Early-onset ischaemic stroke: analysis of 58 polymorphisms in 17 genes involved in methionine metabolism

Early-onset ischaemic stroke: Analysis of 58 polymorphisms in 17 genes involved in methionine metabolism

Betti Giusti[1]; Claudia Saracini[1]; Paola Bolli[1]; Alberto Magi[1]; Ida Martinelli[2]; Flora Peyvandi[2]; Maurizia Rasura[3]; Massimo Volpe[4,5]; Luca A. Lotta[2]; Speranza Rubattu[4,5]; Pier Mannuccio Mannucci[2]; Rosanna Abbate[1]

[1]Department of Medical and Surgical Critical Care, University of Florence, Florence, Italy; [2]Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, University of Milan and Department of Medicine and Medical Specialities, IRCCS Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Luigi Villa Foundation, Milan, Italy; [3]Department of Neurology-Stroke Unit, IInd School of Medicine, Sapienza University, Ospedale S. Andrea, Rome, Italy; [4]Department of Cardiology, IInd School of Medicine, Sapienza University, Ospedale S. Andrea, Rome, Italy; [5]IRCCS Neuromed, Pozzilli (Is), Italy

Summary

The hypothesis underlying this study is that variations in genes involved in methionine metabolism may contribute to genetic susceptibility for early-onset ischaemic stroke. We investigated 58 polymorphisms in AHCY, BHMT, BHMT2, CBS, ENOSF1, FOLH1, MTHFD1, MTHFR, MTR, MTRR, NNMT, PON1, PON2, SLC19A1, SHMT1, TCN2, TYMS genes on genomic DNA from 501 young patients who survived ischaemic stroke and 1,211 sex and age comparable controls. Genotype distribution was significantly different between patients and controls for the following SNPs: rs10037045 BHMT, rs682985 BHMT2, rs1051319 CBS, rs202680 FOLH1, rs2274976 MTHFR, rs1979277 SHMT1, rs20721958 TCN2. On multiple logistic regression analysis adjusted for traditional risk factors, rs10037045 BHMT, rs682985 BHMT2, rs1051319 CBS, and rs202680 FOLH1 remained independent risk factors for stroke. After haplotype reconstruction, generalised linear model analyses adjusted for traditional risk factors and using the FDR multiple testing correction showed significant associations between ischaemic stroke and BHMT, CBS, FOLH1, MTR, PON2, TCN2 and TYMS haplotypes. This study identifies significant genetic associations between premature ischaemic stroke and haplotypes in BHMT, CBS, FOLH1, MTR, PON2, TCN2 and TYMS genes involved in methionine metabolism.

Keywords

Ischaemic stroke, methionine metabolism, homocysteine, polymorphism, haplotype

Correspondence to:
Betti Giusti, PhD
Department of Medical and Surgical Critical Care
University of Florence
Viale Morgagni 85, 50134 Florence, Italy
Tel.: +39 0557949420, Fax: +39 0557949418
E-mail: betti.giusti@unifi.it

Financial support:
This work was supported by grants from Italian Ministry of Health "Ricerca Finalizzata 2005: Non-cardioembolic atherothrombotic stroke", "Genopolis" government FIRB project (RBLA038RMA_008), Italian Ministry of Health (IRCCS), Ingenius Hypercare European Program and from Fiorgen Foundation, Florence, Italy.

Received: November 3, 2009
Accepted after major revision: March 16, 2010
Prepublished online: May 10, 2010

doi:10.1160/TH09-11-0748
Thromb Haemost 2010; 104: 231–242

Introduction

Since 1969 (1), substantial evidence has been accumulated linking high homocysteine (Hcy) levels in blood to the risk of cardiovascular disease (2, 3). Raised concentrations of Hcy have been suggested to be a modifiable, independent risk factor for coronary artery disease, stroke, and deep-vein thrombosis (4–7).

Hcy is a branch-point intermediate of methionine metabolism, which can be further metabolised via two alternative pathways: degraded irreversibly through the transsulfuration pathway or remethylated to methionine by the remethylation pathway. Both pathways are B-vitamin-dependent. Genetic defects in genes encoding enzymes involved in Hcy metabolism, or depletion of important cofactors or (co)substrates for those enzymes – including folate, vitamin B(12) and vitamin B(6) – may result in elevated plasma concentrations of Hcy (8, 9). A recent review showed that 23 genes could modulate Hcy levels and that 112 genes are modulated epigenetically by hyperhomocysteinaemia (10). Several polymorphisms in genes coding for enzymes involved in the methionine metabolism have also been suspected to be associated with hyperhomocysteinaemia. Pertaining to some polymorphisms, e.g. C677T and A1298C in 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, A2756G in 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) gene, A66G in 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) gene, T163A in paraoxonase 1 (PON1) gene, G80A in solute carrier family 19 (folate transporter), member 1 (SLC19A1) gene, C776G in transcobalamin II (TCN2), 6bp ins del in thymidylate synthetase (TYMS) gene, contrasting data are available (10). For C677T and A1298C MTHFR polymorphisms, the rare allele is associated

with reduced enzymatic activity (11, 12). Furthermore, methionine metabolism plays a crucial role in the methylation of a wide variety of essential biological substances, including phospholipids, proteins, neurotransmitters, and DNA. Therefore, besides altering levels of the toxic intermediate Hcy and of Hcy-thiolactone (13), polymorphisms/haplotypes of genes involved in the methionine metabolism might determine the improper function of many other biological processes, such as DNA methylation and, hence, the genetic stability and the expression of several important genes (14).

Due to the complexity of genetic mechanisms implicated in methionine metabolism perturbation, this study aimed to evaluate the genetic susceptibility for premature ischaemic stroke conferred by 58 polymorphisms in 17 genes [adenosylhomocysteinase (AHCY), betaine-homocysteine methyltransferase (BHMT), betaine-homocysteine methyltransferase 2 (BHMT2), cystathionine-beta-synthase (CBS), enolase superfamily member 1 (ENOSF1), folate hydrolase 1 (FOLH1), methylenetetrahydrofolate dehydrogenase (MTHFD1), MTHFR, MTR, MTRR, nicotinamide N-methyltransferase (NNMT), PON1, paraoxonase 2 (PON2), SLC19A1, serine hydroxymethyltransferase 1 (SHMT1), TCN2, TYMS] coding for enzymes involved in the methionine metabolism. Polymorphisms selection was performed to address two objectives: to study genetic variants for which consistent or contrasting literature data were available and to reconstruct haplotypes that could permit us a better evaluation of the effect of gene alleles in determining susceptibility to early-onset ischaemic stroke.

