Folate deficiency is not associated with increased mitochondrial genomic instability: results from dietary intake and lymphocytic mtDNA 4977-bp deletion in healthy young women in Italy

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Received on July 30, 2013; revised on November 27, 2013; accepted on November 29, 2013

The mitochondrial DNA (mtDNA) 4977-bp deletion is a biomarker of mitochondrial genomic instability. It is frequently detected in a number of sporadic diseases, and it accumulates in many tissues during aging. Folic acid plays an important role in the maintenance of genomic stability in mammals. The aim of the present cross-sectional study was to characterise the levels of the mtDNA deletion in the lymphocytes of healthy young women, taking into account folate intake, red blood cell (RBC) folate levels and the distribution of the methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism. Folate intake was estimated by a food frequency questionnaire. Determination of the MTHFR C677T polymorphism and of the mtDNA deletion was performed by real-time polymerase chain reaction analysis. A total of 476 women were enrolled. Low levels of deletion were found (mean ΔCt = 1.24). After multivariate analysis, results did not show any significant relationship between age, smoking habits, pregnancy status, nutritional status, inadequate folate intake, folate deficiency, use of folic acid supplements, MTHFR C677T polymorphism and mtDNA 4977-bp deletions. The lack of association between inadequate folate intake, folate deficiency and mitochondrial genomic instability was confirmed also considering reference values of folate based on DNA damage prevention. Our results indicate that mtDNA 4977-bp deletions are maintained at low levels in lymphocytes of young healthy women despite the wide range of variation of folate intakes and folate status. Future studies, carefully designed to address limits and methodological issues related to variation of this biomarker as an effect of different dietary patterns and of folate status, could provide further insight on the specific mechanisms that are acting in lymphocytes of healthy subjects under observed folate intake.

Introduction

Biomarkers that are influenced by nutritional factors offer a potentially important contribution to public health programs because of not only their direct relevance to explaining the effects of diet on the risk of disease but also their potential role in monitoring the effectiveness of preventive programs (1). Biomarkers of early events should be related to, and ideally critical for, the development of the adverse effect that is the basis for the risk assessment (2,3).

Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage than nuclear DNA since it is not protected by histones and it reveals limited capacity for DNA repair (4). Alterations in mtDNA both qualitatively (mutations) and quantitatively (mtDNA copy number) have been associated with many human diseases including neurodegenerative diseases, metabolic diseases and various types of cancer (5,6). Among several mutations that have been reported to be associated with various diseases, a 4977-bp deletion, occurring between two 13-bp direct repeats at positions 13447–13459 and 8470–8482, has attracted great interests since it has also been shown to accumulate in many tissues during aging and it has been used as a mtDNA damage biomarker (5,7). The mtDNA 4977-bp deletion has been detected in fast replicating cells such as blood leukocytes (8,9), but little is known about the relationship between this mutation and folate status (10). Folic acid plays an important role in the maintenance of genomic stability in mammals that are unable to synthesise folic acid de novo; thus, serum levels of this micronutrient are mainly modulated by dietary habits and in addition by the presence of polymorphisms of genes implicated in relevant biochemical pathways. Among these, an important role is played by methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism that modify cancer risk differently depending on folate status (11–18). However, a previous study indicates that folic acid deficiency induces to a comparable extent chromosome loss and breakage irrespective of the MTHFR genotype (19).

In an experimental study in rodents, a folate-dependent accumulation of mtDNA deletions in lymphocytes has been reported after folate deprivation, suggesting that accumulated lymphocytic mtDNA deletions may serve as a molecular biomarker responding to dietary folate deprivation (10). Furthermore, in a case–control study, an increased frequency of lymphocytic mtDNA 4977-bp deletion has been associated with human hepatocellular carcinoma risk; moreover, in the same study, a high frequency of lymphocytic mtDNA 4977-bp deletion was associated with low levels of lymphocytic folate (11).

