Evaluation of plasma homocysteine level according to the C677T and A1298C polymorphism of the enzyme MTHRF in type 2 diabetic adults

Avaliação da homocisteína plasmática de acordo com o polimorfismo C677T e A1298C da enzima MTHRF em adultos diabéticos tipo 2

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ABSTRACT
Objective: To determine plasma homocysteine levels during fasting and after methionine overload, and to correlate homocysteinemia according to methylenetetrahydrofolate reductase (MTHFR) polymorphism in type 2 diabetic adults. Subjects and methods: The study included 50 type 2 diabetic adults (DM group) and 52 healthy subjects (Control group). Anthropometric data, and information on food intake, serum levels of vitamin B₁₂, folic acid and plasma homocysteine were obtained. The identification of C677T and A1298C polymorphisms was carried out in the MTHFR gene. Results: There was no significant difference in homocysteinemia between the two groups, and hyperhomocysteinemia during fasting occurred in 40% of the diabetic patients and in 23% of the controls. For the same polymorphism, there was not any significant difference in homocysteine between the groups. In the Control group, homocysteinemia was greater in those subjects with C677T and A1298C polymorphisms. Among diabetic subjects, those with the A1298C polymorphism had lower levels of homocysteine compared with individuals with C677T polymorphism. Conclusion: The MTHFR polymorphism (C677T and A1298C) resulted in different outcomes regarding homocysteinemia among individuals of each group (diabetic and control). These data suggest that metabolic factors inherent to diabetes influence homocysteine metabolism.

Keywords
Diabetes; homocysteine; methylenetetrahydrofolate reductase; polymorphism

RESUMO
Objetivo: Determinar os níveis plasmáticos de homocisteína de jejum e após sobrecarga de metionina e correlacionar a homocisteinemia com o polimorfismo C677T e A1298C da metilenotetra-hidrofolato redutase (MTHFR) em diabéticos tipo 2. Sujeitos e métodos: O estudo incluiu 50 adultos diabéticos tipo 2 (Grupo DM) e 52 indivíduos saudáveis (Grupo controle). Obtiveram-se os dados antropométricos, de ingestão alimentar, níveis séricos de vitamina B₁₂, ácido fólico e homocisteína plasmática. Os polimorfismos C677T e A1298C foram identificados no gene da enzima MTHFR. Resultados: Não houve diferença na homocisteinemia entre os grupos, embora a hiper-homocisteinemia de jejum tenha ocorrido em 40% dos diabéticos e 23% dos controles. Para o mesmo polimorfismo, não houve diferenças na homocisteinemia entre os grupos de estudo. Nos controles, a homocisteína foi maior entre aqueles com polimorfismos C677T e A1298C. Os diabéticos com polimorfismo A1298C apresentaram menores níveis de homocisteína quando comparados àqueles com polimorfismo C677T. Conclusão: Os polimorfismos da MTHFR (C677T e A1298C) resultaram em resposta distinta na homocisteinemia entre os indivíduos dentro de cada grupo (diabéticos e controles). Os dados sugerem que fatores metabólicos inerentes ao estado diabético influenciam o metabolismo da homocisteína.

Descritores
Diabetes; homocisteína; metilenotetra-hidrofolato redutase; polimorfismo

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Received on Feb/10/2011
Accepted on Aug/30/2012
INTRODUCTION

Cardiovascular involvement in diabetes has been attributed to fluctuations in glycemic levels and to risk factors, such as systemic arterial hypertension, smoking, obesity, insulin resistance, microalbuminuria, and dyslipidemia (1). In addition, elevation in plasma homocysteine levels has been considered an independent cardiovascular risk factor, contributing to increased morbidity and mortality among diabetic patients (2). Endothelial injury secondary to hyperhomocysteinemia may be due to greater production of reactive oxygen species, to platelet activation, to smooth muscle cell proliferation, and to thrombotic phenomena (3,4).

Several factors have been pointed out as determinants of hyperhomocysteinemia, such as folate, vitamin B₆ and vitamin B₁₂ deficiencies (5), impaired renal function (6), hypothyroidism (7), as well as genetic predisposition. Hyperhomocysteinemia has been attributed to genetic defects of the remethylation pathway responsible for the conversion of homocysteine to methionine, or of the transsulfuration pathway, which converts homocysteine to cysteine. The abnormal increase in homocysteine after methionine overload indicates disorders of the transsulfuration pathway (8).