Materials and methods

Study population

Five hundred one unrelated Caucasian young adults (<65 years) who were referred to the a) Angelo Bianchi Bonomi Hemophilia and Thrombosis Center of the University of Milan, Italy, and b) to Department of Neurology, Sapienza University, Sant'Andrea Hospital, Rome, Italy, for thrombophilia screening after a first ischaemic stroke were enrolled in this study. Patient and control populations were in part previously investigated in relation to polymorphisms of genes encoding procoagulant and inflammatory factors (15). The median time interval between the ischaemic stroke and blood sampling was six months (range one month to 10 years); 75% of patients had blood sampled within two years and 64% within one year after the event. Clinical records were reviewed and when the type of stroke was not specified, neurologists who took care of the patients during the acute phase were contacted. The clinical diagnosis was objectively confirmed by computed tomography scans or magnetic resonance or magnetic resonance angiography and intra-arterial angiography. According to TOAST classification, 13.4% of enrolled patients were classified as large artery stroke, 5.2% as cardioembolic, 16.5% as small artery (lacunar), 53.5% as undetermined aetiology and 11.4% as other determined aetiology stroke. All patients underwent a cardiologic evaluation and transthoracic echocardiography and Doppler examination of the neck vessels.

One thousand two-hundred eleven healthy unrelated Caucasian subjects comparable for age and gender were chosen from the whole population of controls made of partners and friends who accompanied patients to the Centre in the same period as patients and agreed to be investigated. Previous thrombosis in the controls was excluded using a validated structured questionnaire (16). The presence, at the time of stroke for patients and at the time of blood sampling for controls, of hypertension, hypercholesterolaemia, diabetes mellitus, and smoking habit (at least five cigarettes daily) was recorded.

The study was approved by the Institutional Review Board of the University of Milan, University of Rome and University of Florence and all subjects gave their informed consent to the study.

DNA extraction

Genomic DNA was isolated from peripheral blood by using FlexiGene kit (QIAGEN, Hilden, Germany).

Genotyping

We studied 58 single nucleotide polymorphism (SNPs) in 17 candidate genes involved in methionine metabolism (for gene sequences and oligonucleotides see our previous papers) (17, 18), according to their demonstrated or putative function based on literature data, localization in the functional region and/or heterozygosity values >0.30, extracted from dbSNP database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp&cmd=search&term=). Thirteen genes (AHCY, BHMT, CBS, FOLH1, MTHFD1, MTHFR, MTR, MTRR, PON1, SLC19A1, SHMT1, TCN2, TYMS) were selected among 23 genes identified by literature mining that they modulate Hcy levels (10), and three genes (BHMT2, NNMT, PON2) were selected on the basis of their involvement in the methionine metabolism, and one gene (ENOSF1) because we genotyped a SNP located at the 3'UTR of the ENOSF1 gene overlapping the 3' end of the TYMS gene. SNPs information were assessed in dbSNP NCBI and ENSEMBL (http://www.ensembl.org/index.html) databases.

SNPs were analysed by using GenomeLab SNPstream genotyping platform and software (Beckman Coulter, Fullerton, CA, USA) according to previously described protocols(17, 18). Briefly, in order to identify highly homologous and repetitive elements, genomic DNA sequences were subjected to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). A specific software (http://www.autoprimer.com) to design the 12 pairs of primers for multiplex PCR and 12 tagged extension primers were used (see our previous papers) (17, 18). After multiplex polymerase chain reaction (PCR) reaction, a multiplex primer extension reaction was

performed. The hybridisation mix containing the primer extension products was added to the SNPware plate, so that each extended primer hybridise in a specific way to one of the unique probes arrayed in each well. The SNPware plate was analysed with the SNPstream array imager. Sample genotype data were automatically generated.

Hcy measurement

Venous blood was collected after an overnight fasting from all subjects. Total Hcy plasma levels were determined by an immunoassay method (FPIA, IMX system, Abbott).

Data analysis

Statistical analysis was performed using the SPSS package v11.5. The Hardy-Weinberg equilibrium was evaluated by the Chi^2 test. Genotype distributions were compared between groups by Chi^2 analysis. We assessed the association between each polymorphism and stroke using different genetic models. The dominant model compares individuals with one or two rare alleles (heterozygotes+homozygotes) with the baseline group of homozygous subjects. The recessive model compares individuals with two rare alleles with the baseline group of heterozygous and homozygous subjects. The additive model assumes that there is a linear gradient in risk between the three genotypes. Categorical variable are expressed as frequencies and percentages. Unless otherwise indicated, data are given as median values and ranges. Comparisons of continuous variables between patients and controls or among genotypes were performed by the non-parametric Mann–Whitney U- or Kruskal-Wallis- test. The association of polymorphisms that were significantly associated to stroke with Hcy plasma levels was estimated by general linear model adjusted for sex, gender, hypertension, diabetes mellitus, dyslipidaemia, smoking habit. Multiple logistic regression analysis was used to estimate odds ratios (OR) and 95% confidence intervals (CI) for ischaemic stroke. In the multiple analysis, ORs were adjusted for sex, gender, hypertension, diabetes mellitus, dyslipidaemia, and smoking habit. In order to reduce the type I error, we applied the false discovery rate (FDR) multiple testing correction in the statistical analyses. A value of $p<0.05$ was chosen as the cut-off level for statistical significance.

Haplotype analysis

In order to prepare datafiles for linkage disequilibrium (LD) and haplotype reconstruction analysis, datafiles were processed in R environment (http://www.r-project.org). Pairwise LD was evaluated by using SNPAlyze software (Dynacom, Kanagawa, Japan). Haplotype reconstruction and frequency estimation were independently performed using the PHASE v2.1 software (19), and R package haplo.stats by Expectation-Maximization strategy (EM algorithm). The haplo.stats package was also used to identify statistically significant associations between haplotypes and disease risks by means of generalized linear models. In order to reduce the type I error, we applied the false discovery rate (FDR) multiple testing correction in generalised linear model analysis.

To evaluate the effect of haplotype combination and interaction between two unlinked regions we used an algorithm implemented in FAMHAP software v16 (20, 21). The FAMHAP interaction analysis does not explicitly test for a specific pre-defined interaction model, but tests for the global hypothesis that none of the considered haplotype interactions shows association with the disease. The algorithm applies multiple testing correction via the minP approach (the p-value of the best marker/region configuration is corrected via Monte-Carlo simulations) (21).

Results

Subjects

Demographic and clinical characteristics of investigated patients and subjects are reported in ▶Table 1.

Single SNP analysis

In ▶Table 2 genotype distributions of all SNP and Chi^2 analysis by additive and dominant/recessive models are reported. Genotype distribution was significantly different between patients and controls by using the two models for the following SNPs: rs10037045 BHMT, rs682985 BHMT2, rs1051319 CBS, rs202680 FOLH1, rs2274976 MTHFR, rs1979277 SHMT1, rs20721958 TCN2 (Table 2).

