

G1793A polymorphisms in the methylenetetrahydrofolate gene: Effect of folic acid on homocysteine levels

Sandra Soares Melo¹, Darlene Camati Persuhn², Mônica S. Meirelles³,
Alceu Afonso Jordao³, Helio Vannucchi³

¹Nutrition Program, University of Vale do Itajaí, Itajaí, SC, Brazil

²Department of Biochemistry, Federal University of Paraná and Center for Health Sciences, University of Vale do Itajaí, Itajaí, SC, Brazil

³Nutrition Division, Department of Clinical Medicine, FMRP, University of São Paulo, Ribeirão Preto, SP, Brazil

Mutations or polymorphisms in the gene of the enzyme methylenetetrahydrofolate (MTHFR) are associated with hyperhomocysteinemia and possibly with an elevated risk for vascular diseases. A study was conducted on 83 individuals with type 2 diabetes in order to determine the allelic and genotypic frequencies of the G1793A mutation and to assess the effect of folic acid supplementation on plasma homocysteine concentrations. The patients were attended by the Diabetes and Hypertension Program – Balneario Camboriú/SC and received daily supplements containing 1 mg of folic acid for 3 months. DNA was previously extracted from leukocytes and the G1793A mutation was detected by PCR-RFLP. Blood samples were collected during the basal period and after supplementation for the determination of homocysteine by HPLC, and of folic acid and vitamin B₁₂ by RIA. The allele frequency for the G1793A mutation was 3.01% and no homozygous individuals with mutant alleles were detected. Hyperhomocysteinemia was diagnosed in 27.71% of the patients, folic acid deficiency in 15.66%, and vitamin B₁₂ deficiency in 7.23%. Plasma homocysteine concentrations were inversely correlated with folic acid ($r = -0.27$, $p = 0.01$) and vitamin B₁₂ ($r = -0.21$; $p = 0.05$) concentrations. The individuals with a heterozygous genotype for the G1793A mutation showed borderlines or deficient values in folic acid and vitamin B₁₂ concentrations compared to individuals with a normal genotype. Hyperhomocysteinemia and the vitamin deficiencies presented by type 2 diabetic individuals, included with a heterozygous genotype for the G1793A mutation in the MTHFR gene, reached normal values by daily folic acid supplementation.

Keywords: B₁₂ vitamin / Folic acid / G1793A / Homocysteine / Methylenetetrahydrofolate reductase

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1 Introduction

The enzyme methylenetetrahydrofolate (MTHFR) reductase plays a fundamental role in homocysteine metabolism by catalyzing the conversion of 5,10-MTHFR to 5-MTHFR. The latter is the predominant form of folate and is a carbon donor for the remethylation of homocysteine to methionine

[1]. Mutations in the MTHFR gene may reduce the activity of the enzyme, with consequent inadequate homocysteine methylation and may cause hyperhomocysteinemia [2].

Excess plasma homocysteine concentrations (hyperhomocysteinemia) may generate the formation of homocysteine thiolactone, which is a highly reactive intermediate that modifies free amino groups of LDL-cholesterol, causing these particles to aggregate and to be phagocytized by macrophages. Homocysteine can also produce lipoperoxidation and platelet aggregation, resulting in fibrosis and calcification of atherosclerotic plaques [3].

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 [4, 5].

Correspondence: Professor Helio Vannucchi, Nutrition Division, Internal Medicine Department, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes 3900, CEP 14049900, Ribeirão Preto, SP, Brazil

E-mail: hvannucc@fmrp.usp.br

Fax: +55-16-36331586

Abbreviation: MTHFR, methylenetetrahydrofolate

C667T polymorphism in the MTHFR gene results in the substitution of an alanine with a valine at position 226 in the protein. This polymorphism was reported in 1995 by Frosst *et al.* [6] and has been extensively studied in terms of its involvement as a genetic risk factor for vascular diseases, although the conclusions are still controversial [7–9]).

