MODELLING OF INCREASED HOMOCYSTEINE IN ISCHAEMIC STROKE

Post-hoc cross-sectional matched case-control analysis in young patients

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ABSTRACT - *Background & Purpose:* Hyperhomocysteinaemia has been postulated to participate in pathogenesis of ischaemic stroke (IS). However, especially in young adults, there is possibility of significantly increased IS risk due to increased 'normal' homocysteinaemia, i.e., 'hidden' ('pathologically dormant') prevalence within a healthy, normally-defined range. We performed a *post-hoc* modelling investigation on plasma total homocysteinaemia (THCY) in gender- and age-matched young patients in the acute IS phase. We evaluated relationships between THCY and prevalence of other potential risk factors in 41 patients vs. 41 healthy controls. *Method:* We used clinical methods, instrumental and neuroimaging procedures, risk factors examination, total plasma homocysteine measurements and other laboratory and statistical modelling techniques. *Results:* IS patients and healthy controls were similar not only for matching variables, but also for smoking, main vitamin status, serum creatinine and lipid profile. Patients with IS, however, had lower vitamin B6 levels and higher THCY, fibrinogen and triglycerides (TGL). At multivariate stepwise logistic regression only increased THCY and TGL were significantly and independently associated with the risk for stroke (72% model accuracy, p_{model}=0.001). An increase of THCY with 1.0 μmol/L was associated with 22% higher risk of ischaemic stroke [adjusted OR=1.22 (95%CI 1.03÷1.44)]. In this way, novel lower cut-off value for HCY of 11.58 μmol/L in younger patients has been revealed (ROC_{AUC}= 0.67, 95CI% 0.55-0.78, p=0.009). *Conclusion:* The new THCY cut-off clearly discriminated between absence and presence of IS (sensitivity>63%, specificity>68%) irrespectively of age and gender and may be applied to better evaluate and more precisely define, as earlier as possible, the young patients at increased IS risk.

KEY WORDS: homocysteine, epidemiology, ischaemic stroke, young patient.

Aumento de homocisteína em acidente vascular cerebral isquêmico: análise *post-hoc* com casos controles em pacientes jovens

RESUMO - *Objetivo:* Hiperhomocisteinemia tem sido postulada como um dos fatores de risco na patogênese do acidente vascular cerebral isquêmico (AVCI). Todavia, em adultos jovens existe a possibilidade de aumento significativo de risco de AVCI devido a aumento "normal" da homocisteinemia, "oculta" (patologicamente adormecida) dentro de uma variação definida como normal. Neste trabalho foi investigado um modelo *post-hoc* de dosagem de homocisteina no plasma (HC) em pacientes jovens com AVCI agudo pareados por gênero e idade. Foi avaliado também relações entre HC e prevalência de outros fatores de risco para AVCI em 41 pacientes e 41 controles normais. *Método:* Foi utilizado exame clínico, procedimentos instrumentais e de neuroimagem, exame de fatores de risco, dosagem da homocisteína no plasma, outros exames laboratoriais e análise estatística. *Resultados:* Não foram encontradas diferenças quanto a presença de fumantes, dosagem de vitaminas, creatinina sérica e perfil lipídico entre os pacientes com AVCI e os controles normais. Todavia os pacientes com AVCI apresentaram diminuição de níveis de vitamina B6 e aumento de homocisteína, fibrinogênio e triglicerídios. A análise multivariada de regressão logís-

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tica mostrou diferenças significativas apenas para HC e trigliceridios independentemente associadas para fatores de risco para AVCI (72% acuracia, p= 0,001). Um aumento de homocisteína de 1,0 μmol/L estava associado com aumento de 22% de risco de AVCI [OR=1,22 (95%IC 1,03-1,44)]. Foi evidenciado portanto um novo valor de *cut-off* para HC de 11,58 μmol/L em pacientes jovens com AVCI (ROC auc=0,67, 95% IC 0,55-0,78, p= 0,009).   *Conclusão:* Este novo valor de *cut-off* para a homocisteína discrimina claramente a ausência ou presença de AVCI (sensibilidade >63%, especificidade >68%) independente do gênero ou idade e deve ser aplicado para uma melhor avaliação precoce de pacientes jovens com risco de AVCI.

