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ORIGINAL ARTICLE

Methylenetetrahydrofolate Reductase Gene Polymorphisms in Burkina Faso: Impact on Plasma Fasting Homocysteine and after Methionine Loading Test

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SUMMARY

In Burkina Faso the levels of plasma homocysteine (Hcy) are lower and the methionine loading tests suggest a more effective Hcy metabolism. The polymorphisms of methylenetetrahydrofolate reductase (MTHFR) showed a relevant difference in the allele frequencies of T MTHFR-677 in young and in old subjects, while the allele frequency of C MTHFR-1298C was comparable in young and old subjects. The aim of this paper was to study the impact of the MTHFR polymorphisms on plasma fasting Hcy and after methionine loading in Burkina Faso. The young subjects with CC MTHFR-677 genotype had levels of Hcy significantly lower than CT and TT subjects. The level of Hcy in subjects who had AA, AC and CC MTHFR-1298 genotypes were comparable. The levels of Hcy after the methionine loading test were significantly higher in CT and TT MTHFR-677 genotype. These results suggest that the genetic situation in Burkina Faso is different from that of other Western countries and this guarantees the maintenance of lower plasma levels of Hcy in young and old Africans. The elevated levels of plasma Hcy in old subjects compared to young subjects, against the low prevalence of the T allele in elderly subjects is discussed. (Clin. Lab. 2007;53:XXX-XXX)

KEY WORDS

Burkina Faso, homocysteine, methionine loading test, MTHFR, C677T, A1298C

INTRODUCTION

People living in Burkina Faso have a minor risk of cardiovascular diseases (1): in 2000-2001, the National Register of The Yalagdo Ouédraogo Hospital of Ouagadougou collected only 2.67% patients with cardiovascular pathologies. In addition, during 1995-2004, the Maternity Unit of the Saint Camille Medical Center registered only 17 newborns with spina bifida among a total of 63,440 live births (0.0267 %). In industrialized countries high plasma homocysteine (Hcy) represents a risk factor for coronary heart disease (2-3), thrombosis (4) and neural tube defects (5), and an association was found between elevated Hcy levels and Down's syndrome (6). These observations agreed with previous studies in Burkina Faso, where the mean values of plasma Hcy were lower in this population than in European adults and children. In African populations the low prevalence of the MTHFR-677 polymorphism, which in Western countries is associated to increased level of plasma Hcy and with stroke, guarantees the maintenance of low plasma levels of Hcy in young African men and women. It is well known that the variability of Hcy levels is more evident in young than in older pe-

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ople related to the MTHFR polymorphisms, and we verified it in a sample belonging to Burkina Faso (7). At the same time the results of the methionine loading test suggest a more efficient Hcy metabolism in African populations living in Burkina Faso and in South Africa (8-9) and suggest the role of a genetic factor in the methylation pathway which influences the plasma Hcy levels.

Individuals who are homozygous for the codon MTHFR-677 polymorphism also show hypomethylation of DNA in peripheral blood leukocytes, an effect that is especially pronounced when folate levels are low (10). In industrialized countries the MTHFR-677 polymorphism may result in the necessity to have a higher folate ingestion to reduce the plasma Hcy and to compensate the decreased folate absorption seen in old people. The homozygosity CC for MTHFR-1298 becomes a risk factor of vascular disorders, especially with the aging of individuals, if associated with a decreased ingestion of folate (11). This polymorphism reduces MTHFR activity and is rarely associated with signifycantly increased plasma Hcy levels (12). The aim of this paper was to determine the impact of the MTHFR-677 and MTHFR-1298 polymorphisms on plasma fasting Hcy and after methionine loading in Burkina Faso.

MATERIALS AND METHODS

Sample Population

The study was carried out in the city of Ouagadougou (Burkina Faso), which is situated in a meso-endemic area of *plasmodium falciparum* malaria with an intense seasonable occurrence of malaria from July to October 2004. All individuals analyzed were adult, living in Ouagadougou and had been previously studied for the MTHFR polymorphisms. They follow the life habits of their own ethnos and usually eat millet or sorghum flour with vegetable sauce and cereals and, once a week, chicken, pork, mutton or beef, never fish, local seasonal fruits, according the traditional habits of country. All were in good health without anamnestic pathologies in the last six months.

