THE C677T THERMOLABILE VARIANT OF METHYLENE TETRAHYDROFOLATE REDUCTASE ON HOMOCYSTEINE, FOLATE AND VITAMIN B12 IN A HEMODIALYSIS CENTER

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Abstract Homocysteine is a risk factor for cardiovascular disease. Mutations in a key enzyme in homocysteine metabolism, methylenetetrahydrofolate reductase, may contribute to hyperhomocysteinemia and alter folate and cobalamin levels. After starting hemodialysis, 10 mg oral folate daily and 500 µg intravenous methylcobalamin once weekly were prescribed to 27 hemodialysis patients (time on hemodialysis > 12 months) and two groups were defined: Group A normal; Group B heterozygous. Initial, third and twelfth month measurements of homocysteine, serum folate and vitamin B12 levels were collected and analyzed. Heterozygous state of methylenetetrahydrofolate reductase prevalence was 48% and homozygosity 4%. Hyperhomocysteinemia was present in both groups. Cobalamin final levels were significantly lower in Group B compared to Group A. Homocysteine, serum folate and cobalamin levels at third and twelfth month were significantly different from baseline levels but non-different between them in both groups. In Group B, vitamin B12 at third month was significantly higher than initial, but final measurements were not different from baseline determinations. In conclusion, the heterozygous prevalence of the enzyme in hemodialysis patients is similar to that reported in the general population; hyperhomocysteinemia is frequent in hemodialysis patients and final levels in heterozygous patients are significantly higher than in normal patients. Cobalamin levels are lower in the heterozygous group. After one year of treatment, homocysteine tends to increase, suggesting a secondary resistance phenomenon to vitamin supplementation in heterozygous patients.

Key words: homocysteine, MTHFR, folate, folic acid, cobalamin, vitamin B12, hemodialysis

Homocysteine (Hcy), an intermediary amino acid formed by the conversion of methionine to cysteine, is an independent risk factor for atherosclerotic vascular disease and recurrent venous thromboembolism, two frequent complications of end-stage renal disease patients1-6. Homocysteine is metabolized by transsulfuration (vitamin B6 acts as a cofactor) and mainly by remethylation (vitamin B12 is the cofactor). In the remethylation pathway, Hcy is remethylated to methionine in a reaction catalyzed by...
methionine synthase; the methyl group comes from the active form of folic acid methyltetrahydrofolate, which therefore acts as a cosubstrate\textsuperscript{7-8}.

Elevations in plasma Hcy can be caused by a variety of disorders: genetic defects, nutritional deficiencies in the vitamin cofactors, and other causes such as renal failure, liver disease or drugs\textsuperscript{6}. Among the genetic defects, the most common cause of genetic hyperhomocysteinemia is due to a thermolabile variant of methylene-tetrahydrofolate reductase (MTHFR) with reduced enzymatic activity\textsuperscript{9,10}, with a prevalence of the homozygous state estimated between 5 to 14 percent in the general population\textsuperscript{11-13} and similar to the 10 percent reported in hemodialysis (HD) patients\textsuperscript{14}.

The enzyme MTHFR is required for the reduction of 5,10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate, thus generating the active folate derivative required for the remethylation of Hcy to methionine\textsuperscript{15,16}. A variant of this enzyme with decreased activity contains an alanine-to-valine substitution at amino acid 677 (MTHFR 677C←T). It is well established that such mutation of the gene coding for 5,10 methylenetetrahydrofolate reductase may predispose hyperhomocysteinemia\textsuperscript{12}.

We decided to determine the prevalence of the variant states of MTHFR in a HD center in Buenos Aires and assess the impact of this mutation on Hcy, serum folic acid (sFA) and vitamin B\textsubscript{12} (vitB\textsubscript{12}) levels when compared with patients lacking this mutation in stable chronic HD patients. Measurements of these variables were made before the first HD was performed in each patient (baseline, To) and at the third month (T3) and twelfth (T12) month postdialysis.

