Blood micronutrients and DNA damage in children

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Abbreviations:

BNC, bi-nucleated cells

CBMNcyt, cytokineses-block micronucleus cytome

CSIRO, Commonwealth Scientific and Industrial Research Organisation

GST, Glutathione S-transferase

MNi, number of micronuclei in binucleated cells

MTHFR, methylenetetrahydrofolate reductase

MTR, methionine synthase

MTRR, methionine synthase reductase

NPBs, binucleated cells with nucleoplasmic bridges

NBuds, number of binucleated cells with nuclear buds

NDI, nuclear division index

RFC, reduced folate carrier

TRF1/TRF2, telomere repeat binding factors 1/2

UV, ultra-violet

XRCC1/3, X-ray repair cross-complementing group 1/3

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Abstract

Scope

Maintenance of normal cellular phenotype depends largely on accurate DNA replication and repair. DNA damage causes gene mutations and predisposes to cancer and other chronic diseases. Growing evidence indicates that nutritional factors are associated with DNA damage in adults; here, we investigate these associations in children.

Methods and Results

We conducted a cross-sectional study among 462 healthy children 3, 6 and 9 years of age. Whole blood was collected and micronutrient levels were measured. The cytokinesis-block micronucleus cytome assay was used to measure chromosomal DNA damage (micronuclei, nucleoplasmic bridges, and nuclear buds) in lymphocytes. Cell apoptosis, necrosis, and the nuclear division index (NDI) were also measured. Nine loci in genes involved in folate metabolism and DNA repair were genotyped. Data were analysed using linear regression with adjustment for potential confounders. Calcium was positively associated with micronuclei and necrosis, and α-tocopherol negatively associated with apoptosis, NDI and nucleoplasmic bridges; lutein was positively associated with nucleoplasmic bridges. α-tocopherol was positively associated with necrosis.

Conclusion

DNA damage in healthy children may be influenced by blood micronutrient levels and DNA repair genotype. Further investigation of associations between nutritional status and genomic integrity in children is needed to shed additional light on potential mechanisms.

Introduction

Maintenance of normal phenotype in cells depends to a large extent on accurate DNA replication, efficient DNA damage-sensing and repair mechanisms, and error-free chromosome segregation during mitosis. Unrepaired or incorrectly repaired DNA damage, including aneuploidy, may predispose to cancer and other chronic diseases in humans. Elevated micronucleus (MNi) frequency in lymphocytes – a biomarker for chromosome breakage and mal-segregation – has been shown to be associated prospectively with an increased risk of cancer in a large cohort study [1], with severe adverse cardiovascular events in coronary artery disease patients [2] and with mortality from cancer or cardiovascular disease in a nested case-control study [3, 4]. DNA damage has also been shown, in cross-sectional studies of adults, to be associated with diabetes [5], mild cognitive impairment, Alzheimer's, and Parkinson's diseases [6, 7].

Oxidative damage to the human genome occurs as a result of normal cellular metabolism [8]. However, environmental factors such as alcohol consumption, smoking and recreational drug use, and exposure to chemical carcinogens and ionising radiation also increase DNA damage in lymphocytes [9-13].

There is also growing evidence that insufficiency of some nutrients increases susceptibility to DNA damage in adults [14-18]. Moderate deficiencies in folate, vitamin B12 and zinc have been shown to be associated with DNA damage in lymphocytes *in vitro* and *in vivo*, and the levels of induced damage are equivalent to those observed after exposure to ionising radiation doses well above the annual exposure limit for radiation workers [19-21]. Moreover, there is evidence that MNi frequency in lymphocytes in young adults with above-average DNA damage levels can be reduced by supplementation with B vitamins and other essential micronutrients [22].

Polymorphisms in genes involved in the transport and metabolism of folate, vitamin B12 and methionine may have an impact on genome damage, and could modify the effects of deficiencies in these vitamins [23-26]. Polymorphisms in genes associated with xenobiotic metabolism and DNA repair have also been shown to affect the adult body's response to genotoxic insult and MNi frequency in lymphocytes [23, 27].

The great majority of studies investigating factors associated with DNA damage have been conducted in adults. The focus of the studies conducted in children to date has been on the effects of environmental pollutants [28], although there have been some reports of increased MNi frequency and chromosome damage among children with significant malnutrition [29, 30], deficient protein intake [31], obesity [32], or low blood levels of vitamin B12 [33] or iron [34].

To our knowledge, associations between a range of blood micronutrient levels and DNA damage in healthy children have not been investigated previously. DNA damage is cumulative and increases linearly with age, both in children [35] and adults [36-38]. If poor nutrition in childhood should contribute to DNA damage in this important growth phase of life, then it may well contribute to risk of cancer and other diseases in later life. In fact, it could be that poor nutrition in childhood has an even greater negative impact on future health through DNA damage than it does in later life. In childhood, a significantly higher proportion of cells are in DNA synthesis phase, during which they may be more prone to insult by insufficiency of micronutrients needed for the synthesis of nucleotides involved in DNA replication, repair and methylation [16]. In addition, the higher rate of DNA replication in childhood increases the probability that faulty copies of DNA will be replicated resulting in a larger number of genetically aberrant cells inhabiting the body into adulthood increasing the risk of cancer and/or premature senescence.

