DOI 10.1002/mnfr.200700114 Mol. Nutr. Food Res. 2007, *51*, 1430 – 1436

Research Article

Hyperhomocysteinemia and polymorphisms of the methylenetetrahydrofolate gene in hemodialysis and peritoneal dialysis patients

Fernanda Aparecida Domenici, Maria T. I. Vannucchi, Livia M. C. Simões-Ambrósio, Helio Vannucchi

Nutrition Division, University of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil

Hyperhomocysteinemia has been documented in chronic renal failure (CRF). Premature as well as progressive occlusive vascular disease is common. Mutations or polymorphisms in the gene of the enzyme methylenetetrahydrofolate reductase (MTHFR), as C677T, A1298C and G1793A, are associated with hyperhomocysteinemia and possibly with elevated risk for vascular diseases. This study was conducted on 89 individuals with renal failure on dialysis to determine the allelic and genotypic frequencies of the mutations in the MTHFR gene and hyperhomocysteinemia. Blood samples were colleted for determination of homocysteine and DNA. The C677T, A1298C and G1793A mutations were detected. This study confirmed the high prevalence of hyperhomocysteinemia in patients on dialysis, which was diagnosed in 76 patients (85.39%) and high incidence of the C677T and A1298C mutation, 42 (47.19%) and 29 (32.58%) patients, respectively. Five patients (5.62%) presented the G1793A mutation and hyperhomocysteinemia. The authors concluded that there was no influence of the polymorphisms on homocysteine levels in these patients.

Keywords: Folic acid / Homocysteine / Methylenetetrahydrofolate reductase / Polymorphisms / Renal failure

Received: March 20, 2007; revised: June 6, 2007; accepted: June 7, 2007

1 Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid generated as an intermediate product of methionine metabolism. There are two remethylation pathways. The first is linked to the folate cycle in which 5,10-methylenetetrahydrofolate reductase (MTHFR) forms 5-methyltetrahydrofolate, which serves as a methyl donor to remethylate Hcy via methionine synthase, a vitamin B12-dependent enzyme. The other remethylation pathway is catalyzed by betaine-homocysteine methyltransferase. Hcy has atherogenic properties on blood vessels and elevated total plasma Hcy is an independent risk factor for cardiovascular disease [1, 2].

The common approach to search for genetic markers of increased susceptibility is to study candidate genes for association with the disease. Recently, hyperhomocysteinemia has emerged as an independent cardiovascular risk factor in subjects with chronic renal failure disease. Moderate hyperhomocysteinemia is present from the early stages of renal failure and increases in parallel to the deterioration of renal function [3, 4].

Mutation in the MTHFR gene reduces its enzymatic activity and causes hyperhomocysteinemia, as well as vascular complications. The gene that codes for MTHFR has been mapped to chromosome 1, region 1p 36.3, and presents 11 exons ranging in size from 102 to 432 bp [5, 6].

A common polymorphism in MTHFR gene, in which a cytidine residue at nucleotide position 677 is replaced by thymidine (C677T), has been described. This results in the substitution of an alanine by valine, which causes the enzyme to become thermolabile and less active [7, 8]. In patients with renal disease this mutation is linked to elevated total plasma Hcy levels in homozygotes compared with heterozygotes or normal individuals [9–11].

The A1298C mutation in MTHFR gene, consisted by transition from A to C in nucleotide 1298 and resulting in alteration of a glutamate codon for alanine, was reported later [12, 13]. Like C677T mutation, this mutation also results in a reduction of enzymatic activity, which is more

Correspondence: Professor Helio Vannucchi, Nutrition Division – University of Medicine of Ribeirão Preto – University of São Paulo, Avenida Bandeirantes, 3900, Bairro Monte Alegre, CEP: 14049-900, Ribeirão Preto, Brazil
E-mail: hvannucc@fmrp.usp.br
Fax: +55-163-633-1586

Abbreviations: CRF, chronic renal failure; **Hcy**, homocysteine; **HD**, hemodialysis; **MTHFR**, methylenetetrahydrofolate; **PD**, peritoneal dialysis

pronounced in mutant homozygous individuals than in heterozygotes, but has been less associated with the risk of vascular diseases than C677T mutation [14].

