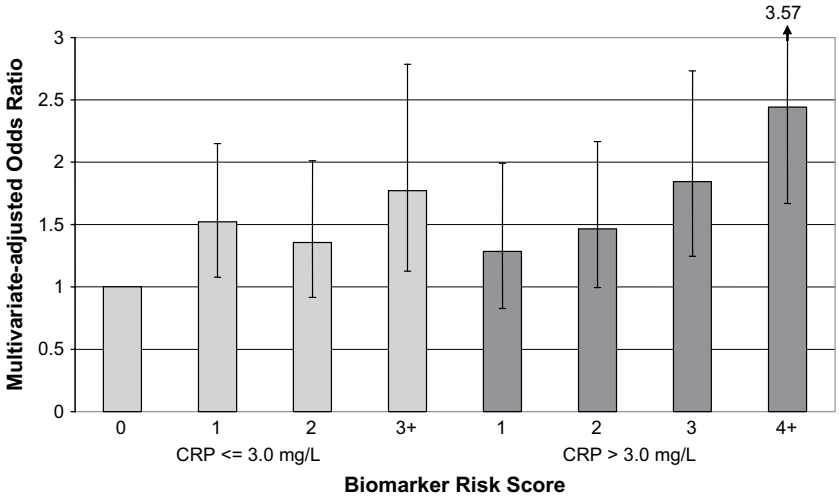

Figure 3. *Multivariate-adjusted analyses of BRS as predictor of incident ischemic stroke, in subgroups stratified by low (<3.0 mg/L) or high (≥3.0 mg/L) CRP level.* Y axis, *Multivariate-adjusted OR.* X axis, *BRS. BRS was defined as number of biomarkers that were above top 25% of distribution among controls. See Table 3 for upper quartile cut points for IL-6, tPA, D-dimer, WBC, neopterin, and homocysteine. In this analyses within CRP subgroups, in computing BRS, one point was assigned for CRP ≥ 3.0 mg/L, whereas for main analyses CRP upper quartile value of 5.2 mg/L was used to define high levels.*

factor screening for guiding CVD prevention efforts,[18] and new CVD risk stratification algorithms that have been proposed include CRP in addition to established risk factors.[20] Our study raises the question of whether multiple biomarkers reflecting inflammation or hemostasis might be useful when measured in addition to CRP. For example, a BRS defined as the total number of inflammation and hemostasis biomarkers that were elevated (i.e., in the upper quartile) revealed an approximate 2-fold gradient in stroke risk among individuals who had high-risk CRP levels (≥3.0 mg/L).[18] Among women with CRP above 3.0 mg/L, nearly 50% had zero or one other elevated biomarkers, and these individuals had a risk of stroke that was similar to those who had CRP below high-risk levels (<3.0 mg/L) but who had elevated levels of one or more other biomarkers. Thus, our data suggest that among individuals with CRP levels in the high-risk category, measurement of other inflammatory and hemostasis biomarkers may be clinically useful to provide additional stratification of stroke risk.

In addition to CRP, this study examined other candidate biomarkers that were selected to reflect a variety of relevant causative pathways including inflammation, atherosclerosis, platelet activity, coagulation, and fibrinolysis. We confirmed that modest elevations in WBC, an acute-phase reactant, may reflect global inflammation and increased stroke risk, as shown previously in this population and others.[21,22] IL-6 is a proinflammatory cytokine and trigger for liver release of CRP, and we confirmed its previously reported association with risk of stroke.[5,7,12] Two of the other biomarkers that we identified as stroke risk factors reflect fibrinolytic activity, including tPA, the main activator of fibrinolysis, and D-dimer, a marker of fibrin turnover. Both have been previously implicated as vascular risk factors.[9,10] Also worthy of note are negative findings for several biomarkers in the current study. For example, a recent meta-analysis of more than 31 prospective epidemiologic studies suggested that elevated levels of fibrinogen, which is involved in inflammation, platelet aggregation, and coagulation cascades, predict the risk of ischemic stroke and other vascular events among healthy adults.[23] However, our data showed no significant association between fibrinogen and stroke among postmenopausal women. This appears to confirm prior findings from the CHS that fibrinogen does not predict stroke among older women, although it does among older men.[24]

Limitations of this study include a lack of data among men and premenopausal women, limiting the ability to generalize results to these groups. We also lacked comparative data for multiple vascular end points in addition to stroke, and had limited statistical power for subgroup analyses by race/ethnicity. It is important to note that this observational study is not able to evaluate whether the identified biomarkers play a causal role or whether their modification may alter risk of stroke. Validation of these results in another population will be important, although we used cross-validation methods to address the lack of an external validation cohort. It is important to note that biomarkers were only measured at baseline, and may have changed during follow-up as a result of biological within-individual variation or initiation of medications that affect inflammation, endothelial function, and coagulation status.

Among several inflammatory and hemostasis-related biomarkers that were studied, we identified elevated CRP level as the strongest independent risk factor for stroke among postmenopausal women. A BRS derived from levels of several biomarkers provided additional useful information for stratifying stroke risk. The findings support the further exploration of multiple biomarker panels for more accurately stratifying an individual's risk of stroke, possibly based on emerging multiplex assay technologies that may reduce technical and cost barriers.[25]

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