In this paper, we report our findings for associations between 16 micronutrients measured in blood and biomarkers of DNA damage and cytotoxicity in 462 healthy children. These micronutrients were selected because they have been shown to be associated with DNA damage in adults [14, 39-47].

We also explored the contributions of common polymorphisms in genes involved in B vitamin metabolism, DNA repair and xenobiotic detoxification.

Materials and Methods

We conducted a cross-sectional study of nutritional factors and DNA damage in children in Western Australia between 2009 and 2011. Parents of healthy children aged 3, 6 and 9 years were invited to participate through mail-outs to childcare centres and schools, and through community-based advertisements. Children with asthma, diabetes, cancer, arthritis or epilepsy were not eligible to take part, as these conditions are known to be associated with higher levels of DNA damage due to endogenous factors or therapy [48-52] , and our aim was to investigate associations with nutritional factors. All eligible volunteers were accepted into the study.

Parents of 464 children provided informed consent: 155 three-year olds, 155 six-year olds and 154 nine-year olds. The nine-year olds were also asked for, and gave, consent. The study was approved by the Human Research Ethics committees of the University of Western Australia (approval number RA/4/1/2333) and the CSIRO Food and Nutrition Division (approval number 10/01).

The study design and methods have been reported previously [53]. Briefly, a phlebotomist visited the child's home and collected 18mL of blood into heparinised vacutainers, and measured their height and weight. On the day of the visit, the parents gave the child a simple breakfast and applied anaesthetic cream an hour before the appointment. Blood samples were

maintained at 5-10°C in Labtop® coolers and couriered to the laboratory within 24 hours of collection. A digital thermometer was used to monitor the temperature during transportation. Approximately 1mL of whole blood was used to measure vitamin B3 (niacin number) and red cell folate. The remaining blood was spun at 3000rpm for 20min at 4ºC and 3.8mL of the plasma collected were used to measure the micronutrients of interest. Red cell folate, plasma folate, and vitamin B12 were measured with a chemiluminescent microparticle folate or vitamin B12 binding protein assay, ARCHITECT™ (Abbott Laboratories, Abbott Park, IL, USA). Vitamin D was measured by an enzyme immunoassay before 2010, and an automated chemilumiescent assay after 2010 (both from Immuno Diagnostic Systems Ltd, Boldon, UK). Quality control and reference standards were used in both assays as required by Australian certified laboratories; the testing laboratory affirmed that the two assays provided comparable data. Vitamin B3 (niacin number), was measured with a validated colorimetric enzymatic assay [54, 55]. α-tocopherol and carotenoids (lutein, retinol, lycopene, α-carotene and β-carotene) were measured by HPLC, and minerals were assessed by ICP-MS (calcium, magnesium and zinc) and ICP-OES (selenium). The volume of blood required to measure plasma vitamin C (9mls) precluded its assessment in young children.

Markers of DNA damage and cytotoxicity in lymphocytes were measured using the cytokineses-block micronucleus cytome (CBMNcyt) assay whole blood culture method as previously described [56]. Lymphocytes were stimulated to divide using phytohaemagglutinin and dividing cells were cytokinesis-blocked in the binucleated stage of mitosis using cytochalasin-B. The DNA damage biomarkers in the CBMNcyt assay (micronuclei, nucleoplasmic bridges and nuclear buds) are expressed in binucleated cells (BNCs) and were measured in 1000 BN cells from each of duplicate cultures. Thus, the DNA damage biomarkers used in this study were: number of micronuclei (MNi) in BNCs, BNCs with nucleoplasmic bridges (NPB) and BNCs with nuclear buds (NBuds). MNi are a

biomarker of chromosome breakage or loss; NPB are a biomarker of dicentric chromosome formation due to DNA strand break misrepair or telomere end fusion; and NBuds are a biomarker of gene amplification or unresolved DNA repair complexes. The frequencies of apoptotic cells and necrotic cells and the nuclear division index (NDI) was also measured by scoring 250 cells from each of the duplicate cultures and determining the ratios of mono-, bi-, and multinucleated viable cells together with necrotic and apoptotic cells. Two experienced observers scored each biomarker visually, and the mean of the two scores was used in the data analysis. The scoring criteria used for all of these biomarkers were as previously described [56].

DNA for genotyping was isolated from whole blood mononuclear cells using a QIAGEN DNeasy Kit with minor modifications to prevent DNA oxidation, as described by Lu et al. [57]. Genotyping was performed at the following loci using Sequenom MassArray Multiplex SNP analysis: *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087), *MTRR* A66G (rs1801394), *XRCC1* Arg399Gln (G28152A) (rs25487), *RFC* G80A (rs1051266), *GST* Mu, *GST* Theta, and *XRCC3* Thr241Met (C18067T) (rs861539). These polymorphisms were chosen because they are known to reduce enzyme activity related to chromosome stability [23, 58] and/or folate metabolism [59, 60].

Salivary cortisol and cotinine levels were both analysed using standard procedures from high sensitivity enzyme immunoassay kits (salivary cortisol and salivary cotinine quantitative EIA kits, Salimetrics LLC, USA). Parents also provided information about relevant exposures (X-rays, medications, immunisations, pest control treatments in the home, sun exposure, parental smoking) in a telephone interview. A standard erythemal UV dose for the child was estimated for each of the 16 weeks prior to blood collection using methods previously described [61].

