The C677T methylenetetrahydrofolate reductase gene mutation does not influence cardiovascular risk in the dialysis population: results of a multicentre prospective study

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Abstract

Background. Although the methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism has been identified as an independent cardiovascular risk factor (CRF) in the general population and among uraemic subjects, the validity of this association remains controversial.

Methods. To verify this hypothesis, we enrolled all subjects on maintenance dialysis treatment from a specific Italian district. We also enrolled, from the same area, 1307 subject to serve as controls. Genomic DNA was obtained and MTHFR C677T gene polymorphisms were determined. After a baseline evaluation, patients were followed-up for 37±13 months, and all cardiovascular events and causes of mortality were recorded.

Results. A total of 461 patients (417 on haemodialysis and 44 on peritoneal dialysis) were investigated, and these included patients with and without cardiovascular diseases at baseline. At enrolment, mean age was 58.8±15.6 years and dialytic age was 82±69 months. Genotype frequencies were not different between controls and uraemics. During the follow-up, the mean mortality rate was 8.81%/year, with cardiovascular events as the most frequent cause of death (n=68, 56.6%). There was no relationship between the MTHFR genotype and cardiovascular morbidity, overall mortality or cardiovascular mortality.

Conclusions. In end-stage renal disease, MTHFR C677T polymorphisms were not associated with cardiovascular disease or mortality.

Keywords: cardiovascular disease; dialysis; genetics; mortality; MTHFR

Introduction

Patients on chronic dialysis are at very high risk for cardiovascular disease. Although this elevated risk may be related to higher prevalences of classic cardiovascular risk factors (CVRFs), such as diabetes, hypertension and related disorders, it may also be due to additional risk factors, including hyperparathyroidism, anaemia, chronic volume expansion, a micro-inflammatory state and hyperhomocysteinaemia [1]. However, the contribution of these factors to cardiovascular mortality is still a matter of debate. A relationship between hyperhomocysteinaemia and cardiovascular risk (CVR) has been reported in the general population [2], and among patients on dialysis who are characterized by markedly high plasma homocysteine (Hcy) levels [3]. In renal replacement therapy (RRT) patients, high Hcy levels have also been associated with high cardiovascular morbidity and mortality [4]. However, it remains uncertain whether this association is truly independent of other CVRFs, such as malnutrition and inflammation. It should nevertheless be emphasized that chronic renal failure patients have an impaired Hcy metabolism and an inverse relationship between the glomerular filtration rate (GFR) and Hcy [5]. Individual Hcy plasma levels may also be influenced by gene polymorphisms coding for enzymes involved in its metabolism. In 1995, Frostick et al. [6] identified a common C677T polymorphism in the gene coding for the 5,10-methylenetetrahydrofolate reductase (MTHFR)
enzyme as a new candidate genetic risk factor for cardiovascular disease [6]. This mutation determines a temperature-related loss of function, with the T allele having an enzyme activity of ~35% of the values observed in individuals carrying the C allele. Among both healthy subjects [6] and patients on RRT [7], the 677TT genotype is associated with significantly higher total plasma Hcy levels than in heterozygotes or in individuals with wild-type C alleles.

In the general population, the MTHFR C677T gene polymorphism has been claimed to be an independent CVRF [6]. Likewise, certain cross-sectional studies have suggested a similar association in dialysis populations [8–10]. To verify this association, we carried out a large, prospective, multicentre study that enrolled all RRT patients in the district of Foggia, a defined area in Southern Italy.

**Subjects and methods**

**Patients**

Between November 1997 and May 1999, we enrolled all patients undergoing RRT that were already on dialysis (prevalent patients) and those who initiated dialysis (incident) in a region of Southern Italy called the district of Foggia. Patients were followed-up until November 2001. Primary renal disease was identified according to the EDTA-ERA (European Dialysis and Transplant Association-European Renal Association) registry code number. We entered 461 patients [244 (52.9%) women and 217 (47.1%) men], aged 21–84 years, into the study. At enrolment, the mean age was 58.8 ± 15.6 years and mean time spent on dialysis was 82 ± 69 months.

Most of the subjects (417 patients) were on haemodialysis, whereas a small group (44 patients) were on peritoneal dialysis. Patients were enrolled in the four dialysis units of the district of Foggia: San Giovanni Rotondo, 152 patients (33.0%); Foggia, 142 patients (30.8%); San Severo, 100 patients (21.7%); and Cerignola, 69 patients (14.5%).

The mean duration of follow-up was 37.2 ± 13.8 (mean ± SD) months. The main characteristics of patients, including primary and renal diseases according to EDTA-ERA Registry codes, and of controls are shown in Table 1.

