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Serum Homocysteine Levels and its Methylenetetrahydrofolate Gene (*MTHFR*) C677t Polymorphism in Patients with Hemodialysis

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Homocysteine plays an important role in cardiovascular disease as an independent risk factor, especially in patients with renal insufficiency. The present study aimed to determine whether Hcy levels, or those of its C677T polymorphism, were associated with higher mortality in patients submitted to chronic hemodialysis treatment. This was a descriptive, prospective study. Chronic renal patients undergoing hemodialysis in the "General Hospital, ISSSTE" Dr. Darío Fernández Fierro, Mexico City were included in the study. Serum homocysteine was analyzed by means of an ELISA test. The primers utilized for *MTHFR* C677T polymorphism identification were the following: F: 5'TGAAGGAGAAGGTGTCTGCGGGA3', R: 5'AGGACGGTGCGGTGAGTG3' and F2: 5'GCAGGGAGCTTTGAGGCTGAC3'. Differences among nominal conditions were evaluated by the Mann-Whitney U-test. Spearman test was used for correlation among variables. Regression, log-linear analysis and receiver operating characteristic (ROC) curves were conducted to evaluate the possible influence on prognosis of Hcy levels and the presence of the *MTHFR* C677T polymorphism. Cox regression and Kaplan-Meier tests were performed to evaluate the Hcy levels influence on survival. In all cases, $p \leq 0.05$ was considered statistically significant. All tests were performed with the SPSS ver. 23 statistical software program. By means of regression analysis ($p = 0.046$) and ROC curve age was the sole significant prognostic variable for the "death". The loglinear analysis did not show any association between the presence of *MTHFR* C677T SNP with the mortality of patients. It was concluded that Hcy levels and the presence/absence of *MTHFR* C677T are not stronger predictors for mortality than the traditional cardiovascular risk factors.

Key words: Chronic renal failure, hemodialysis, homocysteine, *MTHFR* C677T polymorphism, enzyme-linked immunosorbent assay (ELISA), PCR digestion assay

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INTRODUCTION

Homocysteine (Hcy) is a by-product of demethylation sulfur containing essential amino acid (methionine). It is metabolized in two different ways: (1) remethylation to methionine (dependent on vitamin B12 and folic acid) and (2) transsulfuration to cystathionine (dependent on vitamin B6)[1]. While remethylation is the principal determinant of fasting Hcy concentrations, transsulfuration remethylation is responsible for approximately 50% of Hcy metabolism[2].

Experimental studies have shown that hyperhomocysteinemia may induce proliferation of vascular smooth muscle cells and endothelial dysfunction by different mechanisms, such as the production of hydrogen peroxide, abnormal clotting proteins and hemostatic disorders[3-5]. In recent years, a study has shown that Hcy plays an important role in cardiovascular disease (CVD), associated not only with other factors, but also as an independent risk factor[6].

The CVD is the leading cause of death in patients with chronic kidney failure[7]. It is well known that there are several prevalent risk factors for CVD in kidney failure, for example, hypertension and dyslipidemia. Moreover, Type 2 diabetes mellitus (T2DM) is a leading cause of chronic kidney disease (CKD) and the dialysis population co-exists with hypertension.

Without doubt, Hcy is an independent risk factor for CVD in general population and plays a leading role in the development of atherogenesis and vascular thrombosis, especially in patients with renal insufficiency. In this regard, patients subjected to hemodialysis are under the toxic effects of hyperhomocysteinemia[8], present in about 85-90% of these subjects[9].

It has been observed that for every 1 mol L^{-1} of increased Hcy above normal values (10±5 mM L^{-1}), there is a 3% increase in mortality in patients on chronic hemodialysis. Likewise, in chronic kidney failure, hyperhomocysteinemia occurs more frequently than other cardiovascular risk factors when compared with general population and in some reports, it is as high as 83%[10,11].

It is recognized that hyperhomocysteinemia causes are varied and include a genetic component in which single nucleotide polymorphisms (SNP) play a key role. In such, the most studied SNP for its effects on elevated levels of Hcy and the subsequent impact entailed in this, is localized in the C677T position of the *MTHFR* gene, an enzyme essential in the process of the conversion of Hcy into methionine[12,13].