The individuals enrolled for this study were divided as follows: 91 young (50 males and 41 females) and 91 elderly (48 males and 43 females) subjects. Mean age was 35.9+/-5.5 and 62.6+/-9.4 years respectively. The health balance was evaluated by visiting the subjects and collecting blood samples for Hb, glucose, urea nitrogen, serum creatinine, cystatin C, iron, transaminases, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides. The serum folate, vitamin B_6 and vitamin B_{12} levels were also included in the panel of laboratory tests. Each subject filled in a clinical form comprising all parameters proving the health status and functionality of organs. Weight, height, blood pressure, ECG, heart rate and ventilation rate were also controlled. The BMI (body mass index) was calculated for all subjects with the formula weight/height^2 (Kg/m^2). Normal laboratory parameters were considered as essential inclusion criteria for all participants. No variation of these parameters > 2 SD was admitted. A controlled diet of 1.4-1.6 Kcal was prescribed to all participants, assuring that the alimentation habits of each participant in the two weeks before the beginning of the study would be respected. The protocol of this study was reviewed and approved by the Human Ethics Committee of the Medical Center St Camille. All subjects gave their written consent to the study according to the guidelines of the Declaration of Helsinki.

Collection, processing and storage of blood samples

Blood samples (10 mL of peripheral blood: 5 mL in a plain tube and 5 mL in an EDTA tube) were collected in the morning after an overnight fast. The diet was typical for Burkina Faso and no restriction was prescribed. Tubes containing EDTA blood were centrifuged at 1500 g for 10 min at 4 °C, while tubes containing blood without an additive were left to stand at room temperature for 30 min. Plasma and serum were then separated and stored at -80 °C (in 250 μL aliquots).

Routine hematological study

Clinical chemistry tests were performed by the central laboratory of the Centre Medical St. Camille of Ouagadougou using standard methods. We considered the upper reference limit for serum creatinine to be 1.4 mg/dl and used 2 SD from the normal value as the limit for other laboratory parameters. The laboratory values of the subjects had to be within the established limits to be included in the study.

Homocysteine dosage and methionine loading test

The blood samples were collected in sterile tubes containing EDTA after overnight fasting and were considered as basal sample (time 1). Thereafter, L-methionine (100 mg/kg body wt) dissolved in 200 mL orange juice was administered orally and blood samples were taken after 4 and 8 hrs. All blood samples were chilled on ice and plasma and cells were immediately separated within 1 h. The plasma samples and the packed cells were stored at –80 °C and sent in dry ice to the Department of Clinical Biochemistry, Catholic University, Rome, Italy, where plasma tHcy was measured with an isocratic HPLC system and fluorescence detection (l_{ex} = 385 nm, l_{em} = 515 nm) (13).

During the loading test and in the following 12 hrs the clinical condition of the subjects was monitored and all participants were permitted free access to water and non-carbonated soft drinks during the test period. After the last blood sample (8 hrs) had been taken they received dinner according to their family tradition. Side effects of the methionine loading test were monitored during the test.

Table I: Differences between methylenetetrahydrofolate reductase C677T and A1298C genotypes in plasma Hcy levels and after methionine loading test. Values are means ± SD and plasma levels were expressed in μmol/L. P-value was significant (P = 0.003) only for CC versus CT in young subjects after methionine loading test.

	C677T			A1298C		
20-45 years	**CC (n=78)**	**CT (n=12)**	**TT (n=1)**	**AA (n=72)**	**AC (n=15)**	**CC (n=4)**
Hcy (μmol/L)	5.9 ± 1.94	6.88 ± 1.33	9.52	6.0 ± 1.67	6.08 ± 1.59	6.19 ± 1.78
Hcy after methionine Test (μmol/L)*	12.72+/2.14	14.73+/-1.73	16.04	13.02 ± 2.11	12.99 ± 2.24	13.49 ± 2.15
60-90 years	**CC (n=85)**	**CT (n=6)**	**TT (n=0)**	**AA (n=73)**	**AC (n=8)**	**CC (n=10)**
Hcy (μmol/L)	18.9 ± 7.02	18.7 ± 1.97	-	18.50 ± 6.76	21.04 ± 5.22	18.06 ± 3.10
Hcy after methionine Test (μmol/L)*	25.14 ± 3.57	27.12 ± 2.23		25.45 ± 2.99	26.24 ± 2.12	26.05 ± 2.46

* This value is referred to the 8-hour blood sample.

