ACCELERATED PAPER

Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults

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We performed a cross-sectional study (n = 49 males, 57 females) and a randomized double-blind placebo-controlled dietary intervention study (n = 31/32 per group) to determine the effect of folate and vitamin B12 (B12) on DNA damage (micronucleus formation and DNA methylation) and plasma homocysteine (HC) in young Australian adults aged 18–32 years. None of the volunteers were folate deficient (i.e. red blood cell folate <136 nmol/l) and only 4.4% (all females) were vitamin B12 deficient (i.e. serum vitamin B12 <150 pmol/l). The cross-sectional study showed that (i) the frequency of micronucleated cells (MNCs) was positively correlated with plasma HC in males (R = 0.293, P < 0.05) and (ii) in females MNC frequency was negatively correlated with serum vitamin B12 (R = -0.359, P < 0.01) but (iii) there was no significant correlation between micronucleus index and folate status. The results also showed that the level of unmethylated CpG (DNA) was not significantly related to vitamin B12 or folate status. The dietary intervention involved supplementation with 3.5× the recommended dietary intake (RDI) of folate and vitamin B12 in wheat bran cereal for three months followed by ten times the RDI of these vitamins via tablets for a further three months. In the supplemented group, MNC frequency was significantly reduced during the intervention by 25.4% in those subjects with initial MNC frequency in the high 50th percentile but there was no change in those subjects in the low 50th percentile for initial MNC frequency. The reduction in MNC frequency was significantly correlated with serum vitamin B12 (R = -0.49, P < 0.0005) and plasma HC (R = 0.39, P < 0.006), but was not significantly related to red blood cell folate. DNA methylation status was not altered in the supplemented group. The greatest decrease in plasma HC (by 37%) during the intervention was observed in those subjects in the supplemented group with initial plasma HC in the high 50th percentile, and correlated significantly with increases in red blood cell folate (R = -0.64, P < 0.0001) but not with serum vitamin B12. The results from this study suggest that (i) MNC frequency is minimized when plasma HC is below 7.5 µmol/l and serum vitamin B12 is above 300 pmol/l and (ii) dietary supplement intake of 700 µg folic acid and 7 µg vitamin B12 is sufficient to minimize MNC frequency and plasma HC. Thus, it appears that elevated plasma HC, a risk factor for cardiovascular disease, may also be a risk factor for chromosome damage.

Introduction

Folic acid and vitamin B12 play an important role in DNA metabolism (1). Folic acid is required for the synthesis of dTMP from dUMP. Under conditions of folic acid deficiency dUMP accumulates and as a result uracil, instead of thymine, is incorporated into DNA (2). There is good evidence suggesting that excessive misincorporation of uracil in DNA not only leads to point mutation but may also result in the generation of single- and double-stranded DNA breaks, chromosome breakage and micronucleus formation (3,4). Folic acid and vitamin B12 are also required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation (5). Deficiencies in folic acid and vitamin B12 therefore lead to (i) elevated DNA damage rate and altered methylation of DNA, both of which are important risk factors for cancer (3–5) and (ii) an increased level in homocysteine (HC*) status, an important risk factor for increased risk of cardiovascular disease (6). These same defects may also play an important role in developmental and neurological abnormalities (3,4).

The blood levels of folate and vitamin B12 required to prevent anaemia and hyperhomocysteinemia are properly defined, however, it is still uncertain whether such accepted levels of sufficiency are in fact adequate to minimize chromosome damage rates and optimize DNA methylation status. We have therefore performed a series of studies to investigate the interrelationship between DNA damage in somatic cells and blood status for folate, vitamin B12 and HC. As a marker of chromosome damage, we have used the cytokinesis-block micronucleus method in lymphocytes, which has been shown in numerous studies to be a reliable and sensitive biomarker of chromosome breakage and chromosome loss that occurs spontaneously (7) or as a result of elevated exposure to genotoxins (8). The importance of identifying dietary factors that minimize DNA damage rate is underscored by recent evidence from two epidemiological prospective studies indicating that a reduced level of chromosome damage in lymphocytes is a relevant biomarker of reduced future cancer risk (9,10).

