Methylenetetrahydrofolate Reductase (MTHFR) from Mediterranean to Sub-Saharan Areas

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Abstract: There are differences in the allele frequency of MTHFR polymorphism between Western and African population. The aim of this study is to determinate the prevalence of MTHFR C677T and A1298C polymorphisms in young and old people living in different areas from Mediterranean to sub-Saharan areas. The observed vs expected genotype frequencies of 677T were in Hardy Weinberg equilibrium, with the exception of old Sardinian subjects (P=0.02). Calculation of 677T allele frequency in young and old African subjects (8% and 3%, respectively) indicated that the 677T allele was disadvantaged in old Africans (P=0.02). The difference among young and old Sardinians and Sicilians were not significant at the same degree (43% vs 37% P=0.07 and 46% vs 42% P=0.28, respectively). However, the reproducible trend that showed the prevalence of 677T allele in the young subjects of the three studied areas confirms the disadvantage of this polymorphism with the age. There was a significant difference (P=0.005) on the observed vs expected frequency of 1298C homozygosity in African old subjects compared to younger ones, while the observed vs expected genotype frequencies were in equilibrium in young and old Sardinian and Sicilian subjects. The frequencies of 1298C and 1298A alleles were comparable between young and old African, Sardinian and Sicilian subjects. The lower frequency of 677T allele in old African, Sardinian and Sicilian subjects compared to young ones and the absence of TT genotype among old African subjects, should be considered as a consequence of an elevated mortality of 677T carriers.

Key words: MTHFR, Sardinia, Sicily, Burkina Faso, young people, old people, nutrition, folic acid, malaria

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INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is needed for methionine synthase to convert homocysteine (Hcy) to methionine[1]. Its key function in Hcy metabolism makes this enzyme a hot point in the mechanism which associates hyperhomocysteinemia (HHcy) with the cardiovascular risk and other pathologies where the role of folate is largely documented[2]. The MTHFR gene is polymorphic, with single nucleotide variants within codon 677 in exon 4 (CT), which causes an alanine to valine substitution, and within codon 1298 in exon 7 (AC) which determines a substitution of glutamate by an alanine residue[3,4]. The codon 677 variant encodes a thermolabile enzyme with reduced activity that leads to an increase of plasma Hcy level[5]. Individuals who are homozygous for the codon 677 polymorphism show hypomethylation of DNA in peripheral blood leukocytes, an effect that is especially pronounced when folate levels are low[6]. In western countries the C677T polymorphism may result in the necessity to have a higher folate ingestion to reduce the plasma HHcy and it has been considered, especially in North Europe, a risk factor responsible of neural tube defects and 21 trisomy[3,6,7]. The homozygosity CC for 1298 polymorphism becomes a risk factor of vascular disorders, especially with the individual's aging, if associated with a decreased ingestion of folate[8]. Also this polymorphism reduces MTHFR activity and has been reported to be a risk factor for neural tube defects (NTD)[9], but this mutation is rarely associated with significant increasing of plasma Hcy levels[10]. There are differences in the frequencies of this allele in Western and African countries, which suggest some selective mechanism which act prevalently in young age[11-13]. A selective role of malaria could be supported by the homocysteine (Hcy) involvement in the *Plasmodium falciparum* metabolic cycle [14]. The aim of this study is to determinate the prevalence of 677T and

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1298C alleles of MTHFR in young and old people living in different countries from Mediterranean to sub-Saharan areas and to correlate the prevalence of MTHFR polymorphisms with social and economical status, nutritional characteristics, endemia or pregressed endemia for malaria.

