RESEARCH ARTICLE

Homocysteine and Small Vessel Stroke: A Mendelian Randomization Analysis

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Objective: Trials of B vitamin therapy to lower blood total homocysteine (tHcy) levels for prevention of stroke are inconclusive. Secondary analyses of trial data and epidemiological studies suggest that tHcy levels may be particularly associated with small vessel stroke (SVS). We assessed whether circulating tHcy and B vitamin levels are selectively associated with SVS, but not other stroke subtypes, using Mendelian randomization.

Methods: We used summary statistics data for single-nucleotide polymorphisms (SNPs) associated with tHcy (n = 18), folate (n = 3), vitamin B_6 (n = 1), and vitamin B_{12} (n = 14) levels, and the corresponding data for stroke from the MEGASTROKE consortium (n = 16,952 subtyped ischemic stroke cases and 404,630 noncases).

Results: Genetically predicted tHcy was associated with SVS, with an odds ratio of 1.34 (95% confidence interval [CI], 1.13–1.58; $p = 6.7 \times 10^{-4}$) per 1 standard deviation (SD) increase in genetically predicted tHcy levels, but was not associated with large artery or cardioembolic stroke. The association was mainly driven by SNPs at or near the *MTHFR* and *MUT* genes. The odds ratios of SVS per 1 SD increase in genetically predicted folate and vitamin B_6 levels were 0.49 (95% CI, 0.34–0.71; $p = 1.3 \times 10^{-4}$) and 0.70 (95% CI, 0.52–0.94; $p = 0.02$), respectively. Genetically higher vitamin B_{12} levels were not associated with any stroke subtype.

Interpretation: These findings suggest that any effect of homocysteine-lowering treatment in preventing stroke will be confined to the SVS subtype. Whether genetic variants at or near the *MTHFR* and *MUT* genes influence SVS risk through pathways other than homocysteine levels and downstream effects require further investigation.

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The B vitamins folate, vitamin B_6, and vitamin B_{12}, play an essential role in the metabolism of homocysteine (Fig 1). Insufficient levels of either of these vitamins can lead to increased blood levels of total homocysteine (tHcy), which has been epidemiologically linked to increased risk of cardiovascular disease.[1–4] However, the causality of the association has been disputed because a number of randomized controlled trials (RCTs) have failed to show a benefit of homocysteine-lowering therapy with B vitamins on coronary heart disease, total cardiovascular disease, and all-cause mortality,[2,5–7] although a recent primary prevention trial reported a reduction in incidence of ischemic stroke.[8] Furthermore, recent meta-analyses have indicated that B vitamin supplementation significantly reduces the risk of any stroke by 10% compared to placebo.[7,9]

Most RCTs assessed the benefit of homocysteine-lowering therapy on overall stroke only. Ischemic stroke can be caused by multiple pathologies, which result in distinct subtypes, the most common of which are large artery stroke (LAS) attributed to atherosclerosis, cardioembolic stroke (CES), and small vessel stroke (SVS). If the association with homocysteine is specific to one particular subtype, then failure to examine treatment effects on specific subtypes could have resulted in diluted or null results, despite treatment benefits for that subtype.

Several lines of evidence suggest that homocysteine may be more strongly associated with the SVS subtype. A secondary analysis of the largest trial of homocysteine-lowering therapy in patients with stroke or transient ischemic attack, VITATOPS (The VITAmins TO Prevent Stroke), showed a marginally significant treatment effect for SVS, but not other stroke subtypes.[10] A magnetic resonance imaging (MRI) substudy embedded in the same trial showed that