Our preliminary studies in young men had indicated that there was a significant negative correlation between the micronucleus frequency in lymphocytes and plasma vitamin B12 status (11). Results from our more recent studies in men aged 50–70 years have shown that the micronucleus index is negatively correlated with vitamin B12 in subjects who are not vitamin B12 deficient as defined by accepted clinical parameters and that the micronucleus index is significantly and positively correlated with plasma HC status in men who are not folate or vitamin B12 deficient (11,12). These studies suggested that the plasma levels of HC and vitamin B12 that

*Abbreviations: BNCs, binucleated cells; CBMN, cytokinesis-block micronucleus; FOLB12 group, group taking folate and vitamin B12 supplement; HC, homocysteine; MTHFR, methylene-tetrahydrofolate reductase; MNCs, micronucleated cells; PHA, phytohaemagglutinin; R1, round 1; R2, round 2; R3, round 3; RDI, recommended dietary intake; VIT C, vitamin C.
correspond to minimization of chromosome damage require better definition.

With a view to defining optimal folate and vitamin B12 status in terms of genetic stability, we have measured the DNA methylation status and micronucleus index in lymphocytes of young males and females and related these results to indices of folate and vitamin B12 status in blood, including HC status. We have also performed a randomized, placebo-controlled, double-blind intervention study with a combined supplement of folic acid and vitamin B12 as we reasoned that intake of both these vitamins may need to be optimized to reduce DNA damage and plasma HC. To our knowledge, this is the first study reporting the interrelationship of chromosome damage rate, DNA methylation, HC, folate and vitamin B12 status in young Australians.

Materials and methods

Volunteer recruitment

The study was advertised in the local newspapers. The advertisement described the aim of the study, the duration and nature of the intervention and specified that volunteers aged between 18 and 35 years who were not taking vitamin supplements would be given priority. Responding volunteers were initially asked to provide details relating to their address, contact number, age, family history of cancer, gender, smoking status, intake of vitamin supplements with specific emphasis on B vitamins, history of chronic disease, medication, current medical therapy, their level of commitment, availability during the study period and whether they had an objection to donating a blood sample. Volunteers who (i) were supplementing their diets with folic acid and vitamin B12 and other B vitamins, (ii) were epileptic or on anticonvulsant therapy (anti-folate drugs), (iii) had a past or present personal history of cancer, especially if they were on anti-folate therapy or (iv) had a history of pernicious anaemia, were excluded from the study. Volunteers were given a detailed information sheet about the project describing the nature of their involvement and a consent form to sign if they were still interested in participating. Of the 162 volunteers responding to the advertisement 18 were unsuitable in terms of the exclusion criteria detailed above and 39 of the suitable volunteers declined to participate in the study. Only volunteers who had signed the consent form were included in the study. The volunteers were grouped into pairs that were matched for age, gender, smoking status, former use of vitamins and multivitamins, chronic disease condition, family history of cancer and medication. One member of each pair was then randomly assigned to the placebo group and the other member assigned to the supplement group. The study was approved by the Human Ethics Committee, CSIRO Division of Human Nutrition. Before the study commenced each volunteer was asked to complete a food frequency questionnaire, developed at CSIRO (13), as general background information. The coffee and tea consumption levels of volunteers were estimated from data in the food frequency questionnaire.

Cereal and tablets

The cereal was supplied by Kellogg (Australia) Pty Ltd. Supplemented and unsupplemented cereal was delivered to our laboratory one month before the commencement of the study. The cereal was of the ‘All Bran’ variety consisting of either packets of normal unsupplemented cereal (control) or packets of cereal supplemented with folic acid and vitamin B12. The packets with the folate and vitamin B12 supplements were indistinguishable from packets without the supplement. The folate and vitamin B12 supplement in each cereal packet was 700 µg of free folate and 7 µg of vitamin B12 per 40 g of cereal, which is equivalent to 3.5× the Australian recommended dietary intake (RDI) of these vitamins. The cereal was composed of 63% wheat bran, 23% corn bran, 10% wheat flour and the rest consisting of salt, calcium carbonate, sodium bicarbonate, sucrose, annatto colour and caramel colour. The tablets were provided by Blackmores Ltd (Balgowlah, NSW). The tablets supplied were either placebo tablets or tablets supplemented with free folic acid and vitamin B12. The level of supplementation per tablet was 2000 µg free folic acid and 20 µg vitamin B12, which is equivalent to 10× the Australian RDI for these vitamins. The base ingredients of the tablets consisted of microcrystalline cellulose, calcium phosphate, soy polysaccharide, magnesium stearate and trace amounts of iron oxide. The latter was used to make the placebo and folate tablets indistinguishable by colour. Volunteers were intended to consume one placebo or supplemented tablet per day depending on the group to which they were assigned during the last three months of the intervention trial. Analysis by the Australian Government