As suggested from experimental studies in rodents (10), we hypothesised that folate intake and folate status may have an impact on lymphocytic mtDNA 4977-bp deletion levels in healthy humans and thus on mitochondrial genomic instability. Besides, MTHFR genotype should be taken into account in order to study the relationship between folate status and lymphocytic mtDNA 4977-bp deletion.

Our cross-sectional study was designed in order to evaluate the suitability of the mtDNA 4977-bp deletion as an effect biomarker to measure the potentially modifiable biological events that take place under usual folate intake, in real-life dietary habits. Therefore, we characterised the levels and the
range of variation of the lymphocytic mtDNA 4977-bp deletion in healthy young women, taking into account folate intake, red blood cell (RBC) folate level and the distribution of the MTHFR C677T polymorphism.

Materials and methods

Study subjects
During a 12-month period, a total of 476 healthy women of childbearing age, referred to the Laboratory of the S. Bambino Hospital, Catania, Italy, an obstetric centre for preconceptional, prenatal and post-partum care, were enrolled in the study. All women gave their informed consent to participate in the study. The study protocol was approved by the ethics committee of the involved hospital. The study design was described in greater detail elsewhere (20). Briefly, demographic, obstetrical history, anthropometric and lifestyle (including smoking) data were collected by trained epidemiologists. Anthropometric measurement, such as weight and height, were taken using standard measurement equipment. From these, body mass index (BMI) was calculated for each subject, based on criteria from the World Health Organization (21). Pre-pregnancy BMI was based on self-reported pre-pregnancy weight.

Intake of folic acid supplements was derived from specific questions addressing the use of dietary supplements during the periconceptional period and pregnancy. Furthermore, natural folate from foods, i.e. folate intake, during the previous month, was estimated by a previously validated semi-quantitative food frequency questionnaire (FFQ) (20). Prevalence of inadequate folate intake was determined by comparing folate intake with the estimated average dietary requirements (22).

RBC folate levels and MTHFR C677T genotyping determinations
Peripheral blood samples were taken from each woman, after a 12-h fasting period, and collected in EDTA containing tubes and stored at −80°C until analysis. RBC folate levels were measured in the whole blood samples with a commercially available kit (Elecys Folate III Test System, Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s recommendations (23). Folate deficiency was defined as RBC folate <305 nmol/l (22).

Genomic DNA was extracted from whole blood using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare) according to the manufacturer’s protocol and stored at −20°C. Determination of MTHFR C677T polymorphism was performed as previously described (20) using TaqMan allelic discrimination assay, with the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Real-time polymerase chain reaction analysis of mtDNA deletions
mtDNA 4977-bp deletion was determined, by coamplifying the mt ND1 gene with a rarely deleted region and the mt ND4 gene, which is commonly absent in the majority of patients with large deletions, according to the method of He et al. (24) by real-time polymerase chain reaction (PCR) analysis. The PCR primers and the fluorogenic probe for the ND1 region and for the ND4 region were described elsewhere (11). PCR amplification was carried out in a 50 μl reaction volume consisting of TaqMan Universal Master Mix, 200 nmol/l of deletion ND4 primer, 100 nmol/l of each ND1 primer, 100 nmol/l of each mtDNA deletion probe and the ND1 probe primer. The cycling conditions included an initial phase of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 0.5 min at 72°C. The fluorescence spectra were monitored using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). The cycle at which a statistically significant increase in normalised fluorescence was first detected was designated as the threshold cycle number (Ct). The Ct values were used to quantify the relative amount of ND4 to ND1 with the equation \( R = 2^{-\Delta C t} \), where \( \Delta C t = C t_{\text{ND1}} - C t_{\text{ND4}} \). A smaller value of \( \Delta C t \) indicates fewer mtDNA deletions. Furthermore, to quantify the fold change reduction of ND4, the negative inverse of \( R \) was computed (25).