Table II: Biological parameters in young African subjects with different MTHFR genotypes.

		C677T			A1298C		
	Ref. values	**CC**	**CT**	**TT**	**AA**	**AC**	**CC**
Age (yrs)	21 (17-50)	35.2 ± 5.6	37.2 ± 4.4	39.7	36.2 ± 4.6	36.8 ± 4.4	37.7 ± 5.4
BMI (kg/m^2)	25.2 ± 1.4	26.2 ± 3.1	26.3 ± 2.7	26.5	26.6 ± 3.1	26.4 ± 2.8	26.5 ± 3.0
Total cholesterol (mmol/L)	5.5 ± 1.6	6.3 ± 1.4	6.3 ± 1.3	6.4	6.5 ± 1.3	6.2 ± 1.4	6.3 ± 1.5
LDL cholesterol (mmol/L)	4.2 ± 1.2	4.2 ± 1.1	4.1 ± 1.1	4.0	4.2 ± 1.3	4.4 ± 1.3	4.1 ± 1.1
HDL cholesterol (mmol/L)	1.3 ± 0.4	1.2 ± 0.5	1.1 ± 0.4	1.3	1.2 ± 0.4	1.3 ± 0.5	1.2 ± 0.3
Triglycerides (mmol/L)	1.8 ± 0.1	2.1 ± 1.6	2.2 ± 2.1	1.9	2.0 ± 1.6	2.2 ± 2.3	1.9 ± 1.4
Serum creatinine (μmol/L)	72.5 ± 0.11	63.2 ± 9.5	61.4 ± 8.7	63.5	64.2 ± 9.0	63.4 ± 8.9	62.1 ± 9.2
Cystatin C (mg/L)	0.67 ± 0.04	0.68 ± 0.07	0.66 ± 0.05	0.67	0.65 ± 0.07	0.66 ± 0.08	0.67 ± 0.08
Serum folate (ng/mL)	6.5 ± 2.5	5.9 ± 2.2	5.8 ± 2.3	5.6	5.9 ± 2.5	5.9 ± 2.5	5.8 ± 2.6
Serum vitamin B_6 (μg/L)	6.8 ± 2.7	6.6 ± 2.98	6.7 ± 2.6	6.6	6.6 ± 2.6	6.8 ± 2.5	6.7 ± 2.7
Serum vitamin B_{12} (ng/L)	423 ± 38.2	633.0 ± 341.4	632.7 ± 323.3	641.4	624.5 ± 328.2	626.6 ± 341.6	634.4 ± 326.4
Systolic blood pressure (mm Hg)	132 ± 4	132.0 ± 4.2	134.0 ± 3.6	137.4	135.7 ± 4.4	137.4 ± 4.2	135.3 ± 4.6
Diastolic blood pressure (mm Hg)	80 ± 3	82.0 ± 3.4	80.3 ± 3.4	81.4	82.6 ± 3.7	82.5 ± 3.5	81.5 ± 3.6

Table III: ***Biological parameters in old African subjects with different MTHFR genotypes.***