Analytical Laboratories using AOAC microbiological methods indicated that (i) 40 g of the supplemented cereal contained 2.7× the RDI of folate and 4.2× the RDI of vitamin B12 and (ii) a supplemented tablet contained 9.0× the RDI of folate and 16.1× the RDI of vitamin B12. Levels of free folic acid and vitamin B12 were undetectable in the placebo cereal and placebo tablets.

Tablet only

The intervention design adopted was a double-blind, randomized, placebo-controlled, prospective trial (Figure 1). One volunteer in each matched pair was randomly assigned to the placebo (control) group and the remaining volunteer was assigned to the folate/vitamin B12 supplement (FOLB12) group. Apart from the cereal and tablets that were provided, volunteers were instructed to keep to their normal diets during the intervention. During the initial 12 weeks of the intervention, volunteers were required to consume one 40 g packet of cereal that either had no supplement or that had been supplemented with folate and B12 depending on the group to which they were assigned. This was followed by another 12 week period during which all volunteers were required to consume one tablet (placebo or supplement depending on the group to which they were assigned) each day. Those volunteers assigned to the placebo cereal in the initial 12 weeks were also given placebo tablets during the last 12 weeks of the intervention. Similarly, those on vitamin-supplemented cereal during the initial 12 weeks received vitamin-supplemented tablets during the second half of the intervention. All packets of cereal and bottles of tablets were coded by a third independent party so that the investigators and volunteers were never aware of the identity of the cereal packets and tablets during the study.

Blood collection and assays

Volunteers donated their blood samples between 8.00 am and 11.00 am, after an overnight fast and before having breakfast, to minimize possible confounding effects by dietary metabolites and diurnal variation. Blood samples (20 ml in heparin and 10 ml in EDTA) were collected immediately before the start of the intervention with cereal [Round 1 (R1)], 12 weeks after the start and immediately before the tablet phase [Round 2 (R2)] and 12 weeks later when the tablet phase ended [Round 3 (R3)]. Blood samples collected in EDTA were immediately refrigerated at 4°C, while the heparin samples were kept at room temperature. Plasma from the EDTA blood sample and whole blood haemolysate (prepared by mixing 100 µl of fresh EDTA blood with a fresh solution of 1 ml 0.4% ascorbic acid solution) were snap frozen in liquid nitrogen and stored at –80°C until analysed. Lymphocytes were isolated from the heparinized blood samples diluted 1:2 with sterile saline using Ficoll Hypaque gradients. The isolated lymphocytes were then washed twice in Hank’s balanced salt solution and resuspended in culture medium before estimating cell concentration using a Coulter counter. All blood samples were processed within 3–4 h of collection. The rest of the lymphocytes were pelleted, snap frozen in liquid nitrogen and stored at –80°C for subsequent DNA isolation and DNA methylation analysis.

