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Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, β-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

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The aim of this study was to determine the association between dietary intake, determined using a food frequency questionnaire, and genome damage in lymphocytes measured using the micronucleus (MN) assay. The study, performed on 190 healthy individuals (mean age 47.8 years, 46% males), also examined whether a supplementation with β-carotene, vitamins C and E along with zinc (ACEZn), in a randomized trial for 6 months, improves genome stability. Multivariate analysis of baseline data showed that (1) the highest tertile of intake of vitamin E, retinol, folic acid, nicotinic acid (preformed) and calcium is associated with significant reductions in MN frequency, i.e. −28, −31, −33, −46 and −49%, respectively ($P < 0.005$) relative to the lowest tertile of intake and (2) the highest tertile of intake of riboflavin, pantothenic acid and biotin was associated with significant increases in MN frequency, i.e. +36% ($P = 0.054$), +51% ($P = 0.021$), and +65% ($P = 0.001$), respectively, relative to the lowest tertile of intake. Mid-tertile β-carotene intake was associated with an 18% reduction in MN frequency ($P = 0.038$); however, the highest tertile of intake (>6400 μg/day) resulted in an 18% increment in MN frequency. Supplementation with ACEZn significantly reduced the MN index by 13% ($P = 0.038$). The study also showed interactive additive effects such as the protective effect of increased calcium intake (−46%) and the exacerbating effect of riboflavin (+42%) on increased genome damage caused by low folate intake. The results from this study illustrate the strong impact of a wide variety of micronutrients and their interactions on genome health, depending on the level of intake.

Introduction

Numerous studies have shown a clear association between diet and cancer risk; however, the precise genetic and epigenetic mechanisms remain unclear (1–4). It is now evident that above average chromosome damage rates, malsegregation of chromosomes leading to aneuploidy and DNA hypomethylation are important cancer-initiating events, which can be quantified using the micronucleus (MN) assay (2,5–8). Various micronutrients play an important role in DNA metabolism and DNA repair (1,2). Therefore, determining the intake levels of micronutrients required to maintain genome stability is an essential step in the definition of optimal diets for the prevention of cancer and other diseases caused by genome damage (1,2,9). Our previous studies and those of others have shown that certain micronutrients such as folate and vitamin B12 may have a significant impact on the MN index in lymphocytes and that these benefits may depend on the genetic profile of the individual (8,10–12).

The formation of MN in dividing cells is the result of chromosome breakage or chromosome malsegregation, and is associated with the chromosomal instability phenotype and aneuploidy seen in cancer (9,13–16). A high MN frequency may be caused by an excessive uracil incorporation into DNA or hypomethylation of centromeric DNA, which leads to chromosome breakage and loss, respectively (17–19). MN may also be induced by strand breaks or base lesions in DNA owing to oxidative stress and alkylation (5,7,9,20) or defects in the chromosome segregation machinery on account of the inappropriate expression of genes involved in cell cycle check points such as, silencing of hCDC4, which results in an excessive cylin E expression (21), or the lack of essential cofactors such as magnesium and calcium required for kinetochore and spindle assembly and DNA repair (22–24). The sensitivity of the MN index to small variations in micronutrient status within the physiological range makes it an excellent biomarker for identifying dietary factors that are essential for genome stability, and for defining their optimal intake levels (2). The food frequency questionnaire (FFQ) is a potentially useful tool to identify specific micronutrients, macronutrients and food groups that have a strong impact on genome stability. This approach has been successfully used in epidemiological studies on diet and cancer and validation studies have demonstrated that there is a good correlation between the estimated dietary intake of micronutrients measured by the FFQ and the blood levels of micronutrients measured by the standard analytical methods (25–27).

The primary aim of this study was to test the hypothesis that intake of specific micronutrients measured using an FFQ is significantly associated with genome stability, measured using the cytokinesis-block micronucleus (CBMN) assay in peripheral blood lymphocytes. The secondary objective was to determine whether supplementation with a mixture of micronutrients required for tissue antioxidant defense reduces the MN index in lymphocytes in humans. The effect of macronutrients, food groups, beverages and energy intake will be reported separately.

Abbreviations: CBMN, cytokinesis-block micronucleus; FFQ, food frequency questionnaire; MN, micronucleus; MTHFR, methylene tetrahydrofolate reductase; PARP, polyADPribose polymerase.

Materials and methods

Study design

A total of 400 individuals were randomly selected from the electoral register of South Australia and invited by mail to take part in the study which was conducted over a period of 12 months. Those undergoing a medical treatment for major current diseases such as cancer and cardiovascular disease and pregnant women were excluded from the study. One hundred and ninety six individuals volunteered to participate in the study. At the first visit, participants donated a blood sample and trained personnel administered a questionnaire to collect general information about each subject, such as height, weight, smoking habit, occupation, previous diseases and other aspects of lifestyle. Data concerning the usual nutrient intake, including dietary supplements, were assessed by a quantitative FFQ. Participants were required to stop their habitual vitamin supplement intake during the study period. Half the volunteers were randomly selected to take a daily dose, as a tablet, of an antioxidant mixture (ACEZn) supplying 18 mg β-carotene, 900 mg ascorbic acid, 250 mg *d*-α tocopheryl succinate and 12 mg Zn (Beta-ACE, Vitaglow Pty Ltd, Balgowlah, NSW, Australia). The rest of the volunteers were given no supplement and served as controls. The duration of the intervention was 6 months with volunteers providing a second blood sample at the end of the intervention period. A subset of volunteers (12 men and 11 women in the control group; 10 men and 12 women in the ACEZn supplement group) agreed to donate an additional blood sample 3 months into the intervention to assess changes in plasma ascorbic acid, α-tocopherol, β-carotene and zinc. Compliance, determined by counting the number of tablets that were not consumed, was >95%. Of the supplement group, 97 attended the first visit, completed the dietary questionnaire and provided a blood sample and 92 donated a blood sample after completing the intervention period. The corresponding number of participants in the control group were 93 for the first visit and 86 for the final visit 6 months later. Reasons for non-participation were change of residence or illness. The study was approved by the Human Ethics Committee of CSIRO Health Sciences and Nutrition.

Dietary intake measurements

Dietary intake was assessed using a self-administered FFQ based on the original questionnaire described by Baghurst and Record (28). This form of the FFQ is regularly updated to take note of the changing trends in dietary habits and has been used extensively with Australian population samples and national dietary surveys (in 1988, 1993 and 1998). It has been shown to have a high repeatability and consistency with other dietary intake measurement techniques and has demonstrated good reliability when assessed against urinary and protein measures (28–31). The FFQ takes the form of a 20-page booklet including a list of >180 common food and beverage items and questions relating to food preparation and dietary habits. Participants were required to indicate how often each food and beverage was usually consumed per month, week or day. Average daily consumption was based on participants' reports of how often a specified serving size of each food or beverage item was consumed. This information, along with the nutrient composition of the food item/unit weight taken from Australian and British food tables (32,33), allowed participants' daily micronutrient and macronutrient intake to be calculated using the FREQUAN dietary analysis program (28).

