Hyperhomocysteinemia, intima-media thickness and C677T MTHFR gene polymorphism: A correlation study in patients with cognitive impairment

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A B S T R A C T

Objective: Intima-media thickness (IMT) seems associated with risk of Alzheimer disease (AD). Homocysteine (hcy) is a risk factor for vascular diseases and dementia. This study aimed at investigating the possible relationship among IMT, plasma hcy and C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism in relation to cognitive status.

Methods: Sixty-three patients with cognitive impairment and 64 controls underwent biochemical, neuropsychological and carotid ultrasound assessment.

Results: After age and folate adjustment, plasma hcy correlated with both Mini Mental State Examination (MMSE) score (r = −0.7, p < 0.01) and IMT (r = 0.7, p < 0.01). Among patients with cognitive impairment, carriers of TT677 MTHFR genotype had plasma hcy (p < 0.001) and IMT (p < 0.01) values higher than non-carriers.

Furthermore, multiple regression analysis showed that MMSE scores were associated with plasma hcy (β = −0.3, p < 0.01), IMT (β = −0.3, p < 0.01) and TT677 MTHFR genotype (β = −0.3, p < 0.01). Structural equation modelling showed that the relation between hcy levels and MMSE score was partly direct (parameter estimate = −0.6; p < 0.01) and partly mediated by IMT values (parameter estimate = −0.4; p < 0.03). Finally, IMT resulted associated with hypertension (parameter estimate = 0.8; p < 0.0001).

Conclusion: Our findings suggest that TT677 MTHFR genotype promotes plasma homocysteine increase which in turn may favour intima-media thickening in patients with cognitive impairment. Hcy may promote neuronal damage through multiple mechanisms, including a micro-vascular damage, mediated by IMT increase, and a direct neuro-toxic effect.

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1. Introduction

Atherosclerosis is a risk factor for both vascular dementia and Alzheimer disease (AD) [1,2]. Cross-sectional and case-control studies demonstrated that, among other vascular risk factors, elevated plasma levels of homocysteine (hcy) are associated to AD susceptibility [3]. Additionally, in non-demented elderly population, hcy plasma levels are inversely associated with global cognitive performances and specific cognitive skills [4].

Homocysteine results from de-methylation of the S-adenosil-methionine [5]; metabolism of hcy into methionine occurs via one of two re-methylation cycles, whose rate-limiting enzymes are methylenetetrahydrofolate reductase (MTHFR) and betaine hcy methyltransferase. Alternatively, hcy can be metabolized to cysteine via cystathionine betasynthase. MTHFR requires folate and vitamin B12 as cofactors, while cystathionine betasynthase requires vitamin B6 [5].

Homocysteine plasma levels vary widely in the general population and can be affected by a number of factors, including folate and vitamin B12 [6]. Plasma hcy can also be influenced by the polymorphism of the MTHFR gene characterized by a base substitution from C to T on residue 677. T677 allele occurs in 35% of caucasian populations, with up to 10–20% TT homozygotes [7]. Carriers of the TT677 MTHFR genotype frequently exhibit elevated plasma hcy
and, consequently, they are considered at risk for vascular disease and AD [6]. Previous findings in healthy subjects showed a positive correlation between plasma hcy and intima-media thickness (IMT), a marker of systemic atherosclerosis, without any additional effect of TT677 MTHFR genotype [8]. Observations in patients with neurodegenerative disorders are limited [9].

The Rotterdam study demonstrated an association between increased IMT and risk of AD, suggesting that arterial wall thickening might be a marker of cerebro-vascular dysfunction [1]. Recently, a link between progression of carotid atherosclerosis, i.e. IMT increase, and cognitive decline was demonstrated [2]. Despite of these acquisitions, the role of hyperhomocysteinemia on atherosclerosis and AD is not fully understood.

The present study aimed at investigating if hcy plasma levels are associated with IMT values in patients with cognitive decline (AD or amnestic mild cognitive impairment, MCI) and in nondemented controls. Since MTHFR C677T polymorphism might have additional or synergistic effects on the atherosclerotic process, we also investigated the possible effects of this genetic risk factor for hyperhomocysteinemia on the atherosclerotic processes and cognitive decline.