Statistical analyses
Statistical analyses were performed using the software SPSS, version 14.0 (SPSS, Chicago, IL, USA). Differences between continuous variables were tested using Student’s t-test. Correlation between lymphocytic mtDNA deletions and folate intake and RBC folate level were evaluated using Spearman’s correlation coefficient. Associations of all variables with the lymphocytic mtDNA deletions and with the fold change reduction of ND4 were assessed using the \( \chi^2 \) test. Quantitative data (ACI values and fold change reduction of ND4) were converted in qualitative data using the median values as the cut-off point. To measure the association level, the crude odds ratio (OR) and the corresponding 95% confidence interval (95% CI) were calculated. Multivariate logistic regression analysis (backward stepwise–likelihood ratio method) was used to adjust for possible confounding effects and to obtain the adjusted OR with the respective 95% CI. A \( P \) value ≤0.05 was considered statistically significant.

Results

Population characteristics
During the study period, a total of 476 women of childbearing age were enrolled of which 49.4% (n = 235) were pregnant. All women accepted, after giving their informed consent, to donate blood for the present research study. The mean age was 28.4 years (median 27 years, interquartile range: 23–33 years; range: 14–49 years). A total of 42.1% of enrolled women (n = 199) were smokers. Regarding BMI, only 8.8% of women were underweight (n = 42), 56.1% were of normal weight (n = 267) and 35.1% were overweight or obese (n = 167).

Mean folate intake, assessed by the FFQ, thus considering the total folate intake from food excluding folic acid supplements, was 242.9 μg/day (median 219.0 μg/day; range: 47.4–972.8 μg/day). Particularly, mean folate intake was 264.0 μg/day (median 239.4 μg/day; range: 49.4–972.8 μg/day) in pregnant women and 224.2 μg/day (median 204.9 μg/day; range: 47.4–773.0 μg/day) in non-pregnant women (P = 0.001). Mean folate intake did not vary significantly by age (253.0 μg/day in women younger than 27 years, i.e. the median age, and 232.6 μg/day in older women; P = 0.093).

A total of 45.6% (n = 217) of enrolled women reported intake of folic acid supplements or of multi-mineral/multivitamin supplements containing folic acid. Thus, taking into account the intake of folic acid supplements, the prevalence of inadequate folate intake was 45.4% (n = 216) for the overall sample of women, 13.2% (n = 31) in pregnant and 76.8% (n = 185) in non-pregnant women (P = 0.001).

Mean RBC folate level was 322.3 nmol/l (median 291.2 nmol/l; range: 111.9–622.2 nmol/l). Particularly, mean RBC folate level was 372.8 nmol/l (median 364.6 nmol/l; range: 111.9–622.2 nmol/l) in pregnant women and 273.6 nmol/l (median 255.4 nmol/l; range: 114.4–542.5 nmol/l) in non-pregnant women (P < 0.001). Besides, the mean RBC folate level was 303.9 nmol/l in women younger than 27 years (i.e. the median age) and 338.6 nmol/l in older women (P = 0.023). Considering all women, the prevalence of folate deficiency was 52.4%, RBC folate level <305 nmol/l: 33.6% in pregnant women and 70.5% in non-pregnant women, P < 0.001.

The relative frequency of the T mutated allele of the MTHFR C677T polymorphisms was 43.8%. Overall, 20.5% of the subjects were homozygous for the 677TT mutated genotype and 46.6% were heterozygous.

mtDNA 4977-bp deletions in the study subjects
Mean level of lymphocytic mtDNA 4977-bp deletions of the women in study was ΔCt = 1.24 (median 1.09; range: 0.12–2.85). Mean R value was 0.45 (median 0.47; range: 0.14–0.92). Furthermore, the mean fold change reduction of ND4 was −2.55 (median −2.13; range: −7.19 to −1.08).