On multiple logistic regression analysis with stroke as dependent variable and hypertension, diabetes mellitus, dyslipidaemia,

Table 1: Demographic and clinical characteristics of patients with ischaemic stroke and controls.

	Controls (N=1211)	Stroke patients (N=501)	P-value
Age	44.0 (12–65)	44.0 (1–65)	0.659
Sex (male), N (%)	543 (44.8)	228 (45.5)	0.800
Smoking habit, N (%)	316 (26.1)	199 (39.7)	<0.0001
Diabetes, N (%)	4 (0.3)	11 (2.2)	<0.0001
Hypertension, N (%)	88 (7.3)	141 (28.1)	<0.0001
Dyslipidaemia, N (%)	42 (3.5)	105 (20.9)	<0.0001
BMI, (kg/m^2)	23.7 (15.7–45.2)	24.2 (16.5–56.1)	0.029

BMI, body mass index.

Table 2: Genotype distribution and allele frequency of all investigated SNPs in ischaemic stroke and control subjects.

Gene	dbSNP ID or SNP name	Genotypes (%)			Minor allele frequency	Genotypes (%)			Minor allele frequency	P*	P°,§
		Patients (n=501)				Healthy subjects (n=1,211)					
AHCY	rs819146	AA(73.5)	AC(23.5)	CC(3.0)	C=0.148	AA(74.2)	AC(24.3)	CC(1.5)	C=0.136	0.117	0.039§
	rs7271501	CC(97.4)	CG(2.6)	GG(0.0)	G=0.047	CC(98.6)	CG(1.4)	GG(0.0)	G=0.006	0.087	-
BHMT	rs651852	CC(32.4)	CT(48.6)	TT(19.1)	T=0.434	CC(28.5)	CT(50.4)	TT(21.1)	T=0.463	0.260	0.113°
	rs567754	CC(44.3)	CT(43.7)	TT(12.0)	T=0.338	CC(42.3)	CT(45.6)	TT(12.1)	T=0.349	0.777	0.491°
	rs10037045	AA(32.6)	AT(52.5)	TT(14.9)	T=0.412	AA(40.2)	AT(47.7)	TT(12.1)	T=0.359	0.048	0.017°
	rs585800	AA(61.7)	AT(34.1)	TT(4.2)	T=0.213	AA(62.6)	AT(33.0)	TT(4.4)	T=0.209	0.903	0.493°
BHMT2	rs644191	AA(71.0)	AG(27.3)	GG(1.7)	G=0.154	AA(70.3)	AG(27.2)	GG(2.5)	G=0.161	0.689	0.390§
	rs682985	TT(34.7)	TC(44.5)	CC(20.8)	C=0.430	TT(33.6)	TC(50.1)	CC(16.3)	C=0.413	0.039	0.026§
CBS	Pro88Ser	CC(99.4)	CT(0.6)	TT(0.0)	T=0.003	CC(98.9)	CT(1.1)	TT(0.0)	T=0.005	0.353	-
	Ala114Val	CC(99.3)	CT(0.5)	TT(0.2)	T=0.005	CC(97.3)	CT(1.8)	TT(0.9)	T=0.018	0.055	0.017°
	Gly116Arg	GG(100.0)	GA(0.0)	AA(0.0)	A=0.000	GG(99.9)	GA(0.1)	AA(0.0)	A=0.000	0.520	-
	rs1051319	GG(71.2)	GC(24.2)	CC(4.5)	C=0.167	GG(77.2)	GC(21.2)	CC(1.6)	C=0.122	<0.0001	<0.0001§
ENOSF1	rs8423	CC(36.3)	CA(49.3)	AA(14.3)	A=0.390	CC(34.6)	CA(49.6)	AA(15.8)	A=0.406	0.683	0.465§
FOLH1	rs202676	AA(69.5)	AG(28.4)	GG(2.1)	G=0.164	AA(70.4)	AG(27.6)	GG(2.0)	G=0.158	0.904	0.688°
	rs202680	TT(42.9)	AT(42.1)	AA(15.0)	A=0.360	TT(62.2)	AT(32.1)	AA(5.7)	A=0.218	<0.0001	<0.0001°§
MTHFD1	rs2357481	AA(99.0)	AT(1.0)	TT(0.0)	T=0.005	AA(99.7)	AT(0.3)	TT(0.0)	T=0.001	0.082	-
	rs1076991	TT(28.7)	TC(49.5)	CC(21.8)	C=0.465	TT(27.8)	TC(50.5)	CC(21.7)	C=0.470	0.904	0.676°
	rs3783732	AA(98.6)	AT(1.4)	TT(0.0)	T=0.007	AA(99.4)	AT(0.5)	TT(0.1)	T=0.003	0.120	0.087°
	rs8003379	AA(58.1)	AC(35.6)	CC(6.4)	C=0.242	AA(53.5)	AC(39.6)	CC(6.9)	C=0.267	0.221	0.084°
	rs1950902	CC(72.9)	CT(25.1)	TT(2.0)	T=0.146	CC(75.5)	CT(22.8)	TT(1.7)	T=0.131	0.522	0.256°
	rs4902283	CC(99.2)	CT(0.8)	TT(0.0)	T=0.004	CC(99.4)	CT(0.6)	TT(0.0)	T=0.003	0.604	-
	rs2236225	GG(29.1)	GA(51.3)	AA(19.6)	A=0.452	GG(33.1)	GA(48.6)	AA(18.3)	A=0.426	0.276	0.109°
MTHFR	rs1801133	CC(26.0)	CT(47.9)	TT(26.1)	T=0.501	AA(28.5)	CA(48.9)	CC(22.6)	A=0.471	0.252	0.119§
	rs1801131	AA(49.5)	CA(39.3)	CC(11.2)	A=0.308	CC(47.2)	CT(43.7)	TT(9.1)	T=0.309	0.168	0.183§
	rs2274976	CC(94.3)	CT(5.7)	TT(0.0)	T=0.029	CC(92.2)	CT(6.50)	TT(1.3)	T=0.046	0.046	0.012§
	rs4846049	CC(48.7)	CA(41.3)	AA(10.0)	A=0.306	CC(45.5)	CA(44.3)	AA(10.2)	A=0.324	0.469	0.227°
MTR	rs4659725	CC(34.1)	CG(47.9)	GG(17.9)	G=0.419	CC(33.2)	CG(47.4)	GG(19.4)	G=0.431	0.779	0.489§
	rs1805087	AA(67.9)	AG(29.5)	GG(2.6)	G=0.174	AA(67.5)	AG(29.2)	GG(3.3)	G=0.179	0.743	0.441§
	rs2275566	TT(34.7)	TC(46.9)	CC(18.4)	C=0.418	TT(33.2)	TC(47.5)	CC(19.3)	C=0.431	0.801	0.541°
	rs2853522	CC(29.2)	CA(49.3)	AA(21.5)	A=0.462	CC(29.1)	CA(50.7)	AA(20.2)	A=0.456	0.802	0.537§
	rs6676866	CC(29.2)	CA(48.3)	AA(22.5)	A=0.467	CC(27.3)	CA(49.7)	AA(23.0)	A=0.478	0.747	0.447°
MTRR	rs326118	AA(71.3)	AC(25.4)	CC(3.3)	C=0.161	AA(72.5)	AC(24.4)	CC(3.1)	C=0.153	0.866	0.601°
	rs1801394	AA(27.3)	AG(51.7)	GG(21.0)	G=0.468	AA(26.3)	AG(52.5)	GG(21.2)	G=0.474	0.913	0.669°
	rs1532268	CC(37.3)	CT(48.0)	TT(14.7)	T=0.387	CC(40.0)	CT(45.4)	TT(14.6)	T=0.373	0.574	0.308°
	rs2303080	TT(97.2)	TA(2.8)	AA(0.0)	A=0.014	TT(97.3)	TA(2.7)	AA(0.0)	A=0.014	0.878	-
	rs10064631	GG(99.5)	GC(0.5)	CC(0.0)	C=0.003	GG(99.8)	GC(0.2)	CC(0.0)	C=0.001	0.302	-
	rs16879334	CC(97.1)	CG(2.9)	GG(0.0)	G=0.015	CC(96.9)	CG(3.0)	GG(0.1)	G=0.016	0.831	0.899°
	rs8659	AA(41.5)	AT(45.4)	TT(13.1)	T=0.358	AA(37.3)	AT(46.6)	TT(16.1)	T=0.396	0.194	0.133°
NNMT	rs566775	CC(66.3)	CT(30.7)	TT(3.0)	T=0.184	CC(66.8)	CT(30.4)	TT(2.8)	T=0.180	0.964	0.833§
	rs10891639	CC(59.9)	CA(36.1)	AA(4.0)	A=0.222	CC(64.8)	CA(31.3)	AA(3.9)	A=0.196	0.196	0.076°
	rs4646335	TT(68.2)	TA(28.4)	AA(3.4)	A=0.176	TT(69.0)	TA(27.6)	AA(3.4)	A=0.172	0.949	0.754°
	rs3819100	TT(51.1)	TC(40.8)	CC(8.1)	C=0.285	TT(54.1)	TC(38.3)	CC(7.6)	C=0.268	0.528	0.259°