		C677T			A1298C		
	Ref.values	**CC**	**CT**	**TT**	**AA**	**AC**	**CC**
Age (yr)	65 (51-90)	60.2 ± 9.6	61.2 ± 8.4	---	60.2 ± 9.6	61.2 ± 8.4	62.7 ± 9.4
BMI (kg/m^2)	26.2 ± 1.5	26.4 ± 3.2	26.4 ± 2.9	---	26.3 ± 3.0	26.2 ± 2.7	26.4 ± 3.1
Total cholesterol (mmol/L)	5.8 ± 1.4	6.2 ± 1.2	6.2 ± 1.1	---	6.2 ± 1.1	6.2 ± 1.2	6.1 ± 1.1
LDL cholesterol (mmol/L)	4.4 ± 1.3	4.3 ± 1.1	4.2 ± 1.0	---	4.3 ± 1.2	4.2 ± 1.2	4.1 ± 1.2
HDL cholesterol (mmol/L)	1.2 ± 0.4	1.3 ± 0.4	1.2 ± 0.3	---	1.3 ± 0.5	1.2 ± 0.4	1.3 ± 0.4
Triglycerides (mmol/L)	1.9 ± 1.5	2.0 ± 1.5	2.2 ± 2.0	---	2.1 ± 1.6	2.1 ± 2.1	1.9 ± 1.3
Serum creatinine (μmol/L)	72.5 ± 9.8	69.7 ± 12.2	68.9 ± 11.8	---	67.9 ± 12.1	68.1 ± 12.3	68.1 ± 11.5
Cystatin C (mg/L)	0.85 ± 0.10	0.85 ± 0.20	0.86 ± 0.21	---	0.87 ± 0.24	0.86 ± 0.22	0.85 ± 0.19
Serum folate (μg/L)	6.5 ± 2.5	5.8 ± 2.7	5.8 ± 2.5	---	5.7 ± 2.7	5.8 ± 2.6	5.8 ± 2.7
Serum vitamin B_6 (μg/L)	6.2 ± 2.5	5.9 ± 3.2	6.0 ± 3.1	---	5.8 ± 3.2	5.9 ± 3.5	6.1 ± 3.2
Serum vitamin B_{12} (ng/L)	423 ± 38.2	665.4 ± 461.2	657.5 ± 432.8	---	634.7 ± 436.3	641.7 ± 437.9	658.5 ± 445.2
Systolic blood pressure (mm Hg)	152 ± 4	149.0 ± 24.1	151.3 ± 27.5	---	149.5 ± 25.3	147.9 ± 26.1	148.3 ± 25.9
Diastolic blood pressure (mm Hg)	82 ± 3	78.0 ± 11.8	80.4 ± 12.4	---	81.1 ± 12.2	79.7 ± 12.0	81.0 ± 11.8

Other biochemical studies

Serum folate, vitamin B_6 and vitamin B_{12} levels were measured at the Clinical Biochemistry Laboratory of the Catholic University, Rome, Italy.

Serum vitamin B_{12} was measured by microparticle enzyme immunoassay and serum folate by ion capture assay on an AxSYM Analyzer (Abbott Diagnostics, Abbott Park, Illinois, USA). The inter-assay coefficient of variation was 1.7 for both vitamin B_{12} and folate. A folate level < 5 ng/mL was considered as hypofolatemia, and a serum vitamin B_{12} level < 157 pg/mL was considered to be low.

Vitamin B_6 was measured by HPLC using a commercially available kit (Immunodiagnostik, Bensheim, Germany). We considered vitamin B_6 values < 5 ng/mL to be low.

Cistatin C was also measured at the Clinical Biochemistry Laboratory of the Catholic University, Rome, Italy, by using a C PET kit (Dako, Italy). Values ranging from 0.69 to 2.30 mg/L were considered normal.

Statistical analyses

All laboratory parameters are reported as mean and SD, and the differences were compared with Student's T test or the Mann Whitney test, when required. $P < 0.05$ was considered significant.

RESULTS

The levels of plasma Hcy and the results of the methionine loading test, divided on the basis of the MTHFR-677 and MTHFR-1298 genotypes and classes of age, are shown in Table I. No TT-MTHFR 677 genotypes were found among the elderly individuals, whereas only one was present among the group of young people. No significant differences in the plasma Hcy levels were found in either group CC, CT and TT MTHFR-677 genotypes of young and old individuals. This difference was not observed in the elderly group. On the contrary, the level of Hcy after the methionine loading test in young individuals was significantly higher in the CT ($P = 0.003$) and the TT genotype when compared with the CC MTHFR-677 genotype. This difference was not statistically significant comparing CC and CT in old subjects. The level of Hcy in subjects who were AA, AC and CC MTHFR-1298 genotypes was similar compared within the groups. No statistically significant difference was found.

