Transcobalamin 2 variant associated with poststroke homocysteine modifies recurrent stroke risk

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ABSTRACT

Objectives: The Vitamin Intervention for Stroke Prevention trial found an association between baseline poststroke homocysteine (Hcy) and recurrent stroke. We investigated genes for enzymes and cofactors in the Hcy metabolic pathway for association with Hcy and determined whether associated single nucleotide polymorphisms (SNPs) influenced recurrent stroke risk.

Methods: Eighty-six SNPs in 9 candidate genes (*BHMT1, BHMT2, CBS, CTH, MTHFR, MTR, MTRR, TCN1,* and *TCN2*) were genotyped in 2,206 subjects (83% European American). Associations with Hcy measures were assessed using linear regression models assuming an additive genetic model, adjusting for age, sex, and race and additionally for baseline Hcy when postmethionine load change was assessed. Associations with recurrent stroke were evaluated using survival analyses.

Results: Five SNPs in the transcobalamin 2 (*TCN2*) gene were associated with baseline Hcy (false discovery rate [FDR]–adjusted $p = 0.049$). *TCN2* SNP rs731991 was associated with recurrent stroke risk in the low-dose arm of the trial under a recessive model (log-rank test $p = 0.009$, hazard ratio 0.34). Associations with change in postmethionine load Hcy levels were found with 5 SNPs in the cystathionine β-synthase (*CBS*) gene (FDR-adjusted $p < 0.031$).

Conclusions: *TCN2* variants contribute to poststroke Hcy levels, whereas variants in the *CBS* gene influence Hcy metabolism. Variation in the *TCN2* gene also affects recurrent stroke risk in response to cofactor therapy. ***Neurology***® **2011;77:1543–1550**

GLOSSARY

AA = African American; **EA** = European American; **FDR** = false discovery rate; **Hcy** = homocysteine; **LD** = linkage disequilibrium; **LS** = least-squares; **MAF** = minor allele frequency; **NIHSS** = NIH Stroke Scale; **SNP** = single nucleotide polymorphism; **3′UTR** = 3′untranslated region; **VISP** = Vitamin Intervention for Stroke Prevention; **VNTR** = variable number tandem repeat; **WGA** = whole-genome amplification.

Recurrent stroke is the most important modifiable predictor of death or disability at 5 years after first stroke,[1] and 54% of those having a recurrent stroke will be disabled.[2] The Vitamin Intervention for Stroke Prevention (VISP) trial enrolled patients with cerebral infarction and homocysteine (Hcy) levels in the top quartile for the US population.[3] Subjects were randomly assigned to a high or low dose of folic acid, vitamin B_6, and vitamin B_{12}. There was a persistent and graded association between baseline Hcy level and vascular outcomes in both treatment groups. Subgroup analyses of the VISP trial showed that baseline fasting Hcy was comparable to postmethionine load test Hcy or change in postmethionine load Hcy in predicting risk of both recurrent stroke and symptomatic coronary heart disease.[4,5]

A number of studies have found evidence that mild to moderate hyperhomocystinemia may result from genetic variations that alter enzymatic activity in the remethylation or transsulfuration pathways. Associations with Hcy have been reported with genes for methylenetetrahydro-

Supplemental data at www.neurology.org

Supplemental Data

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Table 1 Demographic and trait characteristics of genotyped individuals from VISP trial