A new mutation in exon 11 was recently identified. The mutation occurs at position 1793 and a G substitution with A is observed, resulting in alteration of the translation of an arginine to a glutamine. The authors also reported the prevalence of this allele in different ethnic populations, revealing some variability in allele frequency according to the group studied [15].

The aim of the present study was to determine the allele and genotype frequencies of the C677T, A1298C and G1793A polymorphisms in the MTHFR gene in renal chronic failure patients with hyperhomocysteinemia.

2 Materials and methods

2.1 Patients

Initially the study was conducted with 89 CRF patients, 55 patients on hemodialysis (HD) and 34 patients on peritoneal dialysis (PD). Thirty-six patients were women (40.45%) with a mean age of 55 ± 16.32 years, and 53 patients were men (59.55%) with a mean age of 59.66 ± 14.76 years.

The patients were on dialysis at Nephrology Service of Ribeirão Preto, Brazil. Before the experiment, the patients were informed about the purpose of the study and the type of sample to be collected and gave written consent to participate in the study. The study was approved by the Ethics Committee of the University Hospital, University of Medicine of Ribeirão Preto, University of São Paulo. Patients with infectious diseases, acute inflammatory conditions, alcoholism, renal transplant, neoplasias were excluded from the study. Patients were given supplementation with 1 mg of folic acid three times a week.

2.2 Experimental design

Patients were first submitted to clinical evaluation and blood samples were obtained after a 12-h fast for the extraction of genomic DNA and for the determination of homocysteine.

From 89 patients, 13 (14.61%) were excluded due to normal values of the homocysteine. The DNA of 76 patients with hyperhomocysteinemia was extracted for verification of polymorphisms C677T, A1298C and G1793A in the gene of the enzyme methylenetetrahydrofolate reductase.

The control group consisted of 29 apparently healthy individuals without indication of renal injury.

2.3 Laboratory analyses

Plasma samples were stored frozen at –70°C until analysis. Plasma homocysteine levels were measured with IMMULITE® 1000 in a DPC assay which is an immunoassay using reagents and an antibody. Homocysteine determination involved a preliminary manual sample pretreatment step. Homocysteine in plasma sample was released from its binding proteins and converted to S-adenosyl-homocysteine (SAH) by an off-line 30-min incubation at 37°C in the presence of S-adenosyl-L-homocysteine hydrolase and DTT. The treated sample and alkaline phosphatase-labeled anti-SAH antibody were simultaneously introduced into a test unit containing an SAH-coated polystyrene bead. During a 30-minute incubation, the converted SAH from the patient sample competes with immobilized SAH for binding alkaline phosphatase-labeled anti-SAH antibody conjugate. Unbound enzyme conjugate was removed by a centrifugal wash. Substrate (dioxyethane) was added, and the procedure continued as described for typical immunoassays in the Operator's Manual. Values <12 mmol/L were adopted as the normal range for plasma homocysteine levels.

2.4 Extraction of genomic DNA from leukocytes

Approximately 10 mL of peripheral blood was collected from each individual after a 12-h fast into tubes containing EDTA as anticoagulant, and the genomic DNA of leucocytes was extracted by the method of Miller *et al*. [16]. DNA was resuspended in 200 µL of milli-Q water and stored under refrigeration (4°C) for subsequent identification of genetic mutation.

2.5 Detection of C677T, A1298C and G1793A mutation in the MTHFR gene

The presence of C677T, A1298C and G1793A mutation in gene encoding the MTHFR enzyme was determined by PCR-restriction fragment length polymorphism (RFLP).

Primers for the C677T mutation were 5′TGAAGGAGAAGGTGTCTGCGGGA3′ and 5′AGGACGGTGCGGTGAGAGTG3′ and the parameters used for the reaction have been described by Arruda *et al*. [17]. The amplified 198-bp fragment was digested with restriction enzyme *Hinf I* (Promega®) for 3 h at 37°C and then submitted to 1.5% agarose gel electrophoresis (Gibco-BRL®) and ethidium bromide staining for visualization with a UV transilluminator. Digestion of 198-bp fragment with the *Hinf I* enzyme resulted in two bands of 175 and 23 bp for homozygote 677TT while for the normal 677CC no digestion occurred. In the presence of the 677CT heterozygote three bands of 198, 175 and 23 bp were visualized.

