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ALZHEIMER'S DISEASE IN BRAZILIAN ELDERLY HAS A RELATION WITH HOMOCYSTEINE BUT NOT WITH MTHFR POLYMORPHISMS

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ABSTRACT - *Objective:* To investigate the association between total plasma homocysteine concentration, C677T and A1298C polymorphisms in MTHFR gene and Alzheimer´s disease (AD) development. *Method:* Forty-three patients with probable (63%) and possible (37%) AD and 50 non-demented controls were evaluated. Groups did not differ as to gender, age, scholar years, diabetes, alcohol and coffee intake and physical activity. Total plasma homocysteine (Hcy) levels were determined by HPLC and genotyping for MTHFR by PCR/RFLP. Mann-Whitney "U" test was used to compare quantitative variable, Fisher-Freeman-Halton test to compare genotypes and allele proportions and Chi-square test to other qualitative variables. *Results:* AD patients presented higher total plasma Hcy levels than controls and the difference was statistically significant. No differences in the C677T and A1298C MTHFR polymorphisms distributions were found between patients and controls. Plasma homocysteine concentration did not change with MTHFR genotypes. *Conclusion:* Our data confirms the association between increased plasma Hcy concentration and AD and suggests that neither C677T nor A1298C MTHFR polymorphisms contributed to genetic susceptibility for AD in elderly individuals in the Northeast of Brazil.

KEY WORDS: homocysteine, MTHFR, Alzheimer's disease.

A doença de Alzheimer em idosos brasileiros tem relação com homocisteina mas não com polimorfismos MTHFR

RESUMO - *Objetivo:* Investigar a associação entre a concentração plasmática total de homocisteína (Hcy), os polimorfismos C677T e A1298C do gene MTHFR e o desenvolvimento da Doença de Alzheimer (AD). *Método:* Foram avaliados 43 pacientes com doença de Alzheimer possível (37%) e provável (63%) e 50 controles não dementes, não divergentes quanto ao sexo, idade, anos de escolaridade, diabetes, consumo de álcool e de café e vida sedentária. Os níveis plasmáticos de homocisteína foram determinados por HPLC e a genotipagem para MTHFR por PCR/RFLP. A comparação dos níveis de homocisteína foi realizada pelo teste "U" Mann-Whitney, a comparação das proporções dos genótipos e alelos pelo teste de Fisher-Freeman-Halton e as demais variáveis qualitativas, pelo teste do qui-quadrado. *Resultados:* Os pacientes AD apresentaram níveis mais elevados de Hcy plamática total do que os controles e a diferença entre os grupos foi estatisticamente significante. Não houve diferença nas distribuições genotípicas C677T e A1298C entre pacientes e controles. A concentração de Hcy não variou com os genótipos. *Conclusão:* Nossos dados confirmam a associação de concentração elevada de Hcy plasmática com DA e sugerem que os polimorfismos C677T e A1209C não contribuem para a susceptibilidade genética a DA em idosos do Nordeste do Brasil.

PALAVRAS-CHAVE: homocisteína, MTHFR, doença de Alzheimer.

Metilenetetraidrofolate reductase (MTHFR) is an enzyme of the folate metabolism that reduces 5,10-metilenetetraidrofolate (5,10-mTHFR) to 5-metiltetraidrofolate (5-mTHF), an important co-factor to homocysteine (Hcy) methylation. Mutations in MTHFR gene (C677T and A1298C) result in aminoacids substitutions that lead to a decreased enzyme activity, reducing the 5mTHF availability[1,2]. These muta-

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tions have got polymorphic proportions in human populations[3]. As a consequence of the MTHFR dysfunctions, an increased Hcy level in plasma has been expected which, in turn, produces a cytotoxic effect[4]. Hcy belongs to a thiol group and it might produce self-oxidations resulting in oxygen specimens such as hydrogen peroxide and radical anionic superoxides, probably responsible for cerebral oxidative stress in Alzheimer´s disease (AD) and other neurodegenerative disorders[5]. AD patients have brain atrophy characterized by neurofibrillary tangles, senile plaques and neuronal cell loss. Neurofibrillary tangles are insoluble phosphorylated helical filaments (PHF) deposits which derive from tau protein hyperphosphorylated that lost property to polymerise tubulin[6]. The ruptures in cytoskeleton derived from these alterations contribute to neuronal death[7]. Senile plaques are extracellular deposits of β-amyloid material made from proteolytic fragments of a larger precursor, the β-amyloid precursor protein (APP)[8].

