Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects

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Gene–environment interactions play an important role in folate metabolism, with a potential impact on human health. Deficiencies in the uptake of key micronutrients and variant genotypes can affect the folic acid cycle, modulating methyl group transfer in key processes and leading to increased cancer risk and Down syndrome incidence. So far, the significance of folate status and metabolic genotypes on baseline levels of DNA damage in normal individuals has not been fully elucidated. In this study, the possible modulation of SCE, micronuclei and tail moment values in peripheral lymphocytes by plasma levels of folic acid, homocysteine and vitamin B12, and by the methylenetetrahydrofolate reductase (MTHFR) C677T and methionine synthase reductase (MTRR) A66G polymorphisms was investigated in 191 healthy subjects. The results obtained show a highly significant (P = 0.001) positive association between plasma levels of vitamin B12 and frequencies of both SCE and high frequency cells (HFC, above 90th percentile) in smokers. No significant effect was observed in non-smokers. Moreover, after correction for age, gender and GSTM1 genotype, a significant association (P = 0.026) between the MTRR 66GG variant genotype and higher micronucleus rates was observed. Tail moment values were not affected by any of the independent variables considered. Overall, the results obtained suggest that both folate status and relevant metabolic genotype can influence background levels of DNA damage in normal subjects. The significant association observed in smokers between plasma vitamin B12 and SCE frequencies may highlight the effect of methylation status on DNA damage and repair, although the role of other, unidentified dietary factors cannot be ruled out. At the same time, micronucleus data indicate that the MTRR 66GG variant may represent another individual trait of relative genomic instability, thus supporting epidemiological data on increased risk of Down syndrome conception in MTRR 66GG subjects.

Introduction

Among the factors associated with increased risk of cancer, diet accounts for an attributable risk estimated at ~20–40% (1). The impact of diet on human health is due both to the exposure to carcinogenic contaminants and to the abnormal uptake of key micronutrients involved in DNA maintenance (2).

In this respect, the metabolic pathway of folate, which is impaired by an unbalanced diet, is thought to influence DNA stability in two different ways (3,4) (and references therein). The first is related to the role of folate in one carbon unit transfer during de novo synthesis of nucleotides. Low levels of 5,10-methylenetetrahydrofolate (5,10-methylene-THF), the cofactor of thymidylate synthase, depress thymidylate synthesis, leading to an increased dUMP/dTMP ratio and increased dUTP misincorporation in DNA. The removal of dUTP by DNA-glycosylase may lead to single and double strand breaks; furthermore, the unbalanced nucleotide pool resulting from inefficient thymidylate synthase activity can increase DNA misrepair, contributing to the overall level of DNA damage in the cell. The second way in which folate metabolism may affect DNA maintenance involves the production of S-adenosyl methionine (SAM), the methyl donor of most methylation processes, including CpG methylation. 5,10-Methylene-THF, after conversion into 5-methyltetrahydrofolate (5-methyl-THF) by methylene-tetrahydrofolate reductase (MTHFR), provides the methyl group for methylation of homocysteine to methionine, the precursor of SAM, by methionine synthase (MTR). Thus, low intracellular 5,10-methylene-THF is associated with low SAM production; this may lead to DNA undermethylation, a cause of abnormal gene expression (5) and chromosome segregation (6).

Evidence supports the key role of folate status in human health. An unbalanced diet with reduced uptake of folic acid was associated with increased risk of colon cancer (7). In studies on human donors, low levels of serum folic acid were associated with a higher misincorporation of uracil into DNA as well as with increased incidence of micronuclei in peripheral lymphocytes (8). Micronuclei were also positively correlated to serum homocysteine levels, a marker of folic acid deficiency (9,10). The role of folic acid in DNA integrity was also demonstrated in in vitro studies, where low folic acid concentrations in the medium produced higher uracil misincorporation, micronuclei and DNA strand breaks as detected by comet assay (11,12).