PALAVRAS-CHAVE: homocisteína, epidemiologia, acidente vascular cerebral isquêmico, paciente jovem.

Hyperhomocysteinaemia has been postulated to participate in the pathogenesis of ischaemic stroke (IS)[1] with 19% lower stroke risk for 25% lower homocysteine levels (i.e., for 3 μmol/l), however, a clear cut-off value in young patients has not been reported. Recently, normal homocysteine levels in healthy young population with mean age under 40 were defined[2]. A previous study[3] has shown the association of increased levels of plasma homocysteine (HCY), triglycerides, fibrinogen and decreased levels of vitamin B6 levels in young patients with IS; only smoking and male gender were associated with increased HCY. Of note, the increased HCY has been suspected as IS risk factor – some studies revealed a dose-effect relationship with cerebrovascular disease (CVD), peripheral-vascular disease (PVD) or ischaemic heart disease, and other – of ischaemic stroke[4-7]. The increased total HCY (THCY) was shown to potentate atherogenesis and had thrombogenic effects[8]. Although sustained by acquired factors such as nutrition, lifestyle, associated diseases and medications, genetic factors may also contribute to independent increase of THCY[9-12]. Homocysteine is a sulphur amino acid of the methionine metabolism, being metabolised to methionine (with folate and vitamin B_{12}) or cystathionine (with pyridoxal-5'-phosphate). Both mechanisms are coordinated by S-adenosylmethionine, which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase (MTHFR) reaction and as an activator of cystathionine β-synthase (CBS). Protein disulfide-bound HCY accounts for >80% of THCY and the remaining is found as low molecular weight disulfide forms (i.e., symmetrical disulphide homocysteine and mixed disulphide homocysteine-cysteine). Less than 2% of HCY in circulation is present in free reduced form. The free thiol can undergo a reversible conversion to homocysteine thiolactone but it is present in very minor amounts in plasma, probably at nanomolar levels due to non-specific enzymatic hydrolysis[10].

We may suggest that when increased, the total HCY may represent, even within the normal reference range, an independent risk factor for cerebrovascular pathologies. Notably, it may be hypothesized that the amelioration of homocysteine levels may delay or even prevent the onset of stroke. However, most publications give a normal reference range from 5 to 15 μmol/L. Hyperhomocysteinaemia may arise from disrupted homocysteine metabolism and may be defined as moderate from 15 to 30 μmol/L, intermediate – from 30 to 100 μmol/L and severe – above 100 μmol/L[11-12]. Severe hyperhomocysteinaemia is due to rare genetic defects resulting in deficiencies in CBS, MTHFR, or in enzymes involved in methyl-B_{12} synthesis and homocysteine methylation. More frequent moderate hyperhomocysteinaemia, as seen in fasting conditions, is due to mild impairment in the methylation pathway (i.e. folate or B_{12} deficiencies or MTHFR thermolability). Post–methionine-load hyperhomocysteinaemia may be due to heterozygous CBS defect or B6 deficiency[6]. Most frequent are the heterozygous enzyme mutations leading to such folic acid, B_6 and B_{12} deficiencies with moderate to mild hyperhomocysteinaemia and increased risk of vascular diseases[13]. Low nutritional intake of folic acid, B_6 and B_{12}, renal diseases, some medications as well as advanced age do contribute to increased plasma THCY[14]. Increased fasting plasma THCY (e.g., by 5 μmol/L) may lead to double risk of vascular events and correlates strongly with carotid artery atherosclerosis[15-16]. However, especially in young adults, there is a possibility of significantly increased risk for ischaemic stroke due to an increased 'normal' homocysteinaemia, i.e., 'hidden' ('pathologically dormant') within the healthy, normally-defined range.

To formally test this possibility, we performed a *post-hoc* modelling investigation on the plasma THCY in 41 gender- and age-matched young patients in the acute phase of ischaemic stroke. We evaluated the relationship between THCY and the prevalence of other potential risk factors in 41 patients versus 41 healthy controls (41 matched pairs). The results of this post-hoc modelling approach formed the basis of the present report.