Epidemiological studies have reported an association between increased plasma Hcy level and AD[9,10] and indicate a relationship between hyperhomocysteinemia and MTHFR polymorphisms or between homozygous genotype 677TT and DA[11,12]. Researches in AD which use animal models have demonstrated that folate deficiency and higher level Hcy increase sensibility to β-amyloid toxicity in neurons, PHF and hyperphosphorilated tau protein deposits and apoptosis[13]. In spite of these evidences, contradictory data in the literature indicate that the role of elevated Hcy and MTHFR polymorphisms as contributors factors to the etiopathogenesis of the disease remains unclear[14-16].

Hyperhomocysteinemia such as in AD result from a complex interaction of acquired and genetic factors which may vary according to ethnicity, environmental factors and genetic background. So, this study was carried out to investigate if C677T and A1298C MTHFR polymorphisms as well as increased plasma Hcy level play as risk factors to AD in elderly individuals in the Northeast of Brazil.

METHOD

Subjects – The studied group consisted of 42 individuals (7 males and 35 females) of which, 69% were white people, with ages ranging from 56 to 86 years (mean age 73.8 years, SD=7.2). Of these total, 63% were probable AD and 37% possible AD, according to NINCDS-ADRDA criteria[17]. Patients were selected from the Behavorial Neurobiology Unit - Department of Neurology - University of Pernambuco (Brazil). The control group consisted of 50 individuals (13 males and 37 females) of which 40% were white people, aged 61-89 (mean age 73.9; SD=6.5). They were selected in the community to which the University belongs. Individuals who had undergone vitamin or hormonal therapy were bedridden or had hyperthension were excluded from the study.

Although Brazil is a racial melting pot and the assignment of race, a very subjective issue in this country, patients and controls were classified as non- white (40%), just by visual inspection, whenever traces of racial mixture were evident. Individuals had different proportions of white, black and indigen blood.

Data regarding formal education, family history of dementia, diabetes, smoking habit, alcohol intake and no physical activity were collected through interviews.

This study was approved by the Ethnics Committee of the State University of Pernambuco and a written consent was obtained from every individuals participating in the study.

Plasmatic homocysteine and genotyping – Total Hcy concentrations in plasma were measured by HPLC with fluorescence detection. Procedures for the sample preparation were reported by Pfiffer et al.[18]. DNA was isolated by using a Wizard Genomic DNA Purification kit (Promega) and the genotyping protocols for detection of the MTHFR mutations were the ones described by Frosst et al.[19] for C677T and by Skibola et al.[20] for A1298C.

Statistical analysis – Previous to statistical analysis proper, data were tested for normality (Kolmogorov-Smimov test). Since distributions failed to meet this criterion, Mann-Whitney "U" test was used to compare groups as to quantitative variable; Fisher-Freeman-Halton test to compare genotype and allele proportions; Chi-square test to other qualitative variables. Null hypothesis was rejected when $p<0.05$. The Minitab 3.0 and Stat Exact Statistical softwares were employed for data processing.

RESULTS

Table 1 illustrates the biosocial-demographic data collected and only race/color, family history of dementia and smoking habits showed significant differences between AD and control. Since some these features are recognized as risk factors for vascular diseases[21] and possible AD has vascular components, we decided to compare possible and probable AD and did not find differences between them except in family history (probable AD, 55%; possible AD, 7.7%; p=0.002). Because there were no statistical differences as to other factors, possible and probable AD patients were analyzed together.