In addition to dietary uptake of micronutrients such as folic acid and vitamin B12 (the coenzyme of MTR), also some genetic polymorphisms may affect folic acid metabolism, modulating its effect on human health. Both the polymorphisms of MTHFR C677T and the methionine synthase reductase (MTRR) A66G (13) were associated, in some studies, with increased maternal risk of having a child with Down syndrome (14,15), as well as with increased risk of neonatal spina bifida (16). On the other hand, lower colon cancer risk was associated with the MTHFR 677TT genotype (17,18), and reduced risk of...
childhood and adult leukaemia was attributed to subjects with the MTHFR mutations 677C → T or 1298A → C (19,20). Both variant forms of MTHFR have reduced enzymatic activity (21), and are supposed to give their protective effects by decreasing the conversion of 5,10-methylene-THF into 5-methyl-THF. Higher 5,10-methylene-THF levels would favour the conversion of uracil into thymidine, thus reducing the incorporation of the former base into DNA and consequently DNA damage due to dUTP misincorporation. Such protection however only occurs with normal folate uptake, whereas unbalanced diet makes variant carriers as susceptible as wild-type to cancer (17), maybe because the availability of 5-methyl-THF for biological methylation becomes critical, balancing the beneficial effect of reduced uracil misincorporation (7). In line with this hypothesis, higher risk of gastric cancer was associated with the MTHFR 677TT and 677CT genotypes in a Chinese population with dietary folate deficiency (22).

Actually, the analysis of DNA damage and uracil incorporation in normal and MTHFR variant lymphocytes cultured in vitro in medium with poor folic acid did not highlight a direct effect of this polymorphism on DNA integrity (12,23). The same levels of DNA damage and uracil incorporation were observed irrespective of the genotype of donors, and suggested an indirect role of the MTHFR genotype possibly mediated by its interaction with other genetic factors involved in the metabolism of folic acid, or the suppression of the phenotypic expression of the C677T polymorphism mediated by the presence of high levels of riboflavin and methionine in culture medium (23).

In this study, in order to assess the role of metabolic genotypes at the level of DNA damage in healthy individuals, a population enrolled in a previous biomonitoring study (24) was further characterized for the MTHFR and MTRR genotypes. The results of the analysis of biomarkers of genetic damage, i.e. SCE and micronuclei in T lymphocytes and tail moment in leucocytes, were analysed in relation to individual genotype and plasma levels of folate, homocysteine and vitamin B12, by taking into account multiple potential confounding factors related to personal history, life-style and occupation.

Materials and methods

Study population

The study population consisted of 191 officers from the Municipality of Rome (140 males and 51 females, mean age 41.9 years). Information on smoking habit, alcohol consumption, diagnostic X rays, chemical exposure during occupational or recreational activities and family history of cancer (first degree relatives affected), were collected by questionnaire. All patients gave an informed written consent to their participation in the study. All analyses were carried out on anonymous, coded samples.

Sampling

All subjects contributed a single blood donation during the period December 1999 to February 2001. Blood was collected by venipuncture from fasted subjects, and aliquoted in heparinized tubes, used for genotoxicity and haematotoxicological analyses, and in tubes devoid of anticoagulant, used for DNA extraction and amplification. Tubes were transferred to laboratory within a few hours and immediately used for lymphocyte cultures or stored at −80°C until analysis. For each individual, all analyses were carried out using aliquots of the same blood sample.

Genotypic analysis

Study subjects had previously been genotyped for the CYP1A1, CYP2E1, GSTM1, GSTT1 and DT-diaphorase polymorphisms (25). For this study 174 subjects, selected on the basis of samples availability, were further characterized for two polymorphisms of genes of the folic acid metabolic pathway. The methylenetetrahydrofolate reductase (MTHFR) 677C → T mutation produces a thermo labile form of the protein with reduced catalytic activity. In homozygous and heterozygous subjects, the efficiency of conversion of methylenetetrahydrofolate into methyltetrahydrofolate is reduced to 30 and 65% compared with the wild-type (21). Also, the methionine synthase reductase (MTRR) 66A → G mutation is associated to reduced enzymatic activity (26), resulting in lower activation of methionine synthase, the enzyme catalysing the methylation of homocysteine to methionine.