METHOD

Patients' selection – We studied 41 consecutive ischaemic stroke patients referred during the period 2002-2004 to the Clinic of Cerebrovascular Diseases who satisfied the
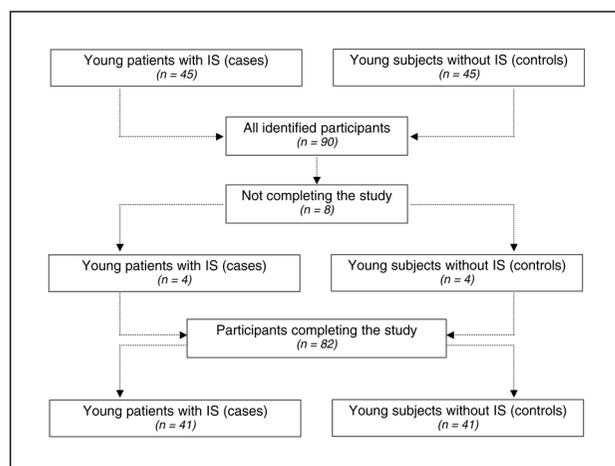
Fig 1. CONSORT flow chart of study design. IS, ischaemic stroke.

selection criteria and provided written informed consent according to the Declaration of Helsinki guidelines. Inclusion criteria were: age <55 years, occurrence of first ischaemic stroke, hospitalization up to 6 hours after the appearance of neurological symptoms and CT-scan-confirmed diagnosis of the ischaemic stroke. Exclusion criteria were: (i) diagnosis of: diabetes mellitus, renal and/or liver insufficiency, diseases of the thyroid gland, malignant tumours, psoriasis or thrombophilia; (ii) recent (up to 3 months back) intake of: folic acid, vitamin B_6, vitamin B_{12} or medications influencing their metabolism. The University Ethical Committee approved the study protocol.

Study design – Forty-five patients with ischaemic stroke were identified as cases (n=45). For each case, one healthy

subject without ischaemic stroke was identified as a control (1:1). The identified controls satisfied the same inclusion/exclusion criteria (n=45). Cases and controls were matched for gender and age (±5 years vs. cases). The subjects were matched by using a dedicated software program for random selection of controls, where each case was matched with the first control as identified on the basis of the criteria described above (Fig 1). After identification and matching, 8 subjects (4 cases and 4 controls) did not provide written informed consent and did not participate and complete the study. Out of 90 identified subjects, 41 cases and 41 controls entered the study.

Clinical and laboratory methods – Each case and control patient who agreed to participate and completed the study underwent an extensive physical and laboratory evaluation with application of a risk factors questionnaire. All relevant demographic, clinical, laboratory and risk factors data were reported in a case record form.

Clinical examination: History of the onset of the cerebrovascular accident and its development till the hospitalization, physical examination, mental status and neurological examination with assessment of neurological deficit (by modified Rankin scale[17]).

Instrumental and neuro-imaging procedures: 12-channel ECG, extra- and transcranial Doppler sonography and brain CT for confirmation of ischaemic stroke diagnosis.

Risk factors questionnaire: Questionnaire survey for presence or absence of known and most frequent cerebrovascular risk factors (arterial hypertension, smoking, dyslipidemia, previous heart diseases).

Total plasma homocysteine (THCY) assessment: Venous blood sampling for THCY determination as primary outcome parameter was performed with EDTA anticoagula-

Table 1. Main characteristics of young patients with ischaemic stroke (cases) and healthy subjects (controls).