Cytokinesis-block micronucleus assay in lymphocytes

Volunteers donated their blood samples between 8.00 and 11.00 am, after an overnight fast and before having breakfast to minimize possible confounding effects by dietary metabolites and diurnal variation. Lymphocytes were isolated from the heparinized blood samples diluted 1:2 with sterile saline using Ficoll Hypaque (Pharmacia) gradients. The isolated lymphocytes were then washed twice in Hanks balanced salt solution and re-suspended in culture medium before estimating the cell concentration using a Coulter counter. The blood samples were processed within 3–4 h of collection. Chromosome damage in peripheral blood lymphocytes was assayed using the original CBMN method for isolated and cultured lymphocytes (34). Briefly, isolated lymphocytes were cultured in McCoy's 5A medium (Sigma) following a stimulation by the mitogen phytohaemagglutinin (PHA). Forty-four hours after PHA stimulation, cytochalasin-B (4.5 mg/ml, Sigma) 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 MN was determined in 2000 binucleated (BN) cells. Scoring criteria for selection of BN cells and MN were as described previously (34,35). Slides were coded with a random number and scored by the same person who was unable to distinguish slides from participants assigned to the control or ACEZn supplement group.

Micronutrient analysis

Plasma α-tocopherol and β-carotene concentrations were measured by HPLC using the method of Yang and Lee (36). Plasma zinc concentration was measured by flame atomic-absorption spectrophotometry using a Perkin Elmer 5000 atomic absorption spectrophotomer (Perkin-Elmer Ltd, Melbourne, Australia) (37). Plasma ascorbate was determined by the ferrozine method (38).

Statistical analysis

A complete univariate analysis was performed on the whole dataset. The intra-individual concordance between MN frequency at the beginning and at the end of the study was evaluated through a linear correlation analysis. The estimated intake of micronutrients was categorized according to tertiles of the observed distribution, and for each item, a univariate comparison between tertiles was carried out.

The role of the dietary intake of specific micronutrients on MN frequency was evaluated through a multivariate regression analysis. The analysis was based on data reported by the dietary questionnaire administered at the beginning of the study. The negative binomial distribution was applied since this approach is particularly efficient with count data, and has the property to take into account the effect of over-dispersion, i.e. a phenomenon frequently arising with count data that increases unexplained variance and yields biased standard errors of the parameter estimates (39). An *ad hoc* multivariate model was performed for each micronutrient. A stepwise procedure beginning with the full model, i.e. a model that includes all available variables (age, gender, BMI, smoking habit, occupation and dietary variables including energy intake), was fitted to data, removing/inserting at each step the variable(s) that are less/more correlated with MN frequency. To control for the inflation of estimated regression coefficient variances owing to the presence of multicollinearity, we evaluated the marginal contribution of each factor to the variance explained by the model taking into account the correlation with other predictors. Covariates selected in this way were then added/removed in new models until stable estimates were obtained. Covariates inflating standard errors of the effect of nutrients were filtered out.

The effect of each dietary item on genome stability is described as the percent variation of the mean MN frequency in the 2nd and 3rd tertiles compared with the 1st tertile of micronutrients frequency distribution. A 95% confidence interval is provided along with data point estimates of the effect. Standard statistical procedures for model checking such as residuals analysis, Cook influence statistic and leverages were applied.

The same statistical model used to quantify the effect of micronutrients was used to evaluate the presence of a protective effect of ACEZn supplementation. MN frequency in the group supplemented with ACEZn antioxidants was compared with the non-supplemented control group, by fitting the negative binomial regression model to MN frequency measured at the end of the study, adjusting for potential confounders and for the baseline MN frequency measured at the beginning of the study. Given the observational setting of the study, no specific correction procedure was applied to take into account the issue of multiple comparison. Study results were corroborated by evaluating the biological plausibility and internal consistency of results (dose–response, consistency of estimates in different models, etc.), and by ranking single nutrients by the size of their impact on MN frequency.

Univariate analysis was carried out with SPSS for Windows statistical software (40), while negative binomial regression models were fitted to the data using STATA statistical software (41).

Results

The demographic, dietary and genome damage rate characteristics of subjects in the study before the ACEZn intervention and the genome damage rate at the end of the ACEZn intervention are listed in Table I. There were no significant baseline differences between the control and treatment group with respect to age, BMI, MN frequency as well as energy, micronutrient and food group intake.

The relationship between MN frequency and individual characteristics other than dietary intake was analysed first (Table II). The results of this analysis show a significantly lower MN frequency in males relative to females, by a factor of 40% ($P < 0.001$) and a significant increment in MN frequency with each decade of age ($P < 0.004$). MN frequency increased progressively with age to a maximum of a 359% increment in those older than 60 years, relative to those aged

Table I. Description of the main characteristics of the study subjects and of dietary habit (from the food frequency dietary questionnaire)

	Assignment to ACEZn supplementation						All subjects		
	No			Yes					
	N	Mean	SD	*N*	Mean	SD	*N*	Mean	SD
MN frequency at the start of the study (‰)	97	18.65	21.18	93	18.68	11.37	190	18.67	17.06
MN frequency at the end of the study (‰)	92	16.03	16.05	86	13.95	9.48	178	15.02	13.29
Age (years)	97	47.94	16.51	93	47.57	14.03	190	47.76	15.31
BMI (kg/m^2)	96	23.96	3.49	92	24.68	3.82	188	24.31	3.66
Energy (kJ)	96	8816.31	2405.33	93	8495.08	1977.15	189	8658.25	2205.10
Cereals (g/day)	97	171.83	92.64	93	171.83	66.42	190	171.83	80.66
Meat (g/day)	97	86.43	38.82	93	92.83	39.69	190	89.56	39.28
Vegetables (g/day)	97	322.14	136.08	93	341.20	146.00	190	331.47	140.97
Dairy (g/day)	97	426.32	247.67	93	415.62	213.03	190	421.08	230.82
Confectionery (g/day)	97	65.12	48.31	93	54.49	43.10	190	59.92	46.02
Fruits (g/day)	97	374.15	268.76	93	363.42	222.14	190	368.90	246.45
Alcohol (g/day)	96	7.59	13.56	93	7.66	14.83	189	7.63	14.16
Vitamin B6 (mg/day)	96	1.74	0.51	93	1.68	0.48	189	1.71	0.49
Vitamin B12 (μg/day)	96	4.53	3.22	93	4.15	2.55	189	4.34	2.91
Vitamin C (mg/day)	96	145.63	80.04	93	137.53	62.18	189	141.64	71.74
Vitamin D (μg/day)	96	2.25	1.49	93	2.33	1.10	189	2.29	1.31
Vitamin E (mg/day)	96	9.42	4.66	93	10.33	3.74	189	9.87	4.25
Calcium (mg/day)	96	1096.85	382.19	93	1098.50	365.61	189	1097.67	373.13
Retinol (μg/day)	96	549.58	506.01	93	473.26	454.62	189	512.03	481.66
β-carotene (μg/day)	96	5518.64	2909.96	93	5871.27	3020.59	189	5692.15	2962.29
Riboflavin (mg/day)	96	2.20	0.69	93	2.19	0.66	189	2.19	0.67
Nicotinic acid pre-formed (mg/day)	96	23.44	6.86	93	23.46	7.10	189	23.45	6.96
Folate (μg/day)	96	240.94	73.74	93	241.66	77.80	189	241.30	75.56
Pantothenic acid (mg/day)	96	5.27	1.45	93	5.23	1.40	189	5.25	1.42
Biotin (μg/day)	96	23.34	8.06	93	22.13	7.15	189	22.74	7.63
Males (%)	41	42.3		46	49.5		87	45.8	
Affected by tumor (%)	4	4.3		4	4.4		8	4.3	
Current smokers (%)	14	15.4		13	15.3		27	15.3	

N, number of subjects; SD, standard deviation.