Statistical analysis

Three markers of DNA damage (MNi, NPB, NBuds) were analysed, as well as NDI, and number of apoptotic and necrotic cells. Blood micronutrient levels and genotypes were considered as possible predictors of DNA damage and cytotoxicity. Prior information indicated that DNA damage may be related to age and sex, and also that some blood nutrients are seasonally dependent [62]. Therefore, each multiple linear regression was adjusted for child's age and sex and season of blood collection. Environmental exposures were also assessed as possible predictors of the outcomes under investigation, and included in models where appropriate.

Nutrient variables (except plasma folate) were log transformed to correct slight right skew and were divided by the standard deviation to reduce the scale. The plasma folate assay was not designed to discriminate values over 45nmol/L so this resulted in a heavily left-skewed distribution. As a result, plasma folate was divided into quintiles for analysis, with cut-points at 31.2, 36.5, 40.1, and 43.3 nmol/L. Each nutrient, genotype and environmental variable was assessed for its relationship with each of the above dependent variables by being added to the base model one at a time. Nutrient variables that had a P value ≤ 0.10 for the R-squared change or trend term (for ordinal variables) were assessed for inclusion in a combined model, and removed if the coefficient had a P value ≥ 0.05. Genotype and demographic variables meeting the above criteria were then added. Two-way interactions involving micronutrients, genotypes, age, and sex were also explored. Terms were then excluded from the combined model if they did not produce a significant change in R-squared or (for ordinal variables) have a trend term P value ≤ 0.05, so that the most parsimonious model remained. Significant gene-micronutrient interactions were only included when there was at least some published evidence of their biological plausibility. We aimed to identify the smallest subset of potentially explanatory variables that had good predictive ability for

each of the DNA damage markers. All analyses were conducted in SPSS 22 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

Results

Data on blood micronutrient levels and DNA damage markers were available for 462 children. Anthropometric and demographic measures of interest have been reported previously [53]. In general, most children were in the normal height, weight and BMI ranges according to the 2007 Australian National Children's Nutrition and Physical Activity Survey [63, 64]; 9.3% were underweight and 0.6% overweight. Socioeconomic status of households was generally high; 53% of study children lived in households with >$100,000 income per year (compared with 13% of Australian children population) [65]; and 71% of children had at least one parent with a tertiary education (compared with 43% of Australian adults having a tertiary education) [66] (data not shown).

Mean values for each of the 16 blood micronutrients varied little by sex or age group; these results can be viewed in Supplementary Table S1. One hundred and two (23%) children took folate supplements; these children had higher mean plasma folate (39.4 vs 35.8 nmol/L) and red-cell folate (853.9 vs 791.5 nmol/L) (data not shown in tables).

Some micronutrients were significantly correlated with each other – a full correlation matrix of raw micronutrient values is shown in Supplementary Table S2. There was little variation among DNA damage and cytotoxicity biomarkers by age or sex (Table 1). Genotype frequencies are shown in Supplemental Table S3.

The coefficients of all variables tested for inclusion in the model (with only child's age, sex and season of blood collection also in the model) are shown in Supplemental Table S4.

Table 2 shows the final models for the DNA damage markers. After adjustment for relevant covariates, MNi were positively associated with blood calcium. There was also an apparent

increased likelihood of MNi in children heterozygous for the RFC G80A polymorphism. NPBs were positively associated with blood lutein, and negatively associated with α-tocopherol. Fewer NPBs were seen among children with the homozygous mutant genotype at *XRCC1* Arg399ln. None of the variables was significantly associated with NBuds. NDI was negatively associated with α-tocopherol and red cell folate, while NDI was positively associated with the *MTRR* 66AG genotype. The number of apoptotic cells was negatively associated with α-tocopherol and apoptosis appeared higher in children with the *MTHFR* 677CT genotype. The number of necrotic cells was positively associated with α-tocopherol and calcium. No two-way interactions improved model fit.

Discussion

Our study of 462 children is the largest to date of lymphocyte MNi frequencies measured using the CBMNcyt assay. Mean MNi frequencies in our study (1.5 [95% CI: 1.3-1.7], 1.6 [95% CI: 1.4-1.7] and 1.5 [95% CI: 1.4-1.6] in children aged 3, 6 and 9 years old respectively) were at least 3-fold lower than those from a pooled analysis of 12 independent studies (total 332 children): 5.4 (3.5-8.5) and 5.6 (3.9-7.9) in children aged 1-4 and 5-9 years respectively [35]. These differences may be due to different exposures to environmental and dietary factors, and/or methodological differences in the measurement of MNi frequencies [37].

After adjustment for potential confounders and environmental factors, only three plasma micronutrients (calcium, α-tocopherol and lutein) were associated with MNi and NPBs in our study, and no associations were seen with NBuds. Associations between some blood nutrients and NDI, apoptotic and necrotic cells were also seen but were limited to calcium, α-tocopherol and red cell folate.