Mean levels of lymphocytic mtDNA 4977-bp deletions did not differ significantly by age (quartiles distribution and median value as cut-off), smoking habits, pregnancy status, inadequate folate intake, folate deficiency, use of folic acid supplements...
and homozygous 677TT mutated genotype. However, mean levels of lymphocytic mtDNA 4977-bp deletions were significantly higher in underweight and normal weight than in overweight and obese women considering both ΔCt value (Table I) and fold change reduction of ND4 (data not shown).

Correlation between lymphocytic mtDNA 4977-bp deletions and folate levels, as folate intake estimated by FFQ and RBC folate level, was assayed. A significant, albeit weak, correlation between folate intake and lymphocytic mtDNA 4977-bp deletions (ΔCt value) was showed (r = 0.133, P = 0.004). Furthermore, lymphocytic mtDNA 4977-bp deletions (ΔCt value) were not significantly correlated with RBC folate levels (r = −0.063, P = 0.316).

Univariate analysis was used to assess the association between lymphocytic mtDNA 4977-bp deletions (using the median value of ΔCt as cut-off) and age (using the median value as cut-off), smoking habits, pregnancy status, nutritional status, inadequate folate intake, folate deficiency, use of folic acid supplements and homozygous 677TT mutated genotype. After multivariate logistic regression analysis, results did not show any significant relationship between the above listed factors and lymphocytic mtDNA 4977-bp deletions (Table II). The same results were obtained using, as cut-off, the median value of fold change reduction of ND4 (data not shown).

Discussion

Folate is necessary to nucleotide synthesis and DNA methylation and plays an essential role in women’s and children’s health (12–14). Besides, there is a growing body of epidemiologic evidence that suggests folate deficiency contributes to cancer risk (15–18). Those relationships are modified by the MTHFR gene C677T polymorphisms (16–18).

In order to obtain new insights regarding the association between folate intake, RBC folate level and any variation of the levels of the mtDNA 4977-bp deletion, we studied a cohort of healthy women of childbearing age showing a high prevalence of inadequate folate intake and of folate deficiency. Notably, our study was conducted in a country, Italy, where no folic acid fortification has been introduced, but only supplementation in the periconceptional period is recommended. It is always assumed that pregnant women consume and/or take more folate either through the choices of foods containing folate or as supplements. In fact, in our study, pregnant women have a significantly higher mean folate intake and mean RBC folate level than non-pregnant women.

Somatic mtDNA deletions obtained from samples of venous blood cells may serve as useful biomarkers for the early detection of global genomic instability and cancer risk, as it has been suggested elsewhere (11). We hypothesised that nutritional factors and specifically folate intake and folate status may have an impact on lymphocytic mtDNA 4977-bp deletion levels in humans, particularly in young healthy women, and thus on mitochondrial genomic instability, as suggested from experimental studies in rodents (10).

In our observational study, no association between characteristics of women and the mtDNA 4977-bp deletion levels, as biomarker selected to measure mitochondrial genomic instability, was found. Additionally, no correlation was found when folate intake and RBC folate levels were explored. Furthermore, our data report lower mean levels of the mtDNA 4977-bp deletion (mean ΔCt = 1.24) in lymphocytes of young healthy women when compared with data from the literature, and particularly with those obtained—with the same methodology—in a healthy group of older males and females (mean ΔCt = 3.5, in the control group) (11). Younger age and gender may explain, at least in part, such observed differences. However, the findings regarding an age-dependent increase of mtDNA 4977-bp deletion are still controversial. Very low mtDNA 4977-bp deletion levels were previously reported in peripheral blood lymphocytes of young and elderly subjects and no accumulation of the 4977-bp deletion with increasing age was shown (7,8).