Table II. Effect of the individual characteristics of the study subjects on the baseline MN frequency

	Subjects	Percent variation of MN frequency	95% CI	*P*
Gender				
Females	103	0	—	—
Males	87	−40	(−51/−26)	<0.001
Age				
≤29	24	0	—	—
30–39	32	79	(22/164)	0.003
40–49	49	166	(87/279)	<0.001
50–59	44	250	(142/407)	<0.001
≥60	41	359	(217/564)	<0.001
Smoking habit				
Non-smokers	149	0	—	—
Current smokers	27	11	(−17/47)	0.480
Occupation				
Unemployed	33	0	—	—
Office workers	92	9	(−17/43)	0.524
All other occupations	59	31	(−3/75)	0.075
BMI (kg/m^2)				
≤22.68	63	0	—	—
22.69–25.56	63	11	(−11/38)	0.352
≥25.57	62	7	(−14/35)	0.535

between 20 and 29 years. MN frequency was not significantly related to smoking habit, occupation or BMI.

Analysis of the relationship between micronutrient intake and base line MN frequency identified nine micronutrients (including one mineral) that are significantly related to genome damage rate (Table III). Increased intake of vitamin E, retinol, folate, preformed nicotinic acid and calcium was associated with a dose-related reduction in MN frequency with significant reductions (−28, −31, −33, −46 and −49% respectively, $P < 0.01$) at the highest tertile of intake. β-Carotene intake at the mid-tertile intake level was associated with an 18% reduction in MN frequency ($P = 0.036$) but this effect was not sustained at the highest intake level at which MN frequency appeared to be increased by 18%, relative to the lowest tertile of intake rather than decreasing even further.

In contrast to the protective micronutrients listed above, increasing intake of riboflavin and pantothenic acid from the lowest tertile to the middle tertile resulted in large increments in MN frequency (41%, $P = 0.005$ and 69%, $P < 0.001$, respectively) that were sustained, but to a lesser degree, at the highest tertile of intake. Biotin was the other micronutrient exhibiting a deleterious effect on genome damage rate in response to an increased intake with a 65% increase in MN frequency ($P = 0.001$) at the highest tertile of consumption but with no effect at the mid-tertile.

Table III. Effect of intake level of specific micronutrients on baseline MN frequency

	Tertiles of intake	Subjects	Percent variation of MN frequency[a]	95% CI	P^a
Calcium (mg/day)	≤927.50	63	0	–	–
	927.51–1249.55	63	−18	(−36/5)	0.121
	≥1249.56	63	−49	(−63/−30)	<0.001
Nicotinic acid (pre-formed) (mg/day)	≤20.04	63	0	–	–
	20.05–25.72	63	−26	(−40/−9)	0.004
	≥25.73	63	−46	(−58/−30)	0.001
Folate (μg/day)	≤206.64	63	0	–	–
	206.65–256.49	63	−16	(−32/3)	0.094
	≥256.50	63	−33	(−49/−13)	0.003
Retinol (μg/day)	≤296.37	63	0	–	–
	296.38–457.47	63	−10	(−24/7)	0.233
	≥457.48	63	−31	(−43/−16)	0.001
Vitamin E (mg/day)	≤7.87	63	0	–	–
	7.88–10.71	64	−15	(−28/1)	0.066
	≥10.72	62	−28	(−42/−11)	0.003
β-Carotene (μg/day)	≤4161.32	63	0	–	–
	4161.33–6433.12	63	−18	(−32/−1)	0.036
	≥6433.13	63	18	(−6/48)	0.148
Riboflavin (mg/day)	≤1.84	63	0	–	–
	1.85–2.41	64	41	(11/78)	0.005
	≥2.42	62	36	(−1/85)	0.054
Pantothenic acid (mg/day)	≤4.59	63	0	–	–
	4.60–5.64	64	69	(34/115)	<0.001
	≥5.65	62	51	(6/114)	0.021
Biotin (μg/day)	≤18.86	63	0	–	–
	18.87–25.49	63	7	(−14/33)	0.542
	≥25.50	63	65	(22/123)	0.001

[a]Percent variation and *P*-values refer to comparison with lowest tertile of intake.

We also examined the combined effects of calcium and folate consumption, since extensive epidemiological evidence showed that these dietary factors tend to interact in modifying the risk of cancer (3,22,42,43). Our analysis of combined effects (Figure 1a) shows that a high calcium intake protects against the genome damaging effect of low folate intake and vice versa and that the combination of the highest tertile of intake of calcium and folate results in a 60% lower genome damage rate, relative to a combined lowest tertile intake of these micronutrients. Since our *in vitro* studies suggested that the genome-damaging effects of low folate concentration in the physiological range could be exacerbated by a high riboflavin concentration (8) we also examined the interactive effect of folate and riboflavin intake (Figure 1b). It is clear from this analysis that increased riboflavin intake has a dose-related detrimental effect on genome stability that becomes stronger as folate intake decreases, so that at the lowest tertile of folate intake, there is a 42% increment in genome damage in the highest tertile of riboflavin intake, relative to the lowest tertile of riboflavin intake, but the corresponding effect of increased riboflavin intake in the highest tertile of folate intake is only 30%. Furthermore, there is a 69% reduction in genome damage for the high tertile folate/low tertile riboflavin intake combination, relative to the reverse low tertile folate/high tertile riboflavin intake combination. Both the folate–calcium and folate–riboflavin combination effects tended to be additive rather than multiplicative and they were dose-related.

The efficacy of the ACEZn intervention was tested by measuring changes in the plasma ascorbic acid, α-tocopherol, β-carotene and zinc in a subset of the control group ($n = 23$) and the supplemented group ($n = 22$) after 3 months of antioxidant supplementation (Table IV). There was no change in the control group. In contrast, the plasma ascorbic acid, α-tocopherol and β-carotene in the supplemented group increased by 27% ($P < 0.05$), 55% ($P < 0.01$) and 500% ($P < 0.001$), respectively but there was no change in plasma zinc. With respect to genome damage rate, there was a non-significant 14% decrease in MN frequency in the control group and a larger significant 25% decrease in MN frequency ($P < 0.05$) in the supplemented group at the end of the study relative to baseline values (Table I). The final net effect of treatment, obtained by comparing the supplemented group and control group at the end of treatment, after adjusting for individual characteristics, dietary habit and baseline values of MN frequency in each group, was a 13% significant reduction in MN frequency (95% CI = −1%, −24%, $P = 0.038$). The internal consistency of the MN frequency measurement is reflected in the significant correlation between measurements taken before and after the intervention in both the control and supplemented groups, which was $R = 0.75$ ($P < 0.001$) and $R = 0.77$ ($P < 0.001$), respectively (Figure 2).

