Novel Genomic Loci Influencing Plasma Homocysteine Levels

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Background and Purpose—Genetic factors that influence interindividual variation in levels of plasma homocysteine, a risk factor for vascular disease, are not fully understood. We performed linkage analyses to identify genomic regions that influence homocysteine levels in blacks and non-Hispanic whites.

Methods—Subjects (n=2283) belonged to hypertensive sibships and included 1319 blacks (63±10 years, 70% women) and 964 non-Hispanic whites (61±7 years, 57% women). Fasting plasma homocysteine was measured by high-pressure liquid chromatography. Genotypes were measured at 366 microsatellite marker loci distributed across the 22 autosomes. Plasma homocysteine adjusted for age, sex, body mass index, serum creatinine, and estrogen use (in women) was used in the genetic analyses. Heritability and linkage analyses were performed using a variance components approach.

Results—Mean (±SD) homocysteine levels were 10.4±5.27 μmol/L in blacks and 10.0±2.84 μmol/L in non-Hispanic whites (P=0.58 for difference). Homocysteine levels were significantly (P<0.0001) heritable in blacks (h2=0.70) and in non-Hispanic whites (h2=0.49). Linkage analyses demonstrated significant evidence of linkage (multipoint logarithm of odds ≥3.0) for homocysteine on chromosomes 1q42, 14q32, and 19p13 in blacks and on chromosomes 9q34 and 12q24 in non-Hispanic whites. Tentative evidence of linkage (logarithm of odds 1.3 to 2.0) was present on chromosomes 2q32, 7p15, 8q24, 18q21, and 20p12 in blacks and chromosomes 6q26 and 18q21 in non-Hispanic whites. Four genes in the homocysteine metabolism pathway (MTR, DNMT1, GAMT, and CARM1) were present under 2 of the significant linkage signals in blacks (chromosomes 1q42 and 19p13).

Conclusions—Plasma homocysteine is a significantly heritable trait. Linkage analyses reveal several unique genomic loci that may influence circulating levels of homocysteine and therefore susceptibility to vascular diseases including stroke. (Stroke. 2006;37:1703-1709.)

Key Words: genetic linkage • homocysteine • stroke

Homocysteine is a thiol-containing amino acid intermediate formed during the metabolism of methionine, an essential amino acid. McCully demonstrated the presence of arteriosclerosis in children and young adults with inborn errors of homocysteine metabolism such as cystathionine β-synthase (CBS) deficiency. These disorders are associated with markedly elevated plasma homocysteine levels (>100 μmol/L). Subsequently, mild-to-moderate hyperhomocysteinemia has been associated with increased risk of vascular diseases including stroke.

Knowledge of the genetic determinants of plasma homocysteine levels may facilitate development of new preventive, diagnostic, and therapeutic strategies for vascular diseases. However, genetic factors that influence interindividual variation in plasma homocysteine levels are yet to be fully elucidated although several candidate genes have been identified. Polymorphisms in genes encoding enzymes in the homocysteine-methionine pathway such as methylenetetrahydrofolate reductase (MTHFR; MIM 607093) and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR; MIM 156570) are associated with mild-to-moderate elevations in homocysteine levels. However, these polymorphisms account for <10% of the variance in plasma homocysteine levels and other, as yet unknown genes, likely play a role. Jeong and colleagues performed a segregation analysis that suggested the existence of a codominantly expressed major-gene influencing homocysteine plasma levels even after adjustment for the MTHFR 677 C→T polymorphism. A recent whole-genome linkage scan and haplotype analysis revealed a new candidate gene (NNMT, Nicotinamide N-methyltransferase; MIM 600008) on chromosome 11q23 as a major determinant of plasma homocysteine levels.
Homocysteine levels in 21 extended Spanish pedigrees.