The main genetic causes of hyperhomocysteinemia are mutations resulting in deficiency of the enzymatic activity of methylenetetrahydrofolate reductase (MTHFR), of cystathionine beta-synthase (CBS) and, to a lesser extent, of methionine synthase (MS) (9,10). C677T and A1298C MTHFR gene polymorphisms of enzyme MTHFR interfere with the remethylation pathway and cause an increase plasma homocysteine during fasting (11,12).

The aim of this study was to assess plasma levels of homocysteine under fasting and after methionine overload, besides analyzing hyperhomocysteinemia during fasting, according to enzyme MTHFR gene polymorphism in type 2 diabetes patients and control subjects.

MATERIALS AND METHODS

Subjects

This study was conducted on 50 type 2 diabetes patients (DM group) followed up at the diabetes outpatient clinic of a university hospital. Mean patient age was 48.2 ± 7.6 years, 70% were females and 62% had been diagnosed with diabetes 10 years or less before the study. The Control group consisted of 52 healthy individuals aged 38.9 ± 8.6 years, 57.7% females, with fasting glycemia within the normal range. Subjects who showed active infectious diseases, acute inflammatory disease, and renal insufficiency, were pregnant and nursing, and individuals with mental, hearing and visual disabilities, smokers, alcohol drinkers and subjects taking vitamin supplements were excluded from the study. The protocol was approved by the ethics committee of the institution. All subjects were informed of the purpose of the study and gave their informed consent to take part in the investigation.

Study design

All subjects underwent a nutritional, physical and laboratory examination to rule out any underlying disease. Fasting blood samples were used to determine basal plasma homocysteine levels; glucose, creatinine, folate, and vitamin B₁₂ serum levels; and to identify C677T and A1298C MTHFR gene polymorphism. For the determination of the serum vitamin levels, samples were kept away from light. Plasma and serum samples were placed on ice and transported to the laboratory within 30 min of collection. Blood was centrifuged at 4°C (3,000 rpm, 10 min), frozen and stored at -70°C until analysis.

Right after collection of the fasting blood sample, methionine (MERCK®) was administered orally (100 mg/kg body weight), diluted in 250 mL of mate tea (13). A new blood sample was collected 4 hours later for the determination of plasma homocysteine levels after methionine overload. After the first blood collection, volunteers ate a standardized methionine-poor breakfast and lunch, adding up a total of 0.9 g methionine (14).

Nutritional and clinical evaluation

Nutritional status was assessed by measuring weight, height and abdominal circumference using previously described techniques. Body mass index (BMI) was calculated as weight/height² (kg/m²) and subjects were considered to be overweight or obese when BMI was ≥ 25 kg/m². Normal values for waist circumference measurements were 102 cm for men and 88 cm for women (15). The composition of the habitual diet was based upon the Semi-Quantitative Food Frequency Questionnaire. Folate, vitamin B₆ and B₁₂ intakes were determined over the six-month period preceding the study, by means the software NutWin Profissional® 1.5 (Universidade Federal de São Paulo – Unifesp, São Paulo, Brazil). Glycemia was determined by the orthotoluidine method using a LABTEST® kit, with normal
values below 100 mL/dL. Serum creatinine was determined by colorimetry using a LABTEST® kit, with normal values below 1.5 mg/dL. Folate (reference range 3.0–17.0 ng/mL) and vitamin B₁₂ (reference range 174.0–879.0 pg/mL) were measured by chemoluminescence in a competitive solid phase enzymatic assay using the IMMULITE 2000 kit (DPC® MEDLAB).

**Homocysteine assay**

Plasma homocysteine was measured by automated high performance liquid chromatography (HPLC) with fluorescence detection (SHIMADZU, LC 9A) and a reverse-phase C18 column, using a specific kit (IMMUNDIAGNOSTIK®, Germany). Fasting plasma homocysteine levels of 15.0 μmol/L or less were considered normal (16). The upper normal limits for homocysteine after methionine overload were based on the sum of the mean + 2 standard deviations of the value detected in the control group (4), so that values lower than 44.2 μmol/L were considered normal.

**Analysis of MTHFR gene polymorphism**

Genomic DNA was extracted from peripheral blood leukocytes using the modified salting-out technique described by Miller, Dykes and Polesky (1988) (17). Genomic DNA segments were amplified by PCR. To determine the amplification of PCR products, 1% agarose gel (A-2790, Sigma) electrophoresis was performed using Tris-borate EDTA buffer and ethidium bromide staining. The amplified fragments were analyzed by restriction fragment length polymorphism (RFLP) (18). The C677T and A1298C polymorphisms were analyzed in the gene that encodes the enzyme MTHFR. C677T mutation was detected by amplification of the sequence corresponding to the polymorphic region of exon 4 flanked by the oligonucleotide (12), followed by digestion with the restriction enzyme Hinf I (Promega®). A1298C mutation was determined by amplifying the polymorphic region in exon 7 and by digesting the product with the restriction enzyme Mbo II (BioLab®).