The results of clinical and laboratory parameters are shown in Tables II and III according to their different MTHRF-677 and MTHFR-1298 genotypes. No statistically significant difference was found in age, BMI, serum creatinine and cystatin C in each group. Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were higher, but these parameters remained in the range normal for an African population. Folate and vitamin B_6 were all within the normal range for both age groups. Only the vitamin B_{12} levels were very high in the entire study group compared to the reference values, however, the study subjects had a quite low meat consumption. Correlations among folate, vitamin B_6 and vitamin B_{12} with plasma Hcy levels were found. A significantly higher systolic pressure was, however, observed in the elderly compared to the young group ($P < 0.05$).

DISCUSSION

This study reports the correlation between MTHFR polymorphisms in Burkina Faso comparing two different age groups and the effect of these variants on plasma fasting Hcy and after the methionine loading test.

We previously reported (7) that in this cohort of samples the prevalence of CT MTHFR-677 heterozygotes was clearly lower than previously reported in Western countries. Moreover, the frequency of the C MTHFR-1298 polymorphism was less than half that of Western countries, but in our study the observed number of subjects with the CC MTHFR-1298 genotype was higher ($P = 0.095$) in the old age group, while the number of observed AC MTHFR-1298 subjects was lower than expected (data not shown). The results cannot be easily explained, but if we consider that the prevalence of T MTHFR-677 in African people could be lower as a consequence of the presence of *plasmodium falciparum* (14), it is reasonable to suspect that social and environmental factors could favor or disfavor the selection of MTHFR polymorphisms in Burkina Faso.

It has been demonstrated that in *p. falciparum* malaria infection plasma Hcy is increased (14) and its levels correlate with the severity of malaria. Since plasma Hcy becomes higher in individuals with the T MTHFR-677 mutation as a consequence of folate inhibition and since the malaria parasite utilizes the polyamine metabolism for its growth, an increase of the Hcy levels in the sub-Saharan area should represent an advantage for the parasite, selecting negatively the individual carriers for the T MTHFR-677 mutation. This model is different from the hypothesis that the T MTHFR-677 polymorphism was selected by the deficiency of folate, but on the contrary supports the hypothesis that the deficiency of NADPH, and subsequently the deficiency of glutathione associated with malaria (15) and aging (16-17), could be responsible for the increase of Hcy as a consequence of remethylation inhibition.

In African populations the low prevalence of the T MTHFR-677 polymorphism, which in Western countries is associated with increased Hcy levels and stroke guarantees the maintenance of low plasma Hcy levels in both young African men and women. In fact, only in the subject with TT MTHFR-677 genotype was the level of plasma Hcy found to be higher than previously described by Simpore et al (1), and comparable to the Western population values. Looking at the data of the methionine loading test, we observed a significant increase of plasma Hcy in all young individuals with the

CC, CT and TT MTHFR-677 genotypes. In contrast, this variation after the methionine loading test was not found in AC and CC MTHFR-1298 genotype in either group. It is well known that the Hcy level variations are more evident in young than in older subjects, related to the MTHFR polymorphisms. We confirmed this observation and in this study we found also that the mean levels of plasma Hcy levels were uniformly elevated in the 60-90 year group. Moreover, the combined variation (CV) of plasma Hcy of the CC genotype subjects was clearly higher in the older than in the young group (37 vs 32%). This increase was also supported by the methionine loading test. However, as observed by Spotila et al 2003 (12), the difference between the CC and CT MTHFR-677 genotypes was not significant in elderly subjects, neither basically nor after the methionine loading test. The elevated levels of plasma Hcy in old subjects compared to younger people against the lower prevalence of the T allele in elderly subjects confirmed these results and suggest that the increase of plasma Hcy in old subjects might be due to a different mechanism in old and young subjects. In Western countries the T allele is not underrepresented in elderly people (18), as we observed in the African population.

The C MTHFR-1298 MTHFR polymorphism did not show a similar effect on plasma Hcy in either young or old subjects.

Based on these findings, the genetic background of Burkina Faso is different from Western countries and comparable to other areas of Africa, but in older subjects, the non-genetic causes of hyper-Hcy may have a greater impact and overwhelm the effect of a genetic polymerphism. The elevated level of plasma Hcy and the insignificant variation of plasma Hcy after the methionine loading test in old subjects could be on the contrary only the expression of glutathione and NADPH deficiency, typical of old age. In this condition an increase in plasma Hcy could represent a marker of old age and consequently the direct results of long survival such as has been observed in European centenarians. ´

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