C677T methylene-tetrahydrofolate reductase mutation in type 2 diabetic patients with and without hyperhomocysteinaemia

M Buysschaert¹, JL Gala², A Bessomo¹, MP Hermans¹

**SUMMARY**

**Objective:** The aim of the study was to determine the prevalence of the C677T mutation in a cohort of type 2 diabetic patients with and without elevated total plasma homocysteine (tHcy).

**Methods:** 80 type 2 diabetic patients with hyperhomocysteinaemia (group 1, tHcy: 21.3 ± 6.7 nmol/L) and 50 subjects with normal levels (group 2, tHcy 11.2 ± 2.3 nmol/L) were studied. C677T mutation was assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

**Results:** Homozygosity was present in 23% of patients in group 1 and 8% in group 2 (P < 0.02). No significant difference in heterozygosity frequency was observed between patients with and without hyperhomocysteinaemia. T allele frequency was 0.43 in group 1 and 0.35 in group 2.

**Conclusion:** C677T mutation is frequent in diabetic patients with hyperhomocysteinaemia and could contribute, besides non genetic factors, to increased levels of tHcy.

**Key-words:** Diabetes · MTHFR · Prevalence · Homocysteine · Complications.

Buysschaert M, Gala JL, Bessomo A, Hermans MP. C677T methylene-tetrahydrofolate reductase mutation in type 2 diabetic patients with and without hyperhomocysteinaemia

Diabetes Metab 2004,30,349-54

**RéSUMÉ**

**Objectifs :** Le but de ce travail est de préciser la fréquence de la mutation de la méthylènetetrahydrofolate réductase chez des diabétiques de type 2 avec ou sans élévation de l’homocystéine plasmatique totale (tHcy).

**Méthodes :** 80 patients diabétiques de type 2 avec hyperhomocystéinémie (tHcy : 21,3 ± 6,7 nmol/L) et 50 sujets avec homocysteinémie normale (tHcy : 11,2 ± 2,3 nmol/L) ont été inclus dans l’étude. La mutation C677T a été identifiée par réaction d’amplification génique et polymorphisme de restriction (PCR-RFLP).

**Résultats :** La fréquence de la mutation homozygote est significativement plus élevée chez les diabétiques avec hyperhomocystéinémie que chez ceux caractérisés par un taux normal d’homocystéine (25 vs 8 %, P < 0,02). Par ailleurs, la fréquence des sujets hétérozygotes n’est pas significativement différente dans les deux groupes. La fréquence de l’allèle T est de 0,43 dans le groupe 1 et de 0,35 dans le groupe 2.

**Conclusions :** La mutation C677T homozygote est fréquente dans un groupe sélectionné de diabétiques de type 2 avec hyperhomocystéinémie et peut contribuer à côté d’autres facteurs non génétiques à l’élévation des taux de tHcy.

**Mots-clés :** Diabète · MTHFR · Fréquence · Homocystéine · Complications.
Elevated plasma total homocysteine (tHcy) concentrations are currently viewed as an independent risk factor for atherosclerotic disease in non-diabetic and diabetic subjects [1-11]. Hyperhomocysteinemia can occur as a result of both inherited and acquired factors [12, 13]. Methylene-tetrahydrofolate (THF) reductase (MTHFR), a vitamin B2-dependent enzyme, plays a key role in homocysteine/folate metabolism, by catalysing the conversion of 5-10 methylene-THF to 5-methyl-THF, the latter being used as methyl donor for vitamin B12-dependent re-methylation of homocysteine to methionine [12, 13]. A mis-sense mutation in the MTHFR gene (C to T substitution at position 677 [C677T]) leads to an alanine-to-valine substitution in the enzyme, that renders it thermolabile and less active [14]. This mutation is associated with elevated tHcy levels in homozygous individuals, in particular when folate status is impaired [15, 16].

The frequency of the C677T mutation varies widely among different ethnic groups, and its prevalence in Caucasian diabetic subjects with high plasma homocysteine levels is still unclear. The aim of the present investigation was therefore to determine the prevalence of the C677T mutation in a cohort of type 2 diabetic patients with and without hyperhomocysteinemia.

