Original Contributions

Plasma Homocysteine Concentration, C677T *MTHFR* Genotype, and 844ins68bp *CBS* Genotype in Young Adults With Spontaneous Cervical Artery Dissection and Atherothrombotic Stroke

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Background and Purpose—The role of mild hyperhomocysteinemia as a risk factor for cerebral ischemia may depend on stroke subtype. To test this hypothesis, we undertook a prospective case-control study of a group of patients with spontaneous cervical artery dissection (sCAD), a group of patients with atherothrombotic stroke (non-CAD), and a group of control subjects.

Methods—Fasting total plasma homocysteine (tHcy) concentration, C677T *MTHFR* genotype, and 844ins68bp *CBS* genotype were determined in 25 patients with sCAD, 31 patients <45 years of age with non-CAD ischemic stroke, and 36 control subjects. Biochemical data in the patient groups were obtained within the first 72 hours of stroke onset.

Results—Median tHcy levels were significantly higher in patients with sCAD (13.2 μmol/L; range, 7 to 32.8 μmol/L) compared with control subjects (8.9 μmol/L; range, 5 to 17.3 μmol/L; 95% CI, 1.05 to 1.52; *P*=0.006). Cases with tHcy concentration above the cutoff level of 12 μmol/L were significantly more represented in the group of patients with sCAD compared with control subjects (64% versus 13.9%; 95% CI, 2.25 to 44.23; *P*=0.003); a significant association between the *MTHFR TT* genotype and sCAD was also observed (36% versus 11.1%; 95% CI, 1.10 to 19.23; *P*=0.045). No significant difference in tHcy levels and in the prevalence of thermolabile *MTHFR* was found between patients with non-CAD ischemic stroke and control subjects and between patients with sCAD and non-CAD ischemic stroke. The distribution of the 844ins68bp *CBS* genotype and the prevalence of subjects carrying both the *TT MTHFR* and 844ins68bp *CBS* genotypes were not significantly different among the 3 groups.

Conclusions—Our results are consistent with the hypothesis that increased plasma homocysteine levels and the *TT MTHFR* genotype may represent risk factors for sCAD. In contrast, their role in atherothrombotic strokes remains a contentious issue. (***Stroke***. 2002;33:664-669.)

Key Words: cervical artery dissection ■ cystathionine β-synthase ■ homocysteine ■ methylenetetrahydrofolate reductase

Cervical artery dissection (CAD) accounts for up to one fifth of ischemic strokes occurring in young and middle-aged patients.[1] Despite the increasing clinical awareness and the development of noninvasive investigational tools such as ultrasound techniques and MRI, the pathogenesis of this condition remains unclear, and the potential role of common risk factors for vascular disease is still controversial. However, epidemiological observations suggest that some as-yet-unrecognized predisposing factors could be heritable[2–4] and that CAD might be the result of coexisting genetic and environmental factors.

In the past decade, raised plasma total homocysteine (tHcy) level has emerged as a potential risk factor for the development of vascular disease.[5,6] Mild hyperhomocysteinemia may result from both nutritional and genetic influences. The most common genetic defect associated with raised Hcy is the C-to-T substitution at nucleotide 677 in the coding region of the gene for methylenetetrahydrofolate reductase (*MTHFR*), which is associated with a thermolabile variant of the enzyme with about half-normal activity. Mutations in the cystathionine β-synthase (*CBS*) gene, the key enzyme in the transsulfuration of Hcy to cystathionine, may also be associated

with altered Hcy metabolism and premature vascular disease.[7]

Whether mild hyperhomocysteinemia can be a causative risk factor for ischemic stroke is still controversial. Most retrospective studies found higher mean Hcy levels in patients with arterial thrombosis,[8,9] whereas results from prospective studies indicated smaller or no association. In particular, several findings indicate that Hcy concentrations are not significantly increased during the acute phase of stroke but rise after the event,[10,11] thus prompting speculation that mild hyperhomocysteinemia does not predate ischemic stroke but could be the consequence of tissue damage as such. A possible limitation of most studies is that they have correlated Hcy concentrations with all kinds of strokes without considering the specific subtypes. In line with these observations, Gallai and coworkers[12] recently suggested that mild hyperhomocysteinemia might represent a predisposing condition for spontaneous CAD (sCAD).