Discussion

The results of this study are relevant to the understanding of genome damage in humans and how diet affects this fundamental cause of human disease in four ways. First, they

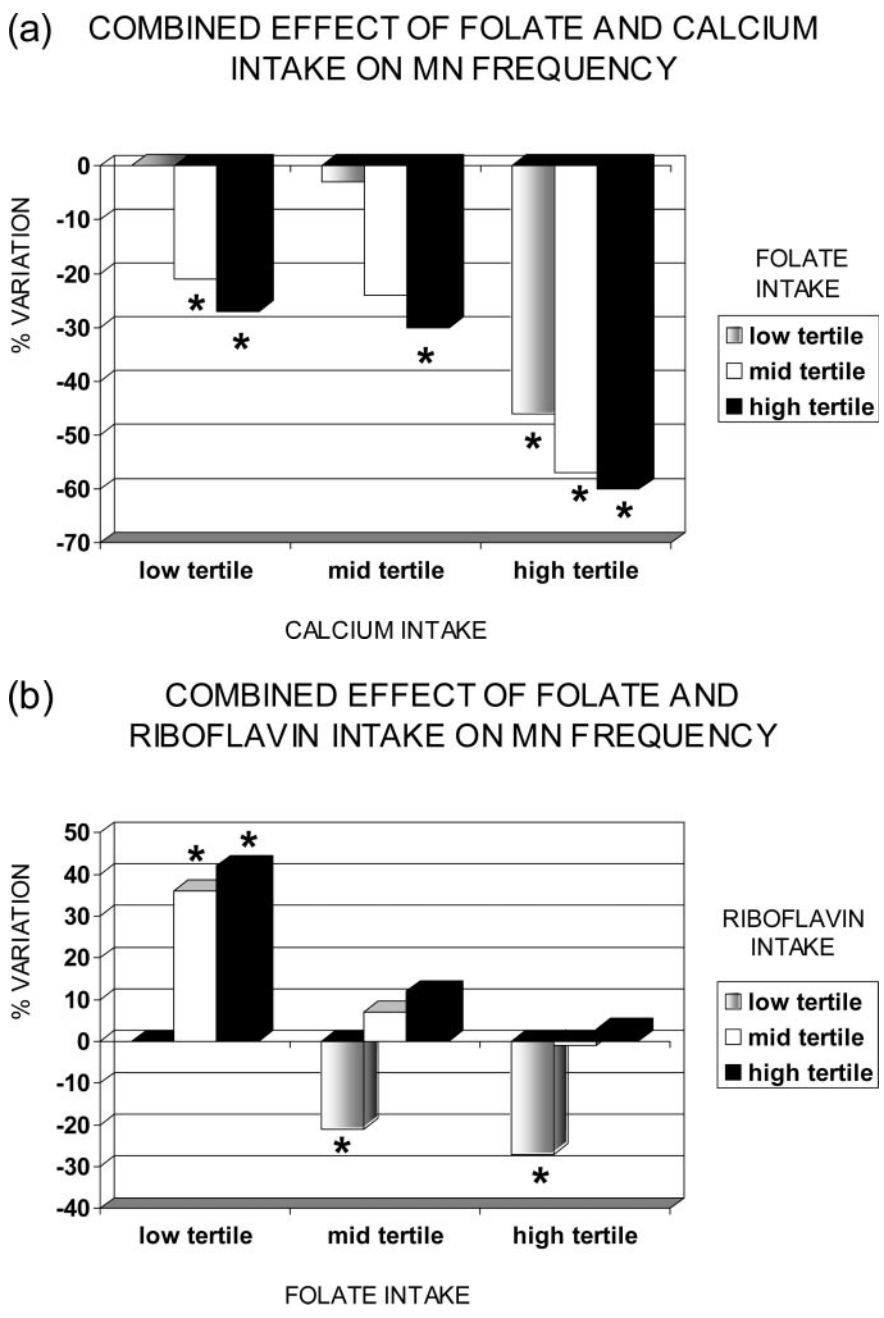

Fig. 1. The combined effect of (**a**) calcium and folate intake and (**b**) riboflavin and folate intake on MN frequency. Results shown are the % variation relative to the combined lowest tertiles of intake in the pair of nutrients examined. $^{*}P < 0.05$ for comparison with the referent value for the combined lowest tertile of intake for the pair of nutrients examined.

confirm the effect of age on MN frequency and therefore the capacity of the CBMN assay to measure in a sensitive manner, the deterioration of chromosomal integrity with age, which is probably due to either (1) increased DNA mis-repair events leading to the formation of acentric chromosome fragments and/or (2) increasing inefficiency in the chromosome segregation machinery (6,9,35,44). The latter may occur owing to defects in cell cycle checkpoints and/or hypomethylation of centromeric satellite DNA leading to an aberrant mitotic spindle formation or defective engagement of chromosomes with the spindle, respectively and subsequent chromosome loss at anaphase and MN formation (2,21,45,46).

Second, they show for the first time significant associations between dietary intake data from a detailed FFQ and genome damage rate measured with the CBMN assay. This observation is consistent with the conclusion of several epidemiological and controlled intervention studies showing that the dietary status has a strong impact on cancer risk and that genome damage rate is, to a large degree, caused by dietary imbalance and micronutrient excess or deficiency (1,2,3,9). Dietary questionnaire data may suffer from inaccuracies of volunteer recall, although significant correlations with blood micronutrient levels are generally reported (27). On the other hand, FFQs can be easily used in population studies, and can provide comprehensive information about food groups, individual foods and micronutrients, making this tool very efficient for studying associations with health outcomes. Micronutrient analysis of blood, although desirable, would be prohibitively

expensive for a preliminary study, although it can be used judiciously as we have done with respect to the ACEZn supplementation study, in which we verified compliance by measuring changes in blood concentration of the supplemented micronutrients in a subset of participants.

Third, this study has identified five micronutrients that appear to be protective against genome instability namely, retinol, vitamin E, folate, pre-formed nicotinic acid and calcium. The results concerning folate are consistent with (1) studies showing that folate deficiency leads to hypomethylation of DNA and excessive incorporation of uracil into DNA, which are two of the known underlying molecular events that cause MN formation (2,18,19,22,47,48) and (2) the observation from controlled intervention studies that folate intakes >200 μg/day are required for chromosomal stability (10,17). To our knowledge, there are no previous data showing an association between dietary calcium and chromosomal instability. However, calcium plays an important role in chromosome segregation (24,49), it restrains cell proliferation, and induces apoptosis and cell differentiation, which may explain, in part, why reduced calcium intake is associated with an increased risk of colorectal cancer (22,42,50). The relevance of the use of the MN index as a biomarker of genome damage and chromosomal instability relevant to cancer (2,9) is also underscored by the observations on the combined effects of calcium and folate intake, which are consistent with epidemiological evidence, showing that the risk for colorectal cancer is further increased when both folate and calcium intake are low (3,22,42,43). The protective effect of preformed nicotinic acid is consistent with its role as a precursor of NAD, the cofactor for polyADPribose polymerase (PARP) function and SIR2 histone deacetylase (51,52). PARP is required for the polyADPribosylation of damaged DNA and for DNA repair, in the maintenance of telomeres, in chromosome segregation, and caspase-dependent apoptosis (53), and SIR2 deacetylation of histones results in more compact chromatin that may be less susceptible to DNA breaks induced by reactive oxygen species (51,52).