SUBJECTS AND METHODS

Subjects: In a period from 1 April to 31 July 2005, 400 subjects in each country (Burkina Faso, Sardinia and Sicily), equally distributed among males and females, were submitted to a physical and clinical examination before they could be considered clinically healthy. All individuals participating to the first selection signed informed consent forms for blood and informative data collection. The local Ethical Committees both in Italy and in Burkina Faso approved this study. The health balance was evaluated by visiting the subjects and collecting blood samples for routine tests. The weight, height, blood pressure, ECG, heart rate and ventilation rate were also controlled. Abnormal laboratory parameters were considered essential exclusion criteria (elevated blood urea nitrogen, serum cholesterol, altered urine analysis, high level of blood pressure, etc) for all participants. No variation of these parameters > 2 SD was admitted. At the end of the examination, 18 African, 5 Sardinian and 26 Sicilian were excluded. Then we collected blood samples of documented healthy subjects without anamnestic pathologies in the last six months:
– 191 African young subjects, 90 males and 101 females, aged 20-45 (average 25) years;
– 191 African old subjects, 89 males and 102 females, aged 60-90 (average 71) years.
– 200 Sardinian young subjects, 89 males and 111 females, aged 28-50 (average 38) years;
– 195 Sardinian old subjects, 95 males and 100 females, aged 60-90 (average 72) years.
– 194 Sicilian young subjects, 95 males and 99 females, aged 22-58 (average 43) years;
– 180 Sicilian old subjects , 81 males and 99 females, aged 60-90 (average 72) years.
All subjects were born from African, Sardinian and Sicilian parents for two generations. Against a slight prevalence of females on males, the power of sample reached the statistical significance for the evaluation of allele frequencies. All young and old individuals of the three areas were matched for sex and age respectively. African subjects, living in a poor social status, usually eat millet or sorghum flower with vegetable sauce and, once a week, chicken, pork, sheep or beef meat, never fish, local seasonal fruits, according to the habits of the country. The folic acid intake was insured by the presence of green vegetable in their diet. All were in good health state. All Sardinian and Sicilian subjects eat according to the Mediterranean diet and consume regularly red wine at lunch and dinner. Also the Mediterranean diet is rich in folates from its high green leafy vegetable content. Their social status was medially characteristic of western countries.

Collection, processing of blood samples: Blood samples (10 mL of peripheral blood: 5 mL in a plain tube and 5 mL in EDTA) were collected in the morning after over night fast. Tubes containing blood in EDTA were immediately 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). Clinical chemistry tests of African subjects were performed by the central laboratory of Centre Medical St. Camille of Ouagadougou using standard methods. The clinical chemistry tests of Sardinians and Sicilians were made in Sassari and Catania private laboratories, respectively, using standard methods. Plasma homocysteine (Hcy), serum folate, vitamin B_6 and vitamin B_{12} levels were measured at the Clinical Biochemistry Laboratory of Catholic University, Rome, Italy. The determination of plasma homocysteine was carried out by HPLC. Daily quality control was carried out by using a control sample prepared from a plasma pool processed at the beginning and at the end of the analytical session. The inter-assay coefficient of variation was 3.6. Plasma Hcy values > 15 μmol/L were considered elevated. Serum vitamin B_{12} was measured by micro particle enzyme immunoassay and serum folate by ion capture assay on an AxSYM Analyzer (Abbott Diagnostics, Abbott Park, USA). The inter-assay coefficient of variation was 1.7% for both vitamin B_{12} and folate. A folate level <11.33 nmol/L was considered hypofolatemia, and a serum vitamin B_{12} level <115.86 pmol/L was considered low. Vitamin B_6 was measured by HPLC using a commercially available kit (Immunodiagnostik, Bensheim, DE). We considered vitamin B_6 values <20.23 nmol/L to be low. Packed peripheral blood containing leukocytes were frozen at -80°C and sent in dry ice to the Laboratory of Institute of Population Genetics, Alghero, Sassari, Italy, for the MTHFR polymorphism study.