**Statistical analysis**

Data were analyzed statistically using the Statistical Analyses System (SAS) software version 9.0 (SAS Institute, Cary, NC). In the nutritional evaluation, categorical variables were compared by the chi-square test and are presented as proportions. We used Student t-test or Mann-Whitney test, according to normality of the variables. Results are presented as mean ± SD (variables with normal distribution) or as median and range (variables with non-normal distribution). The associations between plasma homocysteine concentrations and polymorphism were tested using linear regression models. In order to satisfy the assumptions of the adjusted model, logarithmic transformation of the respective response variables was necessary. The model was adjusted according to age and gender. These results are reported as geometric means with 95% confidence interval (95% CI). The level of significance was set at p ≤ 0.05 for all analyses.

**RESULTS**

**Nutritional and clinical evaluation**

Compared with healthy control subjects, patients of DM group had a higher prevalence of BMI ≥ 25 kg/m² and abdominal circumference exceeding normal values (Table 1). Folate, vitamin B₆ and B₁₂ intakes were similar in both groups. Although there was a significant difference in serum creatinine, folic acid and vitamin B₁₂ between groups, all volunteers were within the normal range regarding these parameters. Even though the DM group was being regularly followed up in the outpatient clinic, their glycemic levels were not properly controlled at the time of the evaluation.

**Plasma homocysteine under basal conditions and after a methionine overload**

There was no significant difference between DM and control subjects in fasting homocysteinemia or after methionine overload (Table 1). Despite the tendency towards higher percentage of individuals with increased fasting plasma homocysteine levels among the diabetic patients (40.0% vs. 23.1%, p = 0.06), hyperhomocysteinemia after the methionine overload was similar in the two groups.

**Homocysteine according to MTHFR gene polymorphisms**

Heterozygote and homozygote C677T mutation frequencies were 49.0% and 13.7% in diabetic patients, versus 48.1% and 7.4% in control subjects, in Hardy-Weinberg equilibrium (p = NS between groups). The heterozygote and homozygote A1298C mutation frequencies were 27.5% and 5.9% in diabetic patients, versus 44.4% and 5.6% in control subjects, respectively (p = NS between groups).
Table 1. General characterization of individuals with diabetes mellitus (DM) and controls subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>DM Group (n = 50)</th>
<th>Control Group (n = 52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>48.2 ± 7.6</td>
<td>38.9 ± 8.6</td>
</tr>
<tr>
<td>Gender (female/male ratio)</td>
<td>35/15</td>
<td>30/22</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>29.5 (22.5-51.7)</td>
<td>26.4 (17.9-40.6)</td>
</tr>
<tr>
<td>BMI ≥ 25 kg/m² [n (%)]*</td>
<td>44 (88.0)</td>
<td>32 (61.5)</td>
</tr>
<tr>
<td>Increased abdominal circumference [n (%)]*</td>
<td>39 (78.0)</td>
<td>21 (40.4)</td>
</tr>
<tr>
<td>Usual nutrient intake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate (mg/day)</td>
<td>193.5 ± 83.2</td>
<td>181.9 ± 63.9</td>
</tr>
<tr>
<td>Vitamin B₆ (mg/day)</td>
<td>1.3 ± 0.5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Vitamin B₁₂ (µg/day)</td>
<td>3.9 (0.6-31.4)</td>
<td>5.4 (1.3-31.0)</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL)*</td>
<td>158.3 (53.0-404.0)</td>
<td>79.6 (62.9-113.3)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)*</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Serum folate (ng/mL)*</td>
<td>9.3 (4.0-16.7)</td>
<td>10.8 (5.7-20.4)</td>
</tr>
<tr>
<td>Serum vitamin B₁₂ (pg/mL)*</td>
<td>409.5 (211.0-1200.0)</td>
<td>334.0 (179.0-887.0)</td>
</tr>
<tr>
<td>Homocysteine preload (µmol/L)</td>
<td>12.8 (3.7-33.3)</td>
<td>11.9 (6.7-37.1)</td>
</tr>
<tr>
<td>Homocysteine afterload (µmol/L)</td>
<td>34.0 (11.4-30.9)</td>
<td>30.1 (13.1-84.0)</td>
</tr>
<tr>
<td>Number with hyperhomocysteinemia (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preload &gt; 15.0 µmol/L</td>
<td>40.0</td>
<td>23.1</td>
</tr>
<tr>
<td>Afterload &gt; 44.2 µmol/L</td>
<td>24.0</td>
<td>13.5</td>
</tr>
</tbody>
</table>

* p < 0.05; mean ± SD for variables with normal distribution; median (minimum-maximum) for variables with non-normal distribution.