Table 2: Continued

Gene	dbSNP ID or SNP name	Genotypes (%)			Minor allele frequency	Genotypes (%)			Minor allele frequency	P*	P°,§
		Patients (n=501)				Healthy subjects (n=1,211)					
PON1	rs854660	TT(43.9)	TA(44.8)	AA(11.3)	A=0.337	TT(39.7)	TA(45.9)	AA(14.4)	A=0.373	0.136	0.108°
	rs662	AA(48.1)	AG(41.1)	GG(10.8)	G=0.313	AA(49.9)	AG(41.0)	GG(9.1)	G=0.296	0.519	0.278§
	rs3917594	GG(100.0)	GA(0.0)	AA(0.0)	A=0.000	GG(99.9)	GA(0.1)	AA(0.0)	A=0.000	0.524	-
PON2	rs11545941	CC(63.5)	CG(33.5)	GG(2.9)	G=0.198	CC(62.2)	CG(34.1)	GG(3.7)	G=0.208	0.720	0.460§
	rs6954345	CC(59.1)	CG(37.3)	GG(3.6)	G=0.223	CC(62.5)	CG(33.9)	GG(3.6)	G=0.206	0.386	0.185°
SLC19A1	rs3177999	TT(34.1)	CT(45.3)	CC(20.6)	C=0.432	TT(30.4)	CT(49.6)	CC(20.0)	C=0.448	0.223	0.129°
	rs1051266	CC(34.5)	CT(44.9)	TT(20.6)	T=0.430	CC(31.0)	CT(49.4)	TT(19.6)	T=0.443	0.211	0.150°
	rs12659	CC(35.9)	CT(44.5)	TT(19.6)	T=0.418	CC(32.0)	CT(47.5)	TT(20.5)	T=0.443	0.282	0.112°
SHMT1	rs638416	CC(33.1)	CG(50.1)	GG(16.8)	G=0.418	CC(32.5)	CG(51.3)	GG(16.2)	G=0.418	0.901	0.810°
	rs1979277	GG(98.5)	GA(1.5)	AA(0.0)	A=0.008	GG(99.5)	GA(0.5)	AA(0.0)	A=0.002	0.048	-
TCN2	rs5749131	GG(36.9)	GA(48.3)	AA(14.8)	A=0.389	GG(36.7)	GA(49.9)	AA(13.4)	A=0.383	0.712	0.447§
	rs1801198	CC(37.5)	CG(47.1)	GG(15.4)	G=0.389	CC(36.9)	CG(49.5)	GG(13.6)	G=0.383	0.520	0.323§
	rs20721958	TT(96.0)	TA(4.0)	AA(0.0)	A=0.020	TT(99.9)	TA(0.1)	AA(0.0)	A=0.000	<0.0001	-
	rs10418	GG(60.9)	GA(33.9)	AA(5.2)	A=0.222	GG(56.6)	GA(38.4)	AA(5.0)	A=0.242	0.216	0.896°
TYMS	rs502396	CC(26.3)	CT(49.3)	TT(24.4)	T=0.490	CC(28.6)	CT(48.9)	TT(22.5)	T=0.470	0.565	0.351°
	rs16430	CC(38.2)	CA(47.5)	AA(14.3)	A=0.381	CC(36.0)	CA(49.5)	AA(14.5)	A=0.393	0.698	0.407°

p*= according to the additive model; p°= according to the dominant model; p§= according to the recessive model. AHCY, adenosylhomocysteinase; BHMT, betaine-homocysteine methyltransferase; BHMT2, betaine-homocysteine methyltransferase 2; CBS, cystathionine-beta-synthase; ENOSF1, enolase superfamily member 1; FOLH1, folate hydrolase 1; MTHFD1, methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase; MTHFR, 5,10-methylenetetrahydrofolate reductase (NADPH); MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; NNMT, nicotinamide N-methyltransferase; PON1, paraoxonase 1; PON2, paraoxonase 2; SLC19A1, solute carrier family 19 (folate transporter), member 1; SHMT1, serine hydroxymethyltransferase 1 (soluble); TCN2, transcobalamin II; TYMS, thymidylate synthetase.

smoking habit and each polymorphism as independent variables, the gene polymorphisms rs10037045 BHMT [OR 1.38 (1.03–2.31 95%CI), p=0.033 for carriers of the rare allele], rs682985 BHMT2 [OR 1.46 (1.07–2.00 95%CI), p=0.017 for rare allele homozygous subjects], rs1051319 CBS [OR 3.75 (1.86–7.50 95%CI), p<0.0001 for rare allele homozygous subjects], and rs202680 FOLH1 [OR 3.00 (2.00–4.48 95%CI), p<0.0001 for rare allele homozygous subjects] were independent risk factors for ischaemic stroke.