Genomic DNA was isolated from whole blood samples using Instagen Matrix (Bio-Rad, Hercules, CA, USA) according to manufacturer’s instructions. The MTHFR C677T mutation was detected after PCR amplification with the appropriate primers. The variant allele was identified by the presence of an 175 bp fragment after digestion with HindIII that leaves uncut the 198 bp wild-type fragment (17). The MTRR A66G mutation was detected after PCR amplification with the appropriate primers that create an artificial Ndel restriction site. The PCR fragment of 66 bp remains uncut in the mutant allele but is digested into 44 and 22 bp fragments in the wild-type allele (16).

Two samples, that failed the amplification and could not be repeated, were not characterized for the MTHFR genotype.

Haematotoxicological analyses

Plasma folic acid and vitamin B12 were measured by a radioimmunoassay (RIA) method (Quantaphase II, Bio-Rad). Red blood cell folate (RCF) could not be determined. On the other hand, the latter mainly represents a storage form of folate, which red cells do not exchange with the extracellular milieu (27), whereas plasma folate is the ready-to-use source of folic acid for lymphocytes, the target cells for DNA damage measurements. Plasma homocysteine was determined by a Microparticle Enzyme Immunoassay (MEIA) method (Abbott Diagnostics, Chicago, IL, USA). Both assays were carried out following the instructions of the manufacturers. All determinations were performed in October to November 2001. No relationship was observed between the duration of storage of blood samples and the levels of plasma markers (data not shown), indicating that the period of storage did not influence analytical determinations. Five plasma samples of the original study group (191 subjects) were no longer available, so haematotoxicological analyses were carried out on 186 subjects only.

Biomarkers of genotoxic damage in blood cells

Data on biomarkers of genetic damage were obtained in the framework of a parallel biomonitoring study on the effects of occupational exposure to urban air pollutants (24,28).

Micronuclei were analysed in cytokinesis-blocked lymphocytes from whole blood cultures. 1000 binucleated lymphocytes were scored on coded slides to evaluate micronucleus frequency. Criteria for selection of binucleated cells and micronuclei were as described by Fenech (29).

For each individual, the frequency of SCEs was determined in at least 80 well-differentiated second metaphases from whole blood cultures. Cells with a number of exchanges greater than the 90th percentile of the overall distribution (nine exchanges per cell) were defined as HFC.

DNA damage was detected in mononuclear leucocytes by single cell gel electrophoresis (Comet assay). Mononuclear cells were isolated from heparinized whole blood samples using Histopaque 1077 (Sigma Chemical, St Louis, MO, USA). Isolated cells were immediately processed in the comet assay following the alkaline protocol developed by Singh et al. (30), with minor modifications described in detail elsewhere (31). DNA damage was quantified by tail moment values calculated by the Casys computerized image analysis system (Synoptic, Cambridge, UK). For each subject, the average tail moment value was determined randomly scoring 100 cells from two slides.

Statistical analysis

Levels of genotoxic damage or plasma folic acid, vitamin B12 and homocyst(e)ine among study groups were compared by the non-parametric Mann-Whitney or Kruskall–Wallis tests. Correlation and regression analyses were performed after natural logarithmic transformation of data in order to stabilize the variance. All the analyses were performed with the SPSS statistical package (version 11.0).

Results

Genotypic analysis

The distribution of the MTHFR and MTRR genotypes within the study population is shown in Table I. Allelic frequencies were in the range described for Caucasian population. Genotype distributions were compatible with the Hardy–Weinberg equilibrium, with a slight, not significant ($\chi^2 P = 0.104$) excess of MTRR heterozygotes.
Plasma markers of folate metabolism

Plasma levels of folic acid, vitamin B12 and homocysteine in the whole study population, and in subjects grouped by gender, smoking habits, alcohol consumption and MTHFR or MTRR genotype, are shown in Table II. All three parameters showed wide inter-individual variability. Average population levels were similar to those recorded in Italian blood donors or in healthy control populations (32,33).

Significantly different plasma homocysteine levels were observed between males and females, and among MTHFR genotypes. Plasma levels of folic acid were significantly modulated by the MTHFR genotypes, and showed a highly significant negative correlation with plasma homocysteine (Table III). Plasma vitamin B12 was significantly decreased in alcohol consumers and, to a lesser extent, in male donors compared with females. The MTRR A66G polymorphism did not significantly influence any of the plasma markers investigated.