Serum B12 and red blood cell folate (haemolysate) were analysed in duplicate using a radiommunoassay kit (Bio-Rad, Quantaphase II) and following the manufacturer’s instructions. Serum for B12 analysis was prepared by removal of fibrin from freshly thawed plasma samples. Haematoctrits of fresh blood samples were also measured in each case to enable the appropriate calculation of red blood cell folate from the results obtained for the haemolysate.
Chromosome damage in peripheral blood lymphocytes was assayed using the original cytokinesis-block micronucleus (CBMN) method for isolated and cultured lymphocytes (14–18). Briefly, isolated lymphocytes were cultured in McCoy’s 5A medium following stimulation by the mitogen phytohaemagglutinin (PHA). Forty-four hours after PHA stimulation, cytochalasin-B (4.5 µg/ml) was added to the cultures to accumulate cells that had completed one nuclear division at the binucleate stage and cells were harvested 28 h later on slides, fixed in methanol and stained using Diff-Quik (Lab-Aids Australia). Two slides were prepared from each culture. The frequency of micronucleated cells (MNCs) was determined in 2000 binucleated cells (BNCs) according to published criteria (18). One-thousand BNCs were scored from one slide by one scorer and another 1000 BNCs were scored from a second slide by the second scorer. The slides for the entire study were scored by the same scorers. The intra-assay coefficient of variation (/CV) for their scores was 32.0 ± 1.4%. To minimize the potential confounding effect of age it was sometimes necessary to adjust MNC frequency using the previously described (11) formula: MNed 25.5y = 25.5 – XS + M, where X = actual age in years, S = slope of the regression line for the relationship between age and MNC frequency, M = actual MNC frequency measured and MNed 25.5y = the MNC frequency adjusted to the value expected at age 25.5 years. Adjustment for the effect of gender on MNC frequency was performed using the ratio of mean MNC frequency in males and females as the correction factor.

Analysis of plasma HIC was performed according to the method of Vester and Rasmussen (19). Briefly, the plasma samples collected in EDTA were snap frozen in liquid nitrogen and stored at –80°C until analysed. They were then thawed, reduced with tri-n-butylphosphine, proteins were precipitated and the sample derivatized with ammonium 7-fluorobenzox-2-oxa-1,3-diazole-4-sulfonate. HIC was detected by HPLC with fluorescence detection. Mercapto-24% greater in females relative to males, (ii) serum vitamin B12 was 36% greater in males relative to females and (iii) red blood cell folate was 21% greater in males relative to females. There was a trend for higher plasma HC in males relative to females but the 9.4% difference did not achieve statistical significance. Analysis of individual data also indicated that 8.7% of females compared with 0% of males were deficient in serum vitamin B12 (<150 pmol/l), and none of the males or females had red blood cell folate levels in the deficient range for anaemia (<136 pmol/l); however, more males (43%) than females (21%) had HC levels above the 10 µmol/l level which is associated with increased cardiovascular disease risk (22). DNA methylation status, cholesterol and vitamin C levels in males and females were not significantly different.

Correlation analysis identified important relationships between MNC frequency and other parameters measured in both males and females (Table II). In both males and females, there was a significant positive correlation between MNC frequency and age (R = 0.51, P = 0.0003 in males, and R = 0.52, P < 0.0001 in females); the slope of the regression line was 0.62 (P < 0.02) for females and 0.27 (P < 0.03) for males. With regard to blood nutrient status, MNC frequency

**Table I.** Base-line data (mean ± 1SE) in males and females

<table>
<thead>
<tr>
<th></th>
<th>Males n = 49</th>
<th>Females n = 57</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25.51 (0.64)</td>
<td>25.61 (0.53)</td>
<td>NS</td>
</tr>
<tr>
<td>MNCs per 1000 BN cells</td>
<td>6.35 (0.39)</td>
<td>7.83 (0.50)</td>
<td>0.017</td>
</tr>
<tr>
<td>Unmethylated Cpg (d.p.m.)</td>
<td>200 (15 270)</td>
<td>186 000 (12 860)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum B12 (pmol/l)</td>
<td>384.1 (14.24)</td>
<td>283.0 (14.53)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Red blood cell folate (pmol/l)</td>
<td>440 (20.46)</td>
<td>363.8 (17.26)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Plasma HC (µmol/l)</td>
<td>9.67 (0.44)</td>
<td>8.84 (0.33)</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/l)</td>
<td>4.68 (0.13)</td>
<td>4.76 (0.13)</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma vitamin C (mg/100 ml)</td>
<td>0.81 (0.04)</td>
<td>0.73 (0.03)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.
was significantly (positively) correlated only with plasma HC ($R = 0.29, P < 0.05$) in males and significantly (negatively) correlated only with serum vitamin B12 in females ($R = -0.36, P < 0.007$). Vitamin C, cholesterol, tea consumption, coffee consumption and smoking status were not significantly related to MNC frequency.