Haplotype analysis

After haplotype reconstruction, generalised linear model analysis adjusted for traditional cardiovascular risk factors showed a statistically significant association between ischaemic stroke and BHMT, CBS, FOLH1, MTR, PON2, TCN2 and TYMS haplotypes (▶Table 3). When we also adjusted for traditional cardiovascular risk factors and Hcy levels, the association with ischaemic stroke remained independent for the following genes: CBS, FOLH1, PON2, TYMS (data not shown).

Haplotype interaction analysis

In order to evaluate the possible effect of haplotype combination between genes, we applied the expectation/maximization algorithm. According to this analysis, significant interactions were observed among haplotypes of the following genes BHMT-CBS, BHMT-FOLH1, BHMT-MTR, BHMT-TCN2, BHMT-TYMS, CBS-FOLH1, CBS-PON2, FOLH1-MTR, FOLH1-TCN2, FOLH1-TYMS, MTR-PON2, MTR-TCN2, PON2-TYMS (see Supplementary Table A available online at www.thrombosis-online.com). In ▶Table 4 a selection of combinations of haplotypes in two different genes with a large difference in the frequency between patients and controls are reported.

Hcy levels

Hcy levels were significantly higher in patients with ischaemic stroke than in controls [9.5 (2.7–65.7) μM vs 8.9 (3.9–49.5) μM; p=0.003]. On logistic regression analysis – adjusted for hypertension, diabetes mellitus, dyslipidemia, and smoking habit – Hcy was a mild but statistically significant and independent risk factor for stroke [OR=1.03 (95%CI 1.01–1.06), p=0.015].

Table 3: Haplotypes reconstruction analysis for each genes and analysis of association with ischaemic stroke by using the generalised linear model (adjusted for sex, gender, hypertension, smoking habit, dyslipidaemia, diabetes) and by using the false discovery rate (FDR) multiple testing correction. In bold are evidenced SNPs that resulted significantly associated with stroke in the single SNP analysis (Table 2); all SNPs in haplotypes are reported from 5′ to 3′ end of the relative gene; *= this SNP is in located at the 3'UTR of the ENOSF1 gene overlapping the 3′ end of the TYMS gene.

Gene	Haplotypes	Frequencies in control subjects	Frequencies in stroke patients	Coefficient	Standard error	P-values
AHCY	a c	0.858	0.847			
	c c	0.136	0.140	–0.003	0.023	1.00
	Rare haplotypes	0.006	0.013	0.108	0.104	0.542
	SNP1=rs819146; SNP2=rs7271501					
BHMT	c c a t	0.136	0.132			
	c c a a	0.085	0.084	–0.026	0.039	0.954
	c c t a	0.276	0.288	0.010	0.031	1.00
	c t a a	0.033	0.041	0.060	0.058	0.568
	t c a a	0.012	0.003	–0.062	0.113	1.00
	t c a t	0.067	0.059	–0.007	0.045	1.00
	t c t a	0.072	0.089	0.027	0.039	0.919
	t t a a	0.304	0.262	–0.029	0.027	0.557
	Rare haplotypes	0.011	0.038	0.250	0.081	0.004
	SNP1=rs651852; SNP2=rs567754; SNP3=rs1003745; SNP4=rs585800					
BHMT2	a t	0.583	0.565			
	a c	0.250	0.281	0.011	0.019	1.00
	g c	0.160	0.153	–0.004	0.023	1.00
	SNP1=rs644191; SNP2=rs682985					
CBS	c c g g	0.857	0.818			
	c c g c	0.120	0.172	0.072	0.023	0.003
	Rare haplotypes	0.022	0.009	–0.051	0.092	1.00
	SNP1=Pro88Ser; SNP2=Ala114Val; SNP3=Gly116Arg; SNP4=rs1051319					
FOLH1	g a	0.154	0.163			
	a a	0.064	0.205	0.225	0.033	$2.36x10^{-11}$
	a t	0.773	0.629	–0.032	0.022	0.265
	Rare haplotypes	0.008	0.0015	–0.138	0.098	0.289
	SNP1=rs202676; SNP2=rs202680					
MTHFD1	a t a a c c a	0.267	0.261			
	a c a a c c a	0.044	0.047	–0.045	0.053	0.746
	a c a a c c g	0.116	0.129	–0.016	0.031	1.00
	a c a a t c a	0.009	0.022	0.132	0.095	0.315
	a c a a t c g	0.036	0.026	–0.046	0.056	0.769
	a c a c c c a	0.085	0.085	–0.008	0.039	1.00
	a c a c c c g	0.169	0.142	–0.035	0.026	0.353
	a t a a c c g	0.180	0.169	–0.003	0.031	1.00
	a t a a t c a	0.014	0.022	0.192	0.116	0.185
	a t a a t c g	0.063	0.069	–0.030	0.041	0.873
	Rare haplotypes	0.013	0.026	0.148	0.087	0.172
	SNP1=rs2357481; SNP2=rs1076991; SNP3=rs3783732; SNP4=rs8003379; SNP5=rs1950902; SNP6=rs49022083; SNP7=rs2236225					