To test the hypothesis that raised Hcy concentration and its genetic determinants are associated with sCAD but not with ischemic stroke of different pathogenesis, we undertook a prospective case-control study of consecutive patients admitted to our department. Fasting levels of tHcy, genotyping for the C677T mutation of the *MTHFR* gene and for the 844ins68bp mutation of the *CBS* gene, were determined in a series of patients with sCAD and compared with those of a group of patients with atherothrombotic stroke (non-CAD) and with those of a group of control subjects.

Subjects and Methods

Consecutive patients admitted to our department between January 1997 and December 2000 were prospectively considered for participation. All subjects in whom clinical and/or duplex ultrasound findings were consistent with the hypothesis of acute CAD underwent neuroradiological investigations to confirm the diagnosis.

Four-vessel conventional angiography and/or MRI (Siemens Magnetom, 1.5 T) and magnetic resonance angiography (MRA; 3-dimensional time of flight) of the brain and neck were included in the diagnostic workup.

The presence of the double-lumen sign (a false lumen or an intimal flap), luminal narrowing with the "string sign," and gradual tapering ending in total occlusion of the lumen (flamelike occlusion) were considered reliable angiographic findings of CAD, whereas a narrowed lumen surrounded by a semilunar-shaped intramural hematoma on axial T1-weighted images was considered the pathognomonic MR sign. Dissections occurring as an immediate consequence of a major trauma were labeled "traumatic" and excluded.

The group of subjects with non-CAD ischemic stroke was selected from a population of consecutive young patients (<45 years of age) suffering first-ever acute cerebral ischemia, after exclusion of the subgroup with CAD-related infarction. Stroke was defined as a sudden loss of global or focal cerebral function that persisted for >24 hours with a probable vascular cause. Patients with CT- and/or MRI-proven cerebral infarction were included. For the purpose of the present study, only subjects with infarction caused by large-vessel atherosclerotic vasculopathy or small-vessel disease were included. This allowed us to restrict the analysis to patients whose stroke occurred as a consequence of similar pathological processes and to focus on the primary role of the arterial wall in the events leading to cerebral infarction. Actually, the hypothesis that both microangiopathy and large-artery atherothrombosis may share common pathophysiological mechanisms is supported by the characteristics of the 2 small-vessel pathologies of major pathogenetic significance to lacune formation. The first one, "lipohyalinosis," is characterized by a distal subintimal degeneration of the small

penetrating arterioles, with evidence of mural foam cells and fibrinoid vessel wall necrosis. The second one affects perforating arteries larger than lipohyalinosis and is pathologically similar to the atherosclerotic process of the large cervicocranial arteries.[13–15]

Extensive ancillary examinations were performed in each subject to assess the mechanism of infarction. All patients underwent a diagnostic protocol, including complete blood cell count, biochemical profile, urinalysis, ECG, and chest roentgenography. Coagulation testing, including prothrombin and activated partial thromboplastin times, antiphospholipid antibodies, fibrinogen, protein C, protein S, activated protein C resistance, and antithrombin III, was carried out at admission in all subjects and at least 3 months later in all except 5 who died after the acute event. Genotyping to detect the G1691A mutation in the factor V gene (factor V Leiden) and the G20210A mutation in the prothrombin gene was also performed, according to a standardized multiplex polymerase chain reaction method.[16] Doppler ultrasonography with frequency spectral analysis and B-mode echotomography of the cervical arteries and transcranial Doppler ultrasonography were also performed in all patients on admission. Conventional angiography and/or MRA were used to investigate extracranial and intracranial vessels. Transthoracic and/or transesophageal echocardiography with intravenous injections of agitated saline with the patient at rest and during the Valsalva maneuver was performed to rule out cardiac sources of emboli.

On the basis of such investigations, patients were classified according to a classification based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria, accommodated and validated for the cause of stroke in the young.[17]

In both sCAD and non-CAD ischemic stroke groups, only the subjects in whom clinical evaluation and collection of blood samples were performed within 72 hours from signs and/or symptoms onset were eligible for the study. Reference control subjects matched to the cases by age in 3-year bands and by ethnic and geographic origin were selected from the list of local general practitioners by random-digit dialing after exclusion of individuals with known history of vascular disease. The study was designed and carried out in observance of the ethical principles established by the local Institutional Guidelines on Clinical Investigation. Written, informed consent was provided by all study participants.