The explanation for the positive association we observed between calcium and MNi is unclear, particularly as increased dietary calcium intake was associated with reduced MNi frequency in adults [67]. However, hypercalcemia is increased in childhood acute lymphoblastic leukaemia and is associated with shorter telomeres, and both of these conditions are linked with increased chromosomal DNA damage which raises the possibility of hypercalcemia-induced sub-clinical genomic pathology [68, 69]. We did not observe inverse associations between MNi and folate or vitamin B12 as previously reported in studies of young adults [70] and older adult males [71]. This may be because 90% of children in our study had plasma concentrations of vitamin B12 close to or above the threshold at which MNi are minimized in adults (>300pmol/L) [20], and 76% of our study participants had a red cell folate concentration above the corresponding threshold (>607 nmol/L) [20] (data not shown).

The inverse association between α-tocopherol and NPBs could be explained by the antioxidant action of vitamin E which may prevent oxidative base damage and/or DNA strand breaks in the telomere sequence. DNA strand breaks may cause telomere shortening, and damaged bases in the telomere sequence may lead to poor binding of proteins, such as TRF1 and TRF2. These are essential for maintaining normal telomere function and prevention of telomere end fusions that cause dicentric chromosome formation and NPBs [72, 73]. However, there is only limited evidence for this *in vitro* [73, 74] and evidence *in vivo* is derived from a cross-sectional study [72]. We observed a positive association between NPBs and lutein, which has not been previously reported, and is difficult to explain given lutein's anti-oxidant properties [75]. However, a recent study showed that lutein sensitises cells to UV-induced DNA damage [76], and UV-induced DNA damage in telomeres is not repaired [77].

α-Tocopherol was inversely associated with apoptosis, possibly because of protection against telomeric shortening as explained above. However, we have no explanation for the apparently positive association of α-tocopherol with necrosis. Similarly, the positive association between calcium and necrosis is difficult to explain unless high plasma calcium is a surrogate for physical inactivity which is associated with increased inflammation and necrosis [78-80]. An alternative explanatory hypothesis might be calcium leakage from cells due to increased necrosis, a relationship that is observed in subjects with cancer but unlikely to be seen in healthy subjects unless there are high levels of membrane damage due to inflammation-induced oxidative stress [81, 82].

Overall, there was little evidence of associations between the genotypes examined and DNA damage markers. There was an apparent reduction in NPB in AA (Gln/Gln) homozygotes for the *XRCC1* Arg399Gln (G28152A) polymorphism. XRCC1 is required for base excision repair of damaged DNA bases and for repair of DNA strand breaks through its role in the ligase step of the repair process [83]. The Arg399Gln (G28152A) polymorphism of *XRCC1* has been shown to be associated with increased chromosome aberrations and micronuclei in those with the (Gln/Gln, AA genotype) [23]. The null association with MNi and apparent weak association with NPB observed in our study are unexpected, and may be due to the relatively low frequencies of MNi and NPB among children who participated in our study. The apparent increase in MNi frequency in children heterozygous for *RFC* G80A is consistent with our previous finding that, in healthy adults, the MNi was increased by 18.1% in the RFC GA/AA genotypes relative to the GG genotype; the MNi frequency was similar in those with GA and AA genotype [58]. Carriage of the A allele of RFC A80G polymorphism was also associated with shorter telomeres in the same cohort [53]; this consistency in reduced DNA integrity in carriers of the A allele increases the probability that this is not a chance finding. RFC is required for the uptake of 5-MeTHF into cells, and it is plausible that

even slight changes in the functional activity of this protein could reduce cellular bioavailability of folate, leading to aberrations in DNA methylation and DNA synthesis and repair; and increased MNi formation [20]. We also found that NDI was positively associated with the *MTRR* 66AG genotype. MTRR reduces oxidised vitamin B12 to its active form. Because functional vitamin B12 deficiency reduces folate bioavailability for DNA synthesis, reduced MTRR activity might impair B12 function and folate metabolism, and reduce DNA synthesis and nuclear division. We cannot explain the association of *MTHFR* 677CT genotype with more apoptotic cells, given that carriage of the MTHFR TT genotype was associated with decreased apoptosis in an *in vitro* lymphocyte study relative to the CC genotype [25].

This is the first reported study of associations between a wide range of blood micronutrient levels and biomarkers of DNA damage and cytotoxicity in healthy children. The strengths of this study are its size, the range of blood micronutrient levels measured, the state of the art methods used to measure markers of DNA damage and cytotoxicity, and that relevant genetic and environmental factors were taken into account. We investigated MNi as well as other previously unexplored biomarkers in the CBMNcyt assay, such as NPB and NBuds. Our findings provide novel reference information about DNA damage levels in healthy children.

Because of our dependence on volunteers, participants in this study were generally from higher socioeconomic backgrounds. While 53% of study children lived in households with >$100,000 income per year, only 13% of children in the wider Australian population do so [65]; and while 71% of children had at least one parent with a tertiary education, only 43% of Australian adults have a tertiary education [66]. As a result, the children in the study were generally well-nourished and the range of micronutrient levels observed may have been relatively narrow, reducing our ability to detect some potential associations. Similarly, our findings may not be generalizable to a broader socio-economic cross-section of the

population. Many variables and interactions were tested in the analysis, so it is possible that some of the associations seen were due to chance, particularly those for which there is no previous evidence or an established mechanism. However, our study was exploratory in nature and we considered it important to present all the findings for readers' information.