Another recent report demonstrated that a novel gene (MMACHC) on chromosome 1p32-34 was responsible for the disorder methy1malonic aciduria cblC type with homocystinuria.13

We assessed heritability of plasma homocysteine levels and performed linkage analyses for homocysteine in blacks and non-Hispanic white subjects participating in the Genetic Epidemiology Network of Arteriopathy (GENOA) study, a multicenter community-based study of hypertensive sitbships that aims to identify genes influencing blood pressure levels and the development of target organ damage attributable to hypertension.14 In the present study, our goal was to identify genomic loci influencing interindividual variation in plasma homocysteine levels and thereby susceptibility to vascular diseases such as stroke.

### Materials and Methods

#### Sample

Recruitment into the initial phase of the GENOA study (9/1995 to 6/2001) has been previously described.15 Between December 2000 and October 2004, participants returned for a second study visit to undergo measurement of risk factors and traits including measurement of plasma homocysteine. Complete genotypic and phenotypic data were available in 2283 participants in phase II (964 in Rochester MN and 1319 in Jackson MS). The study was approved by the Institutional Review Boards of the Mayo Clinic and University of Jackson MS and written informed consent was obtained from each participant.

Blood samples were obtained by venipuncture after an overnight fast. Serum creatinine was measured by an automated spectrophotometric method implemented on a Hitachi 911 Chemistry Analyzer (Roche Diagnostics). Plasma homocysteine was measured using a high-pressure liquid chromatography assay with an interassay coefficient of variation of 5.7% to 7.4%.

#### Genotyping

DNA from all study participants was extracted from 10 mL EDTA-treated blood according to standard procedures. Microsatellite markers (CHLC/Weber screening set 9.0, n=366) were genotyped by standard polymerase chain reaction–based methods by the Mammalian Genotyping Center of the Marshfield Medical Research Foundation. For the Jackson sample, 338 autosomal microsatellite markers (CHLC/Weber screening set 6.0) were genotyped and independently scored by 2 separate individuals at the University of Texas-Houston Health Science Center. For the Rochester sample, 381 autosomal microsatellite markers (CHLC/Weber screening set 9.0) were typed. Marker order and genetic map distances were those provided by the Marshfield Medical Research Foundation (research.marshfieldclinic.org/genetics/). Inconsistencies of the genotypes with pedigree structure were identified by the Lange and Goradia marshfieldclinic.org/genetics/). Inconsistencies of the genotypes with pedigree structure were identified by the Lange and Goradia algorithm as implemented in the PedCheck software.77 Instances that could not be resolved as genotyping errors were considered as missing data. Potential half-sibs, identical twins, and nonsibs were identified using ASPEX and then removed.

#### Statistical Analyses

Before the analyses, we assessed whether the assumption of normality in the distribution of plasma homocysteine levels was violated.18 To reduce skewness, homocysteine levels were log transformed. Log transformed homocysteine values were adjusted for age, sex, body mass index (BMI), serum creatinine, and (in women) estrogen use, before the genetic analysis. Heritability (h^2) was calculated as the proportion of the total phenotypic variance attributable to additive genetic effects.

Linkage analyses were performed using S-plus library ‘multic’19 which uses a variance components approach.20,21 Multipoint identity-by-descent sharing among pairs of relatives were calculated using the SIMWALK2 software program.22 We considered multipoint logarithm of odds (LOD) scores ≥3.00 as statistically significant evidence of linkage, ≥2.00 as suggestive evidence, and ≥1.30 as tentative evidence of linkage.23 These multipoint LOD score thresholds correspond to genome-wide probability values of ≤0.0001, ≤0.001, and ≤0.007 respectively.23

#### Candidate Genes

All genes in the chromosomal regions demarcated by LOD-1 intervals for each linkage signal (≥1.3 LOD) were retrieved from the University of California, Santa Cruz (UCSC) genome annotation (genome.ucsc.edu). Genes were considered to be associated with homocysteine-methionine metabolism if they belonged to: (1) the canonical methionine metabolism pathway (www.genome.jp/kegg/pathway.html) and (2) S-adenosylmethionine (SAM)-dependent methyltransferase family of genes24 (supplemental Table I, available online at http://stroke.ahajournals.org). We also identified genes that interacted with the homocysteine-methionine pathway genes by performing a functional network analysis using the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com). Of the 59 homocysteine-methionine metabolism pathway genes, 40 mapped to genetic networks defined by the IPA tool based on known interactions in the scientific literature.