Material and Methods

Study design

MTHFR status has recently been determined in March 2001 in all patients who were hemodialyzed thrice weekly at the Hospital Británico during the year 2000 and for at least 12 months. Patients who were dialyzed for more than one year and died at the time of MTHFR determination, had frozen serum stored which was later processed for such purpose. Baseline levels, third month (T3) and one year (T12) reported measurements of Hcy, sFA and vitB12 were retrospectively recollected and analyzed.

Patient characteristics

A total of 27 chronic hemodialysis patients were included in this study. Patients were free from malignancy, end-stage chronic heart failure, active liver or thyroid disease, uncontrolled diabetes mellitus and malnourishment and had serum albumin ≥ 3 g/dl and hematocrits ≥ 32%. Patients were consequently divided into three groups according to the MTHFR status (Table 1). Thirteen patients (48%) were normal for the enzyme (Group A); in this group 8 patients (62%) were male, age 59.4±4.6 years and time on HD was 25.2±6.4 months. Causes of end-stage renal disease were: Diabetes mellitus in 2, glomerulonephritis in 5, polycystic kidney disease in 4 and ischaemic nephropathy in 2. Group B consisted of thirteen patients (48%) who were heterozygous; in this group 7 patients (54%) were male, age 64.8±3.5 years, time on hemodialysis was 13.1±1.4 months. Causes of end-stage renal disease were: Diabetes mellitus in 3, glomerulonephritis in 6, polycystic kidneys in 1 patient and ischaemic nephropathy in 3. Group C (4%) included only one patient who was homozygous, male gender, 40 years old and had been on HD for 20 months. He had polycystic kidney disease as the cause of renal failure. Group C was excluded from group comparisons due to small size (n=1). Hemodialysis was performed in a high-flux manner with bicarbonate bath, mean Qd:500 ml/minute and mean Qb:350±50 ml/minute; biocompatible membranes were used: polysulphone F80® (Fresenius Germany), cellulose triacetate FB210® (Nipro, Japan) and CT190G® (Baxter, USA). Each HD session averaged 3.5±0.5 hours thrice weekly.

Biochemical measurements

Homocysteine (normal:10 ± 5 µmol/l) was measured by fluorescent polarization immunoassay, while sFA (normal: >10 ng/ml) and vitB\textsubscript{12} (normal: 200-900 pg/ml) blood levels were determined by radioimmunoassay. All levels were measured predialysis in fasting conditions; baseline levels (To) correspond to those measured at the first HD performed in the patient, while subsequent measurements belong to the third month (T3) and the twelfth month (T12) of dialysis.

DNA extraction and mutation detection

DNA extraction was performed as originally described\textsuperscript{17} from an entire blood sample kept at -20°C. Screening for the MTHFR 677C←T substitution was performed by amplification of a 198-bpDNA fragment and followed by Hinf I digestion, as originally described\textsuperscript{18}.

<table>
<thead>
<tr>
<th>Group</th>
<th>Male</th>
<th>Age (years)</th>
<th>Time on HD (months)</th>
<th>DM</th>
<th>GN</th>
<th>PKD</th>
<th>Isch</th>
<th>Nephr</th>
<th>CHD</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>62</td>
<td>59.4±4.6</td>
<td>25.2±6.4</td>
<td>15</td>
<td>39</td>
<td>31</td>
<td>15</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>54</td>
<td>64.8±3.5</td>
<td>13.1±1.4</td>
<td>23</td>
<td>46</td>
<td>8</td>
<td>23</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>40.4</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
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</tbody>
</table>

Abbreviations: HD, hemodialysis; DM, diabetes mellitus; GN, glomerulonephritis; PKD, polycystic kidney disease; Isch Nephr, ischemic nephropathy; CHD, chronic heart disease; Symbols: *: excluded from study; %, percent
Usual medications prescribed

All patients received erythropoietin (2000-4000 U subcutaneously thrice a week) postdialysis and intravenous iron saccharate to reach a transferrin saturation between 20 and 50%. Most patients are on multivitamins in the predialysis period, but are routinely started on folic acid (10 mg/day orally) and iv methylcobalamin (500 µg/once weekly postdialysis) when admitted to the HD unit at the Hospital Británico.