Trait	Trait value	Low dose for genetic studies	High dose for genetic studies	Rest of VISP	p Value[a]
No.	2,206	1,104	1,102	1,474	
Age, y, mean ± SD, median	67.2 ± 10.7, 68	67.1 ± 10.7, 68	67.4 ± 10.7, 69	64.9 ± 10.8, 65.5	<0.0001
Female gender, n (%)	801 (36.3)	393 (35.6)	408 (37.0)	578 (39.2)	0.0746
Race, n (%)					
Caucasian	1,819 (82.5)	902 (81.7)	917 (83.2)	1,106 (75.0)	<0.0001
African American	265 (12.0)	138 (12.5)	127 (11.5)	280 (19.0)	
Other	122 (5.5)	64 (5.8)	58 (5.3)	88 (6.0)	
High-dose arm of trial, n (%)	1,102 (50)	0 (0)	1,102 (100)	725 (49.2)	0.6476
Baseline homocysteine, μmol/L, mean ± SD, median	13.28 ± 4.81, 12.2	13.31 ± 4.97, 12.1	13.25 ± 4.65, 12.3	13.55 ± 5.38, 12.4	0.2843
Postmethionine homocysteine, μmol/L, mean ± SD, median	29.59 ± 10.07, 27.8	29.54 ± 10.12, 27.6	29.64 ± 10.02, 28.1	30.12 ± 10.80, 28.2	0.2320
Vitamin B_6, nmol/L, mean ± SD, median	42.20 ± 37.22, 33.3	43.58 ± 39.50, 34.4	40.78 ± 34.71, 32.2	42.88 ± 36.45, 34.5	0.5326
Vitamin B_{12}, pmol/L, mean ± SD, median	363.40 ± 221.31, 328	362.08 ± 172.04, 327	364.73 ± 262.06, 329	358.62 ± 239.15, 314	0.0340
Folate, nmol/L, mean ± SD, median	26.19 ± 17.19, 22.7	25.95 ± 16.31, 22.9	26.44 ± 18.04, 22.6	27.58 ± 19.88, 22.9	0.2619
Hypertension, n (%)	1,597 (74.0)	790 (71.8)	807 (73.4)	1,115 (75.8)	0.0285
Diabetes, n (%)	607 (28.0)	325 (29.4)	282 (25.6)[b]	464 (31.6)	0.0081
Smoking history, n (%)					
Current	334 (15.1)	164 (14.9)	170 (15.4)	287 (19.5)	0.0021
Former	1,125 (51.0)	548 (49.6)	577 (52.4)	698 (47.4)	
Recurrent stroke, n (%)	184 (8.3)	95 (8.6)	89 (8.1)	116 (7.9)	0.6088

Abbreviation: VISP = Vitamin Intervention for Stroke Prevention.
[a] Comparison between the VISP genetic sample and the rest of VISP sample using a χ^2 test for categorical variables and Kruskal-Wallis test for continuous variables.
[b] $p < 0.05$ for comparison between the low dose and high dose in the VISP subgroup for genetic analyses using a χ^2 test for categorical variables and Kruskal-Wallis test for continuous variables. The two treatment groups had similar demographic and trait characteristics, except for a slightly lower proportion of diabetes in the high-dose sample ($p = 0.0430$).

folate reductase (*MTHFR*), cystathionine β-synthase (*CBS*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), and cystathionase (*CTH*/*GCT*),[6,7] betaine homocysteine methyltransferase (*BHMT*),[8] and transcobalamin 2 (*TCN2*).[8–10] Our objective was to investigate the spectrum of common variations of genes in the Hcy metabolic pathway for contributions to baseline Hcy measures and determine whether associated SNPs also affect recurrent stroke risk.

METHODS

Standard protocol approvals, registrations, and patient consents. The VISP trial (clinical trial identifier number NCT00004734) was conducted under institutional review board approval at Wake Forest University School of Medicine and each of the clinic sites and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients.

Study design and laboratory measures. VISP was a multicenter, double-blind, randomized controlled clinical trial that enrolled patients aged 35 or older with a nondisabling cerebral infarction (defined as modified Rankin Scale score ≤3) within 120 days of randomization and Hcy levels greater than the 25th percentile for the general population at screening.[3] In August 1997, the eligible Hcy level (25th percentile) was initially >10.5 μmol/L, was modified to >9.5 μmol/L for both sexes on April 7, 1998, and was modified to >9.5 μmol/L (men) and >8.5 μmol/L (women) after April 1, 1999. Subjects were randomly assigned to receive daily doses of the high-dose formulation containing 25 mg pyridoxine (vitamin B_6), 0.4 mg cobalamin (vitamin B_{12}), and 2.5 mg folic acid or the low-dose formulation containing 200 μg pyridoxine, 6 μg cobalamin, and 20 μg folic acid. In December 2002, the VISP Data and Safety Monitoring Board recommended to the National Institute of Neurological Disorders and Stroke that the trial be terminated because of the unlikely event of ever demonstrating a treatment difference. At the time of early trial closure, all participants had been in the study at least 1 year. The average follow-up time was 1.7 years (maximum 2 years).