While our findings provide useful preliminary data on plasma micronutrient levels and DNA damage markers in well-nourished children, future studies involving children with a wider range of ages and nutritional status are needed to shed additional light on these associations.

Author Contributions

Concept and study design: EM, MF, BA, NO'C, MM, NdK; study director and lead writer: EM; statistical analysis: KG, PR; statistical expertize: NdK. All authors contributed to the interpretation of the results and writing the manuscript, and have approved the final version.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Table 1: Mean (SD) for each DNA damage marker by sex and age

	Boys **n=233**	**Girls** **n=229**	**3-year olds** **n=153**	**6-year olds** **n=155**	**9-year olds** **n=154**	**Overall** **N=462**
DNA damage markers						
MNi	1.5 (1.0)	1.5 (1.1)	1.5 (1.1)	1.6 (1.1)	1.5 (1.0)	1.5 (1.1)
NPBs	1.4 (1.0)	1.3 (1.1)	1.4 (1.3)	1.3 (1.0)	1.3 (1.0)	1.3 (1.1)
NBuds	2.7 (1.9)	2.4 (1.7)	2.8 (1.7)	2.6 (1.9)	2.4 (1.7)	2.6 (1.8)
Cytotoxicity markers						
NDI	2.0 (0.2)	2.0 (0.2)	2.0 (0.2)	2.0 (0.2)	2.1 (0.2)	2.0 (0.2)
N Apoptotic cells	1.1 (0.8)	1.3 (1.0)	1.2 (0.9)	1.2 (0.9)	1.1 (0.9)	1.2 (0.9)
N Necrotic cells	10.1 (6.2)	10.7 (7.7)	9.9 (7.0)	10.4 (6.3)	11.0 (7.5)	10.4 (7.0)

Abbreviations: MNi, number of micronuclei in binucleated cells; NPBs, binucleated cells with nucleoplasmic bridges; NBuds, number of binucleated cells with nuclear buds; NDI, nuclear division index.

TABLE 2: Regression coefficients for prediction of DNA damage markers (associated CIs are given in parentheses, with *P*-values beneath).

Term	**MNi**	**NPBs**	**NDI**	**Apoptosis**	**Necrotic cells**
Variability explained by the model (%)	7.6	4.5	7.7	7.6	6.9
Boys[a]	Referent	Referent	Referent	Referent	Referent
Girls	0.006 (-0.19,0.20)	-0.07 (-0.27, 0.14)	-0.03 (-0.07, 0.01)	0.19 (0.03, 0.36)	0.37 (-0.89, 1.64)
	(0.95)	(0.52)	(0.17)	(0.02)	(0.56)
Age 3[a]	Referent	Referent	Referent	Referent	Referent
Age 6	-0.02 (-0.26,0.22)	-0.06 (-0.32, 0.20)	0.01 (-0.04, 0.07)	0.02 (-0.18, 0.23)	-0.40 (-2.01, 1.21)
	(0.85)	(0.67)	(0.60)	(0.82)	(0.63)
Age 9	-0.06 (-0.29, 0.18)	-0.12 (-0.38, 0.15)	0.05 (-0.01, 0.11)	-0.01 (-0.22, 0.19)	-0.05(-1.66, 1.56)
	(0.65)	(0.40)	(0.06)	(0.90)	(0.95)
Season of blood collection	0.03 (-0.06, 0.12)	-0.02 (-0.12, 0.09)	-0.03 (-0.05, -0.01)	0.02 (-0.06, 0.10)	0.87 (0.25, 1.49)
(4-level)[a]	(0.46)	(0.75)	(0.02)	(0.68)	(0.01)
Blood Micronutrients[b]					
Calcium	0.13 (0.03, 0.23)				0.64 (-0.01, 1.29)
	(0.01)				(0.05)

α-tocopherol		-0.14 (-0.26, -0.02) (0.02)	-0.03 (-0.05, -0.01) (0.03)	-0.10 (-0.19, -0.01) (0.03)	1.77 (1.08, 2.46) (<0.001)
Red Cell Folate			-0.04 (-0.06, -0.01) (0.001)		
Lutein		0.14 (0.02, 0.26) (0.02)			
Genotypes					
RFC G80A GG	Referent				
RFC G80A GA	0.22 (0.01, 0.43) (0.04)				
RFC G80A AA	0.06 (-0.22, 0.34) (0.69)				
XRCC1 Arg399Gln GG		Referent			
XRCC1 Arg399Gln GA		-0.09 (-0.31, 0.12) (0.39)			
XRCC1 Arg399Gln AA		-0.35 (-0.69, -0.02) (0.04)			

	(*P* trend 0.05)		
MTRR 66AA		Referent	
MTRR 66AG		0.02 (-0.03, 0.07)	
		(0.53)	
MTRR 66GG		-0.06 (-0.12, -0.01)	
		(0.04)	
XRCC3 T241M CC			Referent
XRCC3 T241M CT			0.08 (-0.18, 0.33)
			(0.56)
XRCC3 T241M TT			0.25 (-0.01, 0.51)
			(0.06)
			(*P* trend 0.02)
MTHFR 677 CC			Referent
MTHFR 677 CT			0.21 (0.04, 0.39)
			(0.02)
MTHFR 677 TT			0.07 (-0.21, 0.36)
			(0.61)