Table I. Mean levels (mean ΔCt) of the mtDNA 4977-bp deletion in the study population by age, nutritional and lifestyle factors and MTHFR C677T polymorphism

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number of women</th>
<th>Mean ΔCt (±SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age first quartile (14–23 years)</td>
<td>124</td>
<td>1.24±0.51</td>
<td>0.67</td>
</tr>
<tr>
<td>Age second quartile (24–28 years)</td>
<td>125</td>
<td>1.28±0.54</td>
<td></td>
</tr>
<tr>
<td>Age third quartile (29–34 years)</td>
<td>111</td>
<td>1.20±0.51</td>
<td></td>
</tr>
<tr>
<td>Age fourth quartile (35–49 years)</td>
<td>105</td>
<td>1.23±0.52</td>
<td></td>
</tr>
<tr>
<td>Age ≤ 27 years (median value)</td>
<td>233</td>
<td>1.28±0.54</td>
<td></td>
</tr>
<tr>
<td>Age &gt; 27 years</td>
<td>232</td>
<td>1.20±0.51</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>196</td>
<td>1.21±0.50</td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>266</td>
<td>1.26±0.54</td>
<td></td>
</tr>
<tr>
<td>Inadequate folate intake</td>
<td>213</td>
<td>1.21±0.49</td>
<td>0.20</td>
</tr>
<tr>
<td>Adequate folate intake</td>
<td>252</td>
<td>1.27±0.55</td>
<td></td>
</tr>
<tr>
<td>Folate deficiency</td>
<td>243</td>
<td>1.04±0.26</td>
<td>0.22</td>
</tr>
<tr>
<td>No folate deficiency</td>
<td>222</td>
<td>0.99±0.26</td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>229</td>
<td>1.24±0.52</td>
<td>0.96</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>236</td>
<td>1.24±0.53</td>
<td></td>
</tr>
<tr>
<td>Underweight and normal weight</td>
<td>201</td>
<td>1.29±0.55</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Overweight and obese</td>
<td>164</td>
<td>1.14±0.44</td>
<td></td>
</tr>
<tr>
<td>Supplement users</td>
<td>212</td>
<td>1.24±0.53</td>
<td>0.97</td>
</tr>
<tr>
<td>Supplement non-users</td>
<td>253</td>
<td>1.24±0.51</td>
<td>0.19</td>
</tr>
<tr>
<td>Homozygous 677TT mutated genotype</td>
<td>95</td>
<td>1.00±0.24</td>
<td></td>
</tr>
<tr>
<td>Other genotypes</td>
<td>370</td>
<td>1.05±0.26</td>
<td></td>
</tr>
</tbody>
</table>

1 Prevalence of inadequate folate intake was determined by comparing folate intake with the estimated average dietary requirements: 520 µg/day for pregnant and 320 µg/day for non-pregnant women (22).

2 Folate deficiency was defined as RBC folate <305 nmol/l (22).

3 Based on criteria from the World Health Organization (21).

4 MTHFR 677CC and MTHFR 677CT.
A recent study has shown that the proportion of mtDNA 4977-bp deletion carriers was similar in elderly and in centenarians individuals, but the individual mutational load was on average much lower in the centenarians than in the elderly, probably reflecting a healthier lifestyle that can be attributed to their very advanced ages, that may result in a lower reactive oxygen species production and thus less overall DNA damage (healthy survivor effect) (9).

Moreover, the low level of mtDNA deletion observed in our study may be due in part to the high turnover of blood cells, which may inhibit the accumulation of genetically abnormal mtDNA (8,26), leading to a dilution effect, due to rapid cytoplasmic division, and a selective effect, by which cells harbouring mtDNA with large-scale deletion are eliminated during growth (27). In addition, a decreased proportion of mtDNA 4977-bp deletion in tumour tissues as compared with corresponding non-tumorous tissues has been observed in different cancers (27,28) and a recent meta-analysis confirmed that an higher frequency of common deletion is detected in cancer patients—cancerous tissues and adjacent non-cancerous tissues—compared with tissues from the healthy controls (6).