Fasting plasma samples for determination of baseline Hcy were obtained at enrollment. Patients were given a dose of methionine (100 mg/ kg body weight) in fruit juice, and a second blood sample was drawn after 2 hours. Recurrent stroke was defined as an acute ischemic stroke of at least 24 hours duration with focal signs and symptoms, without evidence of primary intracranial hemorrhage or other alternative explanation, together with one of the following: a 1-point increase in the NIH Stroke Scale (NIHSS) in a previously normal section or, lacking

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Table 2 Association results for genotyped *TCN2* SNPs with baseline fasting Hcy level[a]

SNP	Major/minor allele	Additive *p* value	FDR-adjusted *p* value	Count			LS mean			Location
				1/1	1/2	2/2	1/1	1/2	2/2	
rs5749131	G/A	0.0051	0.0625	780	1,042	376	12.93	13.29	13.78	Flanking 5′UTR
rs7289549	G/C	0.6543	0.8712	1,681	488	36	13.17	13.31	13.17	Intron
rs9606756	A/G	0.9450	0.9450	1,697	472	34	13.23	13.11	14.12	Coding (I23V)
rs740234	A/G	0.1943	0.4285	1,483	631	88	13.29	12.94	13.14	Intron
rs1801198	C/G	0.0865	0.2756	728	1,031	413	13.05	13.26	13.56	Coding (R259P)
rs9621049	C/T	0.5140	0.8073	1,677	460	35	13.25	13.13	15.00	Coding (S348F)
rs4820886	A/C	0.5163	0.8073	1,698	471	35	13.21	13.09	14.98	Intron
rs4820887	G/A	0.8077	0.9153	1,801	383	20	13.24	13.03	14.43	Intron
rs4820888	A/G	0.0034	0.0494[b]	715	1,050	433	13.65	12.94	12.91	Intron
rs2267164	G/A	0.0293	0.1401	855	1,001	346	13.06	13.23	13.78	Intron
rs2301955	G/A	0.0014	0.0494[b]	835	1,023	335	13.52	13.03	12.63	Intron
rs2301957	G/A	0.0021	0.0494[b]	837	1,028	335	13.51	13.06	12.62	Intron
rs2301958	C/G	0.0083	0.0814	1,352	746	100	13.39	13.00	12.36	Intron
rs1131603	T/C	0.1030[c]	0.3053	1,989	172	2	13.28	12.66	13.25	Coding (L376S)
rs4820889	G/A	0.4223[c]	0.7403	2,023	174	6	13.24	12.90	14.39	Coding (R399Q)
rs2072195	A/T	0.0087	0.0814	1,953	189	23	13.05	13.95	14.48	3′UTR
rs10418	G/A	0.0207	0.1163	1,293	787	116	13.42	13.06	12.53	3′UTR
rs1544468	A/G	0.0027	0.0494[b]	601	1,107	488	13.61	13.12	12.75	Flanking 3′UTR
rs731991	A/G	0.0014	0.0494[b]	602	1,104	489	13.65	13.10	12.76	Flanking 3′UTR
rs5997711	G/A	0.0377	0.1620	778	1,024	346	13.09	13.18	13.82	Flanking 3′UTR

Abbreviations: FDR = false discovery rate; LS = least-squares; SNP = single nucleotide polymorphism; 3′UTR = 3′untranslated region; VISP = Vitamin Intervention for Stroke Prevention.

[a] Values are adjusted for age, sex, and race. Allele 1 is the major allele, and allele 2 is the minor allele, based on allele frequency in the entire VISP sample.

[b] FDR-adjusted $p < 0.05$.

[c] Dominant model shown (minor homozygote count <10).

this, an appropriate new or extended abnormality seen on CT or MRI.

Of the 56 VISP study centers, 46 centers collected blood samples for genetic analyses. DNA was isolated using a Gentra Autopure system (Qiagen, Germantown, MD). Samples underwent whole-genome amplification (WGA) using Repli-g methodology through Molecular Staging Inc. (New Haven, CT) and Qiagen (Hilden, Germany).