2. Patients and methods

2.1. Selection of the subjects

Sixty-three patients with cognitive impairment (45 AD, 18 MCI), were recruited for this study on the basis of the following selection criteria: (1) diagnosis of AD according to NINCDS-ADRDA criteria or (2) of amnestic MCI according to Petersen's criteria [10].

Sixty-four volunteers with no clinical evidence of neurologic disease, matched for age and sex, were also enrolled in this study. All controls denied cognitive symptoms and were screened for cognitive deficit.

Exclusion criteria for both patients and controls were: (1) major vascular disorders (i.e. stroke, coronary ischemic disease, venous thrombosis); (2) chronic alcohol intake or smoking; (3) use of drugs increasing plasma hcy (i.e. phenitoin, levodopa, methotrexate); (4) vitamin supplements intake.

All subjects underwent neuro-psychological evaluation, color-coded duplex examination of neck vessels, biochemical assays, MTHFR C677T genotyping.

The study protocol was approved by our Local Ethic Committee. Signed informed consent was obtained from caregivers and nondemented participant.

2.2. Neuro-psychological evaluation

The Italian version of the Mini Mental State Examination (MMSE) [11] was used as a cognitive screening. Subsequently all recruited subjects were tested with the Mental Deterioration Battery [12], a neuropsychological instrument validated in Italian population.

2.3. IMT assessment

Patients and controls underwent a color-coded duplex examination of neck vessels (IU 22 Philips, Bothell, WA, USA). As previously described [2], IMT was evaluated on the common carotid arteries (CCAs) over ≈1.5 cm proximal to the flow divider, according to standardized guidelines [13]. In brief, IMT was measured at the thickest plaque-free point on the near and far walls with a specially designed computer program. CCA wall thickness was defined as the mean of the maximum wall thickness of the near and far walls bilaterally. The neurosonologist was unaware of the subjects’ clinical characteristics.

2.4. Biochemical measures

Fasting blood samples were collected in the morning at the time of the neuropsychological examination. Plasma was separated according to standard procedure for hcy measurement and was immediately frozen at −80 °C, together with the serum samples. Plasma hcy was determined by HPLC with fluorometric detection, as previously described [14]. t-Homocysteine was used as the external standard and N-acetylcysteine as the internal standard. Plasma folate and vitamin B12 were determined by radioimmunoassay (BioRad Quantaphase II kit; BioRad, Hercules, CA).

Levels of total and LDL cholesterol, triglycerides, creatinine and TSH were performed using commercially available kits (Horiba ABX, Montpellier, France). Our laboratory ranges were <14 μmol/l for hcy, 3–17 ng/ml for folate, 149–986 pg/ml for vitamin B12, 0–200 mg/dl for total cholesterol, 0–180 mg/ml for LDL cholesterol, 0.5–1.1 mg/dl for creatinine, 0–160 mg/dl for triglycerides, 0.4–4 μU/l for TSH.

2.5. MTHFR C677T genotyping

The detection of C677T polymorphism was performed using double-gradient density-acrilamide gel electrophoresis following standard protocol [14].

2.6. Statistical analysis

In the statistical analysis AD and MCI subjects were grouped and considered as cognitive impaired patients (CIP). Continuous variables (biochemical measures, IMT, MMSE scores and age) were analyzed using Student’s t-test between patients and controls, while categorical factors (distribution of sex and MTHFR genotypes) and compliance of Hardy–Weinberg equilibrium for MTHFR genotypes, using chi-square test.

Effect of sex and age on plasma hcy and IMT were evaluated with an analysis of covariance (ANCOVA), including also folate, vitamin B12, creatinine and TSH as covariates (only when plasma hcy was the dependent variable). A forward selection method was used to reduce the number of independent variables included in the final model. After likelihood ratio calculation a variable was excluded if $p \geq 0.1$. In ANCOVA analyses, compliance to normal distribution of residuals was carried out by Shapiro–Wilks test.

Differences in plasma hcy and IMT between patients and controls, grouped according MTHFR genotypes or clinical diagnosis, were evaluated by one-way analysis of variance (one-way ANOVA). Post-hoc analysis within each ANCOVA and one-way ANOVA was carried out with Bonferroni’s adjustment.