Genetic analysis: Genomic DNA was isolated from 2.5 mL of packed peripheral blood as previously described[15]. The C677T and the A1298C mutations in the MTHFR gene were analyzed after polymerase chain reaction (PCR) of genomic DNA. The regions containing the two polymorphisms were amplified separately by using the following primer pairs: C677T forward-CTTGAACAGGTGGAGGCC; reverse-CAAAGAAAAGCTGCGTGATGAT and A1298C forward-GCAAGTCCCCCAAGGAGG; reverse-GGTCCCCACTTCCAGCATC. DNA was amplified by using a PCR thermal cycler (GeneAmp9700, Applied Biosystems, Foster City, CA, USA). Both PCR were carried out in a total volume of 25 mL containing

Table 1: Clinical and laboratory parameters in African fertile and postmenopausal women living in Burkina Faso

Parameters	Young Africans (n. 191) (A)	Old Africans (n. 191) (B)	Young Sardinians (n. 200) (A)	Old Sardinians (n. 195) (B)	Young Sicilians (n. 194) (A)	Old Sicilians (n. 180) (B)
Age (years)	25 (20-45)	71 (60-90)	38 (28-50)	73 (60-90)	43 (22-58)	72 (60-90)
BMI (Kg/m^2)	25.2±1.4*	24.2±2.6	26.1±1.8*	23.2±2.1	25.9±1.9*	22.5±2.0
Blood nitrogen (mmol/L)	2.0±0.45*	2.2±0.33	2.85±0.34*	3.2±0.43	2.79±0.5*	3.3±0.39
Serum cholesterol (mmol/L)	4.56±0.89*	4.51±0.82	4.36±0.75*	4.60±0.83	4.35±0.88*	4.64±0.84
Serum triglycerides (mmol/L)	1.08±0.11*	0.97±0.10	1.10±0.13$	1.13±0.15	1.22±0.14$	1.19±0.15
Serum creatinine (mmol/L)	63.65±9.7*	69.83±14.14	78.4±13.5*	87.8±12.5	75.9±13.2*	88.7±12.4
Cystatin C (mg/L)	0.69 ±0.08*	0.86±0.24	0.70±0.21*	0.88±0.23	0.73±0.22*	0.89±0.21
Hcy (mmol/L)	6.8±4.2*	12.2±5.7	8.5±4.9*	16.2±4.7	8.6±4.6*	16.4±5.2
Serum folate (nmol/L)	13.37±4.98	13.37±5.1	20.4±6.8	21.6±5.7	25.7±7.4	24.3±6.5
Serum Vit B$_6$ (nmol/L)	26.7±11.7	24.7±12.9	28.2±15.3	26.3±12.5	28.4±12.3	27.9±13.0
Serum Vit B$_{12}$ (pmol/L)	614.7±251.2^	527.7±277.8	527.2±255.2	547.5±245.3	538.3±262.1	587.4±255.6
Systolic blood pressure (mm Hg)	132.0±14.2^	139.9±22.5	130.4±22.3*	145.8±24.3	134.3±25.2*	146.3±23.2
Diastolic blood pressure (mm Hg)	82.0±13.4$	79.5±13.6	80.3±13.2$	83.2±12.4	79.9±13.1$	83.8±13.9

Student T Test A vs B * < 0.0001 ^ < 0.001 $ < 0.05

17.5 pmol of each primer, 200 mmol each dNTP/L, 10 mmol Tris-HCl/L (pH 8.3), 1.5 mmol MgCl$_2$/L, 50 mmol KCl/L, 0.75 U of Taq polymerase (Applied Biosystems, Foster City, CA, USA) and 60 ng of template DNA. The reaction conditions for the two fragments were as it follows: initial denaturation at 95°C for 3 min, 32 subsequent cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec and a final extension at 72°C for 10 min. PCR products (10 mL) were digested in a total volume of 20 mL with 2 U HinfI (for nucleotide 677) and with 2 U MboII (for nucleotide 1298) (New England Biolabs Inc, USA). DNA fragments were separated by electrophoresis on a 4% agarose gel in TBE 1X and visualized with ethidium bromide.