The distribution of homocysteinaemia under fasting conditions according to enzyme MTHFR gene polymorphisms in the two groups is shown in Table 2. Considering the same polymorphism, there was no difference in plasma homocysteine levels between diabetic and control subjects. However, plasma homocysteine concentrations were significantly lower in the DM group (p = 0.04) in the presence of C677T polymorphism than in the presence of C677T gene polymorphism. In the Control group, homocysteine levels were higher among individuals with polymorphisms (alone or in combination) than among individuals without polymorphisms.

Table 2. Geometric means and confidence intervals (95% CI) of fasting plasma homocysteine levels (mmol/L) according to MTHFR genotype in the diabetes mellitus (DM) and control groups

<table>
<thead>
<tr>
<th>MTHFR polymorphisms</th>
<th>Plasma homocysteine level (µmol/L)#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Absence C677T or A1298C</td>
<td>9</td>
</tr>
<tr>
<td>C677T (alone)</td>
<td>25</td>
</tr>
<tr>
<td>A1298C (alone)</td>
<td>10</td>
</tr>
<tr>
<td>C677T + A1298C (in combination)</td>
<td>6</td>
</tr>
</tbody>
</table>

# After adjustment for age and gender.

DISCUSSION

In this study, there was a trend towards a greater number of subjects presenting fasting hyperhomocysteinaemia in the DM group than in the Control group. There was no difference in plasma homocysteine levels between diabetic and control subjects according to each MTHFR gene polymorphism. On the other hand, homocysteine levels varied among the different polymorphisms within each study group. Plasma homocysteine concentration was lower among control individuals with no polymorphisms than among individuals with separate or associated polymorphisms. Among diabetic patients, homocysteine levels were lower in the presence of A1298C polymorphism than in the presence of C677T gene polymorphism.

Our results are in agreement with data reported in the literature showing that A1298C polymorphism alone is not associated with hyperhomocysteinaemia (19,20). It has been shown that the effect of this mutation is more relevant when serum folate levels are low (21), or when the mutation is associated with C677T polymorphism (22). When compared with subjects with A1298C polymorphism, homozygotes with C677T polymorphism presented greater reduction of MTHFR activity, lower plasma folate levels, and more marked hyperhomocysteinaemia (20,22,23).

Reduced peripheral resistance to insulin has been considered responsible for lower plasma homocysteine levels in the initial phases of experimental diabetes (26,27), due to increased transsulfuration (28). On the other hand,
variations in serum homocysteine levels were not related with insulin, proinsulin, and insulin sensitivity among the Parkatêjê Indians (29). Hyperglycemia has a hyperosmolar effect that enhances glomerular filtration and may increase urinary excretion of homocysteine (30). This mechanism may have been responsible for the similarity between the DM and Control groups regarding homocysteinema medians in this study.

It is possible that the small sample size limited analysis to MTHFR gene polymorphisms. Epidemiological studies of genetic polymorphism require large samples, especially for the evaluation of hyperhomocysteinemia as a risk factor for chronic complications (2,7,10). On the other hand, even when conducted on a reduced number of patients, clinical studies can point out changes in the metabolic pathways of homocysteine (4,28). The absence of data related to serum concentrations of vitamin B₆ and genetic polymorphisms of other enzymes involved in homocysteine metabolism, especially those of the transsulfuration pathway, are some of the limitations of the present study.

In conclusion, this study shows that homocysteine plasma levels (before or after methionine load) were similar between diabetic patients and healthy controls, although hyperhomocysteinemia was more common among the diabetic patients. The presence of polymorphisms in the MTHFR genes resulted in increased homocysteine plasma levels among control subjects. Among diabetic patients, the only finding was that the A1298C polymorphism resulted in lower homocysteine when compared with C677T. The different outcomes between diabetic patients and control individuals suggest that the diabetic metabolic environment may be involved in the metabolism of homocysteine, regardless of MTHFR polymorphisms.

Grant support: This study was supported by a grant from Conselho Nacional de Desenvolvimento Científico (CNPq), and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

Disclosure: no potential conflict of interest relevant to this article was reported.

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