Variable [unit]*	Healthy subjects (controls)	Ischemic stroke patients (cases)
Number (%)	41 (100)	41 (100)
Gender (%) [m/f]	31 (75.6) / 10 (24.4)	31 (75.6) / 10 (24.4)
Age [years]	45.6±7.5	46.4±7.4
Smoking (%) [yes/no]	27 (65.9) / 14 (34.1)	20 (48.8) / 21 (51.2)
Total homocysteine [μmol/L]	10.6±2.8	13.4±5.5°
Vit B_{12} [pmol/L]	184.4±75.9	187.4±114.8#
Vit B_9 - folates [nmol/L]	16.6±4.2	14.7±4.6
Vit B_6 [nmol/L]	47.2±36.8	27.5±10.5°
Fibrinogen [g/L]	3.06±0.6	3.6±1.1°
Serum creatinine [μmol/L]	85.6±13.9	87.2±21.3#
Total cholesterol [mmol/L]	5.3±0.9	5.4±0.9
HDL-cholesterol [mmol/L]	1.2±0.3	1.12±0.4
LDL-cholesterol [mmol/L]	3.5±0.9	3.5±0.8
Triglycerides [mmol/L]	1.2±0.5	1.6±0.6°

* Notes: Number or frequency (percentage) or mean ±SD, as appropriate; °$p<0.05$ vs healthy subjects by independent sample t-test / chi-square test. #Comparison with the log-transformed values of the patients with ischaemic stroke.

tion (Monovette, Sarstedt), early in the morning in fasting conditions, and preserved on dry ice within one hour. The blood was centrifuged on 4000 rpm for 15 min at 4ºC. After the centrifugation, the plasma was immediately separated from blood cells and stored at –20ºC until analysis. The HCY concentration was defined by modified and validated high performance liquid chromatography (HPLC) method with fluorescence detection based on the transformation of all HCY forms in a free thiol by reduction with sodium borohydride and derivatisation with bromobimane (intra-assay CV<3.4%, inter-assay CV<6.7%; linearity 0-200 μmol/l, r=0.9993; analytical recovery 96-98%). The analysis was performed by HPLC with fluorescence detection (Perkin Elmer, USA), analytical column Supelcosil LC18 at 150 mm x 4.6 mm x 5 μm (Supelco, USA).

Laboratory tests: Blood tests by Coulter STKS (USA); coagulation tests – prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen by Sysmex CA 6000 (Kobe, Japan); tests for glucose, urea, creatinine, total and HDL-cholesterol, triglycerides, liver enzymes (Konelab 60i, Finland), serum vitamin B_{12} and folic acid by MEIA with fluorescence – AxSYM (Abbott, USA); plasma concentration of pyridoxal-5-phosphate (main circulating form of vitamin B_6) – by HPLC with fluorescence (Chromsystems, Germany).

Sample size estimation – The sample size was calculated on the basis of an expected difference in the primary outcome variable (e.g., total plasma homocysteine concentration) between the cases with IS and controls (healthy subjects). Assuming a minimum average fasting THCY concentration of 12.45 μmol/L in young adults with IS and a minimum average difference of 1/3 (i.e., 33.3% or 4.15 μmol/L) between the cases and corresponding controls, it was estimated that to give the study an 80% power to detect such an expected difference as statistically significant at p<0.05, 40 patients per group had to be included. Having made a preliminary estimate of the prevalence of IS patients that would satisfy the inclusion/exclusion criteria of the study from all those referred to the Clinic of Cerebrovascular Diseases, it was predicted that 90 patients (45 cases and 45 controls) needed for the analyses should be identified throughout a screening period of about 24 months (estimated maximum 10% drop-out).

Statistical analyses – The characteristics of the cases and controls were assessed by methods of descriptive statistics, tests of normality and method of percentiles, and the two groups were compared by two-tailed independent sample Student's t-test, χ^2 test or Mann-Whitney test, as appropriate. All variables with complete datasets for each patient were included in the analyses. Prior to the analyses, the variables with skewed distribution were normalized by log-transformation. The associations among different variables listed in Table 1 and the ischaemic stroke were evaluated by univariate analyses. Correlation analysis was done by Pearson's r coefficient. Logistic regression analysis was applied by entry and backward stepwise methods with adjustment for covariate effects (logit link function with likeli-

hood ratio or conditional tests, as appropriate) to those variables that were significantly associated with the ischaemic stroke at univariate analyses, without potential confounders. Logistic curve estimation function was used to fit the regression models. All evaluations were done with SPSS software. Data are mean (S.D. or S.E.) or number and frequency (percentage), unless otherwise stated. The statistical significance of all tests was assumed at p<0.05.