Primers for the A1298C mutation were 5′CTTCTACCTGAAGAGCAAGTC3′ and 5′CATGTCCACAGCATGGAG3′ and the parameters used for the reaction have been described by Hanson *et al*. [18]. The amplified 256-bp fragment was digested with the restriction enzyme *Mbo II* (Bio Labs®) for 3 h at 37°C and then submitted to 1.5% agarose gel electrophoresis and ethidium bromide staining for visu-

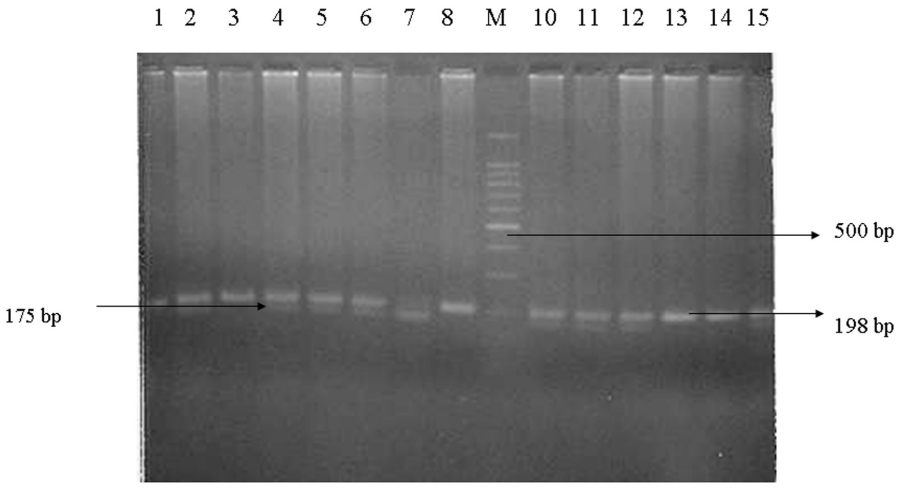

Figure 1. Agarose gel electrophoresis (1.5%) of the PCR products C677T digested with *Hinf I*. M represents the commercial 100-pb marker; Lane 1 DNA was not digested; Lanes 3, 8, 13 and 14, normal patients; Lane 7, homozygous patient; Lanes 2, 4, 5, 6, 10, 11, 12, and 15 heterozygous patients.

Table 1. Prevalence of hyperhomocysteinemia and normal plasma homocysteine levels in renal failure patients with and without C677T, A1298C and G1793A mutation

	Hyperhomocysteinemia[a)]	Without hyperhomocysteinemia	Statistical analysis (p <0.05)[b)]
C677T mutation	42[c)]	6	p = 0.56
Without C677T mutation	34	7	
A1298C mutation	29	9	p = 0.06
Without A1298C mutation	47	4	
G1793A mutation	5	1	p = 1.0
Without G1793A mutation	72	12	

a) Hyperhomocysteinemia: >12 mmol/L.
b) The chi-square test was significant at p <0.05.
c) Absolute number of patients.

alization with a UV transilluminator. Digestion of the 256-bp fragment with the *MboII* enzyme resulted in four bands of 176, 30, 28 and 22 pb for the normal 1298AA and in 204, 176, 30 and 22 bp in the 1298AC heterozygote. In the presence of the mutant homozygous 1298CC genotype three bands of 204, 30 and 22 bp were visualized.

Primers for the G1793A mutation were 5′CTCTGT-GTGTGTGTGCATGTGTGCG3′ and 5′GGGACAGGA-GTGGCTCCAACGCAGG3′ and the parameters used for the reaction have been described by Rady *et al*. [15]. The amplified 310-bp fragment was digested with the restriction enzyme *BsrbI* (BioLabs®) and then submitted to 1.5% agarose gel electrophoresis and ethidium bromide staining for visualization with a UV transilluminator. Digestion of the 310-bp fragment with the *BsrbI* enzyme resulted in two bands of 233 and 77 bp for the normal 1793GG genotype and in three bands of 310, 233 and 77 bp for the G1793A heterozygote. In the presence of the mutant 1793AA genotype, only the 310-bp band was visualized.