In males, the level of unmethylated CpG was significantly and positively correlated with age ($R = 0.57, P < 0.0001$), MNC frequency ($R = 0.39, P = 0.0088$) and plasma cholesterol ($R = 0.33, P < 0.04$); DNA methylation status was not significantly correlated with folate, vitamin B12 or HC levels in the blood. In females, the level of unmethylated CpG was not significantly correlated with age, MNC frequency, plasma cholesterol or blood levels of folate and vitamin B12, but it was significantly and negatively correlated with plasma HC ($R = -0.34, P < 0.02$).

Interrelationships between plasma HC and other measured nutrition-related parameters were investigated. Plasma HC was negatively correlated with serum vitamin B12 ($R = -0.34, P < 0.03$) and red blood cell folate ($R = -0.27, P < 0.06$) in males. In females, plasma HC was more strongly negatively correlated with red blood cell folate ($R = -0.43, P < 0.001$) than with serum vitamin B12 ($R = -0.17, P > 0.05$). Plasma HC was not significantly correlated with plasma cholesterol, plasma vitamin C, tea or coffee consumption in either sex.

**Intervention data**

Only 69.4% male volunteers and 52.6% female volunteers completed the intervention with at least 80% estimated compliance of intake of cereal and tablets; 10 of the volunteers left the study shortly after round 1. Lack of compliance was mainly due to the reluctance of volunteers to eat the ‘All Bran’ cereal on a daily basis. Only results from subjects who achieved at least 80% compliance for both cereal and tablet intake were used in the analysis as it was the aim of the study to identify, with reasonable confidence, the level of above RDI intake of folate and B12 that would minimize DNA damage. The overall compliance for cereal intake and tablet intake in the volunteers whose data were included in the analysis of the intervention study was >98% and >95%, respectively.

As a consequence of the strict selection it was necessary to adopt group comparisons that were not based on the original matched pair design. Data for males and females were combined to maintain a high level of statistical power. The resulting control and supplement (FOLB12) groups were well matched for age and gender (Table III) which are the major confounding factors for the micronucleus index. The control group consisted of 17 males (mean age 24.35 ± 0.96 years) and 14 females (mean age 27.64 ± 0.98 years) and the FOLB12 group consisted of 17 males (mean age 24.88 ± 1.10 years) and 16 females (mean age 26.13 ± 0.97 years).

The results for DNA damage and biochemical measurements for the two groups are also listed in Table III. In the control group there were no statistically significant changes in MNC frequency, DNA methylation, serum vitamin B12, red blood cell folate and plasma HC at R2 and R3 relative to R1 although there was a trend for lower MNC frequency and plasma HC as control volunteers progressed through the intervention (Table III). In the supplemented (FOLB12) group there was a significant reduction in MNC frequency at R2, a significant increase in serum vitamin B12 status at R3, a highly significant increase in red blood cell folate status at R2 and R3, and a highly significant reduction in HC status at R2 and R3 relative to R1 (Table III). There were no significant changes in DNA methylation, cholesterol (data not shown) and vitamin C (data not shown) status during the course of the intervention in both control and FOLB12 groups. In the FOLB12 group the relative change at R2 and R3 compared with R1 for MNC frequency was –15.3 and –8.5% respectively, for serum vitamin B12 it was +11.2 and +15.5% respectively, for red blood cell folate it was +87.8 and +207.5% respectively, and for plasma HC it was –28.6 and –31.7%, respectively. The results for the control group and the FOLB12 group were also compared directly by testing the significance of their differences in the changes observed between R2 and R1 and between R3 and R2 (Table IV). It is evident from this analysis that significant changes in MNC frequency, serum vitamin B12, red blood cell folate and plasma HC in the FOLB12 group occurred mainly during the first phase of the intervention i.e. at R2 relative to R1. Red blood cell folate was the only parameter in the FOLB12 group that changed (increased) significantly relative to the control group during the second phase of the intervention i.e. at R3 relative to R2.