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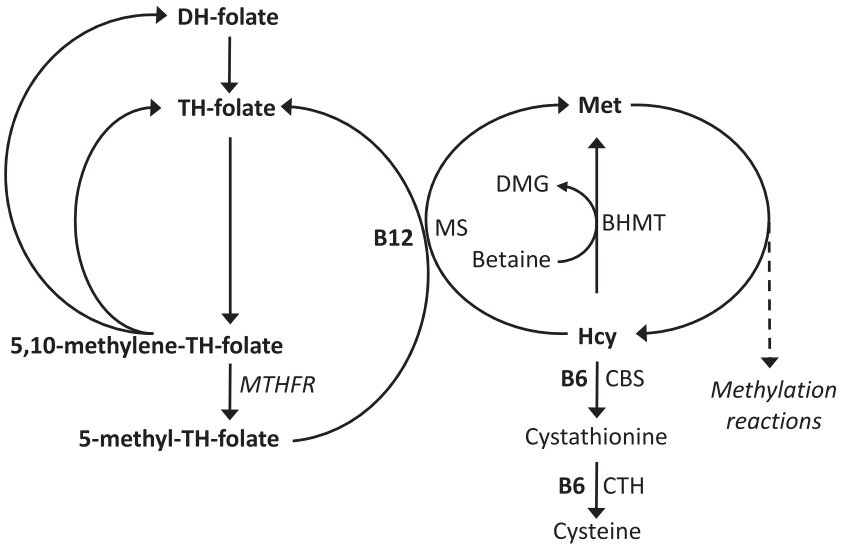

FIGURE 1: Simplified overview of the role of folate, vitamin B_6, and vitamin B_{12} in homocysteine metabolism. In the remethylation pathway, homocysteine is reconverted to methionine by receiving a methyl group from 5-methyltetrahydrofolate, the active form of folate, or betaine. Irreversible removal of homocysteine occurs through the transsulphuration pathway in which homocysteine condenses with serine to form cystathionine. B6 = vitamin B_6; B12 = vitamin B_{12}; BHMT = betaine homocysteine methyltransferase; CBS = cystathionine-β-synthase; CTH = cystathionine-gamma-ligase; DH = dihydro; DMG = dimehtylglycine; Hcy = homocysteine; Met = methionine; MS = methionine synthase (encoded by the *MTR* gene); MTHFR = methylenetetrahydrofolate reductase; TH = tetrahydro.

homocysteine-lowering therapy significantly reduced the progression of white matter hyperintensities (an MRI marker of small vessel disease).[11] Although it did not reduce cognitive decline in the same study,[12] this may be because cognitive testing has been shown to be insensitive to detect changes over a 2- to 3-year period in this cerebral small vessel disease.[13,14] Furthermore, several case-control studies have shown that elevated tHcy levels are a stronger risk factor for SVS than other ischemic stroke subtypes[15–17] and that tHcy levels are higher in patients with cerebral small vessel disease than in healthy controls,[18] although such studies only show associations and cannot exclude confounding.

Mendelian randomization (MR) is an increasingly used technique that can infer causality between exposure and disease through the use of genetic variants, usually single-nucleotide polymorphisms (SNPs), associated with the exposure as proxies for the exposure.[19,20] Use of genetic variants, which are randomly allocated at conception, avoids reverse causality and minimizes confounding by environmental factors in a similar manner as in an RCT.

Here, we used the MR design to determine whether tHcy, folate, vitamin B_6, and vitamin B_{12} levels are associated with ischemic stroke, and whether any association is selective to the SVS subtype, in a sample of 34,217 ischemic stroke cases (including 16,952 subtyped cases) and 404,630 noncases. We also evaluated whether there were associations with coronary artery disease (CAD), which shares pathogenesis with LAS.

Materials and Methods

Data Sources

Summary statistics data for stroke were available from the MEGASTROKE consortium (http://megastroke.org/).[21] We restricted the analyses to studies comprising European-descent individuals to minimize potential bias caused by population stratification. The ischemic stroke data set included 404,630 noncases and 34,217 cases subtyped into SVS (n = 5,386), LAS (n = 4,373), and CES (n = 7,193). Nonsubtyped ischemic stroke cases (n = 17,265) were excluded from the subtype-specific analyses. Ischemic stroke subtyping had been performed using the Trial of Org 10172 in Acute Stroke Treatment criteria.[22]

Summary statistics data for CAD were available from the CARDIoGRAMplusC4D consortium's 1000 Genomes–based genome-wide association meta-analysis of 48 studies, including 60,801 CAD cases and 123,504 noncases (http://www.cardiogramplusc4d.org/).[23] CAD included myocardial infarction (~70% of the total number of cases), acute coronary syndrome, chronic stable angina, or coronary artery stenosis of at least 50%.[23]

Studies contributing data to the MEGASTROKE and CARDIoGRAMplusC4D consortia received ethical approval from relevant institutional review boards. In the present study, we only used summarized (ie, aggregated) data from these two consortia.