The A1298C mutation in the MTHFR gene, which consists of the transition from A to C in nucleotide 1298 resulting in alteration of a glutamate codon for alanine, was reported later [10, 11]. Like the C677T mutation, this mutation also results in a reduction of enzymatic activity, which is more pronounced in mutant homozygous individuals than in heterozygotes, but has been less associated with the risk of vascular diseases than the C677T mutation [12].

Rady *et al.* [13] recently reported a new mutation in exon 11 of this gene detected by screening for SSCP analysis and later by automated sequencing of polymorphic fragments. 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 report the prevalence of this allele in different ethnic populations, revealing some variability in allele frequency according to the group studied.

Some vitamins of the B complex, among them folic acid, function as cofactors and substrates in the metabolism of methionine and homocysteine. Thus, it has been suggested that nutritional status as a single factor or combined with genetic factors may lead to various forms of hyperhomocysteinemia [14, 15].

Considering the importance of MTHFR polymorphisms as possible risk factors for vascular diseases, the high prevalence of vascular diseases among diabetic individuals and the absence of studies investigating the possible involvement of the G1793A mutation in homocysteine metabolism, the objective of the present clinical study was to determine the allelic and genotypic frequencies of this polymorphism and to assess the effect of folic acid supplementation on the plasma concentrations of homocysteine in individuals with type 2 diabetes.

2 Materials and methods

2.1 Population and sampling

The study was conducted on 83 patients who attended the Diabetes and Hypertension Program of the Central Health Unit of the Municipality of Balneario Camboriu – SC/Brazil. The number of 83 patients was calculated statistically on the basis of the total number of patients of this service. The Research Ethics Committee of the Faculty of Pharma-

ceutical Sciences, University of São Paulo, SP approved the study, and by the Research Ethics Committee of the University of Vale do Itajai, SC, and all subjects gave written informed consent to participate. Diabetes mellitus was diagnosed and classified according to the criteria of the Brazilian Diabetes Society [16]. Patients with infectious diseases, acute inflammatory conditions, serum creatinine concentrations >1.4 mg/dL, and patients receiving supplementation with folic acid alone or in combination with other vitamins were excluded from the study.

2.2 Experimental design

The patients were first submitted to clinical and anthropometric evaluation. All of 83 patients studied received a daily capsule containing 1 mg of folic acid (Origin: China-010460. Distributor: Galena-Campinas/SP/(Brazil) for 3 months, but all patients reported to this service in interval periods of 15 days during the entire period of the study. Blood samples were obtained after a 12 h fasting before and after the supplementation period for the extraction of genomic DNA and for the determination of homocysteine, folic acid, and vitamin B₁₂.

2.3 Laboratory analyses

Plasma homocysteine was determined by HPLC using the method described by Katrusiak *et al.* [17]. The calibration curve of the instrument was constructed based on a stock solution containing 200 µM of DL-homocysteine (S-4628-Sigma) in Milli-Q water, at the concentrations 10, 20, and 100 µM. The results obtained were compared with the standard peaks and expressed as µmol/L. Values <14 µmol/L were adopted as the normal range for plasma homocysteine levels, as suggested in various literature reports [18, 19]. Folic acid and vitamin B₁₂ were quantified in plasma by RIA using a Dualcount kit (DPC® Medlab). The reading was taken using a gamma radiation counter with channels for ¹²⁵I (folic acid) and ⁵⁷Co (vitamin B₁₂). The results are expressed as ng/mL for folic acid and as pg/mL for vitamin B₁₂. For the determination of the plasma levels of folic acid and vitamin B₁₂ the values suggested by the kit were used as the normal range, 3.0–17 ng/mL and 200–950 pg/mL, respectively.

2.4 Extraction of genomic DNA from leukocytes

Approximately 5.0 mL of peripheral blood was collected from each individual after a 10–12 h fasting into tubes containing EDTA as anticoagulant, and the genomic DNA of the leucocytes was extracted according to the method described by Miller *et al.* [20]. The DNA was resuspended

in 200 μ L of milli-Q water, autoclaved, and stored under refrigeration for subsequent identification of the genetic mutation.

