Plasma Total Homocysteine Levels and Methylenetetrahydrofolate Reductase Gene Polymorphism in Patients with Type 2 Diabetes Mellitus

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There was no significant difference in tHcy levels between the groups studied. The frequency of MTHFR C677T gene polymorphism observed was similar to that obtained for the general Brazilian population. In patients with type 2 diabetes and hypertension but without impaired renal function, we observed no meaningful correlation between increased tHcy levels and the presence of MTHFR C677T gene polymorphism.

Conclusions: Type 2 diabetics who are homozygous or heterozygous for the MTHFR C677T gene polymorphism showed normal tHcy levels. Our results further suggest that diabetes without an associated adverse risk profile is not an independent correlate of increased tHcy levels.

Introduction

Diabetes mellitus is an important public health problem with a high incidence, affecting quality of life and individuals' survival, and resulting in high management costs. Type 2 diabetes is the most common form of diabetes mellitus, representing approximately 90% of all diabetes mellitus cases worldwide [1]. The aetiology of diabetic vascular complications is multifactorial. Diabetes mellitus is associated with a variety of complications...
including retinopathy, nephropathy, neuropathy, acute myocardial infarction, stroke and peripheral vascular disease. Macrovascular events are more common, while microangiopathy seems to result from functional and metabolic disruption of the small vessels [2]. About 80% of patients with diabetes mellitus die from thrombosis and 75% from cardiovascular events. Endothelial abnormalities play an important role in platelet activation and coagulation initiation in diabetic patients [3]. Hypertension is very common in type 2 diabetic patients [4] and could be accountable for the altered biochemistry indices previously associated with diabetes.

Homocysteine is a sulphur-containing non-protein amino acid and intermediate in the metabolism of methionine. Hyperhomocysteinaemia is recognized as an important independent risk factor for atherosclerotic disease (both arterial and venous) in non-diabetic and diabetic subjects. A number of longitudinal and cross-sectional studies have shown that homocysteine is associated with an increased risk of stroke, carotid artery atherosclerosis and coronary heart disease [5–8]. The rise in plasma total homocysteine (tHcy) levels could be influenced by non-inherited ‘environmental’ determinants, individual genetic background or an interaction between the 2 parameters. Non-inherited factors include age, gender, smoking, renal function, inadequate dietary folate intake, high ingestion of methionine or drugs, e.g., methotrexate, hormones and antiepileptics. Hyperhomocysteinaemia attributed to congenital deficiencies is related to enzyme alterations such as cystathionine β-synthase, methionine synthase and methylenetetrahydrofolate reductase (MTHFR) [9]. The C677T polymorphism [10] in the gene coding for that enzyme is the most common genetic defect associated with moderate hyperhomocysteinaemia. The MTHFR C677T gene polymorphism may lead to enzyme function alteration showing reduced activity at 37°C. Homozygous carriers for this polymorphism show significantly elevated plasma tHcy levels compared with the non-carriers [10].

Hyperhomocysteinaemia in type 2 diabetes is further complicated by vitamin status and impaired renal function. Increased tHcy levels occur in association with marked degrees of renal dysfunction [11, 12]. Homocysteine can accumulate in patients with chronic renal disease due to clearance reduction. The kidneys are responsible for more than 70% of tHcy clearance. Therefore, reduced glomerular filtration rate with age and renal failure have been associated with increased plasma tHcy levels [13]. To date, the association between homocysteine and diabetes mellitus remains unclear, with strong multiple factors that could influence such a relation including genetics, macro- or microangiopathic complications, renal function and nutritional vitamin intake.

The aim of the present study was to determine plasma tHcy levels and frequency of the MTHFR C677T gene polymorphism in asymptomatic healthy volunteers and type 2 diabetic patients with hypertension but without nephropathy. We also addressed the relationship between tHcy levels and the presence of MTHFR C677T gene polymorphism.

