Clinical Nutrition xxx (2017) 1–7

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

Relevance of plasma levels of free homocysteine and methionine as risk predictors for ischemic stroke in the young

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ARTICLE INFO

Article history:
Received 29 November 2016
Accepted 7 July 2017

Keywords:
Free homocysteine
Total homocysteine
Methionine
Premature ischemic stroke

SUMMARY

Background & aims: The debated vascular risk potential of total homocysteine (tHcy), due to failed clinical trials designed on B vitamin supplementation, raises many possible explanations like the higher risk potential of the deleterious, free form of homocysteine (fHcy) or, the unchecked confounding effects of B-vitamins in tHcy-based association studies. Additionally, the cardiovascular risk probability of altered status of the homocysteine precursor, methionine (tMet) could shed light on the causality of association between tHcy and cardiovascular diseases. Hence, we aimed to evaluate the risk associations of elevated plasma levels of tHcy, fHcy and low levels of tMet with premature, ischemic stroke.

Methods: We recruited 171 young, ischemic stroke patients (aged ≤45 years) and 249 age- and gender-matched healthy controls. Plasma levels of fHcy, tHcy, tMet and vitamin B6 were estimated using HPLC coupled with coulometric electrochemical detection. Plasma levels of vitamin B12 and folate were estimated by radioimmunoassay.

Results: Elevated fHcy (>2.9 μmol/L) was independently and strongly associated with the risk of premature, ischemic stroke (OR = 9.62, 95% CI = 3.51–26.40). On the contrary, association between premature ischemic stroke and elevated tHcy (>15.0 μmol/L) was found to attenuate when adjusted for vitamin B6 values (OR = 0.24, 95%, CI = 0.03–1.69). Interestingly, compromised B6-status (<59.2 nmol/l) was found to confer high risk of premature ischemic stroke (OR = 170.80, 95% CI = 58.22–501.06). We could not establish any significant correlation between fHcy and B-vitamin levels (P > 0.05). Low tMet (<13.86 μmol/L) was also not significantly associated with premature, ischemic stroke (OR = 2.53, 95% CI = 0.613–10.38).

Conclusion: Our results indicate significant but not-correlated, independent associations of fHcy and vitamin B6 with risk of premature, ischemic stroke. However, the causality of these associations need prospective and large scale validations. Further, our findings highlight the crucial confounding effects of B-vitamins on risk association between tHcy and premature ischemic stroke.

1. Introduction

Stroke occurring in young adults, which constitutes 10%–14% of total strokes [1,2], has severe socio-economic consequences particularly in developing countries like India. Current understanding of ischemic stroke etiology points towards atherosclerosis as a major contributing factor in young individuals [3]. This indicates a pressing need for the development of appropriate preventive strategies against modifiable and controllable, potentially atherogenic, risk factors such as homocysteine. Although there are studies implicating total homocysteine (tHcy) as a risk factor for ischemic stroke in the young [4,5], the potency and direct causative role of homocysteine in cardiovascular diseases has been doubted owing to failed clinical trials on homocysteine-lowering therapies (HLTs) in disease prevention [6–8]. Further, the doubted causality of hyperhomocysteinemia raises the question whether homocysteine is a biomarker or a risk factor for cardiovascular diseases [9].

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http://dx.doi.org/10.1016/j.clnu.2017.07.005
0261-5614/

Vitamin B supplementation has been considered the mainstay of HLT [10]. B vitamins, namely, vitamin B6 (pyridoxine/pyridoxal-phosphate), vitamin B12, and folate (vitamin B9) play crucial roles in the metabolism of homocysteine and when administered, result in effective lowering of homocysteine [10]. In particular, vitamin B6 and folate have been demonstrated to have regulatory cardiovascular risk potential independent of homocysteine [11,12]. We hypothesize that the debated causality of tHcy for cardiovascular diseases, point towards many possibilities such as confounding effects of B-vitamin deficiencies in tHcy-based association studies [5,13,14]. It is therefore necessary to control the confounding effects of nutritional deficiencies of vitamins B12, B6, and folic acid, when evaluating the cardiovascular risk associated with elevated homocysteine levels [11–14].

Further, the failed clinical trials on tHcy reduction for cardiovascular disease prevention, highlight the unattended higher vascular risk potential of the deleterious, free form of homocysteine (fHcy). Biologically, the potency of tHcy is fractionated into its protein-bound (70%) and the more potent, free, non protein-bound form (fHcy) (30%); the latter includes reduced homocysteine and homocysteine disulfides [15]. It has been demonstrated that the fHcy associates strongly with vascular endothelial dysfunction [16]. Despite its reported high vascular risk potential [16,17], there has been a lack of thorough assessments on the influence of elevated levels of fHcy on cardiovascular risk including ischemic stroke.

Mechanistically, the vascular pathogenicity of elevated homocysteine could derive from the intricately linked, impaired systemic methylation reactions [18]. The emerging role of epigenetic mechanisms in the pathophysiology of cardiovascular diseases support this view [19,20]. Altered status of methionine associated with impaired metabolism of homocysteine, has been considered an indicator of the compromised status of methyl metabolism [18]. We hypothesize that investigations on independent vascular risk potential of altered methionine status, could shed light on the causality of association between tHcy and cardiovascular diseases.