RESULTS

Patients' characteristics – The main clinical and laboratory features of IS patients included in the present study are given in Table 1. As expected, since 41 healthy controls were matched to the 41 patients with IS, no differences in gender and age distributions were observed (31 males vs. 10 females, 45.6±7.5 vs. 46.4±7.4 years, respectively). Only 4 patients in either cohort denied their consent to study participation and were not included. Their characteristics were similar to those of included patients (Fig 1).

According to the clinical data, IS was most frequent in the region of the carotid system, the middle cerebral artery (71.2%), with subacute onset of the neurological deficit being also more frequent. The prevalence of arterial hypertension had been 68.3% while a cardiovascular pathology or ECG changes had been observed in 41.5%. The neurological examination had revealed typical abnormalities in the damaged region, e.g., hemipareses, motor/sensor aphasias. Only a small part of the IS patients (19.5%) had their neurological symptomatics disappearing till 12th day post-IS. Most frequently observed neurological impairments, mainly with paresis symptoms, had a Rankin score of 3 (41.5%). More than one-half of the IS patients had abnormal Doppler sonography findings, while the brain CT scan indicated abnormal findings in 68.3%, those being mainly vascular encephalopathies (46.3%); 28 strokes were lacunar (68.3%) and 13 (31.7%) – nonlacunar infarctions; also, two patients had leukoaraosis (not shown).

Comparative analyses – The IS patients and healthy controls were similar not only for the matching variables, but also for smoking, main vitamin status, serum creatinine and lipid profile (Table 1). Patients with IS, however, had lower vitamin B_6 levels and higher THCY, fibrinogen and triglycerides (TGL). At multivariate stepwise regression analysis (considering smoking and the rest of variables in Table 1 that were significantly associated with IS at univariate analysis), only the increased THCY and TGL were significantly and independently associated with the risk of stroke (Table 2). Of note, the solely increase of

Table 2. Logistic backward conditional regression analysis for prediction of ischaemic stroke (p_{model}=0.001; accuracy 72%).

Independent variable	Odds Ratio (95% CI)	p
Total homocysteine	1.22 (1.03÷1.44)	0.022
Triglycerides	2.59 (1.08÷6.24)	0.032

THCY with 1.0 μmol/L was associated with about 22% higher risk of ischaemic stroke [adjusted OR=1.22 (95% CI 1.03÷1.44)]. The multivariate model revealed the independent role of THCY within a positive non-linear logistic conditional relationship to ischaemic stroke with 72% accuracy (p_{model}=0.001). Since TGL appeared as a second independent predictor, to further investigate the established relationship, we stratified our total population into two sub-cohorts (low-TGL and high-TGL), according to the median of TGL (1.30 mmol/L). Our stratification confirmed the increased mean THCY level in IS cases vs. controls, independently of the TGL levels (i.e., 12.2 and 14.8 μmol/L, Fig 2).

We further produced a univariate logistic equation to fit THCY in order to examine its whole range and predict the whole range of IS probabilities (i.e.,