Multiple studies[14,15] have shown that while homocysteine levels fall with folic acid, cardiovascular events and deaths do not decrease. With these data the use of folate in most patients is discouraged, but doubt remains in patients with terminal CKD. Despite this information, few studies have been done considering the presence of C677T SNP and cardiovascular risk in patients with renal failure. The principal aim of this study was to assess the role of serum Hcy levels and that of the C677T polymorphism on the mortality prognosis in patients treated with hemodialysis.

MATERIALS AND METHODS

This was a descriptive, prospective and transversal study performed at the "Dr. Darío Fernández Fierro" General Hospital, ISSSTE, Mexico City, from May 1st, 2015-April 30, 2016. Following a convenience sampling, randomly chosen patients with CKD on hemodialysis replacement therapy with a minimum of 1 year in the program and an age of >25 years were enrolled. All of the patients were on dialysis three times per week through polysulfone membranes and Kt/V was found at between 1.2 and 1.5. Patients with a history of a previous cardiovascular event (coronary artery disease (CAD), stroke, peripheral artery disease, thromboembolic disease and venous thrombosis) and those with cancer were excluded and those without Hcy measurements were discarded from the final analysis.

A survey was conducted on each of the patients to retrieve information of cardiovascular risk factors, such as alcoholism, age, drug addiction, gender, low physical activity, smoking and a familial history. Patients were measured (m) and weighed (kg) (Torino). Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured with a mercury sphygmomanometer (Baumanometer, USA) after 5 min of rest.

After a 12 h fasting period, venous blood samples were taken in Vacutainer™ tubes for determination of creatinine, glucose, lipid profile, urea, uric acid and blood urea nitrogen (BUN), (Beckman Coulter AU680) and HbA1c. SerumHcy was analyzed at the Universidad Colegio Mayor de Cundinamaca (UCMC), Bogotá, Colombia, by means of an enzyme-linked immunosorbent assay (ELISA) test (Wiener Lab, Argentina).

The DNA extraction from whole blood was performed with the E.Z.N.A.® Blood DNA Kit (Omega Bio-tek, Inc., USA). The DNA obtained was quantified with a NanoDrop™ 2000 spectrophotometer (Thermo Scientific) and was visualized in 2% agarose gels through the Gel Doc XR (Bio-Rad) system. Finally, Quantity One software (Bio-Rad) was employed to quantify the images.

The primers utilized for *MTHFR* C677T polymorphism identification were the following: F: 5'TGAAGGAGAAGG TGTCTGCGGGA3', R: 5'AGGACGGTGCGGTGAGTG3' and F2: 5'GCAGGGAGCTTTGAGGCTGAC3'. Utilizing β-actin as control, the samples were run under the following conditions: at 94°C×3' (1 cycle); (at 94°C×1' and at 60°C×1' and at 72°C×30" (35 cycles), at 72°C×7' (1 cycle) and at 4°C (∞).

In relation to the enzymatic studies, digestion assay of the PCR products was performed with the HINFI enzyme (Promega), 228-bp bands were obtained when the T allele was absent and 172-bp patterns when the CT polymorphism was present (Fig. 1).