2.5 Detection of the G1793A mutation in the gene encoding the MTHFR enzyme

The presence of the G1793A mutation in the gene encoding the MTHFR enzyme was determined by the PCR – restriction fragment length polymorphism (RFLP). Primers 5'CTCTGTGTGTGTGTGCATGTGTGCG3' and 5'GGGACAGGAGTGGCTCCAACGCAGG3' and the parameters used for the reaction have been described by Rady *et al.* [13]. The amplified 310 bp fragment was digested with the restriction enzyme *BsrBI* (Biolabs®) according to manufacturer's instructions and then submitted to 1.5% agarose gel electrophoresis (Gibco-BRL®) and ethidium bromide staining for visualization with a UV transilluminator (Ultra Lum®). 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 370, 233, and 77 bp in the G1793A heterozygote. In the presence of the mutant 1793AA genotype, only the 310 bp band would be visualized [13].

2.6 Statistical analysis

The allelic frequencies were calculated by counting the alleles. The 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 [21, 22]. The correlations between the different variables were calculated by the Spearman nonparametric correlation coefficient. The two-tailed Mann-Whitney test for independent samples was used to evaluate the influence of the genotype for the G1793A mutation on the plasma levels of homocysteine, folic acid, and vitamin B₁₂, both for the baseline period and after supplementation with folic acid. The variables are presented as median and range. The

level of significance was set at $p < 0.05$ for all tests. The GraphPad.Instat software, version 3.0, was used for all analyses.

3 Results

In the present study, blood samples obtained from 83 patients with type 2 diabetes mellitus were analyzed. Forty-six subjects were females (55.42%) with a mean age of 57 ± 9 years, and 37 (44.58%) were males with a mean age of 60 ± 10 years.

After the isolation of chromosomal DNA, the 310 bp fragment containing the G1793A polymorphism of MTHFR was amplified by PCR from all samples. Digestion with the *BsrBI* enzyme revealed that 5 patients (6.02%) presented bands of 310, 233, and 77 bp, which correspond to the heterozygous genotype, while the remaining 78 (93.98%) did not present the 310 bp fragment and were therefore classified as normal homozygotes (Fig. 1). No homozygous individuals with the mutant alleles were observed.

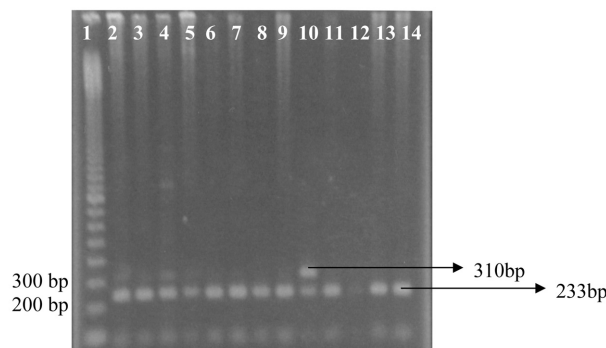


Figure 1. Agarose gel electrophoresis of the PCR products digested with *BsrBI*. Lane 1 represents the commercial 100 bp marker. The fragments of 310 and 233 bp are represented. Patients who presented only the 233 base pair bands (lanes 2–14) were considered to be normal homozygotes and patients who presented the 310 and 233 bp bands (lane 10) were considered to be heterozygotes.