Subjects and Methods

Subjects

Ethical approval was granted for the study by the Research Ethics Committee of the Federal University of Minas Gerais, Belo Horizonte, Brazil. Patients were informed about the nature of the study and consented in line with resolution 196/95 of the Ministry of Health. A total of 53 subjects were studied. These included asymptomatic volunteers (healthy by clinical and laboratory criteria, n = 16), subjects with hypertension (normoalbuminuric and previous diagnosis of hypertension, n = 12), patients with type 2 diabetes (normoalbuminuric and previous diagnosis of type 2 diabetes, n = 7), patients with both type 2 diabetes and hypertension (normoalbuminuric individuals with previous diagnosis of type 2 diabetes and hypertension, n = 18). Patients had hypertension and/or diabetes for more than 1 year at the time of testing. According to their medical records, both hypertension and diabetes were well controlled. At the time of sample collection, all patients showed no other clinical manifestation or laboratory abnormalities. Type 2 diabetic individuals had been diagnosed according to the World Health Organization criteria for the diagnosis of diabetes [14] or were being actively treated for diabetes by hypoglycaemic drugs or insulin. Individuals were classified as hypertensive if systolic arterial pressure was ≥140 mm Hg and diastolic arterial pressure was 90 mm Hg, or if they were on antihypertensive agents [15]. Subjects were excluded from the study if they had chronic renal failure (i.e. a plasma creatinine level ≥2.0 mg/dl) [16], microalbuminuria (i.e. urinary albumin ≥30 mg/g of creatinine), haematuria and bacteriuria, abnormal liver function with alanine aminotransferase, aspartate aminotransferase or alkaline phosphatase levels twice above the upper reference limit [17], a history of coronary disease, stroke, acute infectious illness, or if they were on anticoagulant therapy.

Sample Collections

Fasting blood specimens were collected from both volunteers and patients between 8 and 10 a.m. using a Vacuette system (Greiner Bio-One, Americana, Brazil). Eight millilitres were collected without anticoagulant, 2 ml in fluor-ethylenediamine tetraacetic acid, 5 ml in ethylenediamine tetraacetic acid and 4.5 ml in 3.8% sodium citrate. Two hours after lunch, another 2 ml of blood were taken in fluor-ethylenediamine tetraacetic acid for postprandial glucose. All samples were centrifuged at 2,500 rpm for 20 min, plasma or serum was separated and kept at −70°C for batch-wise analysis. Midstream urine specimens were collected in universal containers, centrifuged at 1,500 rpm for 5 min and
processed within 2 h. Part of the samples were separated and stored at –70°C for posterior determination of microalbuminuria.

Biochemical Assays
All assays were measured using conventional methods and performed according to the manufacturer's recommendations. (1) Plasma tHcy levels were measured using AxSYM® Homocysteine assay (Abbott Laboratories). (2) Glucose, glycated haemoglobin, total cholesterol, high-density lipoprotein cholesterol, triglycerides, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea and creatinine were assessed using Bioclin® kits and BTR-811 equipment (Biotron). (3) Microalbuminuria was determined using Biotecnica® kit and BIO 2000 equipment (BioPlus). (4) Electrolytes Na⁺ and K⁺ were quantified by flame photometry using Corning equipment 400®.

Genetic Analysis
The MTHFR C677T gene polymorphism was analysed using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis as described by Frost et al. [10]. The PCR reaction was performed using the primers 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3'. The PCR reaction conditions consisted of 35 cycles of denaturation to 94°C per 1 min, annealing to 64°C per 30 s and extension to 72°C per 1 min, preceded by an initial step of denaturation to 94°C per 3 min and finished with a step of 72°C per 10 min. The 198-bp PCR product was submitted to digestion with Hinfl (Promega) restriction enzyme. The presence of the polymorphism creates a sequence that Hinfl recognizes and cuts into 2 parts, one at 175 bp and another at 23 bp. Heterozygous individuals showed 3 fragments (198, 175 and 23 bp) while homozygous individuals showed 2 fragments (175 and 23 bp). The digestion product was analysed by polyacrylamide gel electrophoresis (6%), followed by silver staining. The molecular weight standard '1 Kb Plus' (Gibco) was used as reference for the electrophoresis. Fragments with less than 100 bp were not visualized.

Statistical Analysis
Results were entered in a database and analysed by the SigmaStat® software version 1.0. Data were normally distributed (Kolmogorov-Smirnov test); therefore, summary statistics are expressed as the mean ± standard deviation. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the Student-Newman-Keuls multiple comparison test. Correlations were determined using simple regression analysis (r). p < 0.05 was considered statistically significant.

Results
Clinical and Metabolic Characteristics of the Study Population
The clinical and metabolic baseline characteristics of controls, patients with hypertension, patients with type 2 diabetes and patients with type 2 diabetes plus hypertension are summarized in table 1. The type 2 diabetes and hypertension group had a slightly higher mean age and a higher proportion of women than controls or hypertension groups. The body mass index was similar across all groups. The diabetic and hypertension group showed a significant increase in fasting glucose, glycated haemoglobin, postprandial glucose, total cholesterol, low-density lipoprotein cholesterol, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, Na⁺, K⁺ and urine albumin/creatinine index concentrations compared with controls or hypertension groups. However, there was a significant reduction in the high-density lipoprotein cholesterol and creatinine concentrations (table 1).