SNP selection and genotyping. Ninety-six single nucleotide polymorphisms (SNPs) were selected in 9 genes involved in Hcy metabolism: *BHMT1, BHMT2, CBS, CTH, MTHFR, MTR, MTRR, TCN1,* and *TCN2* (table e-1 on the *Neurology*® Web site at www.neurology.org). SNPs were selected in candidate gene loci according to the following criteria: 1) within the proximal and distal 10-kb regions 5′ and 3′ to the given candidate gene (NCBI Build 35); 2) compatibility with the Illumina GoldenGate technology[11] as determined by the Assay Design Tool (TechSupport; Illumina, Inc., San Diego, CA); 3) minor allele frequency (MAF) >0.05 or a tag (r^2 >0.8) for another SNP with MAF >0.05 as determined by applying the multilocus or "aggressive" Tagger option of Haploview v3[12] using International HapMap Project data for CEPH and Yoruban populations (release 19).[13] For *BHMT1, CTH, MTHFR, MTR, MTRR,* and *TCN2*, additional SNPs with prior report of association with a phenotype of interest to the VISP investigators were added (table e-1 a priori candidate SNPs).[6–8,10] Genotyping assays using WGA DNA were conducted by the Custom Genotyping Service of Illumina, Inc using the GoldenGate assay. Although the panel included 1,536 SNPs selected largely from vascular candidate genes, the 9 genes analyzed in the present study were selected specifically as a priori biological candidates for associations with baseline Hcy measures in the VISP trial and have therefore been evaluated separately. Eighty-two (85%) of the 96 SNPs selected in the 9 candidate genes were successfully genotyped at a >96% success rate in 2,206 unique subjects. Reproducibility between replicate samples was >99.99%. Four replicates with both WGA and genomic DNA were included in the dataset; all were 100% concordant for all available genotypes.

TCN2 coding SNP rs1801198 (R259P, C776G)[8–10] was included in the Illumina GoldenGate genotyping panel but failed to genotype successfully. This SNP, together with *TCN2* coding SNPs rs9621049 (S348F) and rs1131603 (L376S) and rs2072195 located in the 3′ untranslated region (3′UTR), were prioritized on the basis of prior association with Hcy or linkage disequilibrium (LD) with SNP rs731991 (associated with recurrent stroke) and subsequently were genotyped using the TaqMan assay.[14] Endpoint allelic discrimination was detected using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Genotyping success rates were >95% for all 4 SNPs.

Statistical analyses. Associations with Hcy measures were evaluated using linear regression models assuming an additive

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Table 3 Association results for genotyped *CBS* SNPs with Hcy level after a 2-hour methionine load test[a]

SNP	Major/minor allele	Additive *p* value	FDR-adjusted *p* value	Count			LS mean			Location
				1/1	1/2	2/2	1/1	1/2	2/2	
rs9982921	G/A	0.3399[b]	0.6636	2,169	32	1	29.20	27.32	25.77	Intron
rs11701048	G/A	0.0005	0.0101[c]	1,833	332	26	29.51	27.71	25.47	Intron
rs9982015	A/G	0.0002	0.0053[c]	1,856	321	20	29.54	27.70	23.93	Intron
rs234715	C/A	0.2900	0.6258	1,282	737	99	29.12	28.87	27.90	Intron
rs2851391	G/A	0.0022	0.0367[c]	694	1,082	411	28.10	29.57	29.88	Intron
rs234709	G/A	0.000037	0.0030[c]	659	1,011	516	30.74	28.61	28.29	Intron
rs234705	G/A	0.1396	0.5986	1,005	937	254	29.54	28.63	28.96	Intron
rs4920037	G/A	0.5356	0.8123	1,340	740	105	29.23	29.08	28.58	Intron
rs6586282	G/A	0.000085	0.0035[c]	1,522	580	66	29.67	28.21	25.87	Intron
rs719037	A/G	0.0065	0.0890	800	1,055	334	28.39	29.45	30.04	Flanking 3′UTR

Abbreviations: FDR = false discovery rate; LS = least-squares; SNP = single nucleotide polymorphism; 3′UTR = 3′untranslated region; VISP = Vitamin Intervention for Stroke Prevention.
[a] Values are adjusted for age, sex, and race. Allele 1 is the major allele, and allele 2 is the minor allele, based on allele frequency in the entire VISP sample.
[b] Dominant model shown (minor homozygote count <10).
[c] FDR-adjusted $p < 0.05$.

genetic model, adjusting for age, sex, and race, and, in addition, for baseline Hcy when postmethionine load change was assessed. A dominant genetic model was used in which the minor allele homozygote count was <10. Because there were few individuals per site (from 1 to 127), analyses were not adjusted for collection center. Further adjustments for smoking, levels of folate, vitamins B_6, and B_{12}, and stroke severity (measured by the NIHSS) were also explored but did not change the conclusions. A least-squares (LS) mean for each genotype, considered the group mean after adjustment for a covariate, is presented.

