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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 B_{12} levels were collected and analyzed. Heterozygous state of methylenetetrahydrofolate reductase prevalence was 48% and homozygozity 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 B_{12} 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 B_{12}, hemodialysis

Resumen ***La variante termolábil C677T de la enzima metileno-tetrahidrofolato-reductasa sobre homocisteína, folatos y vitamina B12 en hemodializados crónicos.*** La homocisteína es un factor de riesgo de enfermedad cardiovascular. Mutaciones en la enzima metilenetetrahidrofolato reductasa pueden contribuir a la hiperhomocisteinemia alterando los niveles séricos de folato y cobalamina. Luego del ingreso a hemodiálisis, se prescribió 10 mg diarios de ácido fólico y 500 µg semanales intravenosos de metilcobalamina a veintisiete pacientes en hemodiálisis (tiempo en hemodiálisis ≥ 12 meses) y se definieron dos grupos: Grupo A normal para la enzima; Grupo B heterocigota. Mediciones iniciales (prediálisis) de homocisteína, folato sérico y vitamina B_{12}, al tercer y duodécimo mes fueron recolectados. El estado heterocigota de la enzima tuvo una prevalencia del 48% y el homocigota un 4%. Los valores iniciales de homocisteína estaban elevados en ambos grupos. Los niveles finales de cobalamina fueron significativamente más bajos en el grupo B. Tanto la homocisteína como el folato y la cobalamina al mes tres y doce fueron significativamente diferentes a los iniciales pero no diferentes entre sí. En el grupo B, la vitamina B_{12} al tercer mes fue significativamente más elevada que la inicial, pero las determinaciones finales no fueron diferentes a las basales. En conclusión, la prevalencia heterocigota de la enzima en los pacientes en hemodiálisis es similar a la de la población general; la hiperhomocisteinemia es frecuente en los pacientes hemodializados y los niveles al año son significativamente más altos en los heterocigotas, si bien dentro de parámetros normales. Los niveles de cobalamina son más bajos en el grupo heterocigota. Al año de tratamiento, la homocisteína mostró una tendencia a elevarse, sugiriendo la existencia de una resistencia secundaria al suplemento vitamínico en los pacientes heterocigotas.

Palabras clave: homocisteína, MTHFR, folato, ácido fólico, cobalamina, vitamina B_{12}, hemodiálisis

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 patients[1-6]. Homocysteine is metabolized by transsulfuration (vitamin B_6 acts as a cofactor) and mainly by remethylation (vitamin B_{12} is the cofactor). In the remethylation pathway, Hcy is remethylated to methionine in a reaction catalyzed by

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methionine synthase; the methyl group comes from the active form of folic acid methyltetrahydrofolate, which therefore acts as a cosubstrate[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[8]. Among the genetic defects, the most common cause of genetic hyperhomocysteinemia is due to a thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) with reduced enzymatic activity[9, 10], with a prevalence of the homozygous state estimated between 5 to 14 percent in the general population[11-13] and similar to the 10 percent reported in hemodialysis (HD) patients[14].

The enzyme MTHFR is required for the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thus generating the active folate derivative required for the remethylation of Hcy to methionine[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[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_{12} ($vitB_{12}$) 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 (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 $vitB_{12}$ (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[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[10].

TABLE 1.– *Patient characteristics*

Group	Male %	Age (years)	Time on HD (months)	DM %	GN %	PKD %	Isch Nephr %	CHD	Deaths
A n=13	62	59.4±4.6	25.2±6.4	15	39	31	15	1	1
B n=13	54	64.8±3.5	13.1±1.4	23	46	8	23	3	2
C* n=1	100	40.4	20	0	0	100	0	0	0

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 especified otherwise. Mann-Whitney U test was used for differences between groups of cuantitative variables. Chi square or Fisher test was used for qualitative variable comparitions; 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. Noteworthy, despite continuous therapy, vitamin B_{12} 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 2.– *Intergroup results*

Group	Homo normal To	cyst 10±5 T3	eine µmol/l T12	Serum normal To	Folic >10 T3	Acid ng/ml T12	Vita normal To	min 200-900 T3	B12 pg/ml T12
A	21.7±1.5	12.8±1.1	12.7±0.9[a]	22.9±6.9	349.9±93.7	372.1±93.8	1673±495	2756±569	3643±846[b]
B	23.2±2.9	16.1±2.0	16.3±0.9[a]	30.2±16.5	178.8±39.6	235.8±52.2	1507±92	2358±587	1505±293[b]
C*	64	11	23	2.4	321	295	481	1094	2316

*Symbols: To: baseline levels; T3: three months postdialysis; T12: twelve months postdialysis; * : excluded*
[a] P=0.014 [b] P=0.039

TABLE 3.– *Intragroup differences*

Measurement	Period	Group A	Group B
Homocysteine (µmol/l)	To-T3	21.7±5.3 vs 12.8±3.9; P=0.002	23.1±10.7 vs 16.1±7.3; P=0.009
Homocysteine (µmol/l)	To-T12	21.7±5.3 vs 12.7±3.3; P=0.003	23.1±10.7 vs 16.3±3.5; P=0.039
Serum Folic Acid (ng/ml)	To-T3	22.9±32.4 vs 349.9±337.9; P=0.001	30.2±59.5 vs 178.8±142.9; P=0.001
Serum Folic Acid (ng/ml)	To-T12	22.9±32.4 vs 372.1±338.2; P=0.001	30.2±59.5 vs 235.8±188.3; P=0.002
Vitamin B12 (pg/ml)	To-T3	1673±1785 vs 2755±2050; P=0.016	1507±1742 vs 2357±2117; P=0.033
Vitamin B12 (pg/ml)	To-T12	1673±1785 vs 3643±3051; P=0.011	1507±1742 vs 1505±1057; p=ns

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 B_{12} 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[18], 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[9, 10], and such resistance is observed at constant doses of vitamin therapy, being folate the most important vitamin involved in Hcy metabolism[11, 15]. This MTHFR malfunction can partially contribute to the hypomethylation phenomenon described in uremia, by which Hcy levels remain high[19, 20]. One possibility to overcome such enzymatic derangement could be to assess Hcy levels after higher doses of folate supplementation ($\geq$ 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 maintainig 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 B_{12} 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 vitB_{12} 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 heterozygozity and low-normal vitB12 concentrations, albeit in a recent report homozygous subjects carrying the MTHFR C 677T variant have higher folate and vitamin B_{12} requirements[21]. Noteworthy and anecdotically, 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[22]. All six patients lacked antiparietal cell and intrinsic factor antibodies. Again, no association between MTHFR mutations and lower vitB12 levels have been reported previously. Whether MTHFR heterozygozity predisposes to *Helicobacter pylori* superinfection through folic acid deficiency and vitB_{12} 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 tend to increase. This pilot study includes a small group of patients so that all results must be analyzed with caution.

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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 anti mis ojos; sin embargo, estas planicies todas las consideran míseras 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 afincado tan sólidamente en mi memoria?

Charles Darwin (1809-1882)

The Voyage of H. M. S. Beagle