Plasma tHcy Levels and MTHFR Gene Polymorphism
Plasma tHcy levels did not differ significantly between the groups studied – control (n = 16; 9.96 ± 3.42 μmol/l), hypertension (n = 12; 9.88 ± 2.86 μmol/l), type 2 diabetes (n = 7; 8.52 ± 1.53 μmol/l) and type 2 diabetes with hypertension (n = 18; 8.47 ± 2.64 μmol/l). Similarly, we observed no meaningful difference between the subjects studied when tHcy levels were assessed according to the MTHFR C677T gene polymorphism (table 2). The distribution of tHcy levels for all groups studied is shown in figure 1. The MTHFR C677T gene polymorphism is illustrated in figure 2.

Discussion
Hyperhomocysteinaemia is a well-known independent risk factor for atherosclerosis and thromboembolism [5]. However, the American College of Pathologists only recommends homocysteine measurement in patients with documented atherosclerotic disease [18]. Hyperhomocysteinaemia is common in patients on haemodialysis for renal failure [19]. Increased homocysteine levels in such patients could also result from other factors independent of renal diseases, e.g. diabetes mellitus. Diabetic patients with hyperhomocysteinaemia have significantly more macro- and microvascular complications [20]. Pathways of homocysteine clearance are impaired in patients with type 2 diabetes and nephropathy [20].
Fasting plasma tHcy levels were similar in type 2 diabetic patients and age-sex-matched non-smoking healthy volunteers [12]. Diabetics showed hyperhomocysteinaemia only after methionine loading with normal fasting concentrations [21, 22]. Patients with diabetes exhibit significant increases in plasma tHcy levels, especially where
Table 1. Clinical and metabolic characteristics of the groups studied

<table>
<thead>
<tr>
<th>Patients (female/male)</th>
<th>Control</th>
<th>Hypertension</th>
<th>Type 2 diabetes</th>
<th>Type 2 diabetes and hypertension</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>52.3 ± 5.4</td>
<td>54.8 ± 5.2</td>
<td>52.1 ± 8.3</td>
<td>57.2 ± 6.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>BMI</td>
<td>25.1 ± 3.9</td>
<td>28.4 ± 3.6</td>
<td>28.1 ± 3.5</td>
<td>28.6 ± 4.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>GLU, mg/dl</td>
<td>91.7 ± 9.3</td>
<td>91.0 ± 9.9</td>
<td>154.6 ± 55.2</td>
<td>167.6 ± 57.3</td>
<td>p&lt;0.0001, p&lt;0.0001</td>
</tr>
<tr>
<td>GHB, %</td>
<td>6.4 ± 1.5</td>
<td>7.2 ± 2.1</td>
<td>8.6 ± 3.2</td>
<td>8.9 ± 2.9</td>
<td>p=0.02</td>
</tr>
<tr>
<td>GLU PP, mg/dl</td>
<td>102.8 ± 16.6</td>
<td>103.3 ± 19.8</td>
<td>184.7 ± 122.1</td>
<td>151.5 ± 69.2</td>
<td>p=0.01, p=0.04</td>
</tr>
<tr>
<td>TC, mg/dl</td>
<td>180.4 ± 27.9</td>
<td>198.4 ± 44.5</td>
<td>201.7 ± 44.2</td>
<td>190.6 ± 44.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>HDLc, mg/dl</td>
<td>47.5 ± 10.1</td>
<td>40.0 ± 13.2</td>
<td>41.0 ± 7.0</td>
<td>39.6 ± 12.0</td>
<td>p=0.046</td>
</tr>
<tr>
<td>LDLc, mg/dl</td>
<td>116.3 ± 29.8</td>
<td>129.9 ± 39.7</td>
<td>137.4 ± 46.0</td>
<td>117.5 ± 37.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>TGL, mg/dl</td>
<td>82.9 ± 45.2</td>
<td>142.5 ± 69.2</td>
<td>116.7 ± 53.1</td>
<td>168.0 ± 75.0</td>
<td>p=0.009, p=0.0004</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>22.5 ± 9.4</td>
<td>18.8 ± 10.6</td>
<td>22.3 ± 8.8</td>
<td>23.6 ± 13.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>27.8 ± 8.3</td>
<td>21.4 ± 4.8</td>
<td>20.3 ± 6.5</td>
<td>23.5 ± 13.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>ALP, U/l</td>
<td>29.5 ± 9.2</td>
<td>38.5 ± 10.0</td>
<td>39.7 ± 15.7</td>
<td>40.9 ± 11.4</td>
<td>p=0.02, p=0.003</td>
</tr>
<tr>
<td>Na+, mEq/l</td>
<td>139.6 ± 6.2</td>
<td>142.8 ± 6.5</td>
<td>137.7 ± 6.6</td>
<td>138.6 ± 7.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>K+, mEq/l</td>
<td>3.8 ± 0.5</td>
<td>4.1 ± 0.4</td>
<td>3.7 ± 0.5</td>
<td>4.5 ± 0.7</td>
<td>p=0.02, p=0.03</td>
</tr>
<tr>
<td>URE, mg/dl</td>
<td>22.5 ± 5.5</td>
<td>22.0 ± 7.6</td>
<td>23.9 ± 9.5</td>
<td>26.1 ± 10.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>CRE, mg/dl</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>p=0.03, p=0.03, p=0.003</td>
</tr>
<tr>
<td>Alb U/Cr, mg/g</td>
<td>5.5 ± 2.9</td>
<td>9.1 ± 7.9</td>
<td>5.1 ± 2.5</td>
<td>8.8 ± 6.6</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Results are shown as the means ± standard deviations. n.s. = Not significant; BMI = body mass index; GLU = fasting glucose; GHB = glycated haemoglobin; GLU PP = postprandial glucose; TC = total cholesterol; HDLc = high-density lipoprotein cholesterol; LDLc = low-density lipoprotein cholesterol; TGL = triglycerides; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; URE = urea; CRE = creatinine; Alb U/Cr = urine albumin/creatinine index. Significance levels between the groups are indicated as follows: superscripts a and b indicate comparison versus control; superscripts c and d indicate comparison versus hypertension; superscript e indicates comparison versus type 2 diabetes.