Other Covariates				
BMI z-score	-0.15(-0.24, -0.06) (0.001)			
Had fever[c]	-0.27 (-0.47, -0.06) (0.01)			
Professional pest control used[c]	0.47 (0.14, 0.79) (0.01)			
UV exposure 6 weeks before blood collection		-0.01(-0.01, -0.001) (0.03)		
UV exposure 12 weeks before blood collection				0.01 (0.00, 0.01) (<0.01)

[a] Child's age, sex and season of blood collection (winter, autumn, spring, summer) are entered into all models.
[b] Coefficients of micronutrients are for each logged standard deviation increase
[c] In the four months before blood collection

Supplementary Table S1: Mean (SD) for each blood micronutrient level by sex and age

Micronutrient	Boys	Girls	3-year olds	6-year olds	9-year olds	Overall
Zinc (μmol/L)	14.4 (1.9)	14.7 (1.7)	13.9 (1.7)	14.7 (1.9)	15.0 (1.6)	14.6 (1.8)
Calcium, total (mmol/L)	2.31 (0.12)	2.32 (0.10)	2.28 (0.09)	2.33 (0.13)	2.34 (0.10)	2.31 (0.11)
Magnesium (mmol/L)	0.80 (0.04)	0.80 (0.04)	0.79 (0.05)	0.80 (0.04)	0.80 (0.05)	0.80 (0.04)
Calcium/Magnesium ratio	2.9 (0.2)	2.9 (0.2)	2.9 (0.2)	2.9 (0.2)	2.9 (0.2)	2.9 (0.2)
Vitamin B12 (pmol/L)	534.0 (209)	554.8 (227)	585.6 (260)	548.7 (197)	499.6 (183)	544.3 (218)
Plasma Folate (nmol/L)	36.6 (6.8)	36.5 (7.9)	38.7 (6.0)	36.7 (7.4)	34.4 (7.8)	36.6 (7.3)
Red Cell Folate (nmol/L)	830.3 (268)	777.9 (295)	829.1 (278)	827.3 (293)	756.9 (273)	803.9 (283)
Vitamin D (nmol/L)	91.5 (29.1)	85.7 (34.4)	82.5 (25.5)	92.4 (35.6)	90.8 (33.1)	88.6 (32.0)
Selenium (μmol/L)	1.13 (0.13)	1.11 (0.13)	1.08 (0.15)	1.12 (0.11)	1.14 (0.13)	1.12 (0.13)
Niacin Number (NAD/NADP * 100)	173.2 (27.2)	181.9 (31.0)	182.9 (28.0)	175.7 (30.4)	173.5 (29.6)	177.7 (29.5)
Lutein (μmol/L)	0.44 (0.25)	0.45 (0.26)	0.35 (0.21)	0.46 (0.21)	0.53 (0.30)	0.45 (0.25)
Retinol (μmol/L)	1.14 (0.23)	1.17 (0.25)	1.15 (0.24)	1.14 (0.25)	1.17 (0.22)	1.15 (0.24)
α-Tocopherol (μmol/L)	23.1 (11.8)	24.2 (12.6)	20.0 (9.5)	25.1 (12.5)	25.9 (13.5)	23.7 (12.2)
Lycopene (μmol/L)	0.52 (0.32)	0.54 (0.32)	0.39 (0.22)	0.58 (0.31)	0.64 (0.36)	0.53 (0.32)
α-Carotene (μmol/L)	194.1(156)	198.3 (165)	164.3 (130)	210.2 (180)	213.6 (162)	196.2 (160)
β-Carotene (μmol/L)	0.55 (0.34)	0.58 (0.41)	0.53 (0.29)	0.57 (0.40)	0.59 (0.42)	0.56 (0.38)

Supplemental Table S2 Correlation coefficients (Spearman's rho) between blood micronutrient measures

	Zinc	calcium	Magnesium	Ca/mg ratio	Plasma folate	Red cell folate	Lutein	Vitamin B12	Vitamin D	Selenium	Niacin Number	Retinol	α−tocopherol	Lycopene	α−carotene	β−carotene
Zinc		0.33**	0.18**	0.09	0.07	0.02	0.16**	0.06	-0.03	0.27**	-0.04	0.07	0.24**	0.19**	0.11	0.09
calcium			0.27**	0.44**	-0.19**	-0.20**	0.26**	-0.03	0.09	0.31**	0.05	0.03	0.16**	0.12*	0.05	0.04
Magnesium				-0.69**	-0.04	-0.12*	0.04	-0.05	0.01	0.18**	0.05	-0.05	0.08	0.03	0.04	0.02
Ca/mg ratio					-0.11*	-0.05	0.12*	0.02	0.04	0.03	0.003	0.03	0.02	0.03	0.01	0.003
Plasma folate						0.41**	-0.04	0.15**	-0.07	-0.10*	0.13*	0.03	0.12*	0.08	0.17**	0.22**
Red cell folate							-0.07	0.14**	0.04	-0.08	0.06	0.04	0.04	0.04	0.10	0.11
Lutein								-0.05	0.07	0.26**	0.01	0.10*	0.42**	0.45**	0.44**	0.38**
Vitamin B12									-0.05	-0.06	0.01	0.06	0.11*	0.01	0.06	0.12**
Vitamin D										0.11*	0.03	0.05	-0.10*	0.02	0.06	-0.04
Selenium											-0.10*	0.19**	0.01	0.06	0.13**	0.05
Niacin Number												0.01	-0.03	-0.04	-0.01	0.04
Retinol													0.03	0.07	0.12*	0.14**
α−tocopherol														0.47**	0.24**	0.33**
Lycopene															0.39**	0.47**
α−carotene																0.80**
β−carotene																