2.6 Statistical analysis

Allelic frequencies were calculated by counting the alleles for the C677T, A1298C and G1793A mutation. Matching of genotypic frequencies with Hardy-Weinberg equilibrium was determined using the chi-square test (p >0.05 = equilibrium, and p <0.05 = disequilibrium). The GENEPOP Program, version 3.2, was used for these analyses [19]. Chi-square test was used to determine the association between the presence or absence of hyperhomocysteinemia and mutation in all renal failure patients. The level of significance was set at p <0.05 for all tests. GraphPad Instat software, version 3.0, was used for the analyses.

3 Results

From 89 patients, 13 (14.61%) showed normal plasma homocysteine levels. The median plasma homocysteine concentration of 76 patients that showed hyperhomocystei-

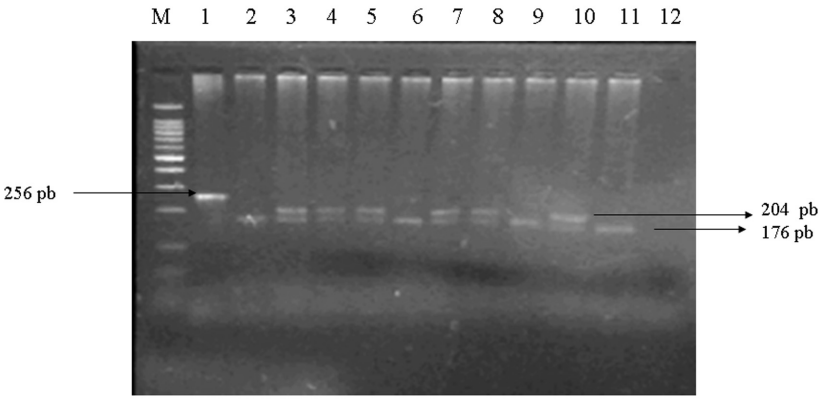

Figure 2. Agarose gel electrophoresis (1.5%) of the PCR product A1298C digested with *Mbo II*. M represents the commercial 100-pb marker; Lane 1 DNA was not digested; Lanes 2, 6, 9, and 11, normal patients; Lanes 3, 4, 5, 7, 8, and 10 heterozygous patients; Lane 12, negative control.

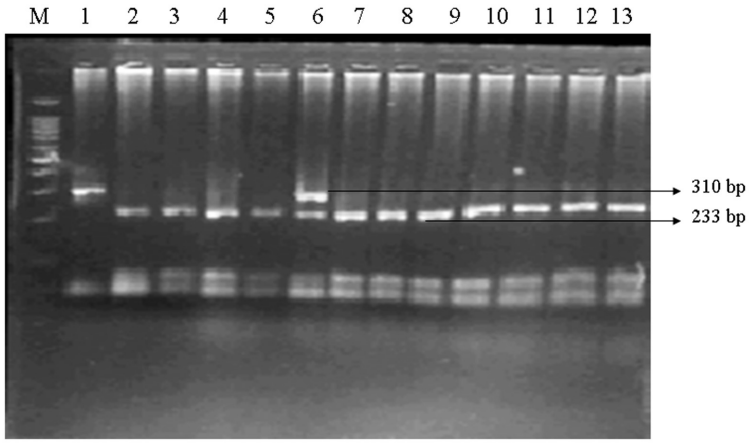

Figure 3. Agarose gel electrophoresis (1.5%) of the PCR product G1793A digested with *Bsrb I*. M represents the commercial 100-pb marker; Lane 1 DNA was not digested; Lanes 2, 6, 9, 11 normal patients; Lanes 3, 4, 5, 7, 8, and 10 heterozygous patients; Lane 12, negative control.

nemia was 26.1 ± 14.2 µmol/L. The control group showed normal levels of the homocysteine (10.20 ± 2.44 µmol/L).