#### Results

Subject characteristics are shown in Table 1. Plasma homocysteine levels were not significantly different in the 2 ethnic groups (P=0.58). The sample of 1319 black subjects in Jackson MS belonged to 674 sibships and included 1135 sibpairs; there were 312 sibships of size 1, 200 sibships of size 2, and 162 sibships of size 3 or greater. The sample of 964 non-Hispanic whites in Rochester, Minn, belonged to 390 sibships and included 1054 sibpairs; there were 51 sibships of size 1, 212 sibships of size 2, and 127 sibships of size 3 or greater.

Likelihood-ratio tests indicated significant heritability (P<0.0001) for plasma homocysteine in each ethnic group. After adjustment for age, sex, BMI, serum creatinine, and estrogen use (in women), more than two-thirds of the residual variance in plasma homocysteine in the blacks (h^2=0.70) and nearly half of the residual variance in plasma homocysteine in blacks.

### Table 1. Sample Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Blacks, n=1319</th>
<th>Non-Hispanic Whites, n=964</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women, % (n)</td>
<td>70.4 (928)</td>
<td>57.0 (549)</td>
</tr>
<tr>
<td>Age, y</td>
<td>62.9±9.5</td>
<td>60.9±9.6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>31.5±6.6</td>
<td>30.5±5.9</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>150.7±22.1</td>
<td>140.4±18.4</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>79.5±10.9</td>
<td>73.9±9.1</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>10.4±5.27</td>
<td>10.0±2.84</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>0.90±0.33</td>
<td>0.90±0.27</td>
</tr>
<tr>
<td>Hypertension, % (n)</td>
<td>78.9 (1041)</td>
<td>75.6 (729)</td>
</tr>
<tr>
<td>Diabetes, % (n)</td>
<td>28.6 (377)</td>
<td>15.9 (153)</td>
</tr>
<tr>
<td>Smoking, % (n)</td>
<td>40.5 (534)</td>
<td>50.2 (484)</td>
</tr>
<tr>
<td>Estrogen use, % (n)</td>
<td>16.5 (217)</td>
<td>24.0 (231)</td>
</tr>
</tbody>
</table>

BP indicates blood pressure.

Table entries are means±SDs for quantitative traits or percentages (counts) for categorical traits.
non-Hispanic whites ($h^2=0.49$), was attributable to additive genetic effects.

The results of the genome-wide linkage scan to identify genomic regions influencing homocysteine levels are shown in Figures 1 and 2. Chromosomal regions where LOD scores exceeded 1.3 are summarized in Table 2. In blacks, statistically significant evidence of linkage (LOD ≥ 3) for plasma homocysteine was present on chromosomes 1q42, 14q32, and 19p13 (Figure 1). The microsatellite markers around these linkage signals, the corresponding genetic distances, and physical locations of the linkage signals are listed in Table 2. Tentative evidence of linkage (LOD 1.3 to 2.0) was noted on chromosomes 2q32 (LOD = 1.45, 182.9 centimorgan [cM]), 7p15 (LOD = 1.82, 34.7 cM), 8q24 (LOD = 1.71, 125.3 cM), 18q21 (LOD = 1.84, 71.3 cM), and 20p12 (LOD = 1.39, 33.7 cM). In non-Hispanic whites, statistically significant evidence of linkage was present on chromosomes 9q34 and 12q24 (Figure 2, Table 2) and tentative evidence of linkage on chromosomes 6q26 (LOD = 1.43, 161.3 cM) and 18q21 (LOD = 1.44, 85.3 cM).