Table I. Distribution of MTHFR and MTRR genotypes within the study population

<table>
<thead>
<tr>
<th>Locus (allele)</th>
<th>Genotype</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR (677 C → T)</td>
<td>CC</td>
<td>52 (30.2)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>79 (45.9)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>41 (23.8)</td>
</tr>
<tr>
<td>MTRR (66A → G)</td>
<td>AA</td>
<td>40 (23.0)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>101 (58.0)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>33 (19.0)</td>
</tr>
</tbody>
</table>

Table II. Plasma levels of folic acid, homocysteine and vitamin B12 in the study population

<table>
<thead>
<tr>
<th>Gender</th>
<th>Folic acid ± SD (ng/ml)</th>
<th>Homocysteine ± SD (µmol/l)</th>
<th>Vitamin B12 ± SD (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (138)</td>
<td>4.15 ± 1.82</td>
<td>14.88 ± 6.04***</td>
<td>407.4 ± 131.5</td>
</tr>
<tr>
<td>Women (48)</td>
<td>4.56 ± 1.99</td>
<td>10.86 ± 3.37</td>
<td>460.4 ± 155.5</td>
</tr>
<tr>
<td>Smoking habits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non smokers (123)</td>
<td>4.23 ± 1.72</td>
<td>13.29 ± 3.80</td>
<td>417.8 ± 137.8</td>
</tr>
<tr>
<td>Smokers (63)</td>
<td>4.29 ± 2.16</td>
<td>15.02 ± 8.35</td>
<td>427.4 ± 144.0</td>
</tr>
<tr>
<td>Alcohol consumptionb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abstinent or moderate (129)</td>
<td>4.27 ± 1.65</td>
<td>13.33 ± 4.05</td>
<td>434.9 ± 135.2**</td>
</tr>
<tr>
<td>Drinkers (40)</td>
<td>3.87 ± 1.76</td>
<td>15.08 ± 5.19</td>
<td>355.6 ± 105.3</td>
</tr>
<tr>
<td>MTHFR (C677T)</td>
<td>CC (51)</td>
<td>4.66 ± 2.06*</td>
<td>12.66 ± 2.86***</td>
</tr>
<tr>
<td></td>
<td>CT (76)</td>
<td>4.19 ± 1.79</td>
<td>12.38 ± 3.03</td>
</tr>
<tr>
<td></td>
<td>TT (40)</td>
<td>3.95 ± 1.99</td>
<td>18.33 ± 9.34</td>
</tr>
<tr>
<td>MTRR (A66G)</td>
<td>AA (38)</td>
<td>4.54 ± 1.70</td>
<td>14.35 ± 9.58</td>
</tr>
<tr>
<td></td>
<td>AG (90)</td>
<td>4.18 ± 2.12</td>
<td>13.74 ± 4.33</td>
</tr>
<tr>
<td></td>
<td>GG (32)</td>
<td>4.25 ± 1.51</td>
<td>13.89 ± 3.99</td>
</tr>
<tr>
<td>Total</td>
<td>Mean (186)</td>
<td>4.25 ± 1.87</td>
<td>13.86 ± 5.75</td>
</tr>
<tr>
<td></td>
<td>Min–Max</td>
<td>1.40–14.90</td>
<td>5.93–64.00</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>3.80</td>
<td>12.50</td>
</tr>
</tbody>
</table>

*In parentheses, number of subjects.

bSeventeen subjects did not provide this information.

Correlation between plasma markers and biomarkers of genetic damage

The incidence of genetic damage (SCE, micronuclei and comet tail moment) in the study population was previously to be unaffected by the occupational exposure to pollutants; however, smoking habits were associated with significantly higher SCE and HFC rates, and both gender (female) and ageing increased micronucleus frequency. No clear association was observed between metabolic polymorphisms and genotoxic damage in blood cells (24,28).