Table 3: Continued

Gene	Haplotypes	Frequencies in control subjects	Frequencies in stroke patients	Coefficient	Standard error	P-values
MTHFR	c c c a	0.271	0.260			
	c a c a	0.016	0.019	0.044	0.068	0.942
	c a c c	0.220	0.192	–0.029	0.024	0.419
	c c t a	0.017	0.016	–0.014	0.068	1.00
	t a c c	0.434	0.464	0.022	0.019	0.508
	Rare haplotypes	0.041	0.046	0.109	0.052	0.062
	SNP1=rs1801133; SNP2=rs1801131; SNP3=rs2274976; SNP4=rs4846049					
MTR	g a c a a	0.395	0.403			
	c a c a a	0.026	0.012	–0.109	0.052	0.064
	c a t a a	0.041	0.044	0.051	0.044	0.440
	c a t c c	0.329	0.352	–0.011	0.019	1.00
	c g t c c	0.165	0.169	–0.002	0.023	1.00
	g a t c c	0.015	0.003	–0.150	0.067	0.047
	Rare haplotypes	0.029	0.015	–0.093	0.061	0.232
	SNP1=rs4659725; SNP2=rs1805087; SNP3=rs2275566; SNP4=rs2853522; SNP5=rs6676866					
MTRR	a g c t g c a	0.235	0.241			
	a a c a g g t	0.013	0.013	0.043	0.073	1.00
	a a c t g c t	0.221	0.188	–0.046	0.025	0.127
	a a t t g c a	0.146	0.165	0.019	0.029	0.946
	a g c t g c t	0.020	0.012	–0.143	0.074	0.098
	a g t t g c a	0.210	0.203	–0.043	0.026	0.182
	c a c t g c t	0.129	0.139	–0.008	0.027	1.00
	Rare haplotypes	0.026	0.038	0.055	0.066	0.760
	SNP1=rs326118; SNP2=rs1801394; SNP3=rs1532268; SNP4=rs2303080; SNP5=rs10064631; SNP6=rs16879334; SNP7=rs8659					
NNMT	c c t t	0.515	0.511			
	c a a c	0.054	0.049	–0.041	0.045	0.683
	c a t c	0.060	0.085	0.063	0.036	0.149
	c a t t	0.062	0.051	–0.029	0.045	0.973
	c c a c	0.096	0.097	0.008	0.031	1.00
	c c t c	0.029	0.021	–0.032	0.072	1.00
	t a a c	0.015	0.023	0.055	0.099	1.00
	t c t c	0.010	0.013	0.029	0.113	1.00
	t c t t	0.145	0.135	–0.0016	0.032	1.00
	Rare haplotypes	0.013	0.015	0.174	0.173	0.593
	SNP1=rs566775; SNP2=rs10891640; SNP3=rs4646335; SNP4=rs3819100					
PON1	t a g	0.335	0.359			
	a a g	0.371	0.328	–0.035	0.019	0.130
	t g g	0.287	0.303	–0.003	0.020	1.00
	Rare haplotypes	0.006	0.009	0.082	0.119	0.889
	SNP1=rs854660; SNP2=rs662; SNP3=rs3917594					
PON2	c c	0.792	0.783			
	g g	0.205	0.208	0.022	0.020	0.490
	Rare haplotypes	0.002	0.009	0.522	0.176	0.005
	SNP1= rs11545941; SNP2= rs6954345					

Table 3: Continued

Gene	Haplotypes	Frequencies in control subjects	Frequencies in stroke patients	Coefficient	Standard error	P-values
SCL19A1	t c c	0.531	0.549			
	c t c	0.022	0.024	0.029	0.056	1.00
	c t t	0.421	0.407	–0.008	0.016	1.00
	t c t	0.019	0.010	–0.125	0.062	0.081
	Rare haplotypes	0.006	0.009	0.056	0.095	1.00
	SNP1=rs3177999; SNP2=rs1051266; SNP3=rs12659					
SHMT1	c g	0.573	0.578			
	g g	0.425	0.414	–0.021	0.021	0.574
	Rare haplotypes	0.0025	0.007	0.081	0.127	0.940
	SNP1= rs638416; SNP2=rs1979277					
TCNII	g c t g	0.338	0.338			
	a c t a	0.019	0.015	–0.024	0.069	1.00
	a c t g	0.039	0.024	–0.056	0.054	0.548
	a g t g	0.329	0.332	–0.0016	0.021	1.00
	g c t a	0.214	0.212	–0.024	0.026	0.663
	g g t g	0.050	0.051	–0.045	0.048	0.650
	Rare haplotypes	0.009	0.027	0.225	0.099	0.044
	SNP1=rs5749131; SNP2=rs1801198; SNP3=rs20721958; SNP4=rs10418					
TYMS	t c a	0.453	0.461			
	c a c	0.379	0.354	–0.009	0.018	1.00
	c c a	0.149	0.144	0.006	0.025	1.00
	t a c	0.015	0.022	0.011	0.070	1.00
	Rare haplotypes	0.002	0.018	0.218	0.089	0.028
	SNP1=rs502396; SNP2=rs16430; SNP3=rs8423*					

Among the haplotypes significantly associated with ischaemic stroke (Table 3), only the rare haplotypes of the BHMT gene [coefficient 22.48 (SE 4.86), p<0.0001] and the aa haplotype of the FOLH1 gene [coefficient 1.75 (SE 0.59), p=0.003] significantly modulated Hcy levels, whereas all the other haplotypes were not associated with increased or decreased Hcy levels.

Discussion

In this study we demonstrated that haplotypes in seven genes (BHMT, CBS, FOLH1, MTR, PON2, TCN2 and TYMS) coding for enzymes involved in the methionine metabolism are susceptibility factors for ischaemic stroke in a large cohort of patients who developed premature disease and that their association is only partially dependent on the role of haplotypes in modulating Hcy levels.

Due to the complexity of regulatory mechanisms of methionine metabolism, the contemporary evaluation of several polymorphisms in different genes was thought to be an efficient tool for understanding the genetic bases of stroke.

Haplotype analysis allowed us to identify alleles in which polymorphisms per se associated with the disease were included, but haplotypes helped to better identify subjects either at risk or protected: e.g. for FOLH1 gene, rs 202680 A allele frequency is 0.22 for controls and 0.36 for patients, whereas aa haplotype frequency is 0.064 for controls and 0.205 for patients. This issue could explain, at least in part, the frequent inconsistency of data observed also in wide study populations concerning the role of SNPs.

Moreover, even if haplotypes in seven genes were associated with an altered susceptibility to ischaemic stroke, only the miscellanea of rare haplotypes in BHMT, PON2, TCN2 and TYMS genes were associated with an increased risk of early-onset ischaemic stroke. This finding suggests that several different mutations of low frequency in these four genes might contribute to an increased risk of stroke by altering either their function or their expression.

A positive association between plasma Hcy and risk of stroke has been repeatedly demonstrated by case-control and cohort studies (4, 5), and our data are in keeping with these observations. At present, few data are available on stroke genetic susceptibility sustained by polymorphisms in genes known or hypothesised to modulate Hcy levels and/or to alter the methionine cycle. Among these polymor-

Table 4: Selection of some interactions between haplotypes in different genes obtained by haplotype interaction analysis.