**Patients and methods**

**Patients**

Two groups of type 2 diabetic in- and outpatients were studied. All patients were followed in the Department of Diabetology of our University Hospital. Group 1 included 80 consecutive patients with increased total homocysteine levels (tHcy > 15 μmol/l). A second group (group 2; 50 diabetic patients) was characterised by normal fasting values of homocysteine (between 5.0 and 15.0 μmol/l). The threshold for tHcy was selected from published cut-off values for high and normal tHcy levels [13, 17].

**Assays**

Total homocysteine was measured in the fasting state on un-heparinised plasma, using a fluorescence polarization immunoassay on a IMX Analyser (Abbott Diagnostic) on samples centrifuged within two hours following sampling, and assayed the same day. Methodology details are described in previous papers [8, 18, 19]. Vitamin B12 and folic acid were determined by radio-immunoassay methods, as were plasma C-peptide and TSH concentrations. Plasma lipids and creatinine were measured using conventional methods. HbA1c was determined by an ion-exchange HPLC. Creatinine clearances values were calculated by the Cockcroft-Gault formula. Homeostasis model assessment (HOMA, 2.1 version's software, Diabetes Research Laboratories, Oxford) of insulin sensitivity and b-cell function were detailed in Hermans, [20] and Buysschaert al. [8].

- The C677 - T genotypes were determined by polymerase chain reaction (PCR) of the genomic DNA and digestion of the PCR product with the Hinfl restriction enzyme. The C-to-T substitution creates indeed a Hinfl restriction site. A 198-bp fragment from exon 4 and intron 4 of the MTHFR gene was amplified by PCR using a previously described sense primer [14] and a modified anti-sense mutagenic primer as follows:

  **Sense** 5’-TGAAGGAGAAGGTGCTCGGGGA-3’ 
  **Antisense** 5’-AGGACCGGTCGGGT(GAGTC)GGGG-3’

Sequence modifications brought to the published anti-sense primer are underlined. As indicated between brackets, the TC nucleotides introduce a Hinfl restriction site in both the mutated or WT amplicons, whereas the GG nucleotides increase the stability of primer DNA binding. The interest of such modifications brought to the original anti-sense primer was to control the quality of the enzymatic digestion and avoid false-negative results in case of unsuspected digestion failure. The PCR cycles were carried out in a model 2400 thermocycler (Applied Biosystems) using Ampli Taq Gold (Applied Biosystems). The protocol included a first denaturation step at 95°C for 10 minutes, followed by two PCR cycles (94°C for 40 sec, 58°C for 40 sec and 72°C for 70 sec), and 30 PCR cycles (94°C for 40 sec, 62°C for 40 sec and 72°C for 70 sec). The PCR products were digested with the Hinfl restriction enzyme at least for 4 h according to the manufacturer’s recommendation. Digested, ethidium bromide-stained DNA amplicons were visualised after electrophoresis on agarose gel. After enzymatic digestion, three fragments (23- 159-, and 16-bp) were yielded in mutated amplicons. The latter being used as methyl donor for vitamin B12-dependent re-methylation of homocysteine to methionine [12, 13].

- Determination of the MTHFR genotype of diabetic subjects with or without hyperhomocysteinemia, given subjects’ informed consent, was approved by the University and Hospital Ethics Committee (Ref 2003/05/03/83).

**Statistics**

Results are presented as mean ± 1 SD or as proportions (%). Differences between respective means were assessed by T test for unmatched samples, or by non-parametric methods when necessary. Differences between respective proportions were evaluated using Chi² test. Differences between means or proportions were considered statistically significant at P values < 0.05.

**Results**

**Table I** shows the clinical characteristics of both groups of patients with (group 1) and without (group 2) increased tHcy. Mean tHcy levels (μmol/L) were 21.3 ± 6.7 and 11.2 ± 2.3 in group 1 and group 2, respectively.
Group 1 subjects were older by an average of six years (70 ± 10 vs 64 ± 11 years in group 2, P < 0.005). The subjects were otherwise comparable with regards to sex ratio, diabetes duration, positive family history for diabetes or cardiovascular disease, smoking and caffeine intake status, as well as to blood pressure levels. BMI and waist circumferences were also similar. There were no significant differences in diabetes treatment allocation between groups. Patients in group 1 were more frequently treated with a fibrate drug, namely fenofibrate.