Simple correlation analysis between plasma markers and genotoxicity end-points indicated that micronuclei were positively correlated with folic acid plasma level, and negatively with homocysteine concentration (Table III). Moreover, both SCE and HFC were positively correlated with plasma vitamin B12. No correlation was observed between plasma markers and comet assay parameters (tail length and tail moment), or with the two parameters of lymphocyte proliferation (PRI, proliferation index and NDI, nuclear division index).

Effects on biomarkers of genetic damage: regression analysis

Mean values of genotoxicity biomarkers in relation to MTHFR and MTRR genotypes are shown in Table IV. No statistically significant difference was observed comparing group means.

To further investigate the possible modulation of genotoxic damage by the folic acid pathway, a forward stepwise multiple regression analysis of data was carried out. This analysis allowed the estimation of how much of the total variance of...
genotoxicity biomarkers was explained by the independent variables considered. To this aim, a statistical model is constructed, accounting for the possible inter-correlation between variables and excluding those not significantly associated to the genetic end-points.

In the regression model, smoking habits, plasma vitamin B12 and the CYP1E1 genotype explained 20% of the variance of SCE (natural logarithmic transformed) (Table V). When the positive association between vitamin B12 and SCE highlighted by the regression analysis was compared separately in smokers and non-smokers by simple regression, SCE were found to increase significantly ($P = 0.001$) with B12 levels only in smokers (Figure 1), suggesting an interaction between vitamin B12 and smoking habits. Similarly ~20% of HFC variability was explained by the same three factors (data not shown).

Multiple regression with micronuclei as dependent variable produced a significant model explaining 15% of variance. In addition to three already identified variables (age, gender, GSTM1 polymorphism) (28), also the MTRR polymorphism contributed to the model (Table V). Plasma homocysteine, although correlated to micronuclei (Table III), was not included in the model, indicating that the observed correlation was secondary to the effect of gender on the modulation of both micronuclei and homocysteine levels (Table II). In order to further investigate the influence of the MTRR polymorphism on micronucleus frequency, residual values after regression with age, gender and GSTM1 were calculated. As shown in Figure 2, micronucleus residuals were slightly higher (~30%) in carriers of the GG variant with respect to AG and AA variants ($P = 0.026$, Mann–Whitney test). No significant regression model explaining the variance of tail moment values was obtained by using the same independent variables.

Finally, in order to assess the possible effects of the concurrent presence of multiple extreme values of plasma markers, the nine subjects with highest putative protective values, being in the highest tertile for plasma folic acid and vitamin B12, and in the lowest for homocysteine, were challenged against the 15 subjects in the lowest tertile for both plasma folic acid and vitamin B12, and in the highest homocysteine tertile. The comparison between SCE rates, micronuclei and tail moment in the two groups did not reveal any statistically significant difference (Mann–Whitney $U$ test) (data not shown).

### Discussion

In this study, the role of folic acid metabolism in modulating baseline DNA damage in human lymphocytes was investigated. Genotoxic damage measured by cytogenetic techniques or by the comet assay in 191 healthy individuals was evaluated with respect to plasma levels of folic acid, homocysteine and...
vitamin B12 were observed in cancer patients (37). Moreover, in another study conducted on 300 Finnish patients, a not significant ($P = 0.14$) positive trend between vitamin B12 and cancer risk was reported, with a 41% additional risk in the high vitamin B12 group (38).

Alternatively, the correlation between plasma vitamin B12 and SCE may unravel an association with other unidentified factor(s) acting synergically with tobacco smoke. In this respect, as the main source of vitamin B12 is meat (3), genotoxic heterocyclic aromatic amines and polycyclic aromatic hydrocarbons found in cooked meat might play a role. In particular, two carcinogenic genotoxic heterocyclic aromatic amines (MeIQx and PhIP) commonly found in meat, were demonstrated to be able to induce SCE in chronically fed mice (39,40). As carcinogens in meat and tobacco smoke share similar metabolic activation pathways, both involving the cytochrome P-450 family, the chronic induction of these enzymes by tobacco smoke could augment the effects of the exposures to food carcinogens. Indeed, recent studies show that cigarette smoke enhanced hepatocarcinogenesis and mutagenicity induced by MeIQx (41,42). However, because detailed information on individual food habits was not collected during the recruitment, this association could not be further investigated.