False discovery rate (FDR)[15,16] was used to adjust for the multiple comparisons for the 86 SNPs. It controls the expected proportion of falsely rejected hypotheses (the FDR). Its threshold is determined from the observed *p* value distribution and is adaptive to the amount of significant results in the data. It is less conservative than the commonly used Bonferroni adjustment. FDR-adjusted *p* values were calculated using the procedure multtest in SAS (SAS Institute, Cary, NC).

We assessed associations with recurrent stroke using survival analyses. We initially considered only *TCN2* rs731991 and *CBS* rs234709 and subsequently evaluated the 4 TaqMan-genotyped coding and 3′UTR SNPs rs1801198, rs9621049, rs1131603, and rs2072195. Censoring may occur because of the loss of follow-up or death. First, we used the Kaplan-Meier method to estimate the survival functions for subjects with different genotypes in the 2-year follow-up. The cumulative incidence curve of recurrent stroke was compared using a logrank test. Second, we evaluated whether the selected SNPs were also associated with recurrent stroke using combined and treatment-stratified Cox proportional hazards models. All models were adjusted for age, sex, and race. Note that the interaction between the SNP and treatment was examined before the treatment-stratified models were performed.

RESULTS

Population characteristics. The characteristics of the subpopulation used for this study and the remainder of the VISP population are shown in table 1. The genetic sample is on average 3 years older and more likely to be of European American (EA) origin, compared with participants who did not provide a genetic sample or whose sample was unable to be genotyped. Sites that chose not to collect blood samples for genetic or biomarker studies represented 840 participants from the parent trial, whereas 167 subjects from enrolling sites chose not to participate in the genetic studies. Biases are therefore more likely to reflect nonparticipation in the VISP genetic study by specific sites, rather than individual participation bias. Participants in the 2 arms of the trial investigated in this study are comparable.

Associations with baseline Hcy levels. The 5 SNPs significantly associated with baseline fasting Hcy were located in the gene for the vitamin B_{12} transporter transcobalamin 2 (*TCN2*) (FDR-adjusted $p = 0.0494$) (table 2). Associated *TCN2* SNPs were consistent with Hardy-Weinberg proportions ($p > 0.19$). *TCN2* coding SNPs rs1801198 (R259P), rs9606756 (I22V), and rs4820889 (R398Q) were not associated with baseline Hcy (table 2). In race-stratified analyses (not shown), *TCN2* rs731991 association with Hcy is predominantly driven by EAs (rs731991 A/A genotype LS mean 13.75 μmol/L Hcy, A/G 12.98, G/G 12.76; $p = 0.0014$), although results trend in the same direction in African Americans (AAs) (rs731991 A/A LS mean 14.26 μmol/L Hcy, A/G 13.92, G/G 13.17; $p = 0.31$). Differences in significance between racial groups may be influenced by differing allele frequencies (rs731991 MAF: EA 0.49; AA 0.35) as well as differences in power due to contrasting sample sizes (EA n = 1,770 and AA n = 262 genotyped at this