Statistical analyses: Laboratory parameters are reported as mean and SD and the difference were compared by Student T and Krustal Wallis tests. Allele frequencies for each genotype and the exact confidence intervals on the frequencies were calculated. Expected genotype frequencies were calculated from the allele frequencies and compared to observed frequencies under the assumption of Hardy-Weinberg equilibrium. The difference among allele frequencies and genotype frequencies were compared with appropriate statistical tests (X^2 and Fisher exact test). The Pearson coefficient followed by Fisher exact test was used to test correlations. In all statistical evaluation P<0.05 was considered significant.

RESULTS

In Table 1 the clinical and laboratory parameters are reported for each group of studied subjects. All the parameters, including also homocysteine, folate, Vit B6 and Vit B12, resulted in the normal range for the country of origin with the differences reported already among African and Mediterranean populations[16]. A significant difference was found between young and old people for blood nitrogen, serum cholesterol and triglycerides, creatinine, cystatin C and Hcy. The level of serum folate was lower in African subjects, compared to Sardinians who had lower serum folate levels than Sicilians, but in any case all were in the normal range. The systolic and diastolic blood pressure was higher in all old studied subjects. Only in Hcy levels a difference was noted between males and females subjects of African and Mediterranean origin[16]. The Pearson coefficient and Fisher exact test showed that only the age and creatinine correlate with plasma Hcy (P < 0.001), while the Vit B6 and Vit B12 were independent variables. Paradoxally the plasma Hcy was lower in Africans, where the serum folate level was also lower but in the normal range. The distributions of MTHFR 677T genotype in young and old subjects, living in different countries from sub-Saharan to Mediterranean areas are reported in Table 2. The observed vs expected 677T genotype frequencies were in Hardy Weinberg equilibrium, with the exception of old Sardinian subjects (P=0.02). Calculated frequencies of 677T and 677C alleles suggest that the 677T allele is higher in young compared to old subjects in all the three studied areas, although only the difference between young and old African subjects was statistically significant (P=0.02). The difference between young and old Sardinian subjects had a marginally significant P value (P=0.07), whereas the difference between young and old Sicilian subjects was not significant (P=0.28). The retrospective power calculation showed that the size of samples was sufficient for the significance level obtained in Africans and in Sardinians (64% and 44% respectively), while it was insufficient for Sicilians (19%). The observed number of African subjects with CC 1298 genotype was significantly higher than expected in the old age

Table 2: Distribution of methylenetetrahydrofolate reductase C677T genotypes and alleles frequencies in young and old African, Sardinian and Sicilian subjects.

Subjects	Genotype	Subjects (N.)	Observed (%)	Expected (%)	Allele	Frequency % (95% CI)
Young African	CC677	163	85.71	85.20		
	CT677	26	13.19	14.20	677C	92.00 (88%-94%)
	TT677	2	1.10	0.59	677T	8.00 (4.7%-9.9%)
	Total	191				
Old African	CC677	179	93.41	93.51		
	CT677	12	6.59	6.37	677C	97.00 (89%-95%)
	TT677	0	0.00	0.1	677T	3.00 (1.8%-5.4%)
	Total	191				
Young Sardinian	CC 677	66	33.00	32.48		
	CT 677	96	48.00	49.01	677C	57.00 (52%-62%)
	TT 677	38	19.00	18.48	677T	43.00 (32%-48%)
	Total	200				
Old Sardinian	CC 677	91	46.67	40.11		
	CT 677	65	33.33	46.44	677C	63.00 (58%-68%)
	TT 677	39	20.00	13.44	677T	37.00 (32%-41%)
	Total	195				
Young Sicilian	CC 677	60	30.85	28.86		
	CT 677	89	45.74	49.72	677C	54.00 (49%-59%)
	TT 677	45	23.40	21.41	677T	46.00 (41%-51%)
	Total	194				
Old Sicilian	CC 677	67	37.50	33.78		
	CT 677	74	41.25	48.67	677C	58.00 (53%-63%)
	TT 677	39	21.25	17.53	677T	42.00 (37%-47%)
	Total	180				

group (P=0.005), while the observed vs expected genotype frequencies were in equilibrium in young and old Sardinian and Sicilian subjects. The frequencies of 1298C and 1298A alleles were comparable between young and old African, Sardinian and Sicilian subjects (see Table 3). No difference was found among males and females of each groups in C677T and A1298C polymorphisms.