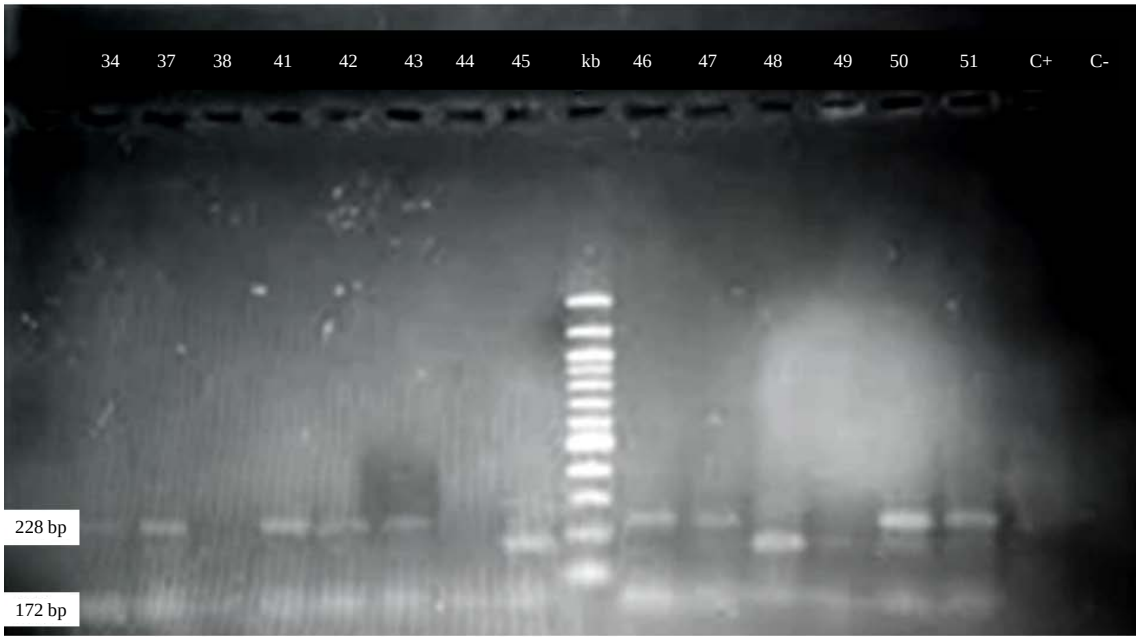

Fig. 1: Enzymatic digestion with the HINFI enzyme. Column 1: Sample 34, Column 2: Sample 34, Column 3: Sample 38, Column 4: Sample 41, Column 5: Sample 42, Column 6: Sample 43, Column 7: Sample 44, Column 8: Sample 45, Column 9: Sample 46, Column 10: Sample 47, Column 11: Sample 48, Column 12: Sample 49, Column 13: Sample 50, Column 14: Sample 51, Column 15: Positive control (C+), Column 16: Negative control (C-)

This protocol was approved by the Ethical and Research Committee of the ISSSTE, Mexico City. In accordance with the National Ministry of Health and the Declaration of Helsinki (Fortaleza, Brazil), this study was classified as zero risk to participants, because it did not involve procedures in addition to those strictly necessary for the highest care standard in the institution's Hemodialysis Unit. All patients signed the informed consent of the hospital.

Statistical analysis: Quantitative variables were expressed as mean±standard deviation (SD) and qualitative variables in frequency and percentage. Differences among nominal conditions were evaluated by the Mann-Whitney U-test. For establishing connection between different parameters, Spearman test was used. Regression, log-linear analysis and receiver operating characteristic (ROC) curves were conducted to evaluate, whether Hcy levels or the presence of the *MTHFR* C677T polymorphism exerted an influence on disease evolution. Cox regression and Kaplan-Meier tests were performed to evaluate the Hcy levels influence on survival. In all cases, $p \leq 0.05$ was considered statistically significant. All tests were performed with the SPSS ver. 23 statistical software program (IBM SPPS 23, USA).

RESULTS

From 53 initially selected patients on hemodialysis, full data was available for 27 of these, who were included in this study. Seventeen (62.96%) patients were males and 10 (37.03%) females. The mean age of the patients was 57.9±14.82 years (range, 25-87 years). Three (11.11%) patients answered to be physically active, nine (33.33%) were active smokers, 15 (55.55%) referred usual alcohol ingestion and two (7.4%) confirmed a drug addiction only.

Regarding the underlying disease, 22 (81.48%) patients had hypertension, 6 (22.22%) had T2DM and five (18.51%) were diabetic, hypertensive and dyslipidemic. the general characteristics of the patients and comorbidities are showed in Table 1. It is interesting to note that besides the patients with known T2DM, three more had hyperglycemia during the study. On the other hand, within the patients with a lipid disorder, seven (25.92%) had mixed dyslipidemia. The laboratory characteristics of the population are depicted in Table 2. Of the 27 samples analyzed, five (18.51%) presented the C677T polymorphism. Although, the Hcy levels showed a trend to be higher in the presence of C677T than in those patients without this SNP (40.8 vs 32.4 μmol L^{-1}) there was no statistical difference.