In a previous cross-sectional study, we did not observe a significant correlation between plasma vitamin E and MN frequency (54); however, a placebo-controlled intervention study showed that above the RDA intake of vitamin E as well as its carrier soyabean oil reduced the MN index by 32%, suggesting that the protective effect of vitamin E may be associated with lipids that afford protection against genome damage (55). Although some studies suggest that vitamin E prevents DNA mutation by reactive oxygen species, other studies report no effects (56). The observation that genome damage was increased at both high and low tertile intake levels of β-carotene relative to mid-tertile intake is consistent with recent data showing a U-shaped dose–response for dietary

Table IV. Plasma micronutrient concentration (μmol/l) before and after ACEZn supplementation for 3 months in a subset of the study participants

Treatment group and sampling time	Ascorbic acid	α-Tocopherol	β-Carotene	Zinc
Control group				
$N = 23$				
Before intervention	67 (20)	31 (9)	0.3 (0.3)	15.9 (2.6)
After 3 months	66 (21)	29 (8)	0.3 (0.2)	14.5 (2.0)
Supplemented group				
$N = 22$				
Before intervention	66 (22)	29 (8)	0.4 (0.2)	15.8 (2.1)
After 3 months	84 (23)[a]	45 (10)[b]	2.0 (1.0)[c]	15.0 (2.8)

Data shown are the mean values with SD in parentheses.
[a]Significantly different from other values in the same column, $P < 0.05$.
[b]Significantly different from other values in the same column, $P < 0.01$.
[c]Significantly different from other values in the same column, $P < 0.001$.

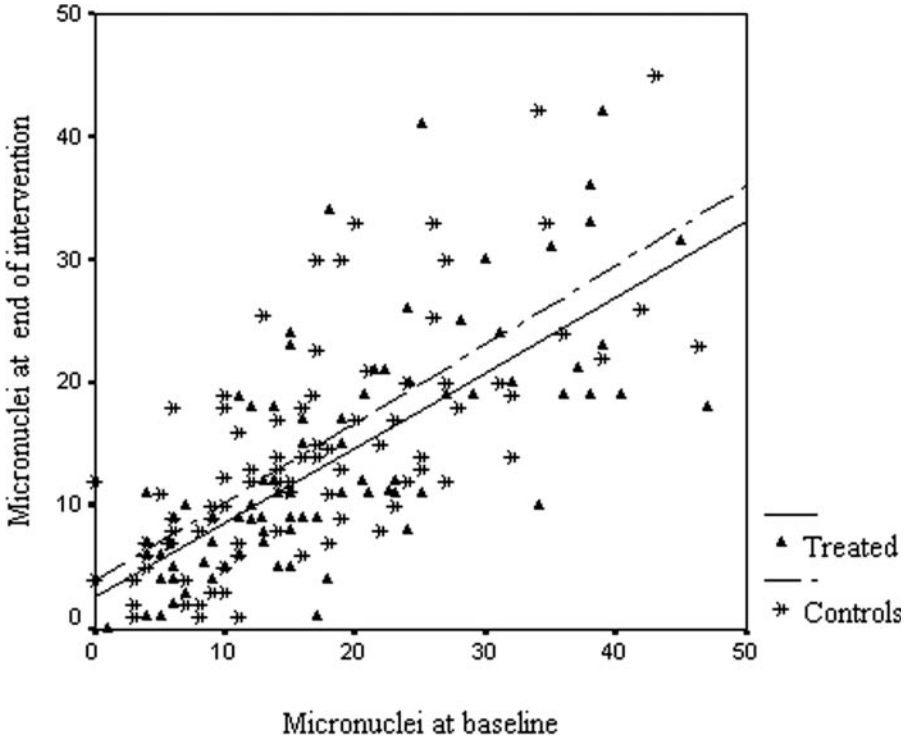

Fig. 2. Correlation of MN frequency (per 1000 BN cells) before and after supplementation in the control group ($R = 0.752$, $P < 0.001$) and the treatment group ($R = 0.769$; $P < 0.001$) illustrating the reproducibility of the assay and the effect of the ACEZn supplement.

carotenoid intake and HPRT mutant frequency in lymphocytes (57), as well as the observation that the intake of β-carotene supplements increased the risk of lung cancer (58). In contrast, we found that there was a steady increasing reduction in MN frequency with increasing retinol intake. Retinol is structurally different from β-carotene, although it can be derived from it and, unlike the latter, it is not a singlet oxygen quencher (59). Although β-carotene can be converted to retinol in the body, free radical attack on β-carotene results in the formation of genotoxic breakdown products that induce MN (60). The protective effect of increased retinol may be due to its one-step conversion to retinoic acid, which has been shown to suppress the inappropriate expression of NFκβ, which leads to inflammation, increased cell proliferation and inhibition of apoptosis (61), all of which may lead to an increased MN expression in lymphocytes (20).

Fourth, we have found significant associations between increased riboflavin, pantothenic acid and biotin concentrations with increased genome instability. We have recently shown, using an *in vitro* model, that elevated riboflavin concentration increased genome instability at a physiological folate concentration (10 nmol/l) observed in moderate folate deficiency (8). Our interpretation of these results was that a high riboflavin concentration could increase the activity of the enzyme methylene tetrahydrofolate reductase (MTHFR), because riboflavin is the precursor of the cofactor FAD for this enzyme, making folate less available for the synthesis of dTMP from dUMP and thus, increasing the risk of uracil incorporation into DNA and the resulting increase in chromosome breakage, breakage–fusion–bridge cycles leading to gene amplification, nuclear budding and MN formation (2,9). The C677T mutation of MTHFR, which reduces its activity, is associated with a reduced risk of colorectal, breast and haematopoietic cancers in a large proportion of studies that investigated this association (43,62,63). These results suggest that increasing MTHFR activity under conditions of suboptimal folate status could be carcinogenic. The observations for the combined effect of riboflavin and folate intake in our *in vivo* cross-sectional study are consistent with these predictions. On the other hand, it is also to be noted that the deficiency of riboflavin is associated with an increased risk for developmental defects, anaemia, neurodegeneration and cervical dysplasia (64). Biotin serves as an essential cofactor for four carboxylases in intermediary metabolism (65–67). Emerging evidence suggests that marginal biotin deficiency, a common feature of both early and later pregnancy, may be teratogenic (65–68). It has been recently shown that intake of a single tablet of 600 μg biotin produced a significant 33% or greater reduction in mitogen-induced proliferation and cytokine-response of lymphocytes *ex vivo* (69). These biomarkers are indicative of a weakened immune response and suggest that this level of intake (i.e. 600 μg) may be close to the tolerable upper intake level, even though this has not been defined yet. Since egg yolk is one of the richest sources of biotin, and because avidin in egg white can cause biotin deficiency, it is uncertain whether the observed association of MN frequency with a higher biotin intake may also reflect the effect of increased avidin intake (65–68). Excessive biotin levels have been shown to induce MN in plant cells (70). Pantothenic acid plays an essential role in the synthesis of fatty acids and membrane phospholipids and the synthesis of amino acids such as leucine, arginine and methionine (71). We could not find any published studies on the effects of pantothenic acid on genome stability or toxicity except for one report, which suggested that calcium pantothenate exhibited a marginal promoting effect in the Balb/c-3T3 assay (72). It is notable that eggs are one of the richest sources of biotin, riboflavin and pantothenic acid (33) and that epidemiological studies conducted in the same city where the cohort of our study resided (Adelaide, South Australia), using a similar dietary questionnaire, have shown an increased risk for colon and pancreatic cancer with increased egg intake (73–75).