Demographic data (age, sex) and history of conventional vascular risk factors, including hypertension, diabetes mellitus, cigarette smoking, and hypercholesterolemia, were obtained from each subject. Hypertension was considered present if systolic blood pressure was >160 mm Hg and diastolic pressure was >95 mm Hg in 2 separate measurements after the acute phase or if the subject was under treatment with antihypertensive drugs before recruitment. The diagnosis of diabetes mellitus was established according to World Health Organization criteria.[18] Cigarette smokers were categorized as current smokers or nonsmokers (the latter included former smokers who had quit smoking for at least 6 months before the study). Hypercholesterolemia was considered present if cholesterol serum levels were >220 mg/dL or if the subject was under treatment with cholesterol-lowering drugs.

In all subjects, we measured fasting plasma tHcy concentration, serum levels of cobalamin (vitamin B_{12}), and folate and carried out genetic analysis of the C677T mutation in the *MTHFR* gene and of the 844ins68bp mutation in the *CBS* gene.

Biochemical and Genetic Analyses

For determination of plasma tHcy levels, venous blood sampling took place in the early morning (before 7 AM) in subjects after overnight fasting. tHcy was measured photometrically after separation with a reversed-phase column. The interassay and intra-assay coefficients of variation for this assay were <7%. Hyperhomocysteinemia was defined as fasting plasma tHcy levels >12 μmol/L.[19] Folate and vitamin B_{12} concentrations were determined in heparinized plasma by routine hospital assays.

Genomic DNA was isolated from −20°C frozen samples of EDTA-anticoagulated whole blood by use of standard DNA extraction. Genotyping for the *CBS* 844ins68bp was performed according to the method of Sebastiao and coworkers.[20] *MTHFR* genotypes

were determined according to the method of Frosst and coworkers[21] using polymerase chain reaction amplification and restriction digestion with *Hin*fI to distinguish mutant from wild-type alleles.

Statistical Analysis

Data were analyzed with the SPSS (version 10.1) program. A value of $P<0.05$ on 2-sided tests was considered significant. A multinomial logistic regression model that included age, sex, hypertension, smoking status, hypercholesterolemia, B_{12}, folate, 844ins68bp mutation in the *CBS* gene, *TT MTHFR* genotype, and tHcy was used to examine the effect of these variables in the prediction of sCAD or non-CAD ischemic strokes. A second model that included the categorical variable hyperhomocysteinemia instead of the continuous variable plasma tHcy was also performed. Finally, the effect of genotypes on the prediction of sCAD or non-CAD ischemic strokes was tested in a further multinomial logistic regression model that included age, sex, hypertension, smoking status, hypercholesterolemia, and *TT MTHFR* or 844ins68bp *CBS* genotype. The variables tHcy, B_{12}, and folate were not considered genotype predictors and thus were not entered into this model. Diabetes mellitus was also not entered into the multiple regression equations because of the low frequency of this condition in the present series. Results are given as 95% CI.

Study Group

Thirty-eight patients with CAD were recruited. Thirteen were excluded from final analysis: 3 owing to traumatic damage of the arterial wall as a consequence of either car accident (n=2) or fight (n=1), 8 owing to late admission (after the first 72 hours from symptoms onset), and 2 because of an unwillingness to participate. Thus, a total number of 25 unrelated patients were included. Intravenous heparin was administered for 7 to 10 days and then changed to oral anticoagulant therapy in all patients admitted in the acute phase. Fasting blood samples were collected within 24 hours of symptoms onset in 12 patients (48%), within 48 hours in 6 (24%), and within 72 hours in the remaining 7 (28%).

Cases with non-CAD ischemic stroke were recruited from a series of 115 unrelated patients after exclusion of subjects with the following mechanisms of infarct: cardioembolism (n=38), thrombophilic disorders (n=14), oral contraceptive use (n=4), nonatherosclerotic vasculopathy other than arterial dissection (n=2; 1 patient with meningovascular syphilis[22] and 1 patient with MELAS, respectively), and drug-related infarct (n=1). Twenty-four patients with stroke of undetermined origin were also excluded. Atherothrombotic vasculopathy and small-vessel disease were the presumed mechanism of infarct in the remaining 32 patients. One of them was referred to our department after the acute phase and thus excluded. Hence, a total number of 31 unrelated patients were enrolled for analysis. Atherothrombotic vasculopathy was considered the cause of infarction in 18 patients. The atherothrombotic mechanism was classified as probable in 10 patients with a stenosis of >60% in the carotid arteries and possible in the remaining 8 patients showing atherosclerotic plaques with no signs of flow abnormalities. Thirteen patients met the criteria for lacunar infarct (probable in 10 and possible in 3). One had signs of previous silent ischemic lesions on brain MRI. Low-dose aspirin as an antiplatelet agent was the treatment of choice for all patients with a presumed atherothrombotic or lacunar infarction. Of these 31 subjects, 17 (54.8%) provided a fasting blood sample within 24 hours, 10 (32.2%) within 48 hours, and 4 (13%) within 72 hours of symptoms onset.