Six (22.22%) patients died, 4 men and 2 women, being the causes of death: Acute myocardial infarction (AMI) (N = 4), septic shock (N = 1) and pneumonia (N = 1). The Mann-Whitney U-test revealed a significant difference in the following situations: Creatinine between genders ($p = 0.027$) and age between those who were alive or dead after 1 year ($p = 0.042$).

Within the data of all the patients, the Spearman test demonstrated an expected positive correlation between age and urea (r^2 = 0.476, p = 0.046), age and BUN (r^2 = 0.471, p = 0.048) and between BUN and urea (r^2 = 0.614, p = 0.007), but Hcy did not show any significant correlation with the evaluated laboratory tests. On the other hand, triglycerides had positive significant correlations with glucose (r^2 = 0.605, p = 0.01), HbA1c (r^2 = 0.614, p = 0.011), urea (r^2 = 0.620, p = 0.008) and age (r^2 = 0.483, p = 0.049).

Of particular concern was the result of age as the sole significant prognostic variable for the "death" with the regression analysis (p = 0.046) as well as with the ROC curve (p = 0.03, [95%CI 0.001-0.306]). The log-linear analysis did not demonstrate any association between the presence either of obesity or SNP and the mortality of our patients.

By performing the same analysis separated for men and women, neither Hcy nor the presence of the C677T polymorphism were identified to be a mortality predictive factor. Finally, the survival analysis for Hcy levels was not significant.

DISCUSSION

Patients with end-stage renal disease (ESRD) are at a high risk of adverse cardiovascular events. In this respect, the data of this survey points out the severity of comorbidities, for example, the high positive correlation between triglycerides and other metabolic parameters (HbA1c, glucose and urea) suggests a high prevalence of metabolic syndrome (cause ESRD) and uremic syndrome (clinical manifestation of ESRD), in both cases it is mandatory to establish a more intensive treatment.

There is controversial data about the role of Hcy, with some authors pointing out that it comprises an important risk factor for cardiovascular morbidity and mortality in patients undergoing dialysis[16-18] and others saying the contrary[19-20].

In a study by Selhub on patients under dialysis with and without CVD, the serum Hcy level was significantly high in patients with CVD compared with patients without this health issue (37.2 vs. 24 μmol L^{-1})[21]. In this study, the mean serum Hcy level of our patients was similar to those of the first group; in contrast, Tamadon *et al*.[22] published lower. The explanation for the difference between the values reported by Tamadon and ours is not clear, but we can exclude T2DM, in that the percentage of affected patients with this disease was similar: 32.33 vs. 37.03%. A possible variable of differences in Hcy levels trends after initiating the replacement treatment might be the dialysis technique, because even the type of membrane demonstrates a decisive role[23]. It is also noteworthy that Hcy is higher in patients with peritoneal disease than in patients on dialysis[24]. An excellent review of the associations between serumHcy level and outcomes in patients with ESRD as well as the associations between genetic polymorphisms of *MTHFR*, *MTR*, *MTRR*, *GCP2*, *RFC1* and *TCN2* and Hcy concentrations in patients with ESRD has been conducted by Wu *et al*.[25] In that review, from 20 studies (years 1995-2006), 15 showed a higher risk for CVD in case of hyperhomocysteinemia and five showed a protective effect Following this line, some contrasting works about Hcy as a cardiovascular risk factor in CKD are shown in Table 3.

Another study showed that supraphysiologic levels of cofactors in Hcy metabolism, including folic acid, 15 mg/day, vitamin B6, 100 mg/day and vitamin B12, 1 mg/day, could maintain the serum Hcy level lower than its normal serum level[21]. However, in the absence of any thrombotic events, there is no definite indication for the treatment of hyperhomocysteinemia[30]. Despite the existence of some strategies for the reduction of the serum Hcy level in patients with CKD, including folate and vitamin supplementation[31], the findings of this survey add to the growing body of evidence from clinical trials exhibiting the lack of any significant effect regarding the serum Hcy level and mortality in these patients[32]. Additional studies, including prospective dietary intervention trials, could provide much needed information on these associations with survival.