Selection of SNPs

We selected all SNPs associated with circulating levels of tHcy, folate, vitamin B_6, or vitamin B_{12} at genome-wide significance threshold ($p < 5 \times 10^{-8}$) in the hitherto largest genome-wide association meta-analyses on tHcy (n = 44,147 individuals)[24] and the B vitamins (n = 37,465 individuals for folate[25]; n = 1,864

individuals for vitamin B_6[26]; and n = 45,576 individuals for vitamin B_{12}[25]) in individuals of European ancestry. Where the specified SNP was unavailable in the stroke or CAD data set, we used a proxy SNP in linkage disequilibrium ($r^2 > 0.9$) with the specified SNP. For one SNP (rs7788053) associated with vitamin B_{12}, no proxy was found. Hence, our analyses included 18 SNPs for tHcy, three for folate, one for vitamin B_6, and 14 for vitamin B_{12} (Supplementary Table 1). We verified that the SNPs within each exposure were independent ($R^2 < 0.1$ in 1000G CEU population). Across the four exposures, two folate-associated SNPs and four vitamin B_{12}–associated SNPs were in linkage disequilibrium with an tHcy-associated SNP (Supplementary Table 1). The SNP associated with vitamin B_6 was not in in linkage disequilibrium with any of the SNPs for the other three exposures.

Characteristics of the tHcy- and B vitamin–associated SNPs and their associations with SVS are shown in Supplementary Table 1. Variance in exposure explained by the SNPs has been estimated to be 4.6% to 5.9% for tHcy,[24] 1.0% for folate,[25] and 6.3% for vitamin B_{12}.[25] The B_6-associated SNP was estimated to explain 1.3% of the variance in vitamin B_6 levels.

Statistical Analysis

The primary analyses were conducted using the inverse-variance weighted method.[27] Where a significant association was observed, we performed sensitivity analyses using the weighted median and MR-Egger methods.[27] The MR-PRESSO method was used to identify potential outliers.[28] In a further sensitivity analysis of tHcy, we included only the lead SNP of each of the 13 tHcy-associated loci.

All results are reported per an approximate 1 standard deviation (SD) increment of each exposure. We prespecified a Bonferroni-corrected significance threshold of $\alpha = 0.0025$ (where $\alpha = 0.05/20$ [four exposures and five outcomes]) to adjust for multiple testing. Associations with p values between 0.05 and 0.0025 were considered suggestive evidence of a possible association. The statistical analyses were conducted using Stata (StataCorp LP, College Station, TX) and the MendelianRandomization package[29] for R (R Foundation for Statistical Computing, Vienna, Austria).

Assessment of Pleiotropy

We assessed whether the tHcy-associated SNPs were associated with potential confounders or macronutrient intake by searching the PhenoScanner database.[30] We looked up associations with the following established or possible risk factors for SVS: systolic and diastolic blood pressure,[31] high-density lipoprotein cholesterol,[32] type 2 diabetes mellitus,[33] and smoking.[31]

Results

Genetically higher tHcy was associated with higher odds of SVS, but not associated with either LAS or CES (Fig 2). The odds ratio (OR) of SVS per 1 SD increase in genetically predicted tHcy levels was 1.34 (95% confidence interval [CI], 1.13–1.58; $p = 6.7 \times 10^{-4}$; Figs 2 and 3). There was suggestive evidence of a possible association between genetically predicted tHcy levels and all ischemic stroke ($p = 0.02$), but no association with CAD ($p = 0.61$; Fig 2).