The study has also shown that ACEZn supplementation does cause a modest reduction in genome damage rate and is consistent with studies suggesting that supplementation with these micronutrients may afford some protection against the DNA damaging effects of oxidants (1,56,76,77). On account of the combination of micronutrients in the supplement, it is not possible to tell whether there were additive/synergistic effects or whether there were counteracting effects. Data from the results of the FFQ showed that vitamin E at the highest tertile of intake was associated with a 28% reduction in MN frequency. However, β-carotene intake >6.4 mg/day was associated with an 18% increase in MN frequency. Given that the ACEZn supplement contained 18 mg β-carotene, it is possible that any beneficial effect from the increased vitamin E intake may have been offset by excess β-carotene, which could explain the relatively low impact (−13%) of the supplement on MN frequency. These concerns highlight the fact that we need to have a better understanding of the dose–response effects of micronutrients (on their own or in combination) on genome stability before formulating vitamin supplement tablets, if significant genome health benefits are to be reliably achieved in individuals and the general population. The lack of a placebo tablet treatment in the control group is a weakness in the design of the study because we cannot exclude the possibility that base ingredients that are commonly used in vitamin tablets, such as calcium phosphate and magnesium stearate, may have also contributed to the observed MN frequency reduction in the treatment group.

In interpreting the data from this study, it is important to note that micronutrients usually exhibit metabolic dose–response effects in which both deficiency and excess can be deleterious (57,58,78–81) and it is probable that in a specific mixed diet, depending on the intake level of an individual, some of the micronutrients may be outside the intake range that is optimal for the prevention of genome instability. The results for β-carotene suggest an optimum between 4000 and 6000 μg per day with a tendency for a marked increase in genome damage at higher or lower intake levels, which is consistent with data suggesting an increased cancer risk with deficiency or supplementation above RDA levels for this vitamin (57,58,76). On the other hand, the apparent genome damage prevention effects associated with vitamin E, retinol, folic acid, preformed nicotinic acid and calcium still increases at the highest tertile of intake, suggesting that an optimum could be achieved at even higher levels of intake or that the maximum beneficial effect is achieved at these levels of intake. For example, the highest tertile of intake for folate was >256 μg/day, which is consistent with a number of studies showing that developmental defects and cancer as well as biomarkers for cardiovascular disease risk, such as homocysteine, are minimized at folate intake levels of 400 μg/day or greater (17,43,82–84). The fact that both vitamin deficiency and excess can increase carcinogenesis is supported by several studies (1,57,78,85) and highlights the acute need for better knowledge of

dose–response relationships between micronutrient intake and genome health.

In conclusion, the results of this study have identified important novel associations between certain micronutrients and genome stability and have generated a series of hypotheses that need to be tested systematically by randomized controlled interventions. These interventions should include dose–responses with a complementary set of genome damage markers so that optimal intake levels for genome health maintenance can be reliably determined.