In the present study, 35.1% of women were overweight or obese, confirming the recently reported prevalence among women representative of the Italian adult population (33.8%) (29). Although obesity has been associated with mitochondrial dysfunction in adipose tissue (30), nutritional status was not an independent factor associated with mtDNA deletion levels in our regression analysis.

Numerous exogenous factors including lifestyle habits and in particular tobacco smoke are known to result in an increase in mtDNA deletion in different tissues (31). In our study, pregnant versus non-pregnant status, obese versus non-obese (either overweight, normal weight or under weight), smokers versus non-smokers and MTHFR 677TT mutated genotype versus MTHFR 677CT/CC genotypes, also when present in several combinations in one individual, might have resulted in an effect on mtDNA deletion levels. After multivariate logistic regression analysis in order to take all the above-mentioned variables into account, no statistically significant influence of any of them on the mtDNA deletion was observed, possibly due to the vast renewal capacity of blood cells and the capability of mtDNA for quick restoration (9).

Very little is known about mtDNA deletions in lymphocytes associated with nutritional factors such as folate especially in healthy individuals. Results from the study of Chou and Huang (10) in lymphocytes of rodents after dietary folate deprivation suggest that accumulated lymphocytic mtDNA deletions may serve as a biomarker of mitochondrial genomic instability responding to dietary folate deprivation as well as depleted cellular folate storage. On the contrary, in our study, no effect of folate deficiency on mitochondrial genomic stability was found. As previously suggested, mtDNA deletion levels in lymphocytes could represent a snapshot of the mutational events that happened in a relatively short period of time before blood sample collection, and inadequate folate intake and folate deficiency represent risk factors that lead to fast but transient short-term changes in leukocyte mtDNA deletion accumulation (9). Furthermore, if a long exposure to folate deprivation is necessary to genomic instability, young age of our population (median age 27 years) may have played a role. In addition, dose of folate intake and blood levels of folate are important issues to be considered. An effect of folate deprivation on genomic instability (i.e. micronuclei frequency) has been clearly demonstrated only in subjects with very low folate intake (≤206.64 μg/day) (32). Blood levels of folate required to prevent anaemia (22) are accurately defined; nevertheless, it is becoming evident that such accepted levels may be much lower than the averaged concentration levels at which DNA damage is minimised (33). Thus, there is an increasing need to establish requirements for preventing damage to both nuclear and mtDNA (34). Lack of association found in the present study was obtained defining inadequate folate intake considering estimated average dietary requirements (520 μg/day for pregnant and 320 μg/day for non-pregnant women) and folate deficiency as RBC folate <305 nmol/l (22). Although it has been reported, from intervention studies in humans, that different biomarkers of DNA damage are minimised when RBC folate concentration is >700 nmol/l (concentration achievable at intake levels more than 400 μg/day folic acid per day) (34), in our study, after multivariate logistic regression analysis, negative results were obtained also considering the last proposed cut-off values (data not shown).

The issue of statistical power is critical in negative studies. Given the lack of reference data regarding the association between folate deficiency and mitochondrial genomic instability in human, we could not assess the statistical power. Our study was clearly underpowered to detect as statistically significant the small effect of folate deficiency suggested by our data (OR 1.52; Table II), and larger follow-up studies are needed to better assess the role of chance. However, our results provide an upper estimate of the effect—if any—of folate deficiency on mitochondrial instability. Furthermore, other factors

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### Table II. Multivariate analysis for mtDNA 4977-bp deletion levels (ΔCt)