*p<0.05 **p<0.01

Supplementary Table S3: Genotype frequencies of polymorphisms investigated in all study children[a]

Polymorphism	Genotype	N	%
MTHFR C677T rs1801133	CC	223	49.1
	CT	187	41.2
	TT	44	9.7
MTHFR A1298C rs1801131	AA	201	44.3
	AC	206	45.4
	CC	47	10.4
RFC G80A rs1051266	GG	174	38.4
	GA	204	45.0
	AA	75	16.6
MTRR A66G rs1801394	AA	128	28.2
	AG	215	47.4
	GG	111	24.4
MTR A2756G rs1805087	AA	285	63.2
	AG	150	33.3
	GG	16	3.5
XRCC1 arg399glm rs25487	GG	194	42.7
	GA	208	45.8
	AA	52	11.5
XRCC3 T241M rs861539	CC	60	13.2
	CT	202	44.3
	TT	194	42.5
GST mu deletion	Null	239	52.3
	M1	218	47.7
GST theta deletion	Null	106	23.2
	T1	351	76.8

[a] Reproduced from: Milne E., O'Callaghan N., Ramankutty P., de Klerk N.H., et al. Plasma micronutrient levels and telomere length in children. *Nutrition* 2015, 31, 331-336, DOI: 10.1016/j.nut.2014.08.005, Supplementary Table 1.

Supplementary Table S4: Preliminary associations[a] (B coefficient, p-value) between all blood micronutrients (LNvalue/1SD), genotype and demographic variables and DNA damage markers.

Variable	Category	MNi	NPBs	Nbuds	NDI	Apoptosis	Necrosis
Micronutrients							
Zinc		-0.08 (0.12)	-0.02 (0.72)	-0.10 (0.23)	-0.01 (0.66)	-0.02 (0.67)	0.003 (0.99)
Calcium		0.09 (0.09)	0.11 (0.03)	-0.15 (0.08)	0.001 (0.93)	-0.01 (0.84)	0.64 (0.06)
Magnesium		0.05 (0.36)	0.03 (0.56)	-0.01 (0.87)	<0.000 (0.99)	-0.03 (0.55)	0.29 (0.38)
Ca/mg ratio		0.01 (0.85)	0.11 (0.03)	-0.11 (0.19)	0.003 (0.82)	0.01 (0.90)	0.21 (0.52)
Vitamin B12		-0.07 (0.19)	0.03 (0.56)	0.07 (0.42)	-0.002 (0.87)	-0.06 (0.20)	0.05 (0.88)
Plasma folate (quintiles)		-0.02 (0.63)	-0.08 (0.03)	0.07 (0.22)	-0.01 (0.09)	-0.06 (0.05)	-0.03 (0.90)
Red cell folate		-0.04 (0.48)	-0.11 (0.03)	0.07 (0.43)	-0.03 (0.003)	0.01 (0.74)	0.50 (0.13)
Vitamin D		0.06 (0.25)	0.06 (0.28)	0.02 (0.84)	-0.01 (0.58)	-0.01 (0.91)	0.06 (0.86)
Selenium		0.03 (0.53)	0.06 (0.22)	-0.06 (0.48)	0.01 (0.44)	-0.002 (0.96)	0.22 (0.51)
Niacin number (nad/nadp*100)		-0.003 (0.96)	-0.02 (0.72)	0.13 (0.17)	-0.01 (0.68)	-0.03 (0.52)	0.06 (0.88)
Lutein		-0.002 (0.97)	0.10 (0.08)	0.10 (0.27)	0.004 (0.74)	-0.07 (0.15)	0.89 (0.01)
Retinol		-0.04 (0.44)	0.02 (0.64)	-0.03 (0.72)	-0.003 (0.82)	0.07 (0.09)	0.17 (0.60)
α-tocopherol		-0.07 (0.22)	-0.10 (0.07)	0.06 (0.52)	-0.03 (0.03)	-0.09 (0.05)	1.73 (<0.001)
Lycopene		-0.05 (0.40)	-0.02 (0.77)	-0.02 (0.81)	-0.01 (0.36)	-0.04 (0.44)	1.03 (0.004)
α-carotene		-0.01 (0.83)	0.05 (0.29)	-0.07 (0.43)	-0.02 (0.13)	-0.04 (0.33)	0.88 (0.007)
β-carotene		-0.04 (0.46)	0.04 (0.44)	-0.06 (0.50)	-0.01 (0.26)	-0.07 (0.12)	0.86 (0.009)
Genotypes							
MTHFR C677T	CC	Referent	Referent	Referent	Referent	Referent	Referent
	CT	-0.04 (0.74)	-0.01 (0.96)	-0.16 (0.37)	0.001 (0.96)	0.21 (0.02)	0.24 (0.74)
	TT	0.02 (0.89)	-0.01 (0.96)	-0.05 (0.86)	0.003 (0.94)	0.14 (0.37)	-0.40 (0.74)
MTHFR A1298C	AA	Referent	Referent	Referent	Referent	Referent	Referent
	AC	-0.06 (0.59)	0.09 (0.40)	-0.07 (0.97)	<0.000 (0.99)	-0.11 (0.21)	-0.62 (0.38)
	CC	0.10 (0.59)	0.09 (0.62)	0.13 (0.64)	-0.01 (0.89)	-0.22 (0.14)	0.88 (0.44)