All the subjects in whom the acute-phase biochemical determinations revealed high fasting levels of tHcy were treated orally with folate supplementation (7.5 mg/d).

Thirty-six subjects were included in the group of control subjects. General characteristics and personal risk factors of the study population are summarized in Table 1. The patient groups more often had hypertension and more often were smokers compared with control subjects. Serum concentration levels of B_{12} and folate were not significantly different among the 3 groups (data not shown). None of the subjects included in the study group had coexisting conditions associated with mild hyperhomocysteinemia (ie, renal impairment, hypothyroidism, and drug therapy).

Among the 25 patients with sCAD, 34 extracranial dissected vessels (20 internal carotid artery dissections and 14 vertebral artery dissections) were found. Seven patients had bilateral dissection, involving the internal carotid artery (2 patients), the vertebral artery (4 patients), and both the internal carotid and vertebral arteries (1 patient). Diagnosis relied on conventional angiography in 19 patients (combined with MRI/MRA in 13) and on MRI/MRA in 6 patients. Five patients had fibromuscular dysplasia. Nineteen patients had cerebral infarction, whereas the remainder had isolated Horner's syndrome caused by internal carotid artery dissection with no evidence of cerebral ischemia. Two patients reported "trivial" trauma such as chiropractic manipulation of the cervical spine (1 patient) and sustained rotation of the head (1 patient) within 1 week preceding the onset of symptoms. The acute event was associated with upper respiratory tract infection in 2 patients and with wind instrument playing in 1 patient.

Results

Data on Hcy concentration are summarized in the Figure. The fasting median plasma tHcy levels were higher in patients with sCAD (13.2 μmol/L; range, 7 to 32.8 μmol/L) compared with control subjects (8.9 μmol/L; range, 5 to 17.3 μmol/L) and patients with non-CAD ischemic stroke (10.9 μmol/L; range, 6 to 30.2 μmol/L). There was a positive relationship between tHcy concentration and sCAD. In particular, a significant difference was found compared with control subjects (95% CI, 1.05 to 1.52; $P=0.006$). This difference remained significant (95% CI, 1.02 to 1.48; $P=0.038$) even after the exclusion of the sCAD cases with no evidence of structural ischemic cerebral lesions (median plasma tHcy concentration in the subgroup with cerebral infarction, 13.5 μmol/L; range, 7 to 22.7 μmol/L). In contrast, although there was a tendency toward a higher median concentration in patients with non-CAD ischemic stroke compared with control subjects, the difference was not statistically significant (95% CI, 0.95 to 1.35). No significant difference was found between the group of patients with sCAD and the group of patients with non-CAD ischemic stroke (95% CI, 0.80 to 1.56).

Similar results were obtained comparing the prevalence of subjects whose plasma tHcy levels were above the normal values in each group. A significant difference (95% CI, 2.25 to 44.23; $P=0.003$) was observed between the group of patients with sCAD (n=16 subjects with abnormal tHcy values; 64%) and the group of control subjects (n=5 subjects with abnormal tHcy values; 13.9%), whereas there was no significant difference between the group of patients with