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References

1. Ames,B.N. and Wakimoto,P. (2002) Are vitamin and mineral deficiencies a major cancer risk? *Nat. Rev. Cancer*, **2**, 694–704.
2. Fenech,M. (2003) Nutritional treatment of genome instability: a paradigm shift in disease prevention and in the setting of recommended dietary allowances. *Nutr. Res. Rev.*, **16**, 109–122.
3. Willett,W.C. (2001) Diet and cancer: one view at the start of the millennium. *Cancer Epidemiol. Biomarkers Prev.*, **10**, 3–8.
4. Bingham,S. and Riboli,E. (2004) Diet and cancer—the European prospective investigation into cancer and nutrition. *Nat. Rev. Cancer*, **4**, 206–215.
5. Tucker,J.D., Eastmond,D.A. and Littlefield,L.G. (1997) Cytogenetic endpoints as biological dosimeters and predictors of risk in epidemiological studies. In Toniolo,P., Boffetta,P., Shuker,D.E.G., Rothman,N., Hulka,B. and Pearce,N. (eds). *Application of Biomarkers in Cancer Epidemiology*. IARC Scientific Publications No. 142, Lyon, France, pp. 185–200.
6. Bonassi,S., Znaor,A., Norppa,H. and Hagmar,L. (2004) Chromosomal aberrations and risk of cancer in humans: an epidemiological perspective. *Cytogenet. Genome Res.*, **104**, 376–382.
7. Fenech,M., Holland,N., Chang,W.P., Zeiger,E. and Bonassi,S. (1999) The HUman MicroNucleus project—An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat. Res.*, **428**, 271–283.
8. Kimura,M., Umegaki,K., Higuchi,M., Thomas,P. and Fenech,M. (2004) MTHFR C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. *J. Nutr.*, **134**, 48–56.
9. Fenech,M. (2002) Chromosomal biomarkers of genomic instability relevant to cancer. *Drug Discov. Today*, **7**, 1128–1137.
10. Fenech,M., Aitken,C. and Rinaldi,J. (1998) Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis*, **19**, 1163–1171.
11. Zijno,A., Andreoli,C., Leopardi,P., Marcon,F., Rossi,S., Caiola,S., Verdina,A., Galati,R., Cafolla,A. and Crebelli,R. (2003) Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects. *Carcinogenesis*, **24**, 1097–1103.
12. Botto,N., Andreassi,M.G., Manfredi,S., Masetti,S., Cocci,F., Colombo,M.G., Storti,S., Rizza,A. and Biagini,A. (2003) Genetic polymorphisms in folate and homocysteine metabolism as risk factors for DNA damage. *Eur. J. Hum. Genet.*, **11**, 671–678.
13. Nowak,M.A., Komarova,N.L., Sengupta,A., Jallepalli,P.V., Shih,IeM., Vogelstein,B. and Lengauer,C. (2002) The role of chromosomal instability in tumor initiation. *Proc. Natl Acad. Sci. USA*, **99**, 16226–16231.
14. Duesberg,P., Li,R. and Rasnick,D. (2004) Aneuploidy approaching a perfect score in predicting and preventing cancer: highlights from a conference held in Oakland in January 2004. *Cell Cycle*, **3**, 823–828.
15. Wang,X., Thomas,P., Xue,J. and Fenech,M. (2004) Folate deficiency induces aneuploidy in human lymphocytes *in vitro*-evidence using cytokinesis-blocked cells and probes specific for chromosomes 17 and 21. *Mutat. Res.*, **551**, 167–180.
16. Gisselsson,D., Bjork,J., Hoglund,M., Mertens,F., Dal Cin,P., Akerman,M. and Mandahl,N. (2001) Abnormal nuclear shape in solid tumours reflects mitotic instability. *Am. J. Pathol.*, **158**, 199–206.
17. Fenech,M. (2001) The role of folic acid and vitamin B12 in genomic stability of human cells. *Mutat. Res.*, **475**, 56–67.
18. Crott,J.W., Mashiyama,S.T., Ames,B.N. and Fenech,M. (2001) MTHFR C677T polymorphism does not alter folic acid deficiency-induced uracil incorporation into primary lymphocyte DNA *in vitro*. *Carcinogenesis*, **22**, 1019–1025.
19. Mashiyama,S.T., Coutemanche,C., Elson-Schwab,I., Crott,J., Lee,B.L., Ong,C.N., Fenech,M. and Ames,B.N. (2004) Uracil in DNA, determined by an improved assay, is increased when deoxynucleotides are added to folate-deficient cultured human lymphocytes. *Anal. Biochem.*, **330**, 58–69.
20. Umegaki,K. and Fenech,M. (2000) Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. *Mutagenesis*, **15**, 261–269.
21. Rajagopalan,H., Jallepalli,P.V., Rago,C., Velculescu,V.E., Kinzler,K.W., Vogelstein,B. and Lengauer,C. (2004) Inactivation of hCDC4 can cause chromosomal instability. *Nature*, **428**, 77–81.
22. Lamprecht,S.A. and Lipkin,M. (2003) Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nat. Rev. Cancer*, **3**, 601–614.
23. Hartwig,A. (2001) Role of magnesium in genomic stability. *Mutat. Res.*, **475**, 113–121.
24. Xu,N., Luo,K.Q. and Chang,D.C. (2003) Ca^{2+} signal blockers can inhibit M/A transition in mammalian cells by interfering with the spindle checkpoint. *Biochem. Biophys. Res. Commun.*, **306**, 737–745.
25. Willet,W.C. (1990) *Nutritional Epidemiology*. Oxford University Press, New York, NY.
26. World Cancer Research Fund, and American Institute for Cancer Research. (1997) *Food, Nutrition and the Prevention of Cancer: a Global Perspective*. American Institute for Cancer Research, Washington, DC.
27. Jacques,P.F., Sulsky,S.I., Sadowski,J.A., Phillips,J.C., Rush,D. and Willett,W.C. (1993) Comparison of micronutrient intake measured by a dietary questionnaire and biochemical indicators of micronutrient status. *Am. J. Clin. Nutr.*, **57**, 182–189.
28. Baghurst,K.I. and Record,S.J. (1984) A computerised dietary analysis system for use with diet diaries or food frequency questionnaires. *Community Health Stud.*, **8**, 11–18.
29. Baghurst,K.I. and Baghurst,P.A. (1981) The measurement of usual dietary intake in individuals and groups. *Trans. Menzies Found.*, **3**, 139–160.
30. Baghurst,P.A., Carman,J.A., Syrette,J.A., Baghurst,K.I. and Crocker,J.M. (1992) Diet, prolactin, and breast cancer. *Am. J. Clin. Nutr.*, **56**, 943–949.
31. Rohan,R.E., Record,S.J. and Cook,M.J. (1987) Repeatability of estimates of nutrient and energy intake: the quantitative food frequency approach. *Nutr. Res.*, **7**, 125–137.
32. Department of Community Services and Health. (1992) *Composition of Foods Australia*. Australian Government Publishing Service, Canberra.
33. Paul,A.A. and Southgate,D.A. (1978) *McCance and Widdowson's 'The Composition of Foods'*, 4th edn. Elsevier, Amsterdam.
34. Fenech,M. (2000) The *in vitro* micronucleus technique. *Mutat. Res.*, **455**, 81–95.
35. Fenech,M. and Morley,A.A. (1986) Cytokinesis-block micronucleus method in human lymphocytes: effect of *in vivo* ageing and low-dose X-irradiation. *Mutat. Res.*, **161**, 193–198.
36. Yang,C.S. and Lee,J.M. (1987) Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies. *J. Nutr. Growth Cancer*, **4**, 19–27.
37. Wilkins,P.J., Grey,P.C. and Dreosti,I.E. (1972) Plasma zinc as an indicator of zinc status in rats. *Br. J. Nutr.*, **27**, 113–120.
38. McGown,E.L., Rusnak,M.G., Lewis,C.M. and Tillotson,J.A. (1982) Tissue ascorbic acid analysis using ferrozine compared with the dinitrophenylhydrazine method. *Anal. Biochem.*, **119**, 55–61.
39. Lindsey,J.K. (1995) *Modelling Frequency and Count Data*. Oxford University Press, Oxford.
40. SPSS for Windows. (2001) Version 11.0. SPSS Inc., Chicago, IL.
41. StataCorp. (2001) Stata Statistical Software: Release 7.0. Stata Corporation, College Station, TX.
42. Giovannucci,E. (2003) Diet, body weight and colorectal cancer: a summary of epidemiological evidence. *J. Womens Health*, **12**, 173–182.