Statistical analyses

Results are expressed as the mean ± standard error of the mean (SEM), unless specified otherwise. Mann-Whitney U test was used for differences between groups of quantitative variables. Chi square or Fisher test was used for qualitative variable comparisons; finally, Wilcoxon signed ranks test was used to compare intragroup results.

Results

Intergroup results

Results are depicted in Table 2

No differences were found with respect to initial Hcy, initial sFA, or serum vitB12 baseline levels; after three months of HD, no significant differences were found between both groups. Finally, after one year of treatment Hcy levels were significantly higher in Group B with respect to Group A. No differences were found regarding SFA levels.

Note worthy, despite continuous therapy, vitamin B₁₂ blood levels were significantly lower in Group B with respect to Group A, although levels were well above normal reference values.

Additionally, regarding thromboembolic events, no significant differences were found between both groups.

In group A, 4 thromboses of arteriovenous accesses were diagnosed during the study (30.7%) versus 6 events in group B (46.2%); in this group 5 thromboses occurred in the arteriovenous accesses and 1 patient had pulmonary thromboembolism. No differences between both populations were observed with respect to clotting complications of the extracorporeal circuit. Finally, 1 patient from group A (7.7%) died due to hypovolemic shock and 2 from group B (15%) of myocardial infarction and chronic heart disease. These differences were non significant.

Intragroup results

Results are shown in Table 3

Group A: Hcy, sFA and vitB12 blood levels were significantly different from their corresponding initial levels, but T3 and T12 measurements were non-different between them.

Group B: Significant reductions were observed in Hcy To-T3 and To-T12 blood levels, but were not statistically

<table>
<thead>
<tr>
<th>TABLE 2.– Intergroup results</th>
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<tr>
<td>Group</td>
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<tr>
<td></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
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<tr>
<td>C*</td>
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</table>

Symbols: To: baseline levels; T3: three months postdialysis; T12: twelve months postdialysis; *: excluded

<table>
<thead>
<tr>
<th>TABLE 3.– Intragroup differences</th>
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<tbody>
<tr>
<td>Measurement</td>
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<tr>
<td>------------</td>
</tr>
<tr>
<td>Homocysteine (µmol/l)</td>
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<tr>
<td>Homocysteine (µmol/l)</td>
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<tr>
<td>Serum Folic Acid (ng/ml)</td>
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<td>Serum Folic Acid (ng/ml)</td>
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<tr>
<td>Vitamin B₁₂ (pg/ml)</td>
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<tr>
<td>Vitamin B₁₂ (pg/ml)</td>
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Results are expressed as the mean ± SD
different between them, although T12 measurements were higher than T3. Significant rises in To-T3 and To-T12 SFA levels were observed. Finally, vitamin B12 T3 concentrations were statistically higher than baseline; T12 were lower than T3 but lacked significant statistical difference, and non-different from initial ones.

Discussion

Our results show that the prevalence of the heterozygous variant of MTHFR in a HD center in Buenos Aires was 48%, similar to the 42.8% reported in a previous study from 418 healthy blood donors in Argentina, demonstrating that this mutation is not associated with renal failure and is not a risk factor to develop end-stage renal disease. To our knowledge, no reported data exist about the prevalence of the thermolabile variant of MTHFR in a HD center in Argentina.