The second finding concerns the possible influence of the MTRR genotype on background frequency of micronuclei. The MTRR gene catalyses the conversion of the inactive form of methionine synthase (MTR) into its active form, by regeneration of methyl(III)cobalamin, the cofactor of MTR. The biochemical effect of the 66 A $\rightarrow$ G substitution (16) is not known in detail. However, a recent study indicates that the variant protein exhibits 4-fold lower activity than the wild-type protein in the reactivation of MTR in vivo (26). Hence, by reducing the level of active MTR, the MTRR variant allele should decrease the availability of SAM, thus leading to DNA hypomethylation. Several data indicate that DNA hypomethylation, particularly in the centromeric region, may be a causative factor of chromosome malsegregation (6), an event that is responsible for the meiotic origin of trisomy 21 and for micronuclear formation. In this respect, epidemiological studies suggest that the GG genotype, alone or in combination with the MTHFR polymorphism, may represent a risk factor for spina bifida (16) and Down conception (14,15). In our study the higher micronucleus frequency recorded in 66 GG variant homozygotes with respect to heterozygotes or wild-type homozygotes is suggestive for a role of this polymorphism in the modulation of chromosome stability. In any case, the influence of the MTRR genotype on micronuclear frequency was small, and only detectable after correction for other independent variables. Detection of centromere sequences or kinetochore protein in micronuclei would allow determination of their origin and to add a mechanistic support to the hypothesis mentioned above.

The observed effect of vitamin B12 on SCE frequency in smokers, and the modulation of micronucleus incidence by the MTRR genotype, point out the influence of both hyper- and hypomethylation on DNA integrity. In cancer cells global DNA hypomethylation, mainly affecting repeated sequences, is the general rule. On the other hand, local hypermethylation is observed in regulatory sequences with consequent transcriptional silencing of key genes for growth control, such as tumor suppressor genes (43). The folic acid pathway has a fundamental role in the maintenance of normal methylation level
into the cell (44). Perturbation of this pathway may reduce global methylation of repeated sequences, including those localized in the centromeric region, and disturbing the apparatus for chromosome segregation (45,46). On the other hand, factors favouring DNA methylation could modulate gene expression or, as suggested by the results of this study, exert an indirect influence on DNA integrity, mediated by the interaction with exogenous factors (47).

Several case-control studies indicate that the MTHFR C677T polymorphism can modify cancer risk (48), and that MTHFR 677TT subjects with low serum folic acid show DNA hypomethylation (49,50). However, no modulation of background genetic damage by the MTHFR polymorphism was observed in this study. Indeed, recent in vitro studies on folate acid deficiency and DNA damage in lymphocytes failed to discriminate subjects with different MTHFR genotype (12,23). These results seem to indicate a marginal influence of the MTHFR genotype on DNA integrity, even though it is possible that unphysiological levels of methionine and riboflavin in culture medium may have biased the results of the in vitro studies.

Previous investigations on the Australian population indicated that micronucelar frequency in men was also significantly increased with increasing plasma homocysteine (9,10). In this study, no significant correlation between micronuclei and homocysteine was observed, once that the effect of other correlated variables (age, gender) was taken into account. However, a direct effect of homocysteine on micronucleus formation in vitro could not be demonstrated (51), and it is possible that in different populations (e.g. Australian versus Italian), plasma homocysteine may be associated with different confounding factors, e.g. related to different dietary habits.

Finally, even though it was recently estimated that the recommended daily intake of micronutrients may be suboptimal for the purpose of protection from genotoxic damage (4), the results of this study indicate that the relationship between genetic integrity and folate status may be complex, modulated by exogenous factors such as tobacco smoke. Yet, both the effect of vitamin B12 on SCE, and that of the MTRR genotype on micronuclei, confirm that folate metabolism plays a critical role in the maintenance of genomic integrity. Further investigation on the relationships between DNA methylation/damage and folate status in different genetic backgrounds are necessary to fully elucidate the genetic consequences of unbalanced folate metabolism.

Acknowledgement

The authors are grateful to Mrs Celestina D’Ascoli for her technical assistance.

References


Received January 20, 2003; revised April 9, 2003; accepted April 10, 2003.