Almost all patients (412 out of 461, 89.37%) were treated with folic acid, while 358 out of 461 (77.65%) were on erythropoietin (α or β) or darbepoietin therapy.

All patients were treated with phosphate binders: the great majority, 258 (55.9%), with calcium-containing binders, others, 108 (23.42%), with calcium-free binders, and the remaining group, 95 (20.6%), with a combination of both drugs.

**Control subjects**

From January 1995 to December 1996, we interviewed 1397 apparently healthy employees from the ‘Casa Sollievo della Sofferenza’ Hospital, San Giovanni Rotondo, Southern Italy. All subjects were Caucasian, and all of their parents and grandparents had been born in the same region. A complete clinical summary was obtained from all subjects by specially trained staff according to a previously described questionnaire [10]. We obtained a total of 1307 apparently healthy subjects without a personal history of disease [739 women (56.5%) and 569 men (43.5%), mean age 37.7 ± 9.0 years] to serve as controls [11].

After approval from the local Ethics Committees, the study was carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects.

**Assessment of mortality and cardiovascular events**

A complete clinical history with emphasis on personal and family history of angina pectoris, myocardial infarction (MI), ischaemic stroke, peripheral arterial disease and vascular risk factors was obtained from all patients by specially trained staff. Hypertension was defined as subjects being treated with antihypertensive drugs at the time of examination or as a mean of two measurements of systolic blood pressure >140 mmHg and/or a diastolic blood pressure >90 mmHg in the sitting position on at least three different occasions. Blood pressure measurements were made in the early morning, after adequate rest. Blood pressure measurements were taken before the second dialysis session of the week in haemodialysis patients, or a random day in peritoneal patients.

Left ventricular hypertrophy was diagnosed by state of the art echocardiography: two-dimensional ECG guided M-mode and Doppler echocardiographic measurements were

<table>
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<th>Table 1. Patient characteristics</th>
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<tr>
<td>Main features and co-morbidity</td>
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<td>n</td>
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<td>Age (years)</td>
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<td>Male/female</td>
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<td>RRT (months)</td>
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<td>PH/HD</td>
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<tr>
<td>Heart failure</td>
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<td>Hypertension</td>
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<td>Arrhythmia</td>
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<td>Myocardial infarction</td>
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performed according to the recommendations of the American Society of Echocardiography (on the day after the second dialysis session of the week in haemodialysis patients). MI was verified by electrocardiogram, by angiographic data when available, and from clinical and laboratory (CK and CK,mb mass) records. Other cardiovascular diseases, such as arrhythmia or valvulopathy, were diagnosed by means of all the above evaluations. Heart failure was diagnosed by means of echocardiographic findings (ejection fraction <35%). Subjects with either a positive history for diabetes mellitus, a fasting blood glucose level >140 mg/dl or 2h post-load plasma glucose levels <200 mg/dl were considered diabetic.

After the initial evaluation, patients were followed for 37 ± 13 months. All MIs or fatal events were reported. Causes of death as well as fatal and non-fatal MIs were classified by means of codes in clinical records that were based on the International Classification of Diseases, 9th revision, clinical modification (ICD-9-CM).

**Determination of genotypes**

Blood samples were collected and DNA was extracted from peripheral blood leukocytes according to standard protocols. Screening for the MTHFR C>T677 substitution was performed by amplification of a 198 bp DNA fragment followed by *Hin*fI digestion, as previously described [7].

**Statistical analysis**

All analyses were performed using the Statistical Package for Social Science (SPSS 10.0 for Macintosh). Differences between means were evaluated by a non-parametric test, whereas differences between proportions were tested by $\chi^2$ statistics. The allele frequencies were estimated by gene counting, and genotypes were scored. The observed numbers of different MTHFR genotypes were compared with those expected for a population in Hardy–Weinberg equilibrium using a $\chi^2$ test. The significance of the difference of observed alleles and genotypes between the groups was tested using the $\chi^2$ analysis after grouping homozygous and heterozygous carriers of the MTHFR C677 allele. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using a Cox proportional hazards stepwise regression model with centre, sex, age, duration of RRT, EDTA-ERA codes and CVRFs at enrolment (MI, heart failure, hypertension, diabetes mellitus, arrhythmia, valvulopathy and left ventricular hypertrophy) included as covariates. CVRFs were introduced separately into the Cox model. Moreover, Kaplan–Meier curves were constructed to show relationships between genotypes and total mortality, cardiovascular mortality and adverse events.

Statistical significance was designated as $P < 0.05$.