The frequencies of the MTHFR genotypes for both loci C677T and A1298C and respective alleles are shown in Table 2. The distributions of the MTHFR genotypes correspond to those expected by Hardy-Weinberg equilibrium in both AD patients and con-

Table 1. Bio-social demographic and clinical data of DA patients and controls.

Variable	Patients (N=42)	Controls (N=50)	p Pac x Control
Sex F,%	83	74	0.2797
Age, mean (DP)	73.8 (SD7.2)	73.9 (SD6.5)	0.6664
Colour, white,%	69	40	0.0101*
Scholar years 0-8,years,%	50	64	0.1759
Dementia familial history,%	40	6	0.0002*
Diabetes,+, %	7	6	0.8250
Smoking habits,+,%	5	24	0.0234*
Alcohol intake, + %	9.5	18	0.2450
Sedentarism, +, %	76.2	60	0.0989

Qualitative variable, by chi-square test and quantitative variable, by Mann-Whitney "U" test. *Statistically significant.

Table 2. Distribution of AD patients and controls according to MTHFR genotypes and alleles proportions.

Genotypes and alleles	AD Patients N (43)	%	Control N (50)	%	p
C677T					
CC	19	44.2	25	50.0	
CT	19	44.2	23	46.0	0.43
TT	5	11.6	2	4.0	
Allele 677T	29	34.0	27	27.0	0.31
A1298C					
AA	21	48.8	27	54.0	
AC	21	48.8	22	44.0	0.84
CC	1	2.4	1	2.0	
Allele 1298C	23	27.0	24	24.0	0.66

Fisher-Freemann-Halton test

Table 3. Homocysteine levels in AD patients and controls according MTH - FR genotypes.

Genotypes	AD patients (N=43) $\overline{X}$±SD	Control (N=50) $\overline{X}$±SD	p
C677T			
CC	18.98±10.2	15.57±6.3	0.22
CT	16.89±4.3	14.50±3.9	0.08
TT	21.29±5.8	22.30±–	–
A1298C			
AA	18.66±7.0	15.11±4.9	0.03
AC	10.00±8.5	15.49±6.9	0.16
CC	17.64±–	18.25±–	–
Total	18.31±7.6	15.34±75.4	0.02

Comparisons: Mann-Whitney "U" test.

trols, indicating that the allelic combinations were made casually. Although the 677TT homozygous frequency was higher in patients (11.6%) than in controls (677TT=4%), as expected, the difference in genotypes distribution was not significant (p>0.05). No statistical differences were observed in A1298C genotypes and alleles, either (p>0.05).

Considering that allelic frequencies vary with ethnicity, white and non-white individuals were examined as to the proportions of genotypes and alleles. Genotype carriers of mutant allele 677T (TT+CT), in relation to 677CC genotype had higher prevalence in white patients (p=0.014). Controls did not present this relation (p=0.77). Allele proportions in white and non-white people were not statistically different (patients, p=0.078; controls, p=0.713).

Hcy concentration was analyzed according to gender, race/colour and age. Males presented the most increased Hcy concentrations in both patients (M= 23.23±8.01; F=17.52±7.30; p=0.004) and control group (M=19.73±7.71; F=13.80±3.35; p=0.0010). No difference between white and non-whites was observed.

The level of total plasma homocysteine was significantly higher in AD patients (18.3 μM/L ± 7.6) than in controls (15.3 μM/L ± 5.4; p=0.02). Hcy was also analyzed according to MTHFR genotypes distributions (Table 3). No significant increase in Hcy level as was theoretically supposed for both 677TT and 1298CC people were observed. An unexpected increase on Hcy level was observed in 1298AA (p=0.03).