Potential Candidate Genes in the Linked Regions
We retrieved genes located in the LOD-1 support interval surrounding each linkage peak using the UCSC human genome annotation database (genome.ucsc.edu); ≈900 genes resided under the 8 linkage signals for blacks, and ≈300 genes resided under the 4 linkage regions for non-Hispanic whites. We identified genes involved in homocysteine-methionine metabolism (listed in supplemental Table I) that were present under the linkage signals (Table 3). For blacks, 5 genes in homocysteine-methionine metabolism pathway were present under the linkage peaks (Figure 3), ie, MTR (chromosome 1q43), MARS2 (chromosome 2q32), CARM1, DNMT1, and GAMT (chromosome 19p13; Table 3). For non-Hispanic whites, we did not identify any of the known genes participating in homocysteine-methionine metabolism under the linkage signals.

We also investigated whether the genes in the homocysteine-methionine pathway interacted with genes under the linkage signals using Ingenuity Pathway Analysis. A gene interaction network consisting of PCSK2, GCG, CBS, CTH, GNMT, and GLS was identified (Table 3, also see network 3 in supplemental Table II, available online at http://stroke.ahajournals.org). In non-Hispanic whites, the chromosome 12q24 region contained RAN (ras-related nuclear protein), a gene that interacts with RNMT, a SAM-dependent methyltransferase (network 5 in supplemental Table II).

Discussion
Several studies suggest that homocysteine may have a role in the etiopathogenesis of a range of cerebrovascular diseases. Not only has plasma homocysteine been associated with stroke but also with silent brain infarcts or cerebral white matter lesions.25
Alzheimer disease, vascular dementia, and cognitive dysfunction. Identifying genomic regions that influence plasma homocysteine may be an initial step toward a better understanding of the genetic architecture of cerebrovascular diseases. In the present study, plasma homocysteine levels were significantly heritable in black and non-Hispanic white subjects belonging to sibships ascertained on the basis of hypertension. Using a variance components linkage analysis approach, we found several unique genomic regions that may influence interindividual variation in plasma homocysteine levels in the 2 ethnic groups. Five genes encoding enzymes or proteins in the homocysteine metabolism pathway were present under the linkage signals for homocysteine levels in blacks and may have contributed to the linkage signals.

At least 3 family-based studies have assessed heritability of plasma homocysteine levels. Souto et al found modest heritability for homocysteine levels (0.24) in 21 Spanish pedigrees ascertained on the basis of thrombophilia. Jee et al found a heritability of 0.47 for homocysteine levels in 661 family members of 112 probands who underwent elective

### TABLE 2. Statistically Significant Linkage Signals for Plasma Homocysteine: Surrounding Markers, Genetic Distances, and Physical Locations

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>LOD</th>
<th>P Value</th>
<th>Markers</th>
<th>Genetic Distance, cM*</th>
<th>Physical Location, MB†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blacks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1q42</td>
<td>3.71</td>
<td>0.0001</td>
<td>D1S549</td>
<td>239.7</td>
<td>216.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D1S547</td>
<td>267.5</td>
<td>238.1</td>
</tr>
<tr>
<td>1q32</td>
<td>3.72</td>
<td>0.0001</td>
<td>D14S617</td>
<td>105.5</td>
<td>91.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D14S1426</td>
<td>125.9</td>
<td>99.7</td>
</tr>
<tr>
<td>19p13</td>
<td>3.71</td>
<td>0.0001</td>
<td>D19S591</td>
<td>9.8</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D19S586</td>
<td>32.9</td>
<td>9.7</td>
</tr>
<tr>
<td><strong>Non-Hispanic whites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9q34</td>
<td>3.04</td>
<td>0.0001</td>
<td>D9S1825</td>
<td>136.5</td>
<td>125.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D9S1838</td>
<td>163.8</td>
<td>138.0</td>
</tr>
<tr>
<td>12q24</td>
<td>3.24</td>
<td>0.0001</td>
<td>D12S2078</td>
<td>149.6</td>
<td>126.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D12S392</td>
<td>165.7</td>
<td>129.8</td>
</tr>
</tbody>
</table>

*Genetic distance is from p-terminus in cM from Marshfield genetic maps (research.marshfieldclinic.org/genetics). †Physical location in the human genome derived from genome.ucsc.edu.