TABLE 1.   General Characteristics of the Study Population

Characteristic	sCAD (n=25)	Non-CAD Ischemic Stroke (n=31)	Control Subjects (n=36)
Age, y	42.3±11.2	39.9±4.2	40.9±9.9
Male sex, n (%)	15 (60)	20 (64.5)	23 (63.9)
Current smoker, n (%)	10 (40)	13 (41.9)	11 (30.6)
Hypertension, n (%)	4 (16)	4 (12.9)	3 (8.3)
Diabetes mellitus, n (%)	1 (4)	2 (6.5)	0
Hypercholesterolemia, n (%)	5 (25)	6 (19.4)	7 (19.4)
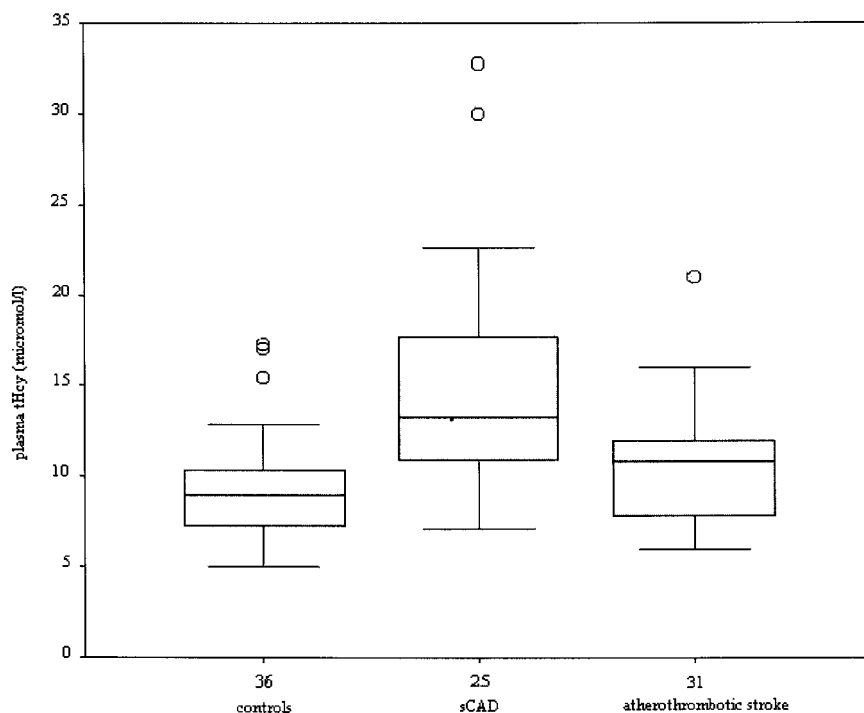
Box plot of fasting total plasma Hcy concentration in the group of patients with sCAD, group of patients with non-CAD ischemic stroke, and group of control subjects.

non-CAD ischemic stroke (n=9 subjects with abnormal tHcy values; 29%) and the group of control subjects (95% CI, 0.50 to 8.77), as well as between the group of patients with sCAD and the group of patients with non-CAD ischemic stroke (95% CI, 0.36 to 63.27). None of the other variables that were entered into the multiple regression equation were significant risk factors for sCAD.

We next investigated the prevalence of the *TT MTHFR* genotype in each group. The homozygous *TT* transition was present in 9 of 25 patients (36%) with sCAD, in 4 of 31 patients (12.9%) with non-CAD ischemic stroke, and in 4 of 36 control subjects (11.1%; Table 2).

There was a significant association between the *TT* genotype and the group of patients with sCAD compared with control subjects (95% CI, 1.10 to 19.23; *P*=0.045). No significant difference in *TT* frequency was observed between the group of patients with non-CAD ischemic stroke and the group of control subjects (95% CI, 0.23 to 5.15) or between the group of patients with sCAD and the group of patients with non-CAD ischemic stroke (95% CI, 0.30 to 58.7).

As opposed to the *TT MTHFR* genotype, the frequency of the 844ins68bp *CBS* mutation (12% in the group of patients with sCAD, 13.9% in the group of controls, and 25.8% in the group of patients with non-CAD ischemic stroke) was not statistically different among the 3 groups after multiple regression analysis (*P*=0.411). Similarly, there was no difference in the frequency of subjects carrying the combination of both the 844ins68bp *CBS* mutation and the *MTHFR TT* genotype (1 in each group).

Discussion

The main finding in the present study is the evidence that both high plasma levels of tHcy and the homozygous *MTHFR TT* genotype are strongly associated with sCAD. In contrast,

neither fasting tHcy levels nor the frequency of the thermolabile form of *MTHFR* significantly differed between patients with non-CAD ischemic stroke and control subjects.

To the best of our knowledge, there have been only sparse reports[23,24] and 1 prospective case-control study[12] of the relationship between fasting tHcy and sCAD. The evidence derived from such study agrees with our results arguing for a strong relationship between high levels of tHcy and arterial dissection. However, that study failed to detect any association between genetic abnormalities in Hcy metabolism and risk of sCAD. No previous studies compared fasting tHcy level and its genetic determinants in stroke patients with primary vascular disorders of different pathogenesis, such as arterial dissection and atherothrombosis of large and small vessels. The raised concentrations of tHcy we found in sCAD patients immediately after the acute event are suggestive of a causal association, whereas, in agreement with the results of previous prospective studies, we observed no evidence of such a correlation in cases of stroke caused by a presumed atherothrombotic process. Taken together, these findings are consistent with the hypothesis that the role played by Hcy in the pathophysiology of cerebral ischemia may depend on the stroke subtypes and the specific underlying mechanism.