DISCUSSION

Of the several biosocial-demographic features analyzed in this study, only ethnic origin (white/non-white), dementia family history and smoking habit were statistically different in AD patients and controls. Smoking habit is an adquired factor which increases the susceptibility for vascular diseases but difference between them could not be attributed to subtypes of AD because probable and possible AD were not different as to the prevalence of smokers and controls had more smokers than patients. It has been stablished that high values of homocysteine and AD result from the interaction of adquired and genetic factors.

There is considerable epidemiologic evidence of increased plasma Hcy levels in elderly people, whether normal or cognitively impairs[22,23], including AD[17,24]. In this communication, we present data supporting the association between Hcy plasmatic concentration and AD. According Postiglione et al.[25] hyperhomocysteinemia is related to the progression and increasing severity of AD. Because inadequate blood levels of folate, B12 and B6 vitamins are responsible for approximately two-thirds of the hyperhomocysteinemia cases[26], the bad nutritional status which accompanies the progressive severity and long duration of AD would, perhaps explain a great number of AD patients with increased Hcy level.

Genetically, we studied the contribution of the polymorphisms C677T and A1298C of the MTHFR gene in plasma concentration of Hcy and in the development of AD, since this enzyme is involved in Hcy metabolism.

The mutation of C677T in MTHFR gene produces an enzyme which has a catalytic activity of 30% and 40% in carriers of TT and CT genotypes, respectively, as compared with CC genotype[27]. In A1298C MTHFR polymorphisms, although the enzyme is not a thermolabile protein, the catalytic activity is also shorter among homozygous CC. Enzymatic deficiency may result from MTHFR polymorphisms and develop AD and hyperhomocysteinemia, but our data did not confirm this hypothesis because no significant difference was observed in genotypes distributions and alleles frequencies between our AD patients and controls.

Because the mixing of white and blacks is more intense in the North and in the Northeast of Brazil (white 29%; non-white 71%) than in other regions of the country and also because genotypes may differ according to ethnicity, the individuals in the two groups were also compared as to race/color. The proportion of CT+TT was significantly higher in white AD patients (72%) than in non-white (~31%; p=0.014). Scientific literature shows that, in fact, the shortest 677TT genotype frequencies are among blacks[28,29]. However, data failed in confirming the same results for controls, maybe due to the smaller proportion of whites in the control group.

In the population from Pernambuco (n= 42 patients; n=50 controls) 677T allele frequencies were 37% in patients and 27% in controls. In the population from Rio Grande do Sul (n=30 patients; n=30 controls), a south Brazilian State where Caucasians are more frequent[30], 677T allele frequencies were 35% in patients and 29% in controls. There was no statistically significant difference between both populations. We suppose that a study involving a bigger sample and a better definition of ethnic origin by molecular markers might account for these contradictories data.

We could not associate MTHFR genotypes with

Hcy, except in 1298AA individuals. Although higher male/female relation may explain some elevated Hcy concentration in controls, 677T allele seems to contribute for this result. In order to test this supposition we excluded 1298AA individuals who were 677TT simultaneously from the data and then, the means were diminished and the difference between patients (15.25 μM/L ± 4.47) and controls (14.54 μM/L ± 3.51) became insignificant (p=0.66).

Our results are in agreement with similar studies in Sweden[31], in UK[32], in Italy[14,15] but association between Hcy and AD is not consensual even in other European and American populations[4,33].

Taking all the above mentioned into consideration, it is possible that others genetic factors involving Hcy metabolism, as mutations on metionina sintase, folate receptor among others, have stronger effect on AD and Hcy than MTHFR loci. Environmental factors such as nutritional deficiency of folate, vitamin B6 and B12 may also contribute to increase the Hcy levels in AD patients as such genetic factors.

As limitations of this study, we can point out the small size of the sample, particularly the number of white people in the control group and the failure to evaluate the nutritional status of patients and controls by determining concentrations of folate, B6 and B12 vitamins in plasma. Our data confirm the association between plasma Hcy level and AD and suggest that C677T and A1298C MTHFR polymorphisms do not contribute to genetic susceptibility for Alzheimer´s disease in elderly individuals in the northeast of Brazil.

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