MB indicates megabase.

### TABLE 3. Candidate Genes Under the Linkage Signals

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Chr</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTR*</td>
<td>5-methyltetrahydrofolate-homocysteine methyltransferase</td>
<td>1</td>
<td>Catalyzes the final step in methionine biosynthesis</td>
</tr>
<tr>
<td>MARS*</td>
<td>Methionine-tRNA synthetase</td>
<td>2</td>
<td>Charges tRNAs with their cognate amino acids</td>
</tr>
<tr>
<td>MFTC</td>
<td>Solute carrier family 25, member 32</td>
<td>8</td>
<td>Binds and transports folic acid</td>
</tr>
<tr>
<td>CARM*</td>
<td>Coactivator-associated arginine methyltransferase 1</td>
<td>19</td>
<td>Regulates transcription/translation</td>
</tr>
<tr>
<td>DNMT*</td>
<td>DNA (cytosine-5)-methyltransferase 1</td>
<td>19</td>
<td>Establishes and regulates of tissue-specific patterns of methylated cytosine residues</td>
</tr>
<tr>
<td>GAMT*</td>
<td>Guanidinoacetate N-methyltransferase</td>
<td>19</td>
<td>Methyltransferase activity</td>
</tr>
<tr>
<td>GCG†</td>
<td>Glucagon</td>
<td>2</td>
<td>Stimulates glycogenolysis and gluconeogenesis</td>
</tr>
<tr>
<td>GLS†</td>
<td>Glutaminase</td>
<td>2</td>
<td>Interacts with GCG</td>
</tr>
<tr>
<td>PCSK2†</td>
<td>Proprotein convertase subtilisin/kexin type 2</td>
<td>20</td>
<td>Processes latent precursor proteins into their biologically active products</td>
</tr>
<tr>
<td>RAN†</td>
<td>Ras-related nuclear protein</td>
<td>12</td>
<td>Essential for translocation of RNA and proteins through the nuclear pore complex</td>
</tr>
</tbody>
</table>

Chr indicates chromosome.

*Genes in the homocysteine-methionine metabolism pathway; †candidates genes for plasma homocysteine identified by pathway analysis using the Ingenuity System.
coronary angiography. A recent study of 51 Dutch families by den Heijer et al\textsuperscript{30} estimated a heritability of 0.34 for homocysteine levels, but the heritability was significantly higher for postmethionine load homocysteine levels (0.68). In the present study, the heritability estimate for homocysteine levels (adjusted for age, sex, BMI, serum creatinine, and estrogen use in women) was 0.70 for blacks and 0.49 for non-Hispanic whites (\(P = 0.0001\)), higher than the heritability estimates from previous studies.

In blacks, the linkage region on chromosome 1q43 (LOD = 3.71) contains the \textit{MTR} gene. \textit{MTR} encodes the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase which catalyzes the final step in methionine biosynthesis and may therefore influence homocysteine levels\textsuperscript{31} (Figure 3). Heterozygous knockout mice for \textit{Mtr} gene have elevated plasma homocysteine and methionine levels compared with wild-type mice.\textsuperscript{31} Mutations in the \textit{MTR} gene result in methyco-


cystinuria, hyperhomocysteinemia, and hypomethioninemia.\textsuperscript{52} To our knowledge, the present study is the first linkage analysis to report \textit{MTR} as a candidate gene influencing interindividual variation in plasma homocysteine levels.