Results for tHcy levels and SVS were similar in sensitivity analyses using the weighted median and MR-Egger methods, although with less-precise estimates (Supplementary Table 2). There was no indication of directional pleiotropy (MR-egger intercept, –0.001; 95% CI –0.039 to 0.037; $p = 0.96$). In the MR-PRESSO outlier test, the SNP near the *MUT* gene was found to be a possible outlier, but the association between tHcy and SVS remained after exclusion of this SNP (Supplementary Table 2). Likewise, the association persisted (Supplementary Table 2) after omission of SNPs associated with one or more potential SVS risk factors or macronutrient intake (Supplementary Table 3). The results were also consistent when restricting the analysis to the lead SNP of each of the 13 tHcy-associated loci (OR, 1.31; 95% CI, 1.08–1.58; $p = 0.005$). However, the association did not remain in a post-hoc analysis excluding the outlying SNP near the *MUT* gene along with the two SNPs at or near the *MTHFR* gene (OR, 1.08; 95% CI, 0.87–1.34; $p = 0.47$). The SNP, rs1801133, in *MTHFR* explains most of the variance in tHcy levels and is also strongly associated with folate levels (Supplementary Table 1).

Genetically predicted folate and vitamin B_6, but not vitamin B_{12}, levels were inversely associated with SVS, but the association with vitamin B_6 levels did not reach significance at the Bonferroni-corrected threshold (Fig 2). In contrast, folate, vitamin B_6, and vitamin B_{12} levels were not associated with LAS, CES, all ischemic stroke, or CAD (Fig 2). The OR of SVS per 1 SD increment in genetically predicted folate and vitamin B_6 levels were 0.49 (95% CI, 0.34–0.71, $p = 1.3 \times 10^{-4}$) and 0.70 (95% CI, 0.52–0.94, $p = 0.02$), respectively. The results for folate were consistent when restricting the analysis to the lead SNP of each of the two folate-associated loci (OR, 0.52; 95% CI, 0.33–0.83; $p = 5.3 \times 10^{-3}$). Weighted median and MR-Egger analyses could not be performed with only three SNPs for folate and one SNP for vitamin B_6. Among the folate-associated SNPs, rs1801133 was associated with systolic and diastolic blood pressure (Supplementary Table 3). The vitamin B_6–associated SNP was not associated with possible SVS risk factors (at p values <0.01).

Discussion

This study showed an overall significant positive association of genetically predicted tHcy levels and an inverse association of genetically predicted folate levels with SVS. Moreover, the association was specific to this subtype and was not present for the other major ischemic stroke subtypes, that is, LAS and CES. We also observed no association between genetically higher tHcy and CAD, consistent with previous studies[24,34] and with the lack of association with LAS in which atherosclerosis also plays a key role. Our