Grouping patients and controls, correlations among biochemical parameters, age, IMT and MMSE were carried out by calculation of Pearson’s product-moment coefficients.

The effect of hypertension, diabetes, dyslipidemia and TT677 MTHFR genotype on IMT and MMSE were explored using linear regression analysis.

First, IMT was regressed according to the presence/absence of conventional vascular risk factors, TT677 MTHFR and plasma hcy. Subsequently, these putative predictors and IMT were included in another regression design with MMSE as dependent variable. To assess whether IMT is an intermediate factor associated with cognitive decline, a structural equation modelling (SEM) with a asymptotically distribution free Gramian procedure was performed, considering conventional vascular risk factors and TT677 MTHFR as exogenous variables, and hcy plasma levels, IMT and MMSE as endogenous variables.

To reduce the number of independent variables in SEM analysis and in both regression models, only those significantly associated with IMT and/or MMSE were included, testing one variable at the time, using simple linear regression.
In the bivariate correlations and in the regression models, MMSE values were ln transformed to optimize the model-fit. All values were considered significant according a threshold of $p < 0.05$.

Most statistics were well powered: referring to a type I error of 0.05, our ability to reject the null hypothesis was >0.8. In particular, observed power for the two-way ANCOVA = 0.9; observed power for IMT-regression model = 0.88; observed power for MMSE-regression model = 0.9.

All the analyses were carried out using Statistica V.6 (Statsoft, USA).

3. Results

Table 1 summarizes clinical, biochemical and genetic variables. No differences in age, sex distribution, lipid profile, vitamin B12, creatinine and TSH levels were observed between the two groups. In contrast, the statistical analysis showed significant differences in IMT, hcy, folate plasma levels and distribution of TT677 MTHFR genotype between patients and controls (Table 1). The relatively small size and the similar prevalence of CT677 MTHFR genotype in the two groups, did not allow to identify a significant $3 \times 2$ association among MTHFR genotypes and the groups ($\chi^2 = 4.95$, df = 2, $p = 0.08$). However, the contrast between presence and absence of TT677 MTHFR clearly indicated an association between this genotype and CIP group.

Moreover, in patients and controls, MTHFR genotypes were compliant to Hardy–Weinberg equilibrium ($\chi^2 = 2.66$, $p = 0.1$ and $\chi^2 = 0.28$, $p = 0.6$, respectively).

Forward stepwise selection excluded from the final ANCOVA model the following variables: sex ($L = -336.9$, $p = 0.3$); TSH ($L = -336.8$, $p = 0.3$); creatinine ($L = -336.4$, $p = 0.6$); vitamin B12 ($L = -336.7$, $p = 0.4$). ANCOVA found an association of plasma hcy with cognitive impairment ($p < 0.0001$), age ($p = 0.04$) and plasma folate ($p < 0.0001$). In this analysis residuals were compliant to normal distribution ($\text{Shapiro–Wilks} = 0.98$, $p = 0.4$).

When testing whether increased IMT values and plasma hcy were associated with cognitive impairment, we found that both IMT values and plasma hcy correlated with MMSE score, even after controlling for age ($r = -0.74$, $p < 0.001$ and $r = -0.7$, $p < 0.001$, respectively: Figs. 1 and 2). In addition, IMT and plasma hcy resulted positively correlated ($r = 0.7$, $p < 0.001$), while no significant effect was found for triglycerides, LDL and total cholesterol concentrations on IMT.

After adjustment for age and plasma folate, one-way ANOVA revealed a significant difference of plasma hcy and IMT between CIP and controls grouped according to MTHFR genotypes (Table 2). Post-hoc analysis demonstrated that CIP carriers of MTHFR TT677 genotype had the highest values of plasma hcy and IMT (Table 2).