Because a high micronucleus frequency could indicate a metabolic problem in the methylation pathways of DNA metabolism, we also analysed the intervention data of the FOLB12 group to establish whether supplementation with folic acid and vitamin B12 produced a more significant reduction in the MNC frequency of those subjects in the high 50th percentile of MNC frequency at R1 relative to those in the low 50th percentile of MNC frequency at R1. To take account
of the confounding effect of age and gender, we classified subjects in the low and high 50th percentile groups after adjusting the MNC frequency at R1 for age and gender. The results (Figure 2; Table V) show that there was a significant 25.4% reduction in MNC frequency at R2 in the group with high initial MNC frequency but there was no further reduction in the micronucleus index at R3. There were no significant changes in MNC frequency during the intervention in the group with low initial MNC frequency. To identify which of the biochemical changes had the strongest relationship with reduction in the MNC frequency, we determined the correlation factors for MNC frequency with plasma HC, serum vitamin B12 and red blood cell folate using the R1, R2 and R3 data for the subjects in the high 50th percentile for MNC frequency at R1. This analysis (Figures 3 and 4) confirmed that MNC frequency was most strongly correlated with serum vitamin B12 ($R = -0.489$, $P < 0.0005$), followed by plasma HC ($R = 0.392$, $P < 0.006$) and red blood cell folate ($R = -0.303$, $P < 0.04$). Because folate, B12 and HC are interrelated we also performed multiple regression to determine their independent contributions to MNC frequency. The results of multiple regression indicated that the $\beta$ value for B12 was $-0.432$ ($P < 0.002$), the $\beta$ value for plasma HC was 0.314 ($P < 0.04$) and the $\beta$ value for red blood cell folate was 0.039 and not significant.

We also examined whether the reduction in plasma HC was more significant in those subjects in the high 50th percentile at R1 relative to those in the low 50th percentile at R1. The results shown in Figure 5 and Table VI clearly show significant reduction in plasma HC in both groups although the relative reduction in plasma HC was greater in those with high initial plasma HC levels. Correlation analysis of the data at R1, R2 and R3
Table V. Comparison of differences between R2 and R1 results and R3 and R2 results for MNC frequency of FOLB12 subjects in the low and high 50th percentile of MNC frequency at R1

<table>
<thead>
<tr>
<th></th>
<th>Difference in MNC frequency per 1000 BNCs between R2 and R1</th>
<th>Difference in MNC frequency per 1000 BNCs between R3 and R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOLB12 subjects in the low 50th percentile for MNC frequency at R1</td>
<td>0.11 ± 0.47</td>
<td>0.55 ± 0.51</td>
</tr>
<tr>
<td>FOLB12 subjects in the high 50th percentile for MNC frequency at R1</td>
<td>−2.36 ± 0.46</td>
<td>0.39 ± 0.62</td>
</tr>
<tr>
<td>p value (Mann–Whitney U test)</td>
<td>0.0013</td>
<td>NS</td>
</tr>
</tbody>
</table>

and R3 for the subjects with high initial plasma HC showed that variation in plasma HC was significantly correlated to red blood cell folate \((R = -0.641, P < 0.0001)\), but it was not significantly correlated to serum vitamin B12 \((R = 0.027, P > 0.05)\). Multiple regression analysis for the relationship of plasma HC with red blood cell folate and serum vitamin B12 gave \(\beta\) values of \(-0.561 (P < 0.00005)\), for red blood cell folate, and 0.068 \((P > 0.05)\), for serum vitamin B12.

Discussion

The study described in this paper represents a detailed investigation of the interplay between red blood cell folate status, serum vitamin B12, plasma HC, chromosome damage and DNA methylation in a group of young Australian adults. These results may provide important insights on the potential for folate and vitamin B12 supplements to reduce chromosome damage rates and alter DNA methylation at CpG sites. The results from this study have implications relating to the reduction of cancer risk and possibly ageing if one takes into account the potential role that chromosome damage rate has in these two processes (23,24). The results of this study may also be of relevance to the current debate on the potential consequences of dietary supplementation with a combination of folic acid and vitamin B12 (25). Two of the key issues in this field of research are as follows: (i) what level of folic acid and vitamin B12 in the blood corresponds with a minimization of chromosome damage? and (ii) what is the optimal level of dietary supplementation with folic acid and vitamin B12 that is necessary to minimize genetic damage rate? The results from this study provide some answers to these questions as explained below.