After isolation of chromosomal DNA 198, 256 and 310-pb fragments containing the C677T, A1298C and G1793A polymorphisms of MTHFR, respectively, were amplified by PCR from all samples. The results presented below refer to patients with hyperhomocysteinemia.

Digestion with the *Hinf I* enzyme revealed that 42 patients (55.3%) presented C677T mutation which of 30 patients presenting 677CT heterozygous genotype and 12 patients presenting the 677TT homozygous genotype (Fig. 1). The control group showed 16 individuals (55.2%) with C677T heterozygous genotype mutation and no homozygous.

A1298C polymorphism was detected in 29 patients (38.2%). Twenty-one patients with A1298C presented the 1298AC heterozygous genotype and 8 patients presented the 1298CC homozygous genotype (Fig. 2). The control group showed 20 individuals (69%) with A1298C mutation which 17 presented the 1298AC heterozygous genotype and 3 presented the 1298CC homozygous genotype.

Digestion with the *BsrB I* enzyme revealed that 5 patients (6.6%) presented mutation and hyperhomocysteinemia and had the heterozygous genotype (1793GA). No homozygous individuals with mutant alleles were observed (Fig. 3). The control group did not show individuals with the G1793A mutation.

The prevalence of the hyperhomocysteinemia and normal plasma homocysteine levels in the patients with at least one mutant allele for polymorphisms C677T, A1298C and G1793A and patients without mutation in the MTHF gene is shown in Table 1. The chi-square test did not demonstrate a significant association between variables.

The genotype distribution and allele frequencies of C677T, A1298C and G1793A polymorphisms are shown in Table 2.

4 Discussion

Chronic renal failure (CRF) is frequently associated with increased plasma levels of Hcy, an amino acid that can be considered a new uremic toxin according to recent evidence [20]. In the present study, we observed a high prevalence of hyperhomocysteinemia (85.39%) in patients with renal failure on dialysis.

Van Guldener *et al*. [21] studied the metabolic pathways of Hcy in patients with renal failure on HD using steady isotopes. Remethylation and transmethylation were decreased in patients compared with normal controls but transsulfura-

Table 2. Genotype distribution and allele frequencies of C677T, A1298C and G1793A polymorphisms in 76 patients with hyperhomocysteinemia

	C677T[a] (%) (*n* = 76)	A1298C[b] (%) (*n* = 76)	G1793A[c] (%) (*n* = 76)
Homozygotes	15.79 (*n* = 12)	10.53 (*n* = 8)	0.0 (*n* = 0)
Heterozygotes	39.47 (*n* = 30)	27.63 (*n* = 21)	6.58 (*n* = 5)
Normal	44.74 (*n* = 34)	61.84 (*n* = 47)	93.42 (*n* = 71)
Allele frequency (%)	C = 64.47 (98/152)	A=75.66 (115/152)	G=96.71 (147/152)
Allele frequency (%)	T = 35.53 (54/152)	C=24.34 (37/152)	A=3.29 (5/152)

a) Hardy-Weinberg equilibrium $p = 0.2422$.
b) Hardy-Weinberg equilibrium $p = 0.2574$.
c) Hardy-Weinberg equilibrium $p = 1$.

tion appeared not to have been affected. The authors believe that the apparent cause of hyperhomocysteinemia in HD may be exactly the alteration of the remethylation pathway due to some factors such as deficiency of folate and vitamin B12, disorders of folate metabolism and decreased activity of the enzymes involved in this pathway resulting from specific genetic mutations.

Metabolic alterations were also considered by Henning *et al*. [22] who simultaneously determined Hcy concentrations and remethylation and transmethylation of the metabolites in patients on dialysis or not. The results showed that transsulfuration increased in parallel with hyperhomocysteinemia but did not reach normal values. On the other hand, remethylation, although sensitive to folate in a certain extent, seems not to be adequately stimulated in CRF, possibly due to the presence of enzymatic inhibition by a still unidentified uremic toxin, and this abnormality is not sufficiently reversed by HD.