	Region1						Region2					Stroke	CTR	OR	P-value
BHMT						**CBS**									**0.0296**
	T	T	A	A			C	C	G	G		0.219	0.268	0.76	
	T	C	A	T			C	C	G	G		0.032	0.051	0.62	
BHMT						**FOLH1**									**0.0012**
	C	C	A	A			A	T				0.049	0.067	0.72	
	C	C	A	A			A	A				0.021	0.004	5.99	
	C	C	T	A			A	A				0.057	0.017	3.54	
	C	C	A	T			A	A				0.019	0.008	2.47	
	T	T	A	A			A	T				0.167	0.246	0.61	
	T	T	A	A			A	A				0.054	0.020	2.82	
	T	C	T	A			A	A				0.017	0.006	2.97	
	T	C	A	T			A	T				0.036	0.049	0.71	
BHMT						**MTR**									**0.0071**
	C	C	T	A			G	A	C	A	A	0.133	0.109	1.25	
	C	C	T	A			C	G	T	C	C	0.051	0.039	1.31	
	C	C	A	T			G	A	C	A	A	0.067	0.046	1.49	
	T	C	T	A			G	A	C	A	A	0.048	0.027	1.85	
	T	T	A	A			C	A	C	A	A	0.005	0.011	0.41	
	T	T	A	A			C	A	T	C	C	0.059	0.112	0.50	
BHMT						**TCNII**									**0.0074**
	C	C	A	A			G	C	T	A		0.009	0.018	0.53	
	C	C	A	T			A	G	T	G		0.059	0.043	1.38	
	C	T	A	A			G	C	T	A		0.003	0.012	0.27	
	T	T	A	A			A	G	T	G		0.072	0.109	0.64	
	T	T	A	A			A	C	T	G		0.003	0.013	0.23	
	T	C	T	A			G	C	T	A		0.031	0.014	2.27	
	T	C	T	A			G	C	T	G		0.041	0.023	1.86	
BHMT						**TYMS**									**0.0119**
	C	C	A	A			C	C	A			0.005	0.014	0.38	
	C	C	T	A			T	C	A			0.154	0.123	1.30	
	T	T	A	A			T	C	A			0.114	0.140	0.79	
	T	T	A	A			C	C	A			0.032	0.047	0.67	
	T	T	A	A			C	A	C			0.081	0.112	0.70	
	T	C	A	T			C	A	C			0.020	0.030	0.65	
CBS						**FOLH1**									**0.0006**
	C	C	G	G			A	T				0.527	0.661	0.57	
	C	C	G	G			A	A				0.171	0.051	3.87	
	C	C	G	C			A	A				0.030	0.006	5.07	
CBS						**PON2**									**0.0197**
	C	C	G	G			G	G				0.163	0.199	0.78	
	C	C	G	C			C	C				0.156	0.092	1.83	
	C	C	G	C			G	G				0.018	0.029	0.62	

Table 4: Continued

	Region1						Region2					Stroke	CTR	OR	P-value
FOLH1						**MTR**									**0.0026**
	A	T				:	C	A	C	A	A	0.011	0.021	0.52	
	A	T					G	A	T	C	C	0.001	0.015	0.08	
	A	T					G	A	C	A	A	0.261	0.307	0.80	
	A	T					C	A	T	C	C	0.203	0.270	0.69	
	A	T					C	A	T	A	A	0.019	0.028	0.68	
	A	A					G	A	C	A	A	0.097	0.022	4.70	
	A	A					C	A	T	C	C	0.075	0.021	3.72	
	A	A					C	A	T	A	A	0.014	0.001	9.87	
	A	A					C	G	T	C	C	0.039	0.012	3.49	
FOLH1						**TCNII**									**0.0304**
	A	T					G	G	T	G		0.036	0.050	0.70	
	A	T					A	C	T	G		0.014	0.027	0.52	
	A	A					A	G	T	G		0.011	0.022	0.47	
	A	A					G	C	T	G		0.019	0.024	0.78	
	G	A					G	C	T	G		0.067	0.045	1.53	
FOLH1						**TYMS**									**0.0004**
	A	T					T	C	A			0.297	0.335	0.84	
	A	T					C	A	C			0.219	0.313	0.62	
	A	A					T	C	A			0.089	0.025	3.80	
	A	A					C	C	A			0.036	0.008	4.57	
	A	A					C	A	C			0.072	0.015	4.94	
MTR						**PON2**									**0.0053**
	C	A	C	A	A		C	C				0.016	0.022	0.73	
	G	A	T	C	C		C	C				0	0.015	0.02	
	G	A	C	A	A		G	G				0.058	0.076	0.76	
	G	A	C	C	C		C	C				0.025	0	291.48	
	C	G	T	C	C		G	G				0.046	0.029	1.58	
MTR						**TCNII**									**0.0114**
	C	A	C	A	A		G	C	T	G		0.007	0.012	0.57	
	G	A	C	A	A		G	C	T	G		0.160	0.132	1.25	
	C	A	T	C	C		A	C	T	G		0.004	0.011	0.36	
	C	A	T	A	A		G	C	T	A		0.003	0.012	0.21	
	C	A	T	A	A		G	C	T	G		0.016	0.008	1.97	
	C	G	T	C	C		G	C	T	A		0.042	0.024	1.76	
	C	G	T	C	C		A	G	T	G		0.078	0.049	1.64	
	C	G	T	C	C		G	C	T	G		0.061	0.047	1.34	
PON2						**TYMS**									**0.0054**
	G	G					C	A	C			0.050	0.082	0.59	

*= multiple testing correction has been applied via the minP approach (the p-value of the best marker/region configuration is corrected via Monte-Carlo simulations) (20, 21).

phisms, the most studied is the C677T in MTHFR gene (rs1801133) (22). Although several studies showed a trend toward an association between stroke and C677T or A1298C MTHFR polymorphisms, data are inconsistent (22, 23). Even if in our population an association between the 677T allele and increased levels of plasma Hcy levels was confirmed ($p<0.0001$, data not shown), C677T and A1298C polymorphisms were not associated with ischaemic stroke. Furthermore, the only MTHFR polymorphism initially associated with stroke (rs2274976), lost this association following adjustment for traditional cardiovascular risk factors. Also haplotype reconstruction analysis in the MTHFR gene showed no association between MTHFR haplotypes and stroke. On the other hand, only haplotypes of two (BHMT and FOLH1) out of seven genes associated with ischaemic stroke did modulate Hcy levels. The BHMT gene encodes a cytosolic enzyme that serves, next to methionine synthase, as a facilitator of methyl group donation for remethylation of Hcy into methionine. Genetic polymorphisms reducing BHMT function may in principle lead to hyperhomocyst(e)inaemia, but such a defect has not yet been observed. Our data demonstrate a role of BHMT gene in altering the susceptibility to stroke, perhaps by modulating Hcy levels. Because the FOLH1 gene encodes a type II transmembrane glycoprotein acting as a glutamate carboxypeptidase, an altered function of this enzyme may be associated with impaired intestinal absorption of dietary folates, resulting in low blood folate and hence to hyperhomocysteinaemia. The rs202680, found to be associated per se or in haplotype with ischaemic stroke, is a samesense polymorphism that shows no linkage disequilibrium with other known FOLH1 polymorphisms in coding and regulatory regions. The aa haplotype of the FOLH1 gene, that was found to be a strong independent risk factor for stroke, is also associated with increased Hcy levels.

Pertaining to the other genes, haplotypes that were associated with stroke did not change Hcy levels. These data suggest that haplotypes of genes of the methionine metabolism play an important role in stroke development independently of their role in influencing Hcy and Hcy-thiolactone levels (13) and that perhaps they act by altering DNA methylation, genetic stability, and gene expression (14). For instance the TYMS gene codes an enzyme that catalyses the methylation of deoxyuridylate to deoxythymidylate using 5,10-methylenetetrahydrofolate (methylene-THF) as a cofactor, a fundamental reaction to maintain the dTMP pool critical for DNA replication and repair. The PON2 gene encodes a protein ubiquitously expressed in human tissues that may act as a cellular antioxidant, protecting cells from oxidative stress.