TABLE 2.   Distribution of the *MTHFR* Genotypes in the 3 Groups

	sCAD (n=25)	Non-CAD Ischemic Stroke (n=31)	Control Subjects (n=36)
MTHFR genotype, % (n)			
TT	36% (9)	12.9% (4)	11.1% (4)
Tt	36% (9)	58.1% (18)	38.9% (14)
tt	28% (7)	29% (9)	50% (18)
T-t allele ratio	0.54:0.46	0.42:0.58	0.31:0.69

Although the exact process leading to sCAD remains elusive, a structural defect of the arterial wall is thought to represent a predisposing condition. Recently, Brandt and coworkers[25] found irregular collagen fibrils and elastic fiber fragmentation in skin biopsies of the majority of subjects from a series of sCAD patients, providing further evidence of a plausible relationship between sCAD and generalized connective tissue disorders and supporting the hypothesis that potential defects in the extracellular matrix of the vessel wall may play a key role in the pathogenesis of arterial dissection. Indirect evidence of an underlying structural defect of the arterial wall is also suggested by the association of sCAD with other vascular disorders.[26–29]

Several biological mechanisms might explain the association between increased levels of plasma tHcy and arterial dissection. Among these, a link between hyperhomocysteinemia and abnormalities in the elastic components of the arterial wall has been reported. In vitro studies demonstrate that high levels of plasma Hcy result in a decrease in the elastin content of the arterial wall as a direct or an indirect consequence of the Hcy-induced activation of metalloproteases[30] and serine elastases.[31] This increased elastolytic activity may result in an opening and enlargement of fenestrae in the medial elastic laminae, leading to a premature fragmentation of the arterial elastic fibers and degradation of the extracellular matrix.[30–32] Hcy has been shown to block aldehydic groups in elastin, thereby inhibiting the cross-linking necessary to stabilize elastin.[33] The cross-linking of collagen may also be impaired.[34] Thus, Hcy may have an influence on the elastic properties of the arterial wall. These findings lead to the hypothesis that the toxic effect of Hcy might be more pronounced in the carotid artery and in the aorta with their many elastic laminas compared with the femoral and brachial arteries in which the muscular layer is thicker. The high frequency of carotid and aortic dissection compared with the low frequency in the femoral and brachial arteries is in line with these findings and indirectly strengthens the hypothesis of a link between increased levels of tHcy and sCAD.

A possible limitation of our study is inherent in the different therapeutic approaches to sCAD with respect to atherothrombotic strokes. However, there is no evidence that either antiplatelets or anticoagulants agents may influence plasma tHcy levels.[35] Furthermore, we measured tHcy concentration in the very acute phase of the vascular event, which is likely to reduce the impact of this potential bias. This also allows us to refute the hypothesis that the raised concentrations of tHcy we observed might be the result of the increase in methylation reactions after tissue injury, which has been suggested to explain the high tHcy levels in patients sampled after the acute phase of stroke.[36]

The association between sCAD and the homozygous *MTHFR TT* genotype is also worth mentioning. It has been reported that ≈5% of patients with spontaneous arterial dissection have at least 1 family member who has had the same disorder.[4] This finding suggests that the underlying structural defects of the arterial wall might be heritable in some cases. As to the 844ins68bp mutation in the *CBS* gene, it has been observed that the heterozygous state, combined with the thermolabile *MTHFR*, may increase the risk of venous and arterial occlusive disease.[37] However, our findings do not support this hypothesis and a relationship between the 844ins68bp mutation in the *CBS* gene, alone or in combination with the thermolabile *MTHFR*, and sCAD seems unlikely.

In conclusion, our data suggest that Hcy may play a differential role in the pathogenesis of vascular disorders, according to the specific mechanism of vascular injury. In particular, increased concentration of plasma tHcy seems to represent a predisposing condition for sCAD, whereas its role in atherothrombotic damage remains a contentious issue. Taking into account the relatively small number of subjects in the present study, we cannot exclude that the lack of significant difference between patients with atherothrombotic stroke and control subjects may be the result of an underpowered comparison. Furthermore, our data point to a relationship between genetic abnormalities in Hcy metabolism and risk of sCAD and suggest a potential association with polymorphisms in other genes.

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