Within the CIP group, simple linear regression demonstrated that both IMT and MMSE scores were significantly associated with plasma hcy ($\beta = 0.6$ and $\beta = -0.7$, respectively; $p < 0.0001$), presence of TT677 MTHFR genotype ($\beta = 0.5$, $p < 0.0001$ and $\beta = -0.6$, $p < 0.0001$, respectively) and hypertension ($\beta = 0.4$, $p < 0.01$ and $\beta = -0.3$, $p = 0.03$, respectively). These variables were included in a multiple regression model that confirmed the presence of a significant association of plasma hcy, TT677 MTHFR genotype and hypertension with IMT (Table 3). Furthermore, multiple regression demonstrated a significant effect of plasma hcy ($\beta = -0.4$, $p = 0.001$) and of TT677 MTHFR genotype ($\beta = -0.4$, $p = 0.003$) on MMSE scores, while hypertension showed a borderline association ($\beta = -0.2$, $p = 0.06$). After including IMT in this regression model, the partial regression coefficients of all the other predictor vari-

### Table 1

<table>
<thead>
<tr>
<th>Demographic, clinical, biochemical and genetic profile of patients and controls.</th>
<th>Patients ($n = 63$)</th>
<th>Controls ($n = 64$)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>$75.7 \pm 9.2$</td>
<td>$75.7 \pm 8.6$</td>
<td>0.9</td>
</tr>
<tr>
<td>M (n; %)</td>
<td>26; 41.3</td>
<td>23; 35.9</td>
<td>0.8</td>
</tr>
<tr>
<td>MMSE</td>
<td>$18.3 \pm 5.0$</td>
<td>$27.3 \pm 1.8$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypertension (n; %)</td>
<td>26; 41.3</td>
<td>23; 35.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Diabetes mellitus (n; %)</td>
<td>8; 12.7</td>
<td>12; 18.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Dislipidemia (n; %)</td>
<td>23; 36.5</td>
<td>24; 37.5</td>
<td>0.6</td>
</tr>
<tr>
<td>IMT</td>
<td>$0.93 \pm 0.14$</td>
<td>$0.75 \pm 0.11$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Biochemical data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hcy (µmol/l)</td>
<td>$19.1 \pm 4.4$</td>
<td>$13.3 \pm 3.5$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>$0.9 \pm 0.2$</td>
<td>$0.9 \pm 0.2$</td>
<td>0.4</td>
</tr>
<tr>
<td>Folate (ng/ml)</td>
<td>$5.2 \pm 1.6$</td>
<td>$5.9 \pm 1.7$</td>
<td>0.02</td>
</tr>
<tr>
<td>Vitamin B12 (mg/dl)</td>
<td>$428.4 \pm 152.7$</td>
<td>$473.9 \pm 164.5$</td>
<td>0.09</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>$118.3 \pm 50.1$</td>
<td>$118.6 \pm 48.5$</td>
<td>0.8</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>$144.7 \pm 24.8$</td>
<td>$143.4 \pm 26.2$</td>
<td>0.8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>$186.4 \pm 47.4$</td>
<td>$188.4 \pm 54.9$</td>
<td>0.9</td>
</tr>
<tr>
<td>TSH (mUI/l)</td>
<td>$1.6 \pm 0.7$</td>
<td>$1.4 \pm 0.8$</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Genetic data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT677 MTHFR allele frequency (%)</td>
<td>51.6</td>
<td>37.5</td>
<td>0.02</td>
</tr>
<tr>
<td>CT677 MTHFR genotype (%)</td>
<td>25; 39.7</td>
<td>24; 37.5</td>
<td>0.6</td>
</tr>
<tr>
<td>CT677 MTHFR genotype (%)</td>
<td>20; 31.7</td>
<td>10; 15.9</td>
<td>0.03</td>
</tr>
</tbody>
</table>

M, male; MTHFR, methylenetetrahydrofolate reductase; hcy, homocysteine; IMT, intima-media thickness.
The association between AD and hyperhomocysteinemia is also corroborated by experimental data demonstrating a relationship between elevated plasma hcy and increased β-amyloid deposition in cultured neurons and in the brain of transgenic models of AD [5,19].