Table 1: General characteristics of the patients and comorbidities

Variables	Values
Age (years)[a]	53.5 ± 15.8
Body mass index (kg m^{-2})[a]	30.4 ± 4.9
Hyperuricemia[b]	5 (18.51)
Hypercholesterolemia[b]	8 (29.62)
Hypertriglyceridemia[b]	7 (25.92)
Hyperglycemia[b]	9 (33.33)
Obesity[b]	10 (37.03)

a: Mean±Standard Deviation, b: Number (%)

Table 2: Laboratory characteristics of the patients

Variables	Mean ±SD
Blood urea nitrogen (mg dL^{-1})	91.9 ± 48.4
Cholesterol (mg dL^{-1})	188.4 ± 91.2
CKD-EPI (mL/min/1.73 m^2)	5.1 ± 2.4
Creatinine (mg dL^{-1})	10.9 ± 4.3
Glucose (mg dL^{-1})	161.3 ± 87.9
HbA1c (%)	8.3 ± 3.0
Homocysteine (μmol L^{-1})	36.6 ± 27.4
Triglycerides (mg dL^{-1})	191.5 ± 142.5
Urea (mg dL^{-1})	151.3 ± 36.2
Uric acid (mg dL^{-1})	6.3 ± 1.1

CKD-EPI: Chronic kidney disease-epidemiology collaboration

Table 3: Studies of homocysteine as a risk factor in chronic kidney disease

Authors	Effects	Comments
Qin *et al*.[26]	Positive	This meta-analysis included 3886 patients
Jardine *et al*.[27]	Negative	10,951 participants in total
Pena and Claro[28]	Negative	This work analyzed the papers from Qin *et al*.[26] and Jardine *et al*.[27]
Levi *et al*.[29]	Positive	A historical prospective study on 3602 subjects

About the *MTHFR* C677T genotype, a meta-analysis of Gao *et al.*[33] concluded that this polymorphism may be associated with an elevated risk for CVD in ESRD, especially among Asians. In contrast, our results did not put the presence of *MTHFR* C677T genotype as a strong determinant for mortality, at least in a short follow up of 1 year. Since the patients with this SNP showed a trend to have higher levels of Hcy, belonging to the range of moderately elevated[34], probably, in this situation, the beneficial effect of reducing Hcy could be confirmed in a more expanded population leading to an expected shrinkage of the standard deviation and reaching significant statistical difference $p \leq 0.05$.

Taken together, these findings need to be interpreted within the context of several inherent limitations, for example, a handful of factors may contribute to the Hcy changes. For example, the Log (tHcy) is directly related to the use of diuretics[35]. In this regard, the drug doses were not supervised; secondly, Hcy is influenced by dietary habits, which were deliberately not recorded. Third and last, the effect of other Hcy-lowering agents, such as vitamin B12, was not analyzed in the current study. These limitations may have compromised our findings. Notwithstanding, the major strength of this study is the evaluation not only of serum Hcy but also of the presence of the *MTHFR* C677T genotype, which offers a better approach to understand the implication over a clinical prognosis than with any of them alone.

CONCLUSION

Based on the result of this study and on previous reports, the effect of Hcy on mortality in hemodialysis patients is not strong enough to consider it as cardiovascular risk factor in ESRD patients, but probably those patients with the *MTHFR* C677T genotype have proclivity to show Hcy levels in range of moderately elevated or severe hyperhomocysteinemia and could be impacted positively with a supplemental and nutritional intervention program to keep Hcy within normal range. This last supposition needs further research.

SIGNIFICANCE STATEMENTS

Patients subjected to hemodialysis are under the toxic effects of hyperhomocysteinemia, present in about 85-90% of these subjects. Hyperhomocysteinemia causes are varied and include a genetic component in which single nucleotide polymorphisms (SNP) play a key role. The results of this study confirms that Hcy does not represent a specific mortality risk in end stage renal disease patients treated with hemodialysis but the role of the presence of *MTHFR*C677T SNP that induces levels in range of moderately elevated or severe hyperhomocysteinemia has still to be elucidated.

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