Table 2. The relationship between MTHFR C677T gene polymorphism and plasma tHcy levels

<table>
<thead>
<tr>
<th>MTHFR (C677T) polymorphism</th>
<th>Total (control/hypertension/ type 2 diabetes/ type 2 diabetes and hypertension)</th>
<th>Plasma tHcy µmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>27 (9/3/5/10)</td>
<td>9.28 ± 3.03</td>
</tr>
<tr>
<td>CT</td>
<td>22 (5/9/2/6)</td>
<td>9.20 ± 2.83</td>
</tr>
<tr>
<td>TT</td>
<td>4 (2/0/0/2)</td>
<td>9.24 ± 2.48</td>
</tr>
</tbody>
</table>

Results are shown as the means ± standard deviations.

There was diabetic nephropathy [23–25]. Impaired glomerular filtration rates caused by chronic hyperglycemia could account for the high plasma tHcy levels observed in the above studies [26, 27]. Chronic hyperglycemia is also known to limit the effect of MTHFR genotype on the tHcy levels [28]. Ndrepapa et al. [29] demonstrated normal plasma tHcy levels in type 2 diabetic patients with a glomerular filtration rate ≥90 ml/min, while increased levels of plasma tHcy were observed in type 2 diabetics with a glomerular filtration rate <90 ml/min. Similarly, plasma tHcy levels in patients with type 2 diabetes were associated with an increase in albumin excretion rate, especially in patients with non-insulin-dependent diabetes [30]. This is probably due to changes in renal function as defined by creatinine clearance [11]. Patients with persistent proteinuria and chronic renal failure showed higher plasma tHcy levels [31, 32]. In contrast, reduced plasma tHcy levels could result from an increase in the activities of hepatic trans-sulphuration pathway, which is involved in the catabolism of homocysteine [33], due to insulin regulation [28, 33, 34]. This highlights the importance of insulin in homocysteine metabolism.

Our study included healthy volunteers (controls), patients with hypertension, patients with type 2 diabetes, and patients with type 2 diabetes and hypertension. The type 2 diabetes and hypertension group had a slightly higher mean age and a higher proportion of women than controls or the hypertension groups. Plasma tHcy levels were significantly elevated in patients with type 2 diabetes and hypertension compared to controls. Additionally, patients with type 2 diabetes had higher plasma tHcy levels than patients with hypertension, even after adjustment for age and gender.