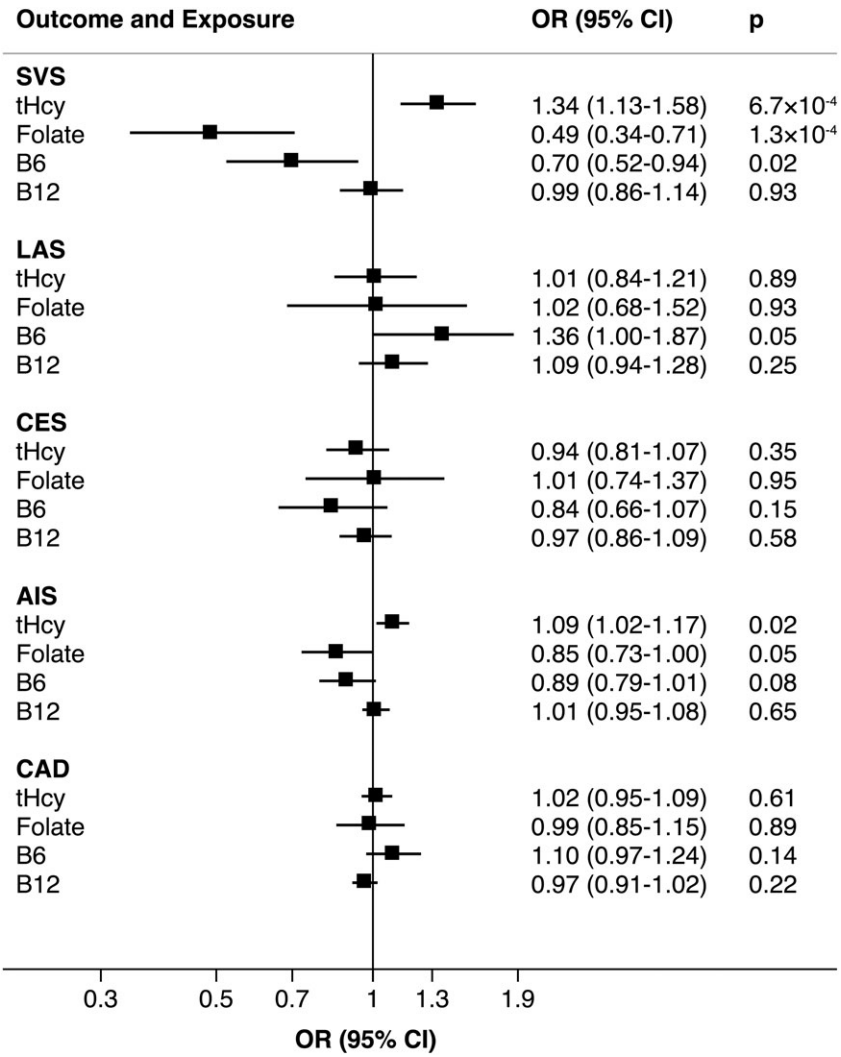

FIGURE 2: Associations of genetically predicted tHcy, folate, vitamin B_6, and vitamin B_{12} levels with ischemic stroke subtypes, all ischemic stroke, and coronary artery disease. The odds ratios (OR) are per genetically predicted 1 SD increase in each exposure. AIS = all ischemic stroke; B6 = vitamin B_6; B12 = vitamin B_{12}; CAD = coronary artery disease; CES = cardioembolic stroke; CI = confidence interval; LAS = large artery stroke; OR = odds ratio; SVS = small vessel stroke; tHcy = total homocysteine.

findings support those from subgroup analyses within VITATOPS[10] and the MRI substudy within that trial,[11] which both suggested a specific treatment effect in the SVS subtype.

Increasing data have implicated endothelial damage as a key process in the pathogenesis of small vessel disease.[35] Experimental studies have shown that elevated tHcy levels can lead to alterations within the blood vessel wall.[36,37] The main mechanisms responsible for these modifications include direct endothelial damage attributed to increased oxidative stress and proinflammatory effects.[37–42] Experimental studies in animals and humans indicate that oxidative or nitrosative stress play a role in reducing the bioavailability of nitric oxide and augmenting endothelial dysfunction caused by hyperhomocysteinemia.[36,37,39,42] Oxidative stress, which may be induced by hyperhomocysteinemia, causes impaired cerebral vascular function by disrupting endothelium-dependent nitric oxide signaling.[43] Cerebral microvessels appears to be more sensitive to hyperhomocysteinemia than extracranial blood vessels,[36] supporting a more prominent role of elevated tHcy levels in SVS than other ischemic stroke subtypes as well as coronary heart disease, for which no benefit of homocysteine-lowering treatment has been observed.[2,5–7]

Previous MR studies of tHcy in relation to stroke have yielded conflicting results. In one study, rs1801133 in the *MTHFR* gene, encoding the key enzyme in homocysteine metabolism (Fig 1), which is strongly associated with tHcy[24] and folate[25] levels, was associated with SVS (n = 1,359 cases), but not with other stroke subtypes.[44] However, another study found no association of a genetic risk score consisting of 18 tHcy-associated SNPs with either SVS (n = 1,894 cases) or other subtypes.[45] We used data from a much larger stroke cohort and also assessed