Peripheral neuropathy and retinopathy were present in 63 and 53% respectively of patients with elevated tHcy, versus 48 and 41% in subjects with normal values (NS). Microalbuminuria and macroproteinuria were evidenced in 24 and 16% respectively of patients in group 1, vs 22 and 15% in group 2 (NS). Plasma creatinine was significantly higher (P < 0.0001) and estimated creatinine clearances lower (P < 0.0001) in individuals with elevated tHcy levels. Macroangiopathy, either as coronary and/or peripheral artery disease (CAD/PAD), was significantly more frequent in patients with than in those without hyperhomocysteinaemia (57 vs 35%, P < 0.02). This difference remained significant following adjustment for age, estimated creatinine clearance, and fibrate use between the two groups. Mean tHcy values between groups were similar in subsets of subjects with estimated creatinine clearances > 40 ml/min and/or free of fibrate therapy (not shown).

As shown in Table II, HbA1c levels were comparable in patients with hyper- and normohomocysteinaemia (8.3 ± 1.4 and 8.3 ± 1.6% respectively), as were fasting lipid levels. Serum folic acid levels were 5 ± 2 vs 7 ± 4 ng/ml in patients of groups 1 and 2 respectively (P < 0.005), while no differences were observed in vitamin B12 levels. Insulin sensitivity (HOMA S) and β-cell function (HOMA B) as assessed by the HOMA model were not significantly different in both groups.

MTHFR mutation in the homozygous state was present in a quarter of patients with hyperhomocysteinaemia, and was significantly more frequent than in subjects of group 2: 25 vs 8% (P < 0.02; Tab III). In contrast, heterozygosity of C677T was found at rates that were not significantly different between groups (35 (group 1) and 53% (group 2)). T allele frequency was 0.425 in group 1 and 0.345 in group 2, the latter value being close to the expected frequency at Hardy-Weinberg equilibrium for a T allelic frequency estimated at 0.30-0.38 in Caucasians [21].
Subjects from the combined groups with homozygous C677C (−/−), heterozygous C677C/C677T (−/+), and homozygous C677T (+/+), genotypes exhibited tHcy values of 16.5 ± 5.9, 16.0 ± 5.4, and 22.8 ± 11.0 μmol/L (P < 0.02 between homozygous C677T and other carriers groups).

Discussion

Several studies have shown that plasma homocysteine is strongly influenced by non-genetic (environmental) determinants such as age, impaired renal function, plasma folates and/or fibrate intake [12, 13, 22-25]. This was confirmed in the present study carried out in type 2 diabetic patients with moderate hyperhomocysteinaemia. We used the standard cut-off values of 5-15 μmol/l to define normal homocysteinaemia levels, as they represent 2 standard deviations in a normal, healthy reference population [13, 17, 26-28], the cut-off value of 15 μmol/l being also close to that used in the Hoorn study, in which hyperhomocysteinaemia was a strong independent risk-factor for mortality, especially in type 2 diabetes [7]. A recent meta-analysis suggests however that a linear dose-response risk relationship exists from 10 μmol/l onwards with no specific threshold, with vitamin determination and/or intervention recommended for plasma homocysteine > 10 μmol/l in high-risk populations [29].

On the other hand, genetic factors could also be involved in marginal/moderate tHcy excess, in particular a C677T mutation leading to a defect in the MTHFR enzyme. Thus, in homozygous C677T subjects (+/+) mean MTHFR activity is only 30-50% of that of non-affected (−/−) individuals [14, 15]. Subsequently, patients with the C677T mutation (+/+) usually have an average 25% higher mean total tHcy than individuals with the wild (−/−) genotype [30]. Our own results in type 2 diabetic individuals are in agreement with the latter data reported in patients with cardiovascular disease and in control subjects [30]. The contribution of C677T mutation in homozygous individuals to the variance in tHcy is estimated to be approximately 9%, compared with 35% that could be attributed to low folate and/or vitamin B12 levels [31].