Table 1. Genotype distribution and allele frequencies of the G1793A polymorphism

Genotype MTHFR G1793A	Ashkenazi Jews ^{a)} (%)	African-Americans ^{a)} (%)	Caucasians ^{a)} (%)	Hispanics ^{a)} (%)	Diabetic individuals studied ^{b)} (%)
Homozygotes	(<i>n</i> = 117)	(<i>n</i> = 97)	(<i>n</i> = 159)	(<i>n</i> = 95)	(<i>n</i> = 83)
Heterozygotes	0.0 (<i>n</i> = 0)	0.0 (<i>n</i> = 0)	0.6 (<i>n</i> = 1)	0.0 (<i>n</i> = 0)	0.0 (<i>n</i> = 0)
Normal	2.6 (<i>n</i> = 3)	6.2 (<i>n</i> = 6)	12.6 (<i>n</i> = 20)	11.6 (<i>n</i> = 11)	6.02 (<i>n</i> = 05)
A allele frequency (%)	97.4 (<i>n</i> = 114)	93.8 (<i>n</i> = 91)	86.8 (<i>n</i> = 138)	88.4 (<i>n</i> = 84)	93.98 (<i>n</i> = 78)
G allele frequency (%)	1.3 (3/234)	3.1 (6/194)	6.9 (22/318)	5.8 (11/190)	3.01 (5/166)
	98.7 (231/234)	96.9 (182/194)	93.1 (276/318)	94.2 (168/190)	96.99 (161/166)

^{a)} [13]

^{b)} Hardy-Weinberg equilibrium $p = 1$.

Table 2. Percentage of hyperhomocysteinemia and marginal or deficient values of folic acid and vitamin B₁₂ found in the total sample of type 2 diabetic individuals evaluated (*n* = 83) subdivided for the G1793A mutation and without G1793A mutation

	Hyperhomocysteinemia	Marginal or deficient folic acid values	Marginal or deficient vitamin B ₁₂ values
Without G1793A mutation (<i>n</i> = 78)	25.30% (<i>n</i> = 21)	12.05% (<i>n</i> = 10)	4.82% (<i>n</i> = 04)
G1793A mutation (<i>n</i> = 5)	40% (<i>n</i> = 02)	60% (<i>n</i> = 03)	40% (<i>n</i> = 02)

Hyperhomocysteinemia: > 14 µmol/L.

Folic acid: deficiency < 3 ng/mL and marginal values of 3–4 ng/mL.

Vitamin B₁₂: deficiency < 200 pg/mL and marginal values of 200–300 pg/mL.

Table 3. Median, minimum, and maximum values for plasma homocysteine, folic acid, and vitamin B₁₂, levels, for the individuals evaluated (*n* = 83) subdivided for G1793A mutation and without G1793A mutation, before and after folic acid supplementation

	Baseline values	Values after supplementation	Statistical analysis (<i>p</i> < 0.05)
Without G1793A mutation (<i>n</i> = 78)			
Homocysteine (µmol/L)	11.00 (5.96–23.94)	7.65 (5.19–13.77)	<i>p</i> < 0.0001
Folic acid (ng/mL)	6.96 (2.07–26.55)	23.46 (10.71–37.46)	<i>p</i> < 0.0001
Vitamin B ₁₂ (pg/mL)	580.64 (149.44–1380.3)	912.04 (430.64–3092.6)	<i>p</i> < 0.0001
G1793A mutation (<i>n</i> = 5)			
Homocysteine (µmol/L)	10.23 (7.16–30.51)	7.87 (6.05–9.18)	<i>p</i> = 0.19
Folic acid (ng/mL)	3.54 (2.42–11.53)	17.40 (11.97–30.97)	<i>p</i> = 0.03
Vitamin B ₁₂ (pg/mL)	315.70 (175.18–707.67)	1046.1 (674.85–1732.0)	<i>p</i> = 0.12

The genotypic data related to the G1793A polymorphism were compared to those observed by Rady *et al.* [13], as shown in Table 1. The study population consisted of 78 individuals (93.98%) of Caucasian ethnic origin, including all heterozygotes diagnosed, 3 Mulattos and 2 Blacks, corresponding to 3.6 and 2.41% of the population, respectively.

The percentages of hyperhomocysteinemia and folic acid and vitamin B₁₂ deficiency can be seen in Table 2, which contains the data for all individuals studied, subdivided for the G1793A mutation and without G1793A mutation.