logistic curve model, p_{model}=0.0055, Fig 3A). The usual 0.5 cut-off of the model probability provided an overall correctness (accuracy) of 66% for the predictions, with sensitivity and specificity above 50% (THCY range 10-14 μmol/l). For this reason, to specify the best predictive cut-off for THCY, we further applied a receiving operator characteristics (ROC) curve analysis (Fig 3B). The ROC analysis confirmed the predictive power of THCY (ROC$_{AUC}$=0.67, 95CI% 0.55-0.78, p=0.009) with the best theoretical cut-off value = 11.58 μmol/L. This univariate consideration of THCY indicated even sensitivity above 70% at the range of cut-off values above 10.00 μmol/l. The THCY cut-off value of 11.58 μmol/L discriminated between the absence and presence of ischaemic stroke (sensitivity>63%, specificity>68%). Among the patients with a THCY ≥11.58 μmol/l, the prevalence of IS was almost 2-fold higher (67% vs. 35%, p=0.004) than in those with THCY below the newly established cut-off. According to the ROC model, there would be no patient without an ischaemic stroke with THCY ≥21.25 μmol/l while all patients with THCY ≤6.20 μmol/l would not suffer an ischemic stroke (so called zero risk, i.e., 100% safety value). Or, *vice versa,* all patients with
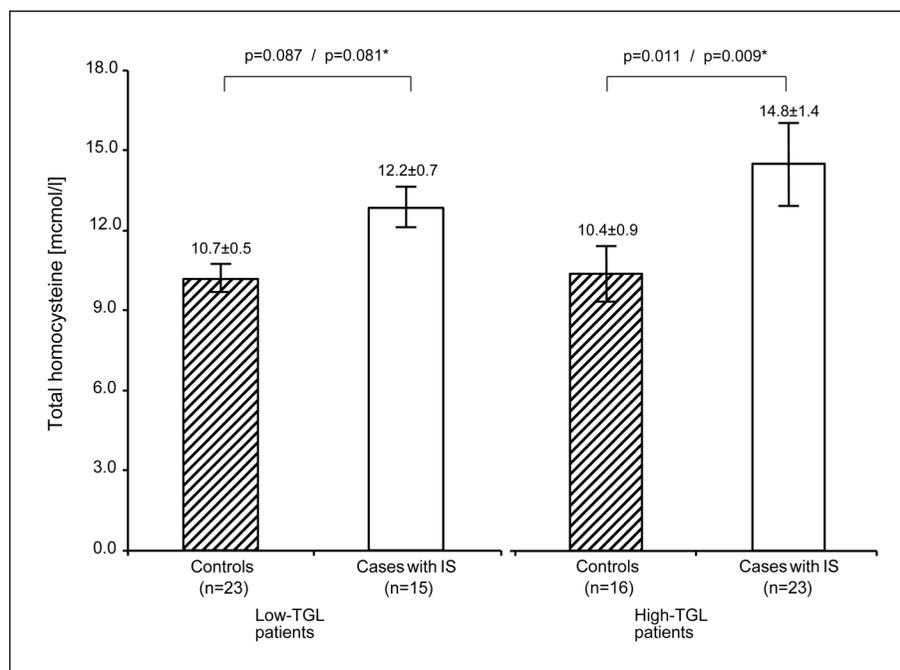

Fig 2. Total plasma homocysteine (THCY) in patients with ischaemic stroke (IS) and control subjects. The comparison was considered according to triglycerides (TGL) concentrations as classified below or above the median level (Me=1.30 mmol/l). Regardless of the TGL level, THCY is always higher in cases with IS than in controls. Left panel, Low-TGL patients (<Me); Right panel, High-TGL patients (≥Me); x-axis, Patient sub-groups (controls vs. cases with IS within low-TGL and high-TGL strata); y-axis, Total homocysteine [μmol/L]; Two-tailed independent sample t-test and Mann-Whitney test (*) of exact statistical significance.
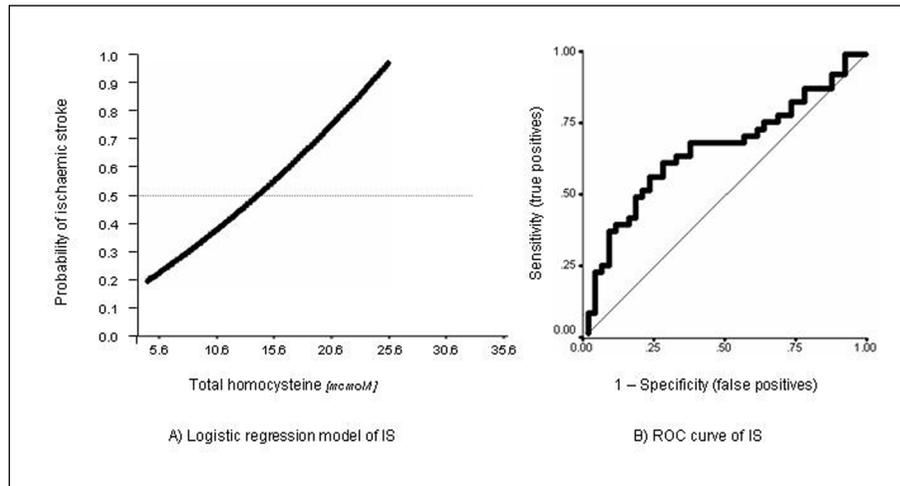
Fig 3. Models of ischaemic stroke (IS) and total plasma homocysteine (THCY) in young adults. (A) Probability of IS expressed as a nonlinear relationship along the THCY range ($F_{model}=8.16$, $p_{model}=0.0055$). x-axis, Total homocysteine [μmol/L]; y-axis, probability of IS (where 0.0=no IS event; 1.0=IS event); horizontal line, cut-off event probability of 0.5. (B) ROC curve of IS versus THCY: for a given THCY level, the ordinate values indicate the corresponding true-positive rate (fraction of IS patients with this THCY) and the abscissa values indicate the corresponding part of the false-positive rate (fraction of controls with this THCY). The inflection point of the curve was chosen as the optimal diagnostic value. The larger area between the ROC curve and the diagonal line reflects the higher degree with which the THCY parameter shows a predictive benefit. x-axis, 1-Specificity; y-axis, Sensitivity. Both estimates are expressed as a proportion of patients without or with IS events (i.e., from 0.00 to 1.00).