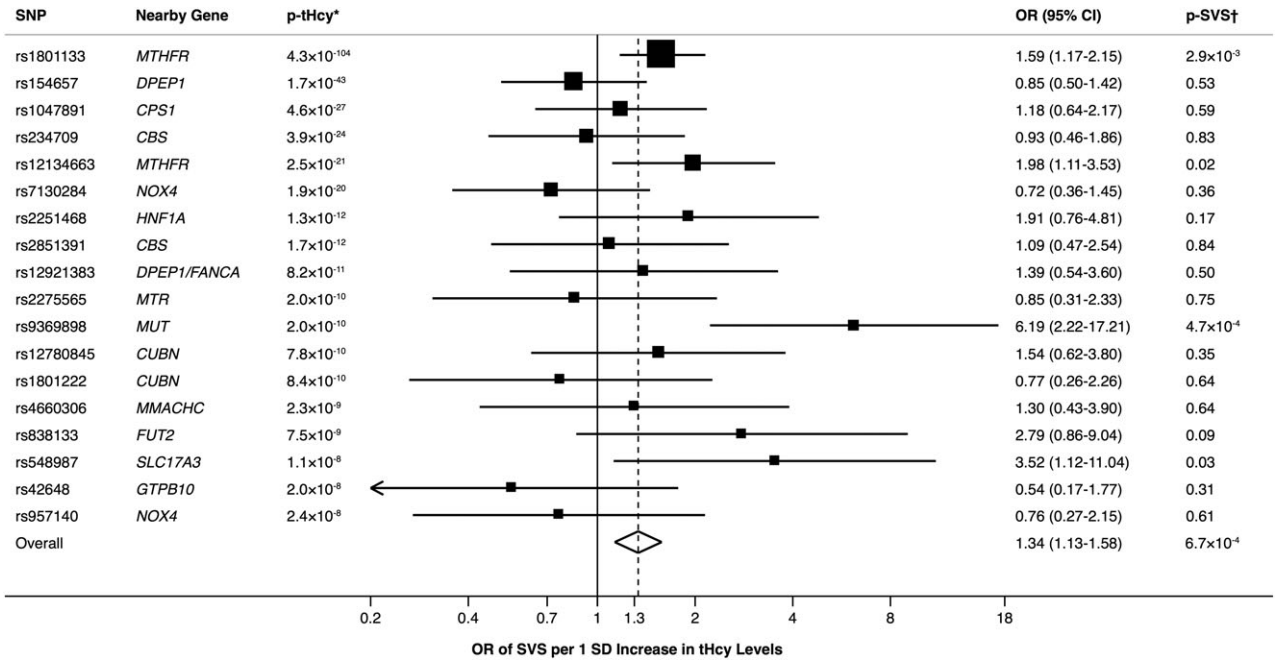

FIGURE 3: Association of genetically predicted tHcy levels with small vessel stroke. The odds ratios (OR) are per genetically predicted 1 SD increase in tHcy. CI = confidence interval; OR = odds ratio; SNP = single-nucleotide polymorphism; SVS = small vessel stroke; tHcy = total homocysteine. *p for the association between the SNP and tHcy; †p for the association between the SNP and SVS.

associations with homocysteine-lowering B vitamins, which were not done in earlier studies.

A recent meta-analysis of RCTs showed that vitamin B therapy may only reduce stroke risk in individuals with normal renal function,[9] which is closely associated with cerebral small vessel disease.[46] In this MR study, we were unable to examine whether genetically predicted tHcy and the B vitamin levels were more strongly associated with SVS in individuals with normal renal function, but this warrants investigation in future studies.

The reason for the lack of association between genetically higher vitamin B_{12} levels and SVS is unclear, but a possible explanation is that vitamin B_{12} levels have little impact on tHcy levels. In a meta-analysis of RCTs, folic acid (synthetic form of folate) supplementation reduced tHcy levels by 25% (95% CI, 23–28) and vitamin B_{12} supplementation produced an additional 7% (95% CI, 3–10) lowering of tHcy levels.[47]