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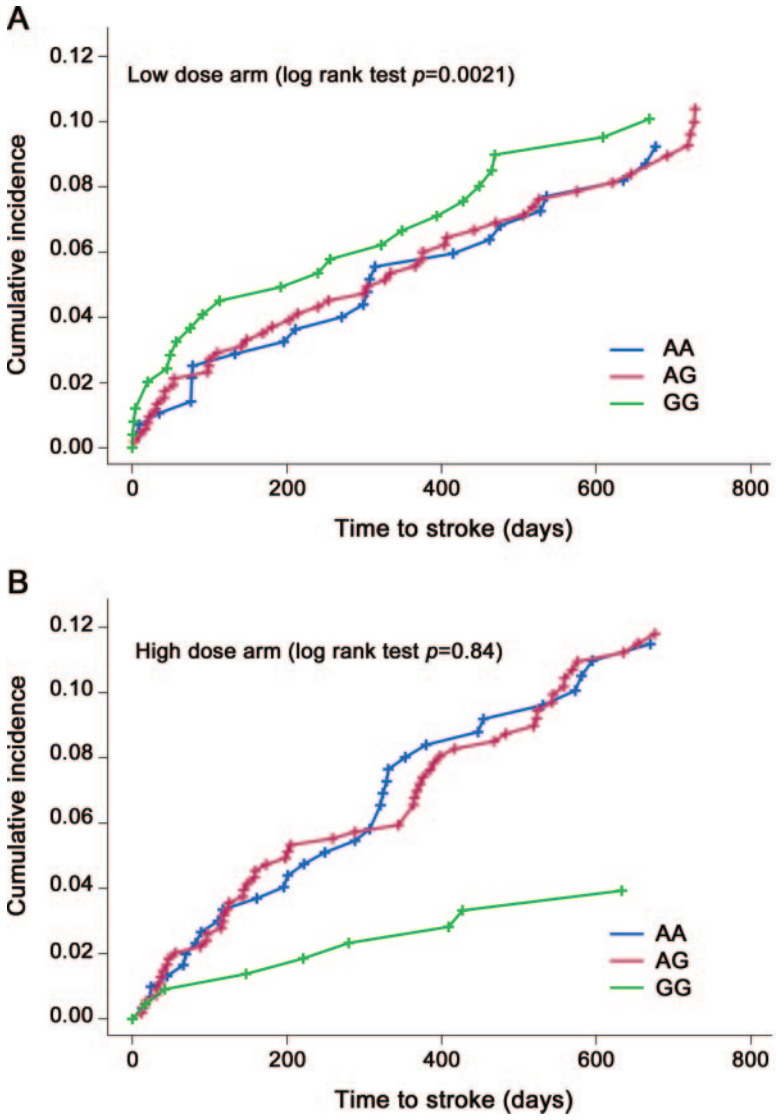
Figure 1 Cumulative incidence curves of recurrent stroke for *TCN2* SNP rs731991, stratified by (A) low-dose and (B) high-dose arms of the trial



Blue line, AA genotype, red line, AG genotype; green line, GG genotype.

SNP). *TCN2* LD structure in the VISP sample is shown in figure e-1.

Associations with postmethionine load Hcy levels. The strongest associations with postmethionine load Hcy levels were with 5 SNPs in the *CBS* gene (FDR-adjusted $p < 0.031$) (table 3). Four of these *CBS* SNPs plus one additional SNP were also associated with change in postmethionine load Hcy level (table e-2). All associated *CBS* SNPs were consistent with Hardy-Weinberg proportions ($p > 0.02$), except for rs234709 ($p = 0.001$).

Associations with recurrent stroke. Given that both baseline fasting Hcy and the methionine load test predicted risk of recurrent stroke,[4,5] we evaluated whether the SNPs most strongly associated with baseline Hcy level, rs731991 (table 2) and rs234709 (table 3), were also associated with recurrent stroke using combined (not shown) and treatment-stratified Cox proportional hazards models. All models were adjusted for age, sex, and race. Survival analysis of *TCN2* rs731991 showed a significant association between AA/AG genotypes and increased risk of recurrent stroke in the low-dose arm of the study. There were 52 recurrent strokes in subjects with the rs731991 AA genotype, 101 in those with the AG genotype, and 30 in those with the GG genotype. Although the additive model was initially tested, subsequent examination of results (figure 1) demonstrated that a recessive model best fits the data (logrank test $p = 0.009$, hazard ratio 0.34). No significant difference was seen in the high-dose arm ($p = 0.92$). The p value for interaction between rs731991 (AA/AG vs GG) and treatment (high vs low) was 0.0087. Forty-four subjects with a B_{12} level of less than 150 pmol/L at baseline, 6 months, or 18 months received supplemental B_{12} during the course of the trial to reduce the likelihood of neurologic consequences. However, only 2 of these subjects experienced recurrent stroke during the course of the trial. Both received supplemental B_{12} only at baseline. Exclusion of these 2 subjects (who carry rs731991 AG and GG genotypes) from survival analyses did not change our conclusions.