**Results**

**Total mortality**

During follow-up, 120 deaths were recorded (27.3%). The mortality rate was 8.81%/year and mean survival was 72.3%. Cardiovascular events were the most frequent cause of death ($n=68$, 56.6%). Other main causes of death were cachexia ($n=29$, 24.1%) or miscellaneous ($n=23$, 19.1%).

Using Cox regression modelling, overall mortality was related to diabetes (HR 2.02; 95% CI 1.36–3.00), heart failure (HR 1.74; 95% CI 1.18–2.76), arrhythmias (HR 1.61; 95% CI 1.05–2.68) and age at admission (HR 1.06; 95% CI 1.04–1.07). In addition, mortality was inversely related to plasma albumin levels ($P=0.001$).

**Cardiovascular mortality and morbidity**

A total of 84 cardiac events were recorded that included 43 cardiac arrests as well as 24 fatal and 17 non-fatal MIs (mean incidence 5.8, 1.68 and 1.19%/year, respectively). Cardiovascular mortality, shown in Table 2, was related to history of diabetes (HR 2.80; 95% CI 1.68–4.66), heart failure (HR 2.29; 95% CI 1.36–3.87), arrhythmias (HR 2.27; 95% CI 1.26–4.09) and age at admission (HR 1.04; 95% CI 1.02–1.06). In addition, there was an inverse relationship with plasma albumin levels ($P<0.001$).

The occurrence of a fatal MI during the follow-up period was positively related to a history of heart failure (HR 4.50; 95% CI 1.87–10.82) and diabetes (HR 4.32; 95% CI 1.74–10.72), and negatively related to plasma albumin levels ($P<0.011$).

When fatal and non-fatal MIs were collapsed together, there was a significant relationship with previous MI (HR 9.11; 95% CI 4.21–19.70) and with diabetes (HR 3.39; 95% CI 1.51–7.61).

**MTHFR C677T polymorphism**

The observed distribution of genotypes was not different from that predicted by the Hardy–Weinberg equilibrium in either patients or controls. In 982

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
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<tr>
<td>Diabetes</td>
<td>2.02</td>
<td>1.36–3.00</td>
<td>Diabetes</td>
<td>2.80</td>
<td>1.68–4.66</td>
<td>Previous MI</td>
<td>9.11</td>
<td>4.21–19.7</td>
</tr>
<tr>
<td>Heart failure</td>
<td>1.74</td>
<td>1.18–2.56</td>
<td>Heart failure</td>
<td>2.29</td>
<td>1.36–3.87</td>
<td>Diabetes</td>
<td>3.39</td>
<td>1.51–7.61</td>
</tr>
<tr>
<td>Arrhythmias</td>
<td>1.61</td>
<td>1.05–2.48</td>
<td>Arrhythmias</td>
<td>2.27</td>
<td>1.26–4.09</td>
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<tr>
<td>Age at entrance</td>
<td>1.06</td>
<td>1.04–1.07</td>
<td>Age at entrance</td>
<td>1.04</td>
<td>1.02–1.06</td>
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apparently healthy subjects from a previous study, the calculated allele frequencies were 55.3 (C allele) and 44.7 (T allele). Genotype frequencies were 30.5 (CC; n = 300), 49.6 (CT, n = 487) and 19.9 (TT, n = 195) [11]. The frequencies in the current study, which were not at variance with those in the previous study, were 30.5 (CC, n = 141), 50.0 (CT, n = 230) and 19.5 (TT, n = 90), and were 55.5 (C allele) and 44.5 (T allele) for allele frequencies.

The three genotypes were not different for time spent on dialysis or age at onset of dialysis. MTHFR C677T polymorphism frequencies were similar in uraemics who died and in survivors, as well as in patients with or without cardiovascular co-morbidity. As a whole, cardiovascular events and MI were not related to the MTHFR genotype (Table 3).

There was no significant relationship between the MTHFR C677T polymorphism and other clinical variables. Using a multivariate survival model, total mortality and cardiovascular mortality and morbidity were not associated with the MTHFR 677 TT genotype (Table 4).

The Kaplan–Meier curves, constructed to show relationships between the genotypes and total mortality, cardiovascular mortality and events, showed that there were no differences between the genotypes (data not shown).

Discussion

The question of whether the MTHFR polymorphism is an independent contributor to CVR has been debated recently. The main finding of the present study was that there was no relationship between the MTHFR C677T polymorphism and CVR in the dialysis population. The TT genotype was not related to cardiovascular co-morbidity at the start of the study, nor to overall mortality, cardiovascular events or mortality recorded during the follow-up.