The analysis of base-line data at R1 indicate that, over a span of 14 years between the ages of 18 and 32 years, micronucleus frequency increases significantly with age and that age can explain ~25% of the observed variation in the lymphocyte micronucleus index, the biomarker for chromosome damage used in this study. This observation is in good agreement with our previous studies showing a positive correlation between micronucleus frequency and age (14,16). The higher micronucleus index in females relative to males is in accordance with our previous studies as well as those of others (14,26,27) and it has recently been shown that this difference is mainly accounted for by the loss of the X chromosome (26,27).

Of the blood nutrients or metabolites measured, only vitamin B12 and HC correlated significantly with the micronucleus index which supports our observations in a similar study with the micronucleus index which supports our observations in a similar study with older men (aged 50–70 years) in whom micronucleus frequency was also significantly correlated with plasma HC \((R = 0.415, P = 0.0086)\) and serum vitamin B12 \((R = -0.315, P = 0.0127)\) even though none of the men were folate or vitamin B12 deficient (12). Together, the data from these two investi-
either the intake level for this vitamin in the population studied was adequate to minimize chromosome damage rate or, as has been suggested by other intervention studies with folate (3,4), the current accepted levels of sufficiency in the blood required to prevent anaemia are also optimal for minimising chromosome damage. Our previous studies with older men showed that dietary supplementation for 4 months with up to 10-fold increase in folate only reduced plasma HC by 11% (from a mean value of 9.33 μmol/l to 8.51 μmol/l) and did not reduce micronucleus frequency. These results together with those in this report suggest that folate supplementation on its own may not be as effective as a combined folate and vitamin B12 supplement regime with regard to lowering of plasma HC and chromosome damage rate.

There was an apparent gender difference with regard to correlations between (i) MNC frequency and plasma HC or serum vitamin B12 and (ii) DNA methylation and age or plasma HC. These differences may be coincidental due to the relatively small sample size or may simply be a consequence of the observed gender differences in serum vitamin B12, red blood cell folate and plasma HC (Table 1). The apparent positive correlation between unmethylated CpG and age in males and the lack of such a correlation in females may be interpreted as indicating an earlier onset of age-related changes in DNA methylation status in males relative to females. It is, as yet, not clear whether HC is directly or indirectly genotoxic. However, it is quite plausible that HC may induce micronucleus formation by indirect mechanisms. For example, experimental studies have shown that accumulation of plasma HC could result in an elevation in the cellular concentration of S-adenosyl homocysteine, a strong inhibitor of methyltransferase (28). It has recently been shown that inhibitors of methyltransferase, such as 5-azacytidine, are able to induce chromosome loss and micronucleus formation in human lymphocytes possibly as a result of undercondensation of heterochromatic regions of chromosomes (29).

The results from our study provide evidence of a significant positive correlation between micronucleus index and the level of unmethylated CpG in males. This is in agreement with the hypothesis that undermethylation of DNA could lead to chromosome loss and micronucleus formation (29). The data for males also support previous reports (30–32) indicating that the level of unmethylated CpG increases with age, although our results, to the best of our knowledge, are the first to show such an effect over only a 14 year age range and in a young adult group. In contrast with the data in males, the results for DNA methylation status in females showed no correlation with age or micronucleus index in our study and revealed a significant negative correlation between plasma HC and the level of unmethylated CpG. The latter relationship does not fit well with the hypothesis that increased plasma HC may inhibit, directly or indirectly, methyl transferase activity which might be expected to result in an increase in the level of unmethylated CpG (28).

The main aim of the intervention study was to test the hypothesis that above RDI intake of folic acid and vitamin B12 may have a significant effect on base-line micronucleus frequencies, DNA methylation status and plasma HC. The results from the intervention study suggest that folic acid and vitamin B12 supplements can be efficiently delivered within cereal and tablets, although the incremental changes in serum vitamin B12 were much smaller than those observed for red blood cell folate. Nevertheless, a key feature of the intervention data is the marked and significant decrease in plasma HC in supplemented males and females and the corresponding significant reduction in the micronucleus index. The largest extent of plasma HC reduction observed in this study for males and females was 32%, on average, at R3 which was considerably greater than the 11% reduction observed in our previous study with older men aged 50–70 years on 2 mg/day folic acid supplementation (12) and the 21% reduction reported by Rasmussen et al. (34) for males and females, aged <30 years, on 10 mg/day folic acid for 7–14 days. None of these studies are strictly comparable, but they suggest that a combination of folic acid and vitamin B12 supplementation may be more efficacious in reducing plasma HC than a folate supplement alone.