Despite both normal and heterozygous patients presented decreased significantly Hcy levels after three months of vitamin supplementation and were non-different between them, Hcy levels in the heterozygous group were significantly higher than in the normal group after one year of treatment, although these final levels were statistically lower than baseline ones. Moreover, albeit T12 levels were non-different from T3, they showed a climbing trend, which may be explained by a secondary resistance of the enzyme to a constant dose of folic acid. These results confirm previous ones which show that such mutation predisposes to higher Hcy levels, and such resistance is observed at constant doses of vitamin therapy, being folic the most important vitamin involved in Hcy metabolism. This MTHFR malfunction can partially contribute to the hypomethylation phenomenon described in uremia, by which Hcy levels remain high. One possibility to overcome such enzymatic derangement could be to assess Hcy levels after higher doses of folate supplementation (≥ 20 mg/day in patients on 10 mg/day) in heterozygous patients. In our center, we have already increased the dose of intravenous methylcobalamin from 500 µg once a week to 500 µg thrice weekly maintaining constant folate daily doses of 10 mg, and no significant reduction in Hcy levels was obtained after six months of therapy. Whether MTHFR heterozygous people are exposed to a higher risk of atherosclerotic complications (coronary heart disease, stroke, etc) or thromboembolic events is to be determined. In our study, these differences were statistically non-significant probably due to the small number of patients included. Likewise, we cannot conclude from this study that T12 Hcy levels in Group B (normal but significantly higher than in Group A) are related to additional cardiovascular or thromboembolic risks.

Curiously, initial Hcy levels were high in all patients despite baseline sFA and vitamin B12 blood levels were normal (Table 2), demonstrating that well above or supra-physiological concentrations of both vitamins must be achieved to lower Hcy significantly. (Normal folate and vitB12 levels could be due to previous multivitamin supplementation even at low doses: average 1 mg oral folate and 200 µg oral cobalamin preparations).

With respect to intragroup comparisons, folate plus vitamin B12 supplementation rapidly and efficiently decreased Hcy in both groups (T3 vs To). In Group B, T12 vitamin B12 levels were non different from initial ones, again showing that the heterozygous population of renal patients is unable to maintain vitB12 levels in the rising pattern that people without MTHFR mutations show after intravenous methylcobalamin supplementation. We have not found any data in the literature reporting any association between MTHFR heterozygosity and low-normal vitB12 concentrations, albeit in a recent report homozygous subjects carrying the MTHFR C677T variant have higher folate and vitamin B12 requirements. Noteworthy and anecdotally, in our study six patients from Group B but no patient from Group A were Helicobacter pylori positive, a recently reported possible cause of cobalamin deficiency. All six patients lacked antiparietal cell and intrinsic factor antibodies. Again, no association between MTHFR mutations and lower vitamin B12 levels have been reported previously. Whether MTHFR heterozygosity predisposes to Helicobacter pylori superinfection through folic acid deficiency and vitB12 malabsorption has not been assessed.

In conclusion, heterozygous MTHFR prevalence in HD patients is similar to that reported in the general population; plasma Hcy in heterozygous patients is significantly higher than in normal MTHFR patients; after one year of therapy with 10 mg daily oral folic acid and 500 µg weekly intravenous methylcobalamin, a secondary resistance phenomenon to vitamin supplementation in MTHFR heterozygous patients is observed, by which Hcy tends to increase. This pilot study includes a small group of patients so that all results must be analyzed with caution.

References

5. Clarke R, Daly L, Robinson K, et al. Hyperhomocys-

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In calling up images of the past, I find the plains of Patagonia frequently pass across my eyes; yet these plains are pronounced by all to be wretched and useless. They can be described only by negative characters: without habitation, without water, without trees, without mountains, they support only a few dwarf plants. Why, then, and the case is not peculiar to myself, have these arid wastes taken so firm a hold on my memory?

Rememorando imágenes del pasado, los llanos de la Patagonia cruzan frecuentemente ante mis ojos; sin embargo, estas planicies todas las consideran miserables e inútiles. Se las puede describir solamente con caracteres negativos: sin morada, sin agua, sin árboles, sin montañas y sólo albergan algunas plantas enanas. Por qué, pues, y el caso no es peculiar mío, estas áridas regiones se han afirmado tan sólidamente en mi memoria?

Charles Darwin (1809-1882)

*The Voyage of H. M. S. Beagle*