Significance levels between the groups are indicated as follows: superscripts a and b indicate comparison versus control; superscripts c and d indicate comparison versus hypertension; superscript e indicates comparison versus type 2 diabetes.
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did not differ significantly between the groups studied (table 1; fig. 1). However, we observed a trend towards decreased plasma tHcy levels in subjects with type 2 diabetes and in those with type 2 diabetes and hypertension. This is in agreement with the finding of Mazza et al. [13] who reported a reduction in plasma tHcy levels in patients with normoalbuminuric type 2 diabetes and without evidence of overt nephropathy when compared with healthy volunteers.

The MTHFR C677T variant is the most frequent genetic cause of moderate hyperhomocysteinaemia, and the frequency of MTHFR gene mutations varies between racial and ethnic groups. Here, among the 53 subjects studied, 7.5% of the MTHFR C677T gene polymorphism cases were homozygous and 41.5% were heterozygous. The prevalence of this polymorphism in Caucasian Brazilians is about 10.3% homozygous and 54.2% heterozygous [35]. The prevalence of either homozygous or heterozygous MTHFR C677T gene polymorphism in diabetic patients and in normal individuals is quite similar [13]. However, homozygous but not heterozygous MTHFR C677T gene polymorphism was more frequent in type 2 diabetic patients with hyperhomocysteinaemia than in diabetics with normal plasma tHcy levels [36].

The presence of MTHFR C677T gene polymorphism correlates with the severity of renal injury in diabetic patients. MTHFR C677T gene polymorphism is a risk factor for diabetic nephropathy in male patients with type 2 diabetes [31, 37]. In addition, it has been suggested that MTHFR gene could be an aggravating factor for diabetic nephropathy, but only in male patients [31]. Type 2 diabetic subjects on haemodialysis who are homozygous for MTHFR C677T gene polymorphism show increased mean plasma tHcy values compared with normal individuals, while the patients with the heterozygous polymorphism show an intermediate increase in mean plasma tHcy levels [19]. Neugebauer et al. [38] and Noiri et al. [37] studied the relationship between C677T gene polymorphism and diabetic nephropathy progression and suggest that diabetic MTHFR C677T gene polymorphism carriers start haemodialysis earlier than diabetic non-carriers. However, in our study, only the hypertension group showed an increased number of heterozygous individuals, with a frequency of 75%. This may be a result of the small sample size evaluated. This finding forms the basis for future studies on the prevalence of hypertension for carriers of this polymorphism.

Subjects homozygous for MTHFR C677T gene polymorphism did not show increased plasma tHcy levels (table 2). Although a poor vitamin B status (folate, vitamin B₆ and vitamin B₁₂) was associated with hyperhomocysteinaemia [29, 36, 39–41], there is no consensus on whether the presence of MTHFR C677T gene polymorphism increases plasma tHcy levels independently or in association with vitamin deficiencies [12, 42, 43]. In addition, recently, it has been shown that therapeutic reduction in the homocysteine level with vitamin B therapy was not associated with a reduction in the incidence of cardiovascular events in patients following myocardial infarction.

Fig. 1. The distribution of plasma tHcy level (µmol/l) in the groups studied. The horizontal lines represent the means while the shaded area shows the normal ranges. HAS = Hypertension; DM2 = type 2 diabetes mellitus.

Fig. 2. MTHFR C677T gene polymorphism polyacrylamide gel silver stained after PCR followed by digestion with Hinfl and electrophoresis. Lane 1 = Standard molecular weight; lane 2 = blank; lanes 3 and 5 = 175-bp band (homozygous carriers); lane 4 = 198- and 175-bp bands (heterozygous carriers); lane 6 = 198-bp band (non-carriers).
or other vascular diseases [44, 45]. The levels of such vitamins in our subjects, or indeed any other vitamin supplement, were not assessed. Despite the small number of homozygous subjects studied, one could suggest that the MTHFR C677T gene polymorphism alone may not be sufficient enough to trigger hyperhomocysteinaemia.

In conclusion, our data are in agreement with recent reports [13, 29]. Notably, it further suggests that patients with type 2 diabetes and hypertension but without nephropathy, whether homozygous or heterozygous for the MTHFR C677T gene polymorphism, do not show elevated levels of tHcy. Thus, the diabetic status alone may not be an independent correlate of increased tHcy levels.

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References

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