A major strength of this study is the MR design. Like RCTs, MR studies avoid reverse causality and minimize confounding by environmental factors. However, unlike RCTs, which usually evaluate the effect of short-term treatment, results from MR studies can be reflective of life-long exposure as genetic variants are fixed at conception. Another important strength is the possibility to examine the association between tHcy and B vitamin levels with SVS in a large sample of over 5,000 SVS cases. RCTs of B vitamin therapy for stroke prevention have only included a few hundred cases (up to approximately 700 cases of any stroke) and did not examine the benefit on specific ischemic stroke subtypes. An additional strength of this study is that most SNPs used as proxies for tHcy and B vitamin levels are located in genes with known functions related to homocysteine, folate, and vitamin B_{12} pathways.[24,25] The SNP associated with vitamin B_6 levels is located near the *ALPL* gene, encoding tissue-nonspecific alkaline phosphatase, which likely is involved in the catabolism of vitamin B_6.[26]

A limitation is that we only had three SNPs as instrumental variables for folate levels and one SNP for vitamin B_6 levels. This resulted in low statistical power in the analyses of these B vitamins. In addition, with only one or three SNPs, sensitivity analyses to explore pleiotropy could not be conducted.

A potential threat to the reliability of the results from an MR analysis is pleiotropy, which occurs when a genetic variant affects more than one phenotype. Our sensitivity analyses based on the weighted median, MR-Egger, and MR-PRESSO methods provided no evidence that pleiotropy explained the observed association between tHcy levels and SVS. Nevertheless, the association did not remain in a post-hoc analysis omitting the three SNPs at or near the *MTHFR* and *MUT* loci of which *MTHFR* is the key gene involved in folate metabolism and also strongly associated with folate levels. Hence, we cannot rule out that the observed association between tHcy levels and SVS is driven by other effects of folate, not mediated through tHcy levels, or other pleiotropic pathways affecting the risk of SVS, but not LAS, CES, or CAD. However, the major SVS

risk factors, such as blood pressure,[31] high-density lipoprotein cholesterol,[32] type 2 diabetes mellitus,[33] and smoking,[31] are also associated with the other stroke subtypes and CAD. Some of those cardiovascular risk factors may influence tHcy levels or mediate the potential adverse effect of higher tHcy levels on SVS. Smokers have higher tHcy levels and lower folate, vitamin B_6, and vitamin B_{12} levels compared to never smokers.[48] Furthermore, raised tHcy levels may cause an increase in blood pressure mediated by damage to vascular smooth muscle and endothelial cells, which, in turn, could lead to a loss of arterial vasodilation and vascular integrity.[36–42,49] Blood pressure may thus be in the causal pathway linking elevated tHcy levels to SVS risk. A previous study showed that rs1801133 in the *MTHFR* gene, which is strongly associated with tHcy and folate levels, was significantly associated with small vessel stroke in hypertensive individuals, but not in normotensive individuals.[44] This gene-environment interaction provides evidence that the association between genetically predicted tHcy levels and SVS is a result of tHcy, because if the association were a result of directional pleiotropy, then it would likely to be present in both hypertensive and normotensive individuals. Moreover, one of the largest RCTs on homocysteine-lowering treatment for stroke prevention showed that folic acid supplementation significantly reduced the risk of stroke in hypertensive individuals.[8]

Another possible source of bias in an MR study is population stratification. We reduced this possible bias by restricting the study populations to individuals of European ancestry. A further shortcoming is that six of the ten studies included in the genome-wide association meta-analysis of tHcy were included in MEGASTROKE. This could lead to model overfitting and bias in the direction of the observational association between tHcy and SVS. However, if genetic associations with tHcy levels were estimated in noncases, then sample overlap would not result in bias or type 1 error inflation.[50] The genome-wide association meta-analyses of folate, vitamin B_6, and vitamin B_{12} did not include studies that were part of MEGASTROKE.

In conclusion, our results provide support for possible associations of tHcy and folate levels with SVS and that these relationships are specific for the SVS subtype. This suggests that any effect of folic acid supplementation in preventing stroke will be limited to the SVS subtype. Whether genetic variants at or near the *MTHFR* and *MUT* genes influence the risk of SVS through pathways other than tHcy levels and downstream effects require further investigation.

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Author Contributions

S.L., M.T., and H.M. contributed to the conception and design of the study; S.L. contributed to the analysis of data, preparing the figures, and drafting the text.

Potential Conflicts of Interest

Nothing to report.

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