The median plasma homocysteine concentration for the patients with hyperhomocysteinemia as a whole was 16.76 µmol/L (range: 14.03–30.51 µmol/L), while the median for the individuals with hyperhomocysteinemia heterozygous for the G1793A mutation was 24.41 µmol/L. A higher percentage of folic acid and vitamin B₁₂ deficiency was identified in individuals heterozygous for the G1793A mutation compared to the study population as a whole.

A negative correlation was observed between basal homocysteine and folic acid concentrations (*r* = −0.27; *p* = 0.01) and between homocysteine and vitamin B₁₂ (*r* = −0.21; *p* = 0.05) and a positive and significant correlation (*p* < 0.0001) was observed between folic acid and vitamin B₁₂ (*r* = 0.75).

Table 3 presents the medians obtained basally and after supplementation for the plasma concentrations of homocys-

teine, folic acid and vitamin B₁₂, subdivided for the G1793A mutation and without G1793A mutation.

Supplementation of the 83 patients studied with 1 mg/day folic acid for 3 months significantly reduced (*p* < 0.0001) by 30.15% the initial median value of homocysteine and no patient continued to have hyperhomocysteinemia after supplementation. Although in the groups of heterozygotes for the G1793A mutation the difference between basal homocysteine values and the values obtained after folic acid supplementation was not statistically significant, the median basal values of homocysteine were higher than those obtained after 3 months of folic acid supplementation (10.23 × 7.87 µmol/L).

The median plasma concentrations of folic acid and vitamin B₁₂ increased after folic acid supplementation, with 100% of the patients showing normal levels of these vitamins, including heterozygotes for the G1793A mutation. However, the changes with increase in plasma folic acid after the supplementation are shown only for individuals with this mutation (Table 3).

4 Discussion

In the present study we observed for the first time the presence of the G1793A mutation in a population of diabetic individuals and we evaluated the effect of folic acid supplementation on the plasma concentrations of homocysteine.

Shen *et al.* [23] studied the incidence of gastric cancer and the presence of mutations of the MTHFR gene in the Chinese population. In the study by Rady *et al.* [13] the ethnic groups involved were Hispanics ($n = 95$), African-Americans ($n = 97$), Ashkenazi Jews ($n = 117$), and Caucasians ($n = 159$). It was observed that 2.6% ($n = 3$) of the Ashkenazi Jews, 6.2% ($n = 6$) of the African-Americans, 11.6% ($n = 11$) of the Hispanics, and 12.6% ($n = 20$) of the Caucasians were heterozygotes. The incidence of polymorphism observed by Rady *et al.* [13] was lower among Jews and African-Americans and higher in the other two ethnic groups.

In the present population, 78 individuals (93.98%) were Caucasians, including all the heterozygotes diagnosed ($n = 5$). However, the frequency of Caucasian heterozygotes detected by Rady *et al.* [13] was the highest (12.6%) and differed from that detected in the present study (6.02%). As shown in Table 1, the frequency of the A allele detected among the diabetic individuals studied was only 3.01%, similar to that detected by Rady *et al.* [13] in the African-American population. The genotypic distribution for the G1793A locus is in Hardy-Weinberg equilibrium, with values of $p > 0.5$. We suggest that the inbreeding rate was reduced in the diabetic individuals studied and the gene and genotype frequency remained constant from generation to generation. Shen *et al.* [23] observed that the frequency of the A allele was low in case (8%) and control (7.3%) individuals and that the G1793A mutation was not associated with the risk of gastric cancer.

The mutant homozygous genotype (AA) was practically absent in the populations studied, with only one homozygote having been detected among the 155 Caucasians studied by Rady *et al.* [13] and two individuals with a diagnosis of gastric cancer among the 320 subjects evaluated by Shen *et al.* [23]. Among the 83 diabetic individuals analyzed here, none presented this genotype, a fact revealing that the A allele is not sufficiently prevalent to be detected in homozygosis.

Moderate hyperhomocysteinemia is frequently associated with the presence of a mutation in the gene coding for MTHFR, with a reduction in the activity of this enzyme [24] and with the phenotypic demonstration of such reduction being frequently influenced by the concentration of vitamins in blood [25, 26].