DISCUSSION

The aim of this study was the estimation of the frequency of MTHFR C677T and A1298C polymorphisms in young and old subjects living in Burkina Faso, Sardinia and Sicily. It has been previously reported that the 677T allele prevalence in African population is lower than in Western countries (6.6%)[11-13]. In European populations the prevalence of the 677T allele ranges from 24% to 40% or higher[17,18]. The frequency of the 1298C allele in African population was reported to be less than half that of Western countries (14% vs 30%)[3,19]. Our results are in good agreement with the above cited literature, and in particular, the 677T and 1298C allele frequencies lie at the higher values of the above reported ranges in Sardinians and in Sicilians, while they are comparable in Africans (see Table 2 and Table 3). Table 2 shows that the C677T polymorphism is in Hardy Weinberg equilibrium in

both young and old subjects. The frequency of 677T allele in old subjects was lower in all groups studied, mainly in Africans and Sardinians. The difference amongst young and old subjects does not reach the statistical significance only in Sicilians, probably because a larger sample size should be needed for so small differences. The observed number of African subjects with CC 1298 genotype was significantly higher than expected in the old age group (P=0.005), while the number of observed 1298C subjects was clearly lower than expected (9.20% vs 25.42%). In Sardinian and Sicilian populations we did not find statistically significant difference between observed and expected values according to Hardy Weinberg equilibrium. The plasma Hcy levels were high in old people both in African and in European subjects (see Table 1) and could correlate with increased creatinine levels as a marker of renal function according our previous findings[20]. On the contrary the plasma Hcy levels were found lower in young Africans than the mean values found in Sardinia and in Sicily and this in accord with our precedent study[16]. This results appear contradictory since we found a higher plasma Hcy level in Mediterranean areas than in Africa, while the folate level was lower in Africa as previously reported by Amouzou et al.[13]. If this is true only the low frequency of MTHFR 677T allele could explain the low plasma Hcy levels in young African The difference in the MTHFR alleles

Table 3: Distribution of methylenetetrahydrofolate reductase A1298C genotypes and alleles frequencies in young and old African. Sardinian and Sicilian subjects.

Subjects	Genotype	Subjects (N.)	Observed (%)	Expected (%)	Allele	Frequency % (95% CI)
Young African	AA1298	151	79.27	77.09		
	AC1298	32	17.07	21.41	1298A	87.00 (84%-90%)
	CC1298	8	3.66	1.5	1298C	13.00 (9%- 16%)
	Total	191				
Old African	AA1298	153	80.46	72.35		
	AC1298	15	9.20	25.42	1298A	85.00 (80%-87%)
	CC1298	23	10.34	2.23	1298C	15.00 (13%-20%)
	Total	191				
Young Sardinian	AA1298	106	53.00	51.84		
	AC1298	76	38.00	40.32	1298A	72.00 (67%-76%)
	CC1298	18	9.00	7.84	1298C	28.00 (24%-32%)
	Total	200				
Old Sardinian	AA1298	86	44.44	49.00		
	AC1298	99	51.11	42.00	1298A	70.00 (65%-74%)
	CC1298	10	4.44	9.00	1298C	30.00 (26%-35%)
	Total	195				
Young Sicilian	AA1298	96	48.94	50.00		
	AC1298	86	43.62	41.45	1298A	71.00 (67%-76%)
	CC1298	12	7.45	8.55	1298C	29.00 (24%-33%)
	Total	194				
Old Sicilian	AA1298	84	46.84	47.6		
	AC1298	78	44.30	42.78	1298A	69.00 (63%-73%)
	CC1298	18	8.86	9.62	1298C	31.00 (27%-37%)
	Total	180				