Five to 12% of the general population exhibit the homozygous C677T defect and the subsequent reduction in MTHFR activity [12, 16, 32]. Heterozygosity (+/-) is seen in

| Table II | Biological characteristics of type 2 diabetic patients with (group 1) and without (group 2) hyperhomocysteinaemia. |
|-----------------|--------------|-----------------|-----------------|-----------------|
|                | Group 1      | Group 2         | P               |
|                | (n = 80)     | (n = 50)        |                 |
| HbA1c (%)      | 8.3 ± 1.4    | 8.3 ± 1.6       | NS              |
| C-peptide (pmol/ml) | 0.82 ± 0.35  | 0.80 ± 0.55     | NS              |
| HOMA S (%)     | 50 ± 25      | 49 ± 26         |                 |
| HOMA B (%)     | 57 ± 44      | 54 ± 46         | NS              |
| creatinine (mg/dl) | 1.2 ± 0.4    | 1.0 ± 0.3       | <0.002          |
| creatinine clearance *(ml/min⁻¹) | 69 ± 31 | 92 ± 32 | <0.0001 |
| cholesterol    | 194 ± 29     | 192 ± 33        | NS              |
| LDL-cholesterol (mg/dl) | 116 ± 26 | 116 ± 30 | NS              |
| HDL-cholesterol (mg/dl) | 47 ± 13 | 46 ± 12 | NS              |
| triglycerides  | 155 ± 75     | 152 ± 61        | NS              |
| TSH (mIU/ml)   | 1.9 ± 3.9    | 1.7 ± 1.2       | NS              |
| folic acid (ng/ml) | 5 ± 2 | 7 ± 4 | <0.0005 |
| vitamin B12 (pg/ml) | 484 ± 377 | 489 ± 290 | NS              |

* Cockroft-Gault estimate

| Table III | Genetic status in patients with (group 1) and without (group 2) hyperhomocysteinaemia. |
|-----------------|--------------|-----------------|-----------------|-----------------|
|                | Group 1      | Group 2         |                 |
|                | (n = 80)     | (n = 50)        |                 |
| homocysteinaemia (μmol/L) | 21.3 ± 6.7 | 11.2 ± 2.3 |                 |
| MTHFR genotype |               |                 |                 |
| C677C / C677C | 40           | 39              |                 |
| C677C / C677T | 35           | 53              |                 |
| C677T / C677T | 25           | 8 *             |                 |

* P < 0.02
a very large segment of the population, namely 43% in an Irish group [33]. The present data in type 2 diabetic patients with normal levels of tHcy (group 2) extend the latter observation: homo- and heterozygosity for C677T were documented in 8 and 53% of the individuals in this group, respectively. In contrast, in selected type 2 diabetic patients with hyperhomocysteinemia, homozygosity was identified in up to 25% of the cohort. In this line, it is of interest to mention that in non-diabetic populations with vascular disease, a higher prevalence of TT homozygosity (14-17%) than in control groups was also reported [12, 26, 34]. Heterozygosity for the C677T mutation was not increased in our patients with hyperhomocysteinemia when compared with those in group 2, and therefore could not account for the increased tHcy levels, as also suggested by other authors [15, 31, 32].

Our diabetic patients with hyperhomocysteinemia were characterised by more frequent macroangiopathy than subjects without increased tHcy. The latter observation confirms and extends our previous reports, in particular in type 2 diabetes [8]. On the other hand, the precise role of the C677T mutation per se, as a candidate genetic risk factor for vascular complications, is still debated [35, 36, 37]. Data from Folsom et al. [38], Bruhlart et al. [39] as well as results from a large meta-analysis confirmed that there was no significant increase in cardiovascular risk in homozygous patients [26]. In the same line, no association was observed between carotid intima-media thickness in type 2 diabetic patients and MTHFR polymorphism [40]. On the contrary, Arai et al. [41] and Hasewaga et al. [42] suggested that the C677T mutation could be a risk factor for macroangiopathy in diabetic subjects. In their meta-analysis, Klerk et al. found that individuals with the MTHFR 677 TT genotype had a significantly higher risk of CAD compared with individuals with the CC genotype, especially when a low folate status was present [43]. As far as microangiopathy is concerned, conflicting data are also reported concerning the influence of MTHFR polymorphism in the development of retinopathy or nephropathy [44-47].

In conclusion, C677T homozygosity but not heterozygosity was more frequent in type 2 diabetic patients with hyperhomocysteinemia than in diabetic individuals with normal tHcy values. Thus, C677T mutation, besides non-genetic factors, could contribute to hyperhomocysteinemia in this selected population. The prevalence of macroangiopathy was higher in individuals with increased levels of homocysteine than in subjects with normal plasma values. Further studies are needed to precise the exact role of C677T mutation in hyperhomocysteinemia and in the development of chronic complications in diabetes. Meanwhile, C677T determination could be useful in diabetic patients resistant to folate supplementation in case of hyperhomocysteinemia.

**References**