No associations with recurrent stroke were observed with putative functional *TCN2* coding SNPs (rs1801198, rs9621049, and rs1131603) or rs2072195. We did not find any relationship between *CBS* SNP rs234709 and recurrent stroke ($p = 0.95$ in the low-dose arm and $p = 0.86$ in the high-dose arm).

DISCUSSION Our results are consistent with previous suggestions that plasma levels of fasting Hcy reflect cobalamin (vitamin B_{12})- and folate-dependent remethylation, whereas levels postmethionine loading reflect pyridoxal 5′-phosphate (vitamin B_6)–dependent transsulfuration.[17]

Transcobalamin 2 is the primary plasma facilitator of cellular uptake of B_{12}.[18] The widely studied *TCN2* C776G (rs1801198, or R259P) mutation[9,10] and A67G (rs9606756, I22V)[10] have been associated with plasma Hcy levels in other studies and influence the proportion of B_{12} bound to transcobalamin.[19] In contrast, in our study, neither SNP was associated with baseline poststroke Hcy measures (table 2). However, 5 other SNPs in the *TCN2* gene, located in intron 7 and flanking the 3′UTR, were associated with baseline fasting Hcy (table 2). Interestingly, rs731991 was not associated with the baseline plasma B_{12} level ($p = 0.83$), although 10 *TCN2* SNPs

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(rs731991, rs2267164, rs1544468, rs1801198, rs482088, rs4820887, rs2301957, rs2301955, rs9606756, and rs740234) showed a significant interaction with B_{12} in determining baseline Hcy (FDR-adjusted $p < 0.05$). The most significant interactions were with rs731991 and rs2267164 (FDR-adjusted $p = 0.00213$). Baseline B_{12} levels may reflect the complex combination of dietary intake, turnover, and excretion rates, whereas interactions may relate to B_{12} plasma transport and bioavailability for participation as a cofactor in the one-carbon metabolic pathway.

The *TCN2* genotype appears to influence the recurrent stroke event rate only in the setting of inadequate B_{12} supplementation (figure 1). The recurrent stroke rate differed by the rs731991 genotype in the low-dose arm despite subjects receiving 3 times the recommended daily allowance for B_{12} (6 μg) and slightly higher B_{12} levels in the genetic substudy compared with the rest of the VISP sample (table 1) and the mean Hcy difference between the 2 homozygote extremes (AA vs GG) at baseline being only 0.89 μmol/L (table 2). In the high-dose arm, supplementation with 400 μg B_{12} potentially overwhelms any putative influence of *TCN2* rs731991 on B_{12} bioavailability because recurrent stroke rates are the same across all groups ($p = 0.84$) (figure 1). An alternative explanation is that, although the majority of subjects (rs731991 A/A and A/G genotypes) see a modest decrease in recurrent stroke risk with B vitamin supplementation, rs731991 G/G carriers' risk for recurrent stroke increases (or they lose their relative protection) with B vitamin supplementation (figure 1). If, for example, this group is more efficient at transporting B_{12}, there are reports of increased vascular disease with high B_{12} levels.[20–22] Further analyses will be required to localize possibly independent regions of association and determine their function.

Methionine loading may expose a latent abnormality of Hcy metabolism in up to 40% of people with a fasting plasma Hcy level in the normal range.[17,23] CBS deficiency is the most common cause of homocystinuria.[24] A common 68-bp insertion at the intron 7-exon 8 boundary of the *CBS* gene (844ins68) present in the heterozygous state in approximately 12% of Caucasians[25] has been associated with lower postmethionine load Hcy levels,[26,27] especially in the presence of low vitamin B_6. A 31-bp variable number tandem repeat (VNTR) that spans the exon 13–intron 13 boundary of the *CBS* gene has also been associated with Hcy and postmethionine load levels via alternative splicing at the exon 13–intron 13 splice junction site, with an increasing number of repeat elements showing a significant increase in plasma Hcy concentrations, especially after methionine loading.[19,28] How associated *CBS* SNPs (table 3) relate to the 68-bp insertion and 31-bp VNTR in terms of LD remains to be explored. Although associated SNP rs6586282 is within intron 12, 244 bp from the VNTR, the most associated SNP, rs234709, is located in intron 2 and a separate LD block (figure e-2).