There have been contrasting findings in the general population, with some studies reporting an association [6], whereas others did not [12]. Among patients on RRT, studies have reported the MTHFR C677T polymorphism as an independent determinant of CVR [8–10], but these were only cross-sectional [8–10] and only one enrolled a large population [8]. It is well known that an inadequate sample size may affect association studies, and that significant associations require confirmation in long-term prospective studies that enrol large and homogenous populations in a defined geographic area. To the best of our knowledge, this is the first large, prospective, long-term follow-up study that addressed these associations.

Serum levels of Hcy are directly related to CVR in both the general population [2] and RRT patients [3]. Because of the well known effects of the TT genotype on Hcy levels, it was predicted that the thermolabile variant of the MTHFR gene would by itself act as a CVRF. Given the synergistic effect of renal insufficiency and the TT genotype to increase Hcy, it is possible that the MTHFR mutation may affect the prevalence of vascular disease and associated mortality in patients on maintenance dialysis.

The present results are in agreement with recent large studies in the general population showing that the C677T mutation is a major cause of mild hyperhomocysteinaemia, but not a factor affecting cardiovascular disease risk [12,13]. These findings indicate that in the RRT population, other superimposed and more relevant risk factors are worthy of further investigation. Very recently, low rather than high total plasma Hcy was found as an indicator of poor outcome in haemodialysis patients [14]. This paradoxical inverse association between poor clinical outcome and levels of total Hcy agrees with the so-called ‘reverse epidemiology’ hypothesis [15]. In light of these

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n/%</th>
<th>Age (years)</th>
<th>Age at start of RRT</th>
<th>Mean dialytic age</th>
<th>% Dialytic age (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>134/30.5</td>
<td>62.5±15.3</td>
<td>60.01±14.72</td>
<td>8.68±5.93</td>
<td>20.13 41.06 38.81</td>
</tr>
<tr>
<td>CT</td>
<td>220/50.0</td>
<td>61.4±15.3</td>
<td>58.47±15.52</td>
<td>7.89±5.42</td>
<td>24.55 42.26 33.19</td>
</tr>
<tr>
<td>TT</td>
<td>86/19.5</td>
<td>60.4±16.7</td>
<td>57.60±17.13</td>
<td>9.33±5.89</td>
<td>24.42 29.07 46.51</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
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<table>
<thead>
<tr>
<th>Genotype</th>
<th>n/%</th>
<th>CV events-all, n/%</th>
<th>MI fatal, n/%</th>
<th>MI non-fatal, n/%</th>
<th>Cardiac arrests, n/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>134/30.5</td>
<td>24/17.9</td>
<td>8/5.2</td>
<td>5/4.5</td>
<td>11/9.0</td>
</tr>
<tr>
<td>CT</td>
<td>220/50.0</td>
<td>46/20.9</td>
<td>12/4.5</td>
<td>9/4.5</td>
<td>25/12.7</td>
</tr>
<tr>
<td>TT</td>
<td>86/19.5</td>
<td>14/16.2</td>
<td>4/3.5</td>
<td>3/4.7</td>
<td>7/9.3</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
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</table>

MTHFR C677T mutation and cardiovascular risk in dialysis

Table 3. Clinical characteristics according to MTHFR C677T genotypes

Table 4. Cardiovascular events according to MTHFR C677T genotypes
findings, it is not surprising that the MTHFR C677T polymorphism, although it increases Hcy levels, is not by itself a CVRF. In very recent studies, similar results were reported in the general population by the Copenhagen City Heart Study [16], and in the dialysis setting by Wrone et al. [17], who confirmed both the reverse relationship between total Hcy and cardiovascular events in end-stage renal disease (ESRD) patients and the lack of difference in event rates between the MTHFR genotypes.

Two important factors in the present study, including the lower crude mortality of the European dialysis population, which was confirmed in the present follow-up, and the routine treatment with folic acid and multivitamin supplements, may have influenced our findings. The 677T allele may increase Hcy levels through decreased formation of 5-methyltetrahydrofolate; however, this increase may be prevented by folate administration.

Finally, a series of papers have indicated that the TT genotype may be a risk factor for the development of ESRD [8,18]. However, Zychma et al. [19] did not show an association between the MTHFR C677T polymorphism and increased risk for development of ESRD, and the present study showed no relationship between the polymorphism and age at the start of RRT or with duration of RRT.

In conclusion, this large, prospective and multicentre study did not support the hypothesis that the MTHFR C677T polymorphism is a CVRF in the dialysis population. It is likely that specific clinical features, such as malnutrition, chronic arterial hypotension, or other biochemical markers such as the insulin resistance present in this high risk group, are important for evaluating the CVR profile in these patients.

Conflict of interest statement. None declared.

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