THCY<21.25 μmol/l have a probability different from 0 to have not or have not had an ischaemic stroke while all patients with THCY>6.20 have a probability (risk) different from 0 to have or have had an ischaemic stroke.

DISCUSSION

In this *post-hoc* analysis of a study cohort of young patients with ischaemic stroke and age- and gender-matched control subjects we modelled the predictive role of the total plasma homocysteine as an important cerebrovascular risk factor (CVRF). Notably, we addressed the relationship of THCY to the occurrence of stroke and established a new, lower cut-off value of 11.58 μmol/L in younger patients. Longitudinal studies are further needed to better clarify the role of the increased total plasma homocysteine, even still being within the usually accepted 'normal' range, in the IS pathogenesis and the potentials and settings for successful prevention by add-on medications and vitamin supplementation. Well-known and clinically defined conventional modifiable and non-modifiable CVRF are arterial hypertension, advanced age, smoking, hyperlipidaemia, co-morbid heart diseases, diabetes, overweight, etc. Recently, however, new important CVRF emerged such as increased THCY,

impaired fibrinolysis, infectious agents and inflammation, sleep-related respiratory disorders, etc.[18-21]. The increased THCY is due to folic acid deficits, mainly in aged patients (> 65 years) with hormonal disorders, impaired metabolism or decreased intake of vitamins and multiple CVRF[22].

The present study confirmed the patterns of a clinically established and instrumentally diagnosed ischaemic stroke in young patients in respect to its most frequent localizations, main focal neurological symptoms, vasculopathies and the prevalence of typical, previously known main CVRF (i.e., arterial hypertension, smoking). The relatively early atherosclerotic patterns in our young IS patients and the related pathological laboratory findings (Table 1) may be considered as modulators that potentate thrombogenesis and lower levels of vitamin B_6 as part of the homocysteine metabolic chain. We found statistically significant differences in some parameters (e.g., fibrinogen) between the IS cases and controls but only THCY and TGL maintained significant and independent role at the multivariate evaluation. It is possible that the increased THCY in more than one-half of our IS patients (e.g., 54%) be also explained by the role of vitamin B_{12} as being part of the THCY metabolism and/or by their eventually impaired renal

function[23-25] in such patients, however, no differences were found in neither vitamin B_{12} nor serum creatinine (Table 1). It was suggested that changes in the thiol redox status and relationships among vitamin B_{12}, vitamin B_6 and folates (vitamin B_9) may lead to increased THCY[26-27]. In our study, however, only vitamin B_6 was significantly lower in IS patients but neither smoking nor vitamin B_9, although different, reached statistical significance as compared to controls. It is quite probable that the younger age of our total study population may have played a role in 'masking' these interferences, if any.