Recently, by evaluating the same panel of polymorphisms, we demonstrated that haplotypes in AHCY, FOLH1, MTHFD1, MTR, NNMT, PON1 and TYMS genes are susceptibility factors for abdominal aortic aneurysm and that this association is independent from their role in modulating Hcy levels (17). The FOLH1 aa haplotype only was a risk factor for both ischaemic stroke and abdominal aortic aneurysm. Perhaps association of the FOLH1 gene with stroke and abdominal aortic aneurysm is part of the common atherosclerotic mechanisms that underlie the two diseases.

All gene associations found in abdominal aortic aneurysm are independent of the role of haplotypes in modulating Hcy levels, whereas in this study at least two haplotypes associated with ischaemic stroke did indeed modulate Hcy levels (BHMT rare haplotypes and FOLH1 aa haplotype). This issue seems of particular interest if we consider that ischaemic stroke is the only atherothrombotic disease for which the effect of folic acid supplementation on the risk of stroke is probably causal (24).

Due to the lack of information about vitamin status, our study cannot definitively establish whether susceptibility to stroke conferred by polymorphisms and haplotypes of genes involved in methionine metabolism are dependent from Hcy levels. On the other hand, these results are not influenced by folic acid food supplementation, that is not regularly implemented in Italy.

Patients enrolled in this study were stroke survivors referred to our attention at a median time interval between the ischaemic stroke and blood sampling of six months. Therefore, we cannot exclude a possible bias due to the loss of died or permanently disabled severe patients.

The present paper lacks of a data replication in an independent cohort. This issue represents a weakness in genetic association studies because results could not be confirmed in other populations.

What is known about this topic?

- A positive association exists between plasma homocysteine and risk of stroke.
- Randomised controlled trials performed to date have failed to demonstrate that lowering by means of B vitamins reduces the risk of stroke.

What does this paper add?

- This paper has identified novel genetic markers associated with premature stroke in seven genes involved in the homocysteine metabolic pathway.
- The previously recognised link between elevated total homocysteine and stroke may be mediated by upstream mechanisms involved in the homocysteine metabolic pathway.

Conclusions

In conclusion, these data have identified novel genetic markers of early-onset ischaemic stroke. The deregulation of the methionine metabolism due to polymorphisms/haplotypes and therefore the association with the premature ischaemic stroke is perhaps due not only to their effect on Hcy levels but also to the alteration of the numerous biological processes in which methionine metabolism plays a crucial role.

Acknowledgments

The authors thank Dr. Marco Ferrari for critical discussion on clinical data and Dr. Anna Evangelista for the helpful technical assistance.

References

1. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 1969; 56: 111–128.
2. Nygård O, Nordrehaug JE, Refsum H, et al. Plasma homocysteine levels and mortality in patients with coronary artery disease. N Engl J Med 1997; 337: 230–236.
3. Verhoef P, Hennekens CH, Malinow MR, et al. A prospective study of plasma homocysteine and risk of myocardial infarction in US physicians. J Am Med Assoc 1992; 268: 877–881.
4. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. Br Med J 2002; 325:1202–1206.
5. The Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. J Am Med Assoc 2002; 288: 2015–2022.
6. Wald DS, Wald NJ, Morris JK, et al. Folic acid, homocysteine, and cardiovascular disease: judging causality in the face of inconclusive trial evidence. Br Med J 2006; 333: 1114–1117.
7. Undas A, Brozek J, Szczeklik A. Homocysteine and thrombosis: from basic science to clinical evidence. Thromb Haemost 2005; 94: 907–915.
8. Lievers KJ, Kluijtmans LA, Blom HJ. Genetics of hyperhomocysteinaemia in cardiovascular disease. Ann Clin Biochem 2003; 40: 46–59.
9. Gellekink H, den Heijer M, Heil SG, et al. Genetic determinants of plasma total homocysteine. Semin Vasc Med 2005; 5: 98–109.
10. Sharma P, Senthilkumar RD, Brahmachari V, et al. Mining literature for a comprehensive pathway analysis: a case study for retrieval of homocysteine related genes for genetic and epigenetic studies. Lipids Health Dis 2006; 5: 1.
11. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995; 10: 111–113.
12. Weisberg I, Tran P, Christensen B, et al. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998; 64: 169–172.
13. Perla-Kajan J, Twardowski T, Jakubowski H. Mechanisms of homocysteine toxicity in humans. Amino Acids 2007; 32: 561–572.
14. Waterland RA. Assessing the effects of high methionine intake on DNA methylation. J Nutr 2006; 136: 1706S-1710S.
15. Rubattu S, Speranza R, Ferrari M, et al. A role of TNF-alpha gene variant on juvenile ischemic stroke: a case-control study. Eur J Neurol 2005; 12: 989–993.
16. Frezzato M, Tosetto A, Rodeghiero F. Validated questionnaire for the identification of previous personal or familial venous thromboembolism. Am J Epidemiol 1996; 143: 1257–1265.
17. Giusti B, Saracini C, Bolli P, et al. Genetic analysis of 56 polymorphisms in 17 genes involved in methionine metabolism in patients with abdominal aortic aneurysm. J Med Genet 2008; 45: 721–730.
18. Giusti B, Sestini I, Saracini C, et al. High-throughput multiplex single-nucleotide polymorphism (SNP) analysis in genes involved in methionine metabolism. Biochem Genet 2008; 46: 406–423.
19. Scheet P, Stephens M. A fast and flexible statistical model for large-scale population genotype data: applications to inferring missing genotypes and haplotypic phase. Am J Hum Genet 2006; 78: 629–644.
20. Becker T, Cichon S, Jonson E, Knapp M. Multiple testing in the context of haplotype analysis revisited: application to case-control data. Ann Hum Genet 2005; 69: 747–756.
21. Becker T, Schumacher J, Cichon S, et al. Haplotype interaction analysis of unlinked regions. Genet Epidemiol 2005; 29: 313–322.
22. Bersano A, Ballabio E, Bresolin N, Candelise L. Genetic polymorphisms for the study of multifactorial stroke. Hum Mutat 2008; 29: 776–795.
23. Sazci A, Ergul E, Tuncer N, et al. Methylenetetrahydrofolate reductase gene polymorphisms are associated with ischemic and hemorrhagic stroke: Dual effect of MTHFR polymorphisms C677T and A1298C. Brain Res Bull 2006; 11: 45–50.
24. Wang X, Qin X, Demirtas H, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 2007; 369: 1876–1882.