Although there was a significant decrease in plasma HC following supplementation, there was no corresponding significant change in DNA methylation. This suggests that the decrease in MNC formation that occurred as a result of the folate/B12 supplementation was through a different mechanism to methylation of DNA. There are two alternative plausible explanations: (i) HC itself may be acting directly or indirectly as a genotoxin but its effects are minimized when plasma concentrations are adequately reduced and (ii) folate/B12 supplementation resulted in a significant reduction in mis-incorporation of uracil in DNA; according to recent reports, uracil misincorporation could contribute to the formation of double-strand breakage of DNA and micronucleus formation (3,4). If the latter explanation was correct then the reduction in MNC frequency should have been more strongly related to increases in folate status because uracil misincorporation increases exponentially with folate deficiency (3,4). However, none of the subjects were folate deficient, base-line MNC frequency was not significantly correlated with red blood cell folate, and the results of multiple regression analysis indicated that the reduction in MNC frequency following folate/B12 supplementation was significantly related to increases in serum vitamin B12 and plasma HC but not to increases in red blood cell folate. The possible effect of folate/B12 supplementation on uracil misincorporation could be investigated using recently developed methods for measuring uracil in the DNA of lymphocytes (3,4,35).
It is interesting that reduction in micronucleus frequency in our study mainly occurred in those subjects who had a high chromosome damage rate and correlated significantly with changes in vitamin B12 and plasma HC. These results suggest that (i) an elevated micronucleus index is an important biomarker associated with defects in metabolic pathways requiring folate or vitamin B12 and (ii) a combination of folate and vitamin B12 supplements, rather than supplementation with each vitamin on its own, is more likely to diminish chromosome damage rates in those with above average levels of chromosome damage. It is apparent from the results obtained that MNC frequency is minimized when plasma HC is <7.5 μmol/l and serum vitamin B12 is >300 pmol/l. These studies also confirm that folate and vitamin B12 supplements produce a more marked reduction in plasma HC in those with above average levels of plasma HC. The greatest reduction in the micronucleus index and plasma HC during the intervention occurred after 3.5× the RDI intake of folic acid and vitamin B12, indicating that supplements above this level are probably not required to minimize chromosome damage rate and plasma HC.

The results from our studies suggest that limitations to HC metabolism are associated with elevated chromosome damage rates. Future studies could be targeted to individuals with specific gene mutations in these pathways e.g., mutations in methylene-tetrahydrofolate reductase (MTHFR) and methionine synthase (MS) genes, although the presence of an elevated plasma HC may in itself be a more practical biomarker for identifying most individuals from a wide spectrum of associated metabolic defects. Such defects are linked with elevated risk for neural tube defects and cardiovascular disease (36,37). It has recently been suggested that the thermolabile (677C→T) MTHFR mutation may be linked with reduced risk for cancer as reduced MTHFR activity might divert folate to the synthesis of thymine, thus preventing misincorporation of uracil into DNA (38). If this hypothesis is correct then one might expect that such individuals should exhibit normal chromosome damage rates but abnormally high plasma HC and possibly reduced DNA methylation status. It would therefore be interesting to determine the validity of this hypothesis and establish whether folate and vitamin B12 supplements may have a profound effect on DNA damage rate in individuals with the thermolabile MTHFR mutation.

The results from this study have confirmed that there is a significant positive correlation between plasma HC and the micronucleus index and a significant negative correlation between the micronucleus index and serum vitamin B12. It appears that the micronucleus index in lymphocytes is minimized when plasma HC is <7.5 μmol/l and serum vitamin B12 is >300 pmol/l. Supplementation with above the RDI intake of folic acid and vitamin B12 not only produces large reductions in plasma HC, a risk factor for cardiovascular disease, but also produced significant reductions in the micronucleus index, a biomarker for genomic instability. It is apparent that reductions in the micronucleus index following folic acid and vitamin B12 supplementation are maximal at a level of 3.5× the RDI and that they mainly occur in those individuals with MNC frequency that were initially above average.

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B vitamins and DNA damage in young Australians


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