Similarly, in autopsy series, an increased frequency of neuritic plaques was observed in AD patients with concomitant atherosclerosis in the Willis polygon, independently of the presence of ischemic lesions [20]. These findings are in line with neuro-radiological studies evidencing a relation between plasma hcy and whole brain and temporal atrophy in presence of minor cerebro-vascular damages not accounting alone for the amount of neuronal loss [21,22]. Moreover, in subjects with coronary heart disease, elevated levels of hcy were associated with a decreased concentration of cerebral N-acetyl-aspartate, a specific marker for neuronal damage, suggesting that hcy has a direct neuro-toxic effect [23].

Our study shows also that TT677 MTHFR genotype exerts its effect on IMT by increasing plasma hcy, even after controlling for potential confounders such as age, other cardiovascular risk factors, and creatinine or folate/vitamin B12 levels. Differently from other studies that failed to detect a significant effect of MTHFR genotypes on IMT, in our study cases and controls presented similar cardio-vascular risk profiles, outlining a very homogeneous population. Moreover, the frequency of T677 MTHFR allele is higher in Italy than in other countries [7]. In particular, while TT677 MTHFR genotype was present in 8% of cases screened by Kelemen et al. [8] we identified this genotype in 31.7% of our patients. This different genetic distribution should be taken into account for a better understanding of our data with respect to previous reports. Additionally, our results are in line with those of Passaro et al. [24], demonstrating

Fig. 3. Path diagram indicating relations among hypertension, MTHFR TT677 genotype, plasma homocysteine, intima-media thickness and MMSE. \( ^* p < 0.0001, ^{†} p = 0.01, ^{‡} p = 0.03 \).
an increase of IMT in relation to TT677 MTHFR genotype in Italian elderly women, and with a meta-analysis reporting an increased IMT in subjects with this genotype [25].

The higher frequency of TT677 MTHFR genotype in our cohort, together with the exclusion of subjects taking vitamin supplements, could also explain why in our sample, plasma hcy was not influenced by vitamin B12, previously described as a determinant of plasma hcy in an AD population [18].

Moreover, we observed a significant association between hypertension and IMT increase but no direct association between hypertension and MMSE score. The relationship between cognitive functions and blood pressure is controversial. In fact, cross-sectional studies showed conflicting results on the association between hypertension and cognitive impairment [26]. In our patients, hypertension showed a slightly significant association with MMSE in simple linear regression analysis. This effect disappeared when hypertension was included in multiple regression analysis, together with IMT, plasma hcy and presence of TT677 MTHFR genotype. This finding could be explained by a possible indirect influence of hypertension on MMSE mediated by atherosclerosis.

SEM analysis supports this hypothesis demonstrating a relation between hypertension, degree of intima-media thickening and severity of cognitive decline. An increased IMT may represent a marker of atherosclerosis also for the cerebral microcirculation. An altered microcirculation might produce diffuse hypoperfusion and chronic hypoxia, ultimately triggering neurodegenerative changes [27].

In our patients, as demonstrated by SEM analysis, the relation between plasma hcy and MMSE score is explained by two casual links: (i) plasma hcy directly affects cognitive status; (ii) the effect of hcy levels on MMSE is partially mediated by the influence of IMT increase on cognitive performances. In particular, we suggest that TT677 MTHFR genotype might identify a particular subgroup of AD patients wherein hyperhomocysteinemia, having a direct neuro-toxic effect and favouring a subclinical micro-vascular damage, may interact with neurodegenerative processes in the development of cognitive decline.

The main limitation of our investigation is the small sample size of the screened cohort, though the statistical power analysis showed a good compliance of number of cases screened for the study design. Moreover, being a case-control study we could not assess the temporal relation between hcy levels, IMT, and MMSE.

5. Conclusions

Our findings suggest that TT677 MTHFR genotype promotes plasma homocysteine increase which in turn may favour intima-media thickening in patients with cognitive impairment. In addition, the results of this study suggest that hcy may promote neuronal damage through multiple mechanisms, including a micro-vascular damage, mediated by IMT increase, and a direct neuro-toxic effect.

Although hcy is determined by a large number of factors, it is a stable measure with little intra-individual variation. Thank to this property, IMT assessment might be a useful measure to select a sub group of cognitively impaired subjects at increased risk for micro-vascular brain injury precipitating the underlying neurodegenerative phenomena.

References