The causes of hyperhomocysteinemia in renal failure are still not clear. However, the possibilities include defective renal or extrarenal metabolism as a result of uremic toxicity. Renal plasma flow is important in homocysteine renal metabolism. Among the consequences of hyperhomocysteinemia in renal failure are impaired protein and DNA methylation, with an alteration in the allelic expression of genes regulated through methylation [23].

The importance of genetic mutations in the performance of the metabolic pathways described above must also be considered. Genetic defects of the enzymes involved in this metabolism can be the cause of hyperhomocysteinemia in patients under treatment.

Among the genetic causes of hyperhomocysteinemia in humans are homozygous or heterozygous deficiencies of enzymes such as cystathione beta synthase (CβS), MTHFR and methionine synthetase (MS) [24].

It has been reported that homozygosity C677T of MTHFR is a strong determinant of plasma Hcy levels in HD patients [25] and that negative correlation between serum Hcy and serum folate is more pronounced in the TT genotype [26]. Therefore, patients with MTHFR mutation may be more susceptible to intracellular deficiency of folate and consequent metabolic dysfunction than individuals without MTHFR mutation.

In the present study the patients with hyperhomocysteinemia showed 55.3% C677T, 38.2% A1298C and 6.6% G1793A polymorphisms. Control group showed normal levels of Hcy and high prevalence of the polymorphisms in MTHFR gene with 55.2% C677T, 69% A1298C and no G1793A mutation. Results of this study showed that patients with renal failure had incidence of polymorphisms C677T and A1298C in the gene MTHFR as high as control group.

In our patients, none effect ($p > 0.05$, using chi-square test) of homozygous or heterozygous C677T, A1298C and G1793A MTHFR polymorphisms was observed on Hcy levels. These patients showed high incidence of hyperhomocysteinemia (85.39%) independent of the presence or not of the polymorphism. Although high prevalence of polymorphisms healthy individuals showed normal levels of the Hcy.

This result may be explained by an increase in plasma Hcy level could be caused by an increased production rate (transmethylation), a decreased rate of removal by transsulfuration or remethylation, or a decrease in excretion of Hcy. Current evidence indicates that the major mechanism for hyperhomocysteinemia in renal failure is a decrease in Hcy removal from the body. However, it is debated whether this effect is result of a decrease in the renal metabolic clearance or a result of extrarenal metabolic changes and the glomerular filtration of Hcy seems to be restricted because of protein binding.

Canepa *et al*. [27] studied C677T polymorphism in children with renal failure and found that 40% had hyperhomocysteinemia and 14% and 5% had folate and vitamin B12 deficiency, respectively. On univariate analysis, the serum Hcy concentration was negatively correlated with the plasma folate concentration ($p < 0.05$) in controls and with glomerular filtration rate ($p < 0.05$) in patients. No effect of MTHFR polymorphism on Hcy levels was observed and the authors explained these results, in addition to the limited number of patients, might partially be explained by the low prevalence of folate deficiency.

Haviv *et al*. [28] studied 120 patients on HD to determine the prevalence of MTHFR C677T and A1298C mutations and their relative association with hyperhomocysteinemia and cardiovascular disease (CVD). The authors found that both the C677T and A1298C mutations were highly prevalent in HD patients, with allele frequencies of 0.41 and 0.27, respectively. The prevalence of CVD in HD patients was 55% and its significant risk factors included, in decreasing order of importance, hyperhomocysteinemia, MTHFR C677T mutation, low serum folate levels, diabetes mellitus, hypertension, and double heterozygote status for both MTHFR mutations (677CT/1298AC). MTHFR A1298C mutation alone and gender were not associated with either hyperhomocysteinemia or increased CVD risk, but the HD patients with homozygous 1298CC and wild alleles 677CC (677CC/1298CC) had a significant increase of Hcy (37.7 ± 12) and a high prevalence of CVD. These results show that hyperhomocysteinemia, serum folate levels and both C677T and A1298C MTHFR mutations are associated with CVD in HD patients.