The modest reduction in total Hcy observed in the VISP trial may be due in part to the addition of folic acid to enriched cereal grain foods in the United States (initiated in 1996 and mandated in January 1998) that coincided with the initiation of the VISP trial,[4] probably reducing the number of participants with high total Hcy who might be most likely to benefit from the intervention.[29] The VISP trial investigators[4] concluded that determinants of Hcy other than folate may be more important in this setting, which would be consistent with our findings.

The extensively studied "thermolabile" *MTHFR* C677T SNP (rs1801133) with reduced enzymatic activity[30] was not associated with Hcy ($p = 0.22$), postmethionine load test Hcy ($p = 0.0104$), or change in postmethionine load Hcy ($p = 0.031$). Associations between *MTHFR* 677TT genotype and mild hyperhomocysteinemia have been detected primarily in the presence of low folate status.[31–33] However, there was no significant interaction between any of the candidate gene SNPs and baseline folate in determining Hcy (FDR-adjusted $p > 0.58$), including *MTHFR* C677T (FDR-adjusted $p = 0.99$). It is possible that folate fortification and the VISP intervention treatments negated the impact of *MTHFR* variation on Hcy in the majority of VISP trial patients.

There are a number of limitations to the present study. Prospective serial analyses of Hcy after ischemic stroke have shown that Hcy increases in the subacute stage and remains stable during the convalescent period.[34–37] Results may not be generalizable to an event-free population or the full spectrum of Hcy values. However, 75% of those screened qualified for the study, and the effect of hyperhomocysteinemia is believed to be linear,[38,39] so results should be generalizable to the population at greatest risk for recurrent stroke. The 4 genomic and WGA replicates had consistent genotypes across all 82 SNPs, suggesting that there was no amplification bias based on SNP location. However, this does not represent a broad sampling of DNAs of differing quality; thus, we cannot rule out possible artifacts introduced by WGA in a subset of samples.

If we had used the conservative Bonferroni correction for the 82 SNP tests ($p < 0.0006$), only 4 associations with postmethionine load (rs11701048, rs9982015, rs234709, and rs6586282) (table 3) and

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Hcy change (rs2851391, rs234709, rs6586282, and rs719037) (table e-2) would be considered significant. Using the FDR approach, we were able to additionally detect significant associations with 5 *TCN2* SNPs (rs4820888, rs2301955, rs2301957, rs1544468, and rs731991) associated with baseline fasting Hcy level, *CBS* SNP rs2851391 associated with postmethionine load, and *CBS* SNP rs9982015 associated with Hcy change.

Varying combinations and doses of folate, vitamin B_6, and vitamin B_{12} have been studied in high-risk populations in large-scale trials with conflicting results, and the exact role of Hcy and the impact of vitamin supplementation on vascular events remains a controversial topic.[40] A multifactorial approach that takes into account genotypes that influence individual responses to enzymatic cofactor therapy may need to be considered to reconcile the results of vitamin supplementation trials. Genetic factors that determine bioavailability of cobalamin, such as *TCN2*, may be important confounders of the effectiveness of cofactor supplementation on recurrent stroke risk.

AUTHOR CONTRIBUTIONS

Dr. Hsu: drafting/revising the manuscript, analysis or interpretation of data, and statistical analysis. E.G. Sides: drafting/revising the manuscript and acquisition of data. Dr. Mychaleckyj: study concept or design and analysis or interpretation of data. Dr. Worrall: drafting/revising the manuscript, acquisition of data, and study supervision. G. Elias: analysis or interpretation of data, acquisition of data, and statistical analysis. Dr. Liu: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, and study supervision. Dr. Chen: analysis or interpretation of data and statistical analysis. Dr. Coull: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data, and study supervision. Dr. Toole: drafting/revising the manuscript, study concept or design, study supervision, and obtaining funding. Dr. Rich: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, and obtaining funding. Dr. Furie: drafting/revising the manuscript, study concept or design, and analysis or interpretation of data. Dr. Sale: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data, study supervision, and obtaining funding.

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