<table>
<thead>
<tr>
<th>Population characteristics and variables</th>
<th>ΔCt below the median value* (N*)</th>
<th>ΔCt above the median value* (N*)</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (below the median value: 27 years)</td>
<td>46.8% (109)</td>
<td>53.4% (124)</td>
<td>0.77</td>
</tr>
<tr>
<td>Age (above the median value: 27 years)</td>
<td></td>
<td></td>
<td>1.09 (0.62–1.89)</td>
</tr>
<tr>
<td>Smokers</td>
<td>46.1% (107)</td>
<td>38.7% (89)</td>
<td>0.92</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
<td>0.97 (0.57–1.65)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>48.9% (114)</td>
<td>49.6% (115)</td>
<td>0.25</td>
</tr>
<tr>
<td>Pregnant</td>
<td></td>
<td></td>
<td>1.68 (0.70–4.03)</td>
</tr>
<tr>
<td>Overweight and obese</td>
<td>38.6% (90)</td>
<td>31.9% (74)</td>
<td>0.32</td>
</tr>
<tr>
<td>Overweight and obese</td>
<td></td>
<td></td>
<td>0.75 (0.43–1.32)</td>
</tr>
<tr>
<td>Inadequate folate intake</td>
<td>46.8% (109)</td>
<td>44.8% (104)</td>
<td>0.55</td>
</tr>
<tr>
<td>Inadequate folate intake</td>
<td></td>
<td></td>
<td>1.52 (0.39–6.00)</td>
</tr>
<tr>
<td>Folate deficiency</td>
<td>52.1% (155)</td>
<td>52.7% (88)</td>
<td>0.18</td>
</tr>
<tr>
<td>Folate deficiency</td>
<td></td>
<td></td>
<td>1.52 (0.83–2.80)</td>
</tr>
<tr>
<td>Supplement non-users</td>
<td>55.8% (130)</td>
<td>53.0% (123)</td>
<td>0.33</td>
</tr>
<tr>
<td>Supplement non-users</td>
<td></td>
<td></td>
<td>0.46 (0.10–2.18)</td>
</tr>
<tr>
<td>Homozygous 677TT mutated genotype</td>
<td>22.4% (59)</td>
<td>18.0% (36)</td>
<td>0.43</td>
</tr>
<tr>
<td>Homozygous 677TT mutated genotype</td>
<td></td>
<td></td>
<td>1.27 (0.71–2.27)</td>
</tr>
</tbody>
</table>

*ΔCt median value was 1.09.

*Number of women.

*OR for ΔCt above the median value.
should be discussed to explain our results. Although different characteristics have been included in the regression model to control and adjust simultaneously for various confounders, other possible uncontrolled factors should be considered, such as preconceptional, peri- or post-partum or infertile status. Furthermore, the mean folate intake was not weighted by season of the FFQ collection in order to control for difference across seasons, even though a recent large study (35) reports no systematic variations for folate intake according to the season of the dietary intake collection also in our country. Besides, although the FFQ administered have been previously validated (20), generally dietary questionnaire data may suffer from inaccuracies of volunteer recall. Additionally, studies are difficult to compare since they differ considerably in terms of the sensitivity of the methodologies applied and the demographic characteristics of the participants, highlighting the need to promote a “gold standard method” to investigate the role of the mtDNA 4977-bp deletion in different observational contexts.

Our results indicate that mtDNA 4977-bp deletions are maintained at low levels in lymphocytes of young healthy women despite the wide range of variation of folate intakes and folate status. We cannot exclude the absence of mtDNA damage in other tissues. To our knowledge, this is the first study conducted in order to explore the association between folate deficiency and mitochondrial genomic instability in young healthy women. Future studies, carefully designed to address limits and methodological issues related to variation of this biomarker as an effect of different dietary patterns and of folate status, could provide further insight on the specific mechanisms that are acting in lymphocytes of healthy subjects under usual folate intake.

**Funding**

This work was supported in part by grants from the University of Catania (Progetti di Ricerca di Ateneo to A.A.).

Conflict of interest statement: None declared.

**References**


32. Fenech, M., Baghurst, P., Luderer, W., Turner, J., Record, S., Ceppi, M. and Bonassi, S. (2005) Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability—results from a dietary intake and micronucleus index survey in South Australia. *Carcinogenesis*, 26, 991–999.