In the present study, the frequency of heterozygous patients of G1793A polymorphism with hyperhomocysteinemia was 6.6%. This mutation has been detected recently and requires clarification regarding its association with hyperhomocysteinemia and with alterations of folic acid metabolism. Rady *et al*. [15], in study on various ethnic groups such as Hispanics, African-Americans, Ashkenazi Jews and Caucasians, observed that 11.6, 6.2, 2.6 and 12.6% of them were heterozygotes, respectively. In the present study, as shown in Table 2, the frequency of the A allele detected among the renal failure patients studied was only 3.29%, similar to that detected by Rady *et al*. [15] in the African American population, which was 3.1%.

Similar results for G1793A polymorphism were also obtained by Melo *et al*. [29] who studied 83 individuals with type 2 diabetes. The authors found only a 3.01% frequency of the A allele among the diabetic individuals and observed that individuals with a heterozygous genotype for the G1793A mutation showed borderline or deficient folic acid and vitamin B12 concentrations compared to individuals with a normal genotype.

The present study showed a high presence of the mutation in MTHFR gene in the in both groups studied (renal failure patients and healthy individuals) but only in the renal failure patients it was observed the presence of the hyperhomocysteinemia. These results of the high levels of the Hcy independent of the genetic factors in these patients can be explained by the metabolic disorder showed in renal failure patients in treatment.

Financial support from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) is acknowledged.

5 References

[1] Graham, I. M., Daly, L. E., Refsum, H. M., Plasma homocysteine as a risk factor for vascular disease, The European Concerted Action Project, *JAMA* 1997, *277*, 1775 – 1781.

[2] Rosenblatt, D. S., Methylenetetrahydrofolate reductase, *Clin. Invest. Med.* 2001, *24*, 56 – 59.

[3] Morimoto, K., Haneda, T., Okamoto, K., Ishida, H. *et al*., Methylenetetrahydrofolate reductase gene polymorphism, hyperhomocysteinemia, and cardiovascular diseases in chronic hemodialysis patients, *Nephron* 2002, *90*, 43 – 50.

[4] Nakamura, T., Saionji, K., Hiejima, Y., Hirayama, H. *et al*., Methylenetetrahydrofolate reductase genotype, vitamin B12, and folate influence plasma homocysteine in hemodialysis patients, *Am. J. Kidney Dis.* 2002, *39*, 1032 – 1039.

[5] Goyette, P., Sumner, J. S., Milos, R., Duncan, A. M. V. *et al*., Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification, *Nat. Genet.* 1994, *7*, 195 – 200.

[6] Goyette, P., Pai, A., Milos, R., Frosst, P. *et al*., Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR), *Mamm. Genome* 1998, *9*, 652 – 656.

[7] Frosst, P., Blom, H. J., Milos, R., Goyette, P. *et al*., A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase, *Nat. Genet*. 1995, *10*, 111 – 113.

[8] Arruda, V. R., Von-Zuben, P. M., Chiaparini, L. C., Annichino-Bizzacchi, J. M. *et al*., The mutation Ala 677-Val in the methylenetetrahydrofolate reductase gene: a risk factor for arterial disease and venous thrombosis, *Thromb. Haemost.* 1997, *77*, 818 – 821.

[9] Bostom, A. G., Culleton, B. F., Hyperhomocysteinemia in chronic renal disease, *J. Am. Soc. Nephrol.* 1999, *10*, 891 – 900.

[10] Kimura, H., Gejyo, F., Suzuki, S., Takeda, T. *et al*., A C677T mutation in the methylenetetrahydrofolate reductase gene modifies serum cysteine in dialysis patients, *Am. J. Kidney Dis.* 2000, *36*, 925 – 933.

[11] Fodinger, M., Buchmayer, G., Heinz, A., Association of two MTHFR polymorphisms with total homocysteine plasma levels in dialysis patients, *Am. J. Kidney Dis.* 2001, *38*, 77 – 84.

[12] Van Der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A. *et al*., A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am. J. Hum. Genet.* 1998, *62*, 1044 – 1051.

[13] Weisberg, I., Tran, P., Christensen, B., Sibani, S., Rozen, R., A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity, *Mol. Genet. Metab.* 1998, *64*, 169 – 172.