In this sense, many reports[14,28] have shown that the deficit in the vitamin status may provoke moderate to mild hyperhomocysteinaemia which may correlate to the progression of atherosclerotic plaques and increased risk of cerebrovascular incidents[7,29], even in younger patients. For instance, Bushnell and collaborators[30] had found the younger age and hyperlipidaemia are independently associated with THCY metabolism. To note, our study has been balanced also for the latter factors since any differences were found for neither age (matched) nor lipid profile between IS patients and controls (Table 1). The established differences of THCY in our young patients have confirmed earlier results[31-33]. For instance, Parnetti and collaborators[34] had reported a higher mean value of 13.02±2.5 μmol/L for THCY in IS patients. Mizrahi and collaborators[8] established a correlation between THCY and conventional CVRF in older patients (mean age 71.2 years) and defined THCY as an independent risk factor; hypertensive patients with hyperhomocysteinaemia had been defined as high-risk group for stroke. Of note, there are discrepancies in reporting such conclusions – according to Meiklejohn and collaborators[35] the THCY levels increase only in the reconvalescent period in IS patients, however, most studies had suggested that THCY may be seen as independent CVRF[7,34,36-38]. As mentioned above, our current study reported that the risk role for THCY was independent also from fibrinogen, although the latter had increased risk-value levels of the estimates for both the mean (controls: 3.06 g/L; cases: 3.60 g/L, see Table 1) and odds ratio (OR$_{univariate}$ =1.99, p=0.023) that were comparable to those in other study populations such as PROCAM or Göteborg cohorts[39]. In our patients, in fact, we found a univariate correlation between THCY and fibrinogen (r=0.29, p=0.011) but the latter was excluded from the multivariate model by the backward procedures since its statistical significance reached a p-value of 0.126 only. It is possible that the effect of fibrinogen in our model had been "masked" by, either the fact that both sub-groups (controls and cases) had increased at-risk fibrinogen levels, or because of the stronger covariate role of triglycerides, or both. Notably, such independent role for THCY in IS risk is in agreement with earlier reports[36-39]. Thus, in these studies, even moderate hyperhomocysteinaemia was considered as important pattern in IS and concrete recommendations for THCY examination in patients with stroke, TIA and aortic atheromas were given. It was also shown that increased THCY levels may be related to worse IS prognosis and outcome[33,40].

According to accepted upper normal level for THCY of 15 μmol/L[41], significant differences in younger IS patients have been reported in previous studies[3,20]. Beyond the IS patients, according to the ECAP definition (THCY>12.1 μmol/L[11]), hyperhomocysteinaemia was defined in more than 30% of 153 healthy volunteers (THCY reference range 5.5-18.5 μmol/L, age 18-65 years, without vitamin deficit)[2]. Also, about 14% of 36 healthy control subjects had THCY>12 μmol/L (THCY range 5.0-17.3 μmol/L, age 40.9±9.9 years)[21]. Therefore, within the present post-hoc analysis, we decided to further search for a lower, potentially significant cut-off, within the usually accepted healthy 'normal' range of TCHY bellow 12.1 μmol/L[11]. Thus, by logistic fitting and ROC analysis, we have established in younger patients and suggest a novel and well-sensitive lower THCY cut-off value (11.58 μmol/L), irrespectively of age and gender, to better discriminate and more precisely define and predict correctly the patients at increased risk of IS. Moreover, in such populations with high background risk of IS as the Bulgarian one, a valid and reliable cut-off level for hyperhomocysteinaemia may have more relevant and informative clinical applications (although still surrounded with much controversy as new and modifiable risk factor for IS[42]) than usually applied 'normal' laboratory reference ranges.

Notably, we addressed the relationship of THCY to the occurrence of stroke and, by exploring a large cohort of age- and gender-matched younger patients, we established a better cut-off value of 11.58 μmol/L. Our finding is a very important contribution that more precisely indicates the presence of 'hidden' ('pathologically dormant') but increased risk prevalence within a usually-defined healthy range of 'normal' homocysteinaemia thus allowing better clinical application of preventive and treatment strategies in younger patient population.

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