43. Bailey,L.B. (2003) Folate, methyl-related nutrients, alcohol, and the MTHFR 677C→T polymorphism affect cancer risk: intake recommendations. *J. Nutr.*, **133**(Suppl. 1), 3748S–3753S.
44. Bonassi,S., Fenech,M., Lando,C., Lin,Y.-P., Ceppi,M., Chang,W.P., Holland,N., Kirsch-Volders,M., Zeiger,E., Ban,S., Barale,R., Bigatti,M.P., Bolognesi,C., Jia,C., Di Giorgio,M., Ferguson,L.R., Fucic,A., Lima,O.G., Hrelia,P., Krishnaja,A.P., Lee,T.K., Migliore,L., Mikhalevich,L., Mirkova,E., Mosesso,P., Müller,W.U., Odagiri,Y., Scarfì,M.R., Szabova,E., Vorobtsova,I., Vral,A. and Zijno,A. (2001) The HUman MicroNucleus project: International data base comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes. I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ. Mol. Mutagen.*, **37**, 31–45.
45. Bharadwaj,R. and Yu,H. (2004) The spindle checkpoint, aneuploidy, and cancer. *Oncogene*, **23**, 2016–2027.
46. Esteller,M. and Herman,J.G. (2002) Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *J. Pathol.*, **196**, 1–7.
47. Huang,S. (2002) Histone methyltransferases, diet nutrients and tumour suppressors. *Nat. Rev. Cancer*, **2**, 469–476.
48. Choi,S.W. and Mason,J.B. (2002) Folate status: effects on pathways of colorectal carcinogenesis. *J. Nutr.*, **132**(8 Suppl), 2413S–2418S.
49. Honda,S., Marumoto,T., Hirota,T., Nitta,M., Arima,Y., Ogawa,M. and Saya,H. (2004) Activation of m-calpain is required for chromosome alignment on the metaphase plate during mitosis. *J. Biol. Chem.*, **279**, 10615–10623.
50. Cho,E., Smith-Warner,S.A., Spiegelman,D., Beeson,W.L., van den Brandt,P.A., Colditz,G.A., Folsom,A.R., Fraser,G.E., Freudenheim,J.L., Giovannucci,E., Goldbohm,R.A., Graham,S., Miller,A.B., Pietinen,P., Potter,J.D., Rohan,T.E., Terry,P., Toniolo,P., Virtanen,M.J., Willett,W.C., Wolk,A., Wu,K., Yaun,S.S., Zeleniuch-Jacquotte,A. and Hunter,D.J. (2004) Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J. Natl Cancer Inst.*, **96**, 1015–1022.
51. Hasty,P. (2001) The impact energy metabolism and genome maintenance have on longevity and senescence: lessons from yeast to mammals. *Mech. Ageing Dev.*, **122**, 1651–1662.
52. Blander,G. and Guarente,L. (2004) The sir2 family of protein deacetylases. *Annu. Rev. Biochem.*, **73**, 417–435.
53. Kirkland,J.B. (2003) Niacin and carcinogenesis. *Nutr. Cancer*, **46**, 110–118.
54. Fenech,M. and Rinaldi,J. (1995) A comparison of lymphocyte micronuclei and plasma micronutrients in vegetarians and non-vegetarians. *Carcinogenesis*, **16**, 223–230.
55. Fenech,M., Dreosti,I. and Aitken,C. (1997) Vitamin-E supplements and their effect on vitamin-E status in blood and genetic damage rate in peripheral blood lymphocytes. *Carcinogenesis*, **18**, 359–364.
56. Claycombe,K.J. and Meydani,S.N. (2001) Vitamin E and genome stability. *Mutat. Res.*, **475**, 37–44.
57. Nyberg,F., Hou,S.M., Pershagen,G. and Lambert,B. (2003) Dietary fruit and vegetables protect against somatic mutation *in vivo*, but low or high intake of carotenoids does not. *Carcinogenesis*, **24**, 689–696.
58. Bendich,A. (2004) From 1989 to 2001: what have we learned about the 'biological actions of beta-carotene'? *J. Nutr.*, **134**, 225S–230S.
59. Olson,J.A. (1996) Vitamin A. In Ziegler,E.E. and Filer,L.J. (eds) *Present Knowledge in Nutrition*. ILSI Press, Washington, DC, pp. 109–119.
60. Alija,A.J., Bresgen,N., Sommerburg,O., Siems,W. and Eckl,P.M. (2004) Cytotoxic and genotoxic effects of beta-carotene breakdown products on primary rat hepatocytes. *Carcinogenesis*, **25**, 827–831.
61. Carlsen,H., Alexander,G., Austenaa,L.M., Ebihara,K. and Blomhoff,R. (2004) Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutat. Res.*, **551**, 199–211.
62. Robien,K. and Ulrich,C.M. (2003) 5,10-Methylenetetrahydrofolate reductase polymorphisms and leukemia risk: a HuGE minireview. *Am. J. Epidemiol.*, **157**, 571–582.
63. Sharp,L. and Little,J. (2004) Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. *Am. J. Epidemiol.*, **159**, 423–443.
64. Powers,H.J. (2003) Riboflavin (vitamin B2) and health. *Am. J. Clin. Nutr.*, **77**, 1352–1360.
65. Mock,D.M., Henrich,C.L., Carnell,N. and Mock,N.I. (2002) Indicators of marginal biotin deficiency and repletion in humans: validation of 3-hydroxyisovaleric acid excretion and a leucine challenge. *Am. J. Clin. Nutr.*, **76**, 1061–1068.
66. Mock,D.M., Quirk,J.G. and Mock,N.I. (2002) Marginal biotin deficiency during normal pregnancy. *Am. J. Clin. Nutr.*, **75**, 295–299.
67. Mock,D.M., Henrich,C.L., Carnell,N., Mock,N.I. and Swift,L. (2002) Lymphocyte propionyl-CoA carboxylase and accumulation of odd-chain fatty acid in plasma and erythrocytes are useful indicators of marginal biotin deficiency. *J. Nutr. Biochem.*, **13**, 462–470.
68. Zempleni,J. and Mock,D.M. (2000) Marginal biotin deficiency is teratogenic. *Proc. Soc. Exp. Biol. Med.*, **223**, 14–21.
69. Zempleni,J., Ricki,M. and Mock,D.M. (2001) *In vivo* biotin supplementation at a pharamcologic dose decreases proliferation rates of human peripheral blood mononuclear cells and cytokine release. *J. Nutr.*, **131**, 1479–1484.
70. Fiume,M.Z. (2001) Cosmetic ingredient review expert panel. Final report on the safety assessment of biotin. *Int. J. Toxicol.*, **20**, 1–12.
71. Pesofsky-Vig,N. (1996) Pantothenic acid. In Zeigler,E.E. and Filer,L.J. (eds) *Present Knowledge in Nutrition*. ILSI Press, Washington, DC, pp. 236–244.
72. Sivak,A. and Tu,A.S. (1980) Cell culture tumor promotion experiments with saccharin, phorbol myristate acetate and several common food materials. *Cancer Lett.*, **10**, 27–32.
73. Baghurst,P.A., McMichael,A.J., Slavotinek,A.H., Baghurst,K.I., Boyle,P. and Walker,A.M. (1991) A case-control study of diet and cancer of the pancreas. *Am. J. Epidemiol.*, **134**, 167–179.
74. Steinmetz,K.A. and Potter,J.D. (1993) Food-group consumption and colon cancer in the Adelaide Case-Control Study. II. Meat, poultry, seafood, dairy foods and eggs. *Int. J. Cancer*, **53**, 720–727.
75. Steinmetz,K.A. and Potter,J.D. (1994) Egg consumption and cancer of the colon and rectum. *Eur. J. Cancer Prev.*, **3**, 237–245.
76. Collins,A.R. (2001) Carotenoids and genomic stability. *Mutat. Res.*, **475**, 21–28.
77. Dreosti,I.E. (2001) Zinc and the gene. *Mutat. Res.*, **475**, 161–167.
78. Bashir,O., Fitzgerald,A.J. and Goodlad,R.A. (2004) Both suboptimal and elevated vitamin intake increase intestinal neoplasia and alter crypt fission in ApcMin/+ mouse. *Carcinogenesis*, **25**, 1507–1515.
79. Walter,P.B., Knutson,M.D., Paler-Martinez,A., Lee,S., Xu,Y., Viteri,F.E. and Ames,B.N. (2002) Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. *Proc. Natl Acad. Sci. USA*, **99**, 2264–2269.
80. Mertz,W. (1981) The essential trace elements. *Science*, **213**, 1332–1338.
81. Mertz,W. (1998) A perspective on mineral standards. *J. Nutr.*, **128**(2 Suppl), 375S–378S.
82. Ashfield-Watt,P., Pullin,C.H., Whiting,J.M., Clark,Z.E., Moat,S.J., Newcombe,R.G., Burr,M.L., Lewis,M.J., Powers,H.J. and McDowell,I.F.W. (2002) Methylenetetrahydrofolate reductase C677T genotype modulates homocysteine responses to a folate-rich diet or low-dose folic acid supplement: a randomised controlled trial. *Am. J. Clin. Nutr.*, **76**, 180–186.
83. van Oort,F.V., Melse-Boonstra,A., Brouwer,I.A., Clarke,R., West,C.E. and Katan,M.B. (2003) Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose–response study. *Am. J. Clin. Nutr.*, **77**, 1318–1323.
84. Wald,N.J., Law,M.R., Morris,J.K. and Wald,D.S. (2001) Quantifying the effect of folic acid. *Lancet*, **358**, 2069–2073.
85. Song,J., Medline,A., Mason,J.B., Gallinger,S. and Kim,Y.I. (2000) Effects of dietary folate on intestinal tumorigenesis in the apcMin mouse. *Cancer Res.*, **60**, 5434–5440.

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