[14] Friedman, G., Goldschimidt, N., Friedlander, Y., Yehuda, A. B. *et al*., A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations, *J. Nutr.* 1999, *129*, 1656 – 1661.

[15] Rady, P. L., Szucs, S., Grady, J., Hundnall, S. D. *et al*., Genetic polymorphisms of methylenetetrahydrofolate reductase and methionine synthase reductase in ethnic populations in Texas; a report of a novel MTHFR polymorphic site, G1793A, *Am. J. Med. Genet.* 2002, *107*, 162 – 168.

[16] Miller, S. A., Dykes, D. D., Polesky, H. F., A simple salting out procedure for extracting DNA from human nucleated cells, *Nucleic Acids Res.* 1988, *1*, 1215.

[17] Arruda, V. R., Zuben, P. M., Chiaparini, L. C., Annichino-Bizzacchi, J. M., Costa, F. F., The mutation Ala677Val in the methylene tetrahydrofolate reductase gene: a risk factor for arterial disease and venous thrombosis, *Thromb. Haemost.* 1997, *77*, 818 – 821.

[18] Hanson, N. Q., Aras, O., Yang, F., Tsai, M. Y., C677T and A1298C polymorphisms of the MTHFR gene: incidence and effects of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease, *Clin. Chem.* 2001, *47*, 661 – 666.

[19] Rousset, F., *J. Evol. Biol.* 2000, *13*, 58 – 62.

[20] Satta, E., Perna, A. F., Lombard, C., Acanfora, F. *et al*., Hyperhomocysteinemia in chronic renal failure, *G. Ital. Nefrol.* 2006, *23*, 480 – 489.

[21] Van Guldener, C., Janssen, M. J. F. M., Meer, K., Donker, A. J., Stehouwer, C. D., Effect of folic acid and betaine on fasting and postmethionine-loading plasma homocysteine and methionine levels in chronic haemodialysis patients, *J. Inter. Med.* 1999, *245*, 175 – 183.

[22] Henning, B. F., Rielzler, R., Tepel, M., Evidence of altered homocysteine metabolism in chronic renal failure, *Nephron* 1999, *83*, 314 – 322.

[23] Perna, A. F., Ingrosso, D., Satta, E., Lombardi, C. *et al*., Homocysteine metabolism in renal failure, *Curr Opin Clin Nutr Metab Care*, 2004, *7*, 53 – 57.

[24] Miner, S. E. S., Evrovski, J., Cole, D. E. C., Clinical Chemistry and molecular biology of homocysteine metabolism: un update, *Clin. Biochem.* 1997, *30*, 189 – 201.

[25] Fodinger, M., Mannhalter, C., Wolfl, G., Pabinger, I., Muller, E. *et al*., Mutation (677 C to T) in the methylenetetrahydrofolate reductase gene aggravates hyperhomocysteinemia in hemodialysis patients, *Kidney Int*. 1997, *52*, 517 – 523.

[26] Kimura, H., Gejyo, F., Suzuki, S., Miyazaki, R., The C677T methylenetetrahydrofolate reductase gene mutation in hemodialysis patients, *J. Am. Soc. Nephrol.* 2000, *11*, 885 – 893.

[27] Canepa, A., Carrea, A., Caridi, L. D., Minniti, R. C., Cerone, R., *et al*., Homocysteine, folate, vitamin B12 levels, and C677T MTHFR mutation in children with renal failure, *Pediatr. Nephrol.* 2003, *18*, 225 – 229.

[28] Haviv, Y. S., Shpichinetsky, V., Goldschimit, N., Abou Atta, I., Benyehuda, A., *et al*., The common mutation C677T and A1298C in the human methylenetetrahydrofolate reductase gene are associated with hyperhomocysteinemia and cardiovascular disease in hemodialysis patients, *Nephron* 2002, *92*, 120 – 126.

[29] Melo, S. S., Persuhn, D. C., Meirelles, M. S., Jordão, A. A., Vannucchi, H., G1793A polymorphisms in the methylenetetrahydrofolate gene: Effect of folic acid on homocysteine levels, *Mol. Nutr. Food Res.* 2006, *50*, 769 – 774.