The G1793A mutation has been detected recently and requires clarification regarding its association with hyperhomocysteinemia and with alterations of folic acid metabolism [13, 27]. In the present study, hyperhomocysteinemia was detected in 27.71% of the diabetic individuals investigated, with 30.44% of these presenting the mild form and 69.56% the moderate form according to the classification of Malinow *et al.* [1]. The median plasma homocysteine

concentrations detected in type 2 diabetic patients were similar to those reported in other studies on diabetic patients and higher than those detected in healthy individuals [28–30].

The present study identified the presence of elevated homocysteine concentrations in 40% of the heterozygotes for the G1793A mutation, a value 12.3% above that detected in the diabetic population studied.

A moderate elevation of plasma homocysteine as a cause of vascular diseases has been a topic extensively debated in recent studies. Wald *et al.* [31] detected a significant association between homocysteine and ischemic heart disease, venous thrombosis, and stroke. Among the various factors determining the concentrations of circulating homocysteine, genetic disorders, metabolic disorders and folic acid and vitamin B₁₂ deficiency have been associated with hyperhomocysteinemia [32].

The folic acid and vitamin B₁₂ deficiency observed in the present study was 15.66% and 7.23%, respectively, among the sample of diabetic individuals tested as a whole. When the mutant A allele was present, 60 and 40% of individuals heterozygous for the G1793A mutation presented borderline values of deficiency of these vitamins (median: 3.50 ng/mL and 315.70 pg/mL). These plasma concentrations did not differ significantly from those observed in individuals without the mutant alleles for the G1793A polymorphism (6.96 ng/mL and 580.64 pg/mL). However, since these results were obtained for only five individuals, an expansion of the series is suggested for the identification of a possible association of folic acid and vitamin B₁₂ deficiency with the G1793A mutation.

Daily supplementation with 1 mg of folic acid was efficient in significantly reducing the plasma concentrations of homocysteine in the study population. However, for G1793A polymorphism, although the basal median homocysteine concentration was reduced after folic acid supplementation ($10.23 \times 7.87 \mu\text{mol/L}$), the difference was not significant, suggesting an additional involvement of vitamin B₁₂ deficiency. As well as folate, remethylation of homocysteine to methionine requires vitamin B₁₂, which has proved to be an effective determinant of plasma homocysteine concentrations in synergy with folate.

Folic acid concentrations increased by almost three and a half times and vitamin B₁₂ by 1.6 times in response to supplementation and 100% of the patients started to present normal folic acid and vitamin B₁₂ concentrations. However, in individuals heterozygous for the G1793A mutation, only folic acid concentrations increased in a statistically significant manner after supplementation with this vitamin.

These results demonstrate that folic acid concentration was not dependent on the genotype or haplotype of other MTHFR polymorphisms that have not been examined, and also not influenced by age, diet, BMI, and sex of these patients (unpublished data).

The apparent causal relationship between genotype for the C677T mutation and plasma folic acid concentrations indicates that in individuals with hyperhomocysteinemia reduced folic acid concentrations are not necessarily attributable to insufficient dietary ingestion of this vitamin but may be due, at least in part, to the direct result of reduced thermolabile MTHFR activity [33].

In conclusion, in the present study no statistical association was identified between the genotype of the G1793A mutation and hyperhomocysteinemia, probably by a small number of individuals, although lower median values of vitamin B₁₂ and folic acid were detected and folic acid supplementation was efficient in normalizing the plasma concentrations of these vitamins.

In view of the importance of MTHFR gene polymorphisms as a risk factor for vascular diseases and of the lack of literature information regarding the relevance of the G1793A mutation, further studies on a larger number of patients considered to be at high risk for the development of vascular disease would be of fundamental importance in order to determine the association between vitamin deficiencies (folic acid and vitamin B₁₂) and the G1793A mutation and the possible effect of combined folic acid and vitamin B₁₂ supplementation on the reduction of plasma homocysteine concentrations in these clinical patients.

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5 References

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