among African and Mediterranean subjects is not easily to be explained, but it is reasonable to suspect that social and environmental factors could advantage or disadvantage the selection of MTHFR genotypes. Probably the adequate folic acid intake in a given population may give rise to an increase in the frequency of the MTHFR 677T allele, whereas an inadequate intake may results in decreased frequency. In this regards the north-to-south increase in the prevalence of the MTHFR 677 allele in Europe is of interest[3,11] and may be influenced by the apparent higher folic acid content of Mediterranean foods compared with northern European foods. Moreover, Rosenberg et al.[21] from haplotype associations suggest also a founder effect to explain the expansion of 677T allele in Western countries. Moreover the association of the allele T with NTD[3], which is found more frequent in the North of Europe (Ireland and The Netherlands), but not in Sicily and France[22], must be considered. The same observation can be made for trisomy (Down syndrome)[6,7]. This considerations suggest that a early adverse event of infancy and no only late events, may affect the allele frequency. It has been also demonstrated that in *Plasmodium falciparum* malaria plasma Hcy reaches high values which correlate with malaria severity[14]. Since polyamine metabolism correlates to Hcy *via S*-adenosylmethionine and plays an essential role in *P.*

falciparum cell proliferation and differentiation[23,24], we hypothesize that an increase of Hcy levels associated to endemic malaria in sub-Saharan area could represent a metabolic advantage for the parasite, selecting prematurely the 677T carriers. In fact, it is not surprisingly to found the 677T allele prevalence in Sardinia, intermediate among Burkina Faso and Sicily, since the two Mediterranean islands have had different pregressed malaria endemia. Moreover the difference among young and old people in C677T polymorphism is statistically documented only in Africa and in Sardinia (see Table 2). The lower plasma Hcy levels in subjects living in Burkina Faso, compared with Western subjects[12], associated to low prevalence of C677T polymorphism and to a low incidence of hearth coronary diseases[25], is not unexpected. In Western countries C677T polymorphism is associated to increased Hcy levels and stroke[26,27], especially in presence of known risk factors like as smoke, alcohol, sedentary life condition, etc[28]. It is obvious to think that the difference among young and old people in Sardinian and Sicilian population could be due to increased mortality of 677T carriers for cardiovascular pathologies. However it is well known that the effect of HHcy on cardiovascular damage related to the MTHFR polymorphism is more evident in young than in old people[8]. The elevated level of plasma

Hcy in old subjects, in presence of a lower prevalence of C677T polymorphism, in addition of higher creatinine levels, should be more probable the expression of glutathione and then NADPH deficiency typical of old age[29,30]. In fact this determines an increase of plasma Hcy through an inhibition of re-methylation, suggesting that non genetic cause of HHcy may have a greater impact and overwhelms the effect of a genetic polymorphism. It is possible that MTHFR genotype might affect behavioral and socioeconomic factors by being associated with polymorphic variants of loci in linkage disequilibrium, that predispose subject with TT genotype at risk of unhealthy behaviors, low socioeconomic status, or abnormal physiological risk factors predisposing to stroke (such as blood pressure or serum cholesterol)[31]. However folate status and other environmental exposures could influence the Hcy concentration, exposing the individuals to cardiovascular events.

CONCLUSION

Going to our malaria hypothesis, old people living in Africa and Sardinia should be selected for 677T alleles before the disappearance of malaria. Studies on the influence of 677T in the malaria outcome will clarify this difference in future. Similar mechanism could explain also the difference that was found some years ago[32] in the prevalence of beta-thalassemia trait among young and old people living in Sicily after the disappearance of malaria with extended use of DDT. The old Sicilians showed higher prevalence of beta-thalassemia trait than younger, since beta-thalassemia protects them from malaria. We think that it will be necessary to compare these results with additional data from other areas where the malaria has never been documented to verify the residual effect of malaria infection in human genomic.

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