The chromosome 19p13 linkage signal in blacks contained 3 SAM-dependent methyltransferases: \textit{DNMT1}, \textit{CARM1}, and \textit{GAMT} (Figure 1). These enzymes are involved in the synthesis of S-adenosyl homocysteine, the precursor of homocysteine, and catalyze formation of a variety of methylated biomolecules\textsuperscript{24} (Figure 3). In non-Hispanic whites, \textit{RAN}, a gene that regulates the binding of \textit{RNMT}, a SAM-dependent methyltransferase, was present under the linkage peak at chromosome 12q24 (Figure 2). Increased activity of intracellular methyltransferases is associated with increased cellular homocysteine.\textsuperscript{24} Recently, a member of the SAM-dependent methyltransferase family, \textit{NNMT} (nicotinamide-N-methyltransferase), was identified as a determinant of plasma homocysteine levels by linkage and haplotype analysis in Spanish pedigrees.\textsuperscript{10} Furthermore, mice deficient in one such methyltransferase—phosphatidylethanolamine N-methyltransferase (\textit{PEMT})—have decreased plasma homocysteine levels.\textsuperscript{33} Thus, SAM-dependent methyltransferases may have an important role in regulating plasma homocysteine levels and thereby in the pathophysiology of homocysteine-associated vascular disease.

Mild-to-moderate hyperhomocysteinemia has been associated with the 677 C\textsuperscript{3} T polymorphism of the \textit{MTHFR} gene.\textsuperscript{8} We found nominal evidence of linkage on chromosome 1p36 between the markers D1S468 and D1S1597 (Figure 1), the genomic region that harbors the \textit{MTHFR} locus (LOD = 0.91, \(P = 0.0204\)). The classic disorder, homocystinuria, results from a mutation in cystathionine \(\beta\)-synthase (\textit{CBS}; MIM 236200),\textsuperscript{34} an enzyme in the trans-sulfuration pathway that catalyzes the conversion of homocysteine to cystathionine\textsuperscript{35} (Figure 3). We did not find a linkage signal on chromosome 21q22 in the region of the \textit{CBS} gene. We also did not find evidence of linkage to recently identified candidate genes for homocysteine levels (\textit{NNMT} and \textit{MMACHC}), nor to regions on chromosomes 13 and 16 with suggestive evidence of linkage for plasma homocysteine levels in 13 Dutch pedigrees.\textsuperscript{36} Furthermore, we found no overlap in the linkage signals between blacks and non-Hispanic whites in the present analyses. 

![Figure 3. Pathways involved in homocysteine metabolism. There are 3 main reaction cycles, ie, remethylation, transmethylation, and transulfuration. Genes labeled with asterisk are located under the linkage signals identified in the present study. For gene names, see supplemental Table I.](http://stroke.ahajournals.org/)

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**Figure 3. Pathways involved in homocysteine metabolism.** There are 3 main reaction cycles, i.e., remethylation, transmethylation, and transulfuration. Genes labeled with asterisk are located under the linkage signals identified in the present study. For gene names, see supplemental Table I.
results suggest genetic heterogeneity of the plasma homocysteine trait. A strength of the present study is inclusion of a black cohort. To the best of our knowledge, the present study is the first to report a genome-wide linkage scan for plasma homocysteine in this ethnic group with increased susceptibility to cerebrovascular diseases such as ischemic stroke.\(^{37,38}\) A limitation of our study is that dietary habits as well as intake of vitamin supplements—factors that may influence homocysteine levels—were not accounted for. Furthermore, measurements of plasma homocysteine were made at least 2 years after the institution of folate supplementation of flour in the United States.\(^{39}\) This may have resulted in underestimation of the genetic signals for homocysteine.

In conclusion, plasma homocysteine is significantly heritable in blacks and non-Black Hispanics ascertained from sibships with essential hypertension. Linkage analyses reveal several quantitative trait loci significant on a genome-wide basis that may influence plasma levels of homocysteine and thereby susceptibility to vascular diseases including stroke. Three genes (DNMT1, GAMT, and CARM1) of the 5 candidate genes for homocysteine metabolism identified under the linkage signals are SAM-dependent methyltransferases, and 1 gene (RAN) regulates a SAM-dependent methyltransferase, suggesting a role for these enzymes in determining plasma homocysteine levels and thereby in the pathophysiology of homocysteine-associated vascular disease. Further work, including fine mapping and haplotype analyses, is needed to confirm that these genes are responsible for the linkage signals and influence plasma homocysteine levels.

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**Disclosures**

None.

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