Hence, based on our hypotheses, we investigated the risk of premature ischemic stroke associated with the crucial metabolites of homocysteine metabolism, tHcy, fHcy and tMet while controlling the confounding effects of B vitamins.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee of the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India. Informed consent was obtained from all healthy and diseased volunteers.

2.2. Study participants

We investigated 171 young stroke patients aged ≤45 years, reporting to the Neurological Services of our hospital. Diagnosis of ischemic stroke was affirmed by cranial computed tomography scan and/or magnetic resonance imaging within 24 h of hospital admission. The Glasgow coma scale (GCS) was used to evaluate the conscious state of the stroke patients during admission [21]. Patients were excluded from the study if diagnosed of i) hemorrhagic stroke ii) infantile hemiplegia iii) neuro-infection with resulting infarction iv) disseminated intra-vascular coagulation v)hypothyroidism vi) renal or liver dysfunction vii) malignancy or any other terminal illnesses. A total of 171 patients were studied for duration of 2 years. The control group consisted of 249 healthy volunteers, free from any pre-existing vascular diseases and matched for age, gender, ethnicity and socioeconomic status.

2.3. Anthropometric and biochemical analyses

Demographic and anthropometric details, inclusive of variables: age, sex, smoking habits, alcohol use, presence of diabetes mellitus and hypertension were acquired from the study subjects. Fasting venous blood was withdrawn within 7 days of onset of ischemic stroke symptoms. Serum triglycerides (TG), total cholesterol (TC), and high density lipoprotein (HDL)-cholesterol concentrations were estimated. Lipid profile was measured on an automated, random access, clinical chemistry analyzer (Olympus AU640, Olympus Singapore Pvt. Ltd., Singapore) using commercial kits (Beckman Coulter, Inc., Clare, Ireland). Total cholesterol was estimated by enzymatic method based on combined actions of cholesterol esterase and oxidase [22], (assay kits, OSR 6116 and 6216). Triglyceride measurement was based on a series of coupled enzymatic reactions including hydrolysis by a combination of microbial lipases [assay kit, OSR 60118] [23]. HDL-C was estimated by an immune-inhibition method (assay kit, OSR 6187). Concentration of low density lipoprotein (LDL)-cholesterol was determined using Friedewald's formula [24]. Levels of vitamin B12 and folic acid were assayed by radioimmunoassay using commercially procured kits (SimulTRAC-SNB, MP Biomedicals, USA).

2.4. Assessment of plasma tHcy, fHcy, tMet and vitamin B6 by high-performance liquid chromatography

Plasma tHcy, fHcy and tMet were measured by reverse-phase high-performance liquid chromatography (HPLC) and coulometric electrochemical detection using a Shimadzu HPLC system (LC-10ADVP, Shimadzu Corporation, Japan) provided with an auto sampler (SIL-HT_A) and a ESA Coulochem III detector (ESA Ins, Chelmsford, MA) [25]. Chromatographic separation was conducted at a flow rate of 1.0 ml/min and a pressure of 120–140 kg/cm^2 (1800–2100 psi), using a reverse phase C18 Supelco column (5 μ, 4.6 × 250 mm, Shimadzu). The active form of vitamin B6 in plasma, pyridoxal 5′phosphate (PLP) was also assayed by reverse-phase HPLC and coulometric electrochemical detection [26].

2.5. Statistical analysis

Data were analyzed using SPSS (V16.0) program. The differences in the distribution between patients and controls, of continuous variables with normal and skewed distributions, were tested by paired 't' test and Mann–Whitney *U* tests, respectively. All P values were 2-sided. Cut offs for elevated tHcy (≥15.00 μmol/l) and fHcy (≥2.90 μmol/l) were defined according to previous reports [27]. Low plasma tMet status was defined based on cut off of ≤13.86 μmol/l (10th percentile values in control population). Cut offs of elevated tHcy/tMet and fHcy/tMet were also based on 10th percentile values in controls (Table 2). Vitamin B6 deficient and suboptimal statuses were defined according to the recommended cut off values of 20 nmol/l [28] and 30 nmol/l [29] respectively. Stroke risks conferred by plasma levels of different total homocysteine, its free form and methionine levels were evaluated by logistic regression analyses. Multivariate analyses were constituted of covariates such as age, gender, smoking, alcohol consumption, hypertension, diabetes mellitus, lipid levels and B vitamin levels (vitamins B6, B12 and folate). Hypertension was characterized as diastolic blood pressure ≥90 mmHg and/or systolic blood pressure ≥140 mmHg [30] and/or use of antihypertensive medication. Diabetes was diagnosed on the basis of fasting plasma glucose values >126 mg/dl or the subjects' self-reported history of diabetes or use of anti-diabetic medication [31]. Coefficients of correlation were calculated using Pearson's/Spearmann's correlation analysis. Logistic regression analyses were carried out to test statistical

Please cite this article in press as: Rudreshkumar KJ, et al., Relevance of plasma levels of free homocysteine and methionine as risk predictors for ischemic stroke in the young, Clinical Nutrition (2017), http://dx.doi.org/10.1016/j.clnu.2017.07.005