RFC G80A	GG	Referent	Referent	Referent	Referent	Referent	Referent
	GA	0.22 (0.05)	0.09 (0.42)	0.13 (0.48)	0.01 (0.77)	0.14 (0.13)	1.13 (0.13)
	AA	0.08 (0.60)	0.14 (0.37)	-0.22 (0.36)	-0.002 (0.95)	0.18 (0.15)	1.51 (0.12)
MTRR A66G	AA	Referent	Referent	Referent	Referent	Referent	Referent
	AG	0.02 (0.88)	0.06 (0.65)	0.09 (0.66)	0.002 (0.93)	0.06 (0.53)	0.84 (0.29)
	GG	-0.01 (0.93)	0.06 (0.67)	0.30 (0.19)	-0.07 (0.02)	0.004 (0.98)	0.71 (0.45)
MTR A2756G	AA	Referent	Referent	Referent	Referent	Referent	Referent
	AG	-0.06 (0.58)	0.02 (0.84)	-0.06 (0.76)	0.02 (0.42)	-0.10 (0.28)	-0.02 (0.98)
	GG	-0.02 (0.96)	-0.20 (0.48)	-0.09 (0.85)	0.01 (0.82)	-0.10 (0.67)	0.65 (0.72)
XRCC1 arg399gln (G28152A)	GG	Referent	Referent	Referent	Referent	Referent	Referent
	GA	-0.18 (0.10)	-0.09 (0.42)	0.16 (0.37)	0.002 (0.92)	0.15 (0.10)	-0.02 (0.98)
	AA	0.06 (0.72)	-0.36 (0.03)	-0.20 (0.49)	-0.05 (0.18)	0.05 (0.75)	0.47 (0.68)
XRCC3 T241M Thr241Met (C18067T)	CC	Referent	Referent	Referent	Referent	Referent	Referent
	CT	0.06 (0.70)	0.11 (0.51)	0.02 (0.95)	0.01 (0.73)	0.12 (0.36)	-0.90 (0.39)
	TT	-0.03 (0.85)	0.10 (0.56)	0.22 (0.40)	-0.02 (0.63)	0.25 (0.06)	0.44 (0.68)
GST mu deletion	Null	Referent	Referent	Referent	Referent	Referent	Referent
	M1	0.10 (0.35)	-0.01 (0.94)	0.11 (0.50)	-0.05 (0.04)	-0.04 (0.59)	0.28 (0.67)
GST theta deletion	Null	Referent	Referent	Referent	Referent	Referent	Referent
	T1	0.07 (0.55)	0.01 (0.96)	0.11 (0.60)	0.01 (0.60)	0.17 (0.09)	-0.34 (0.66)
GST combination	Double Null	-0.02 (0.89)	-0.10 (0.58)	-0.10 (0.72)	0.04 (0.32)	-0.13 (0.34)	1.32 (0.23)
Other covariates							
Mothers age (years)		-0.01 (0.52)	-0.004 (0.65)	-0.03 (0.11)	0.002 (0.39)	<0.000 (0.98)	-0.03 (0.69)
Fathers age (years)		0.01 (0.33)	-0.004 (0.63)	-0.01 (0.34)	0.002 (0.26)	-0.01 (0.36)	-0.06 (0.31)
BMI z score		-0.13 (0.004)	-0.07 (0.12)	0.01 (0.93)	-0.01 (0.53)	-0.02 (0.58)	-0.40 (0.19)
Cotinine (ng/dL)		0.07 (0.38)	0.03 (0.73)	0.01 (0.97)	0.02 (0.17)	-0.06 (0.35)	-0.57 (0.23)
Cortisol (mcg/dL)		-0.24 (0.35)	0.52 (0.04)	0.36 (0.39)	0.11 (0.04)	-0.11 (0.60)	-2.16 (0.20)

Parental education	Some high school	Referent	Referent	Referent	Referent	Referent	Referent
	Complete high	0.44 (0.12)	-0.03 (0.93)	-0.10 (0.84)	-0.09 (0.17)	0.06 (0.79)	0.75 (0.69)
	Trade/technical	0.30 (0.23)	0.20 (0.44)	0.03 (0.94)	-0.02 (0.73)	-0.23 (0.29)	2.30 (0.17)
	University/college	0.28 (0.23)	0.28 (0.23)	0.20 (0.61)	-0.01 (0.88)	-0.03 (0.90)	1.17 (0.44)
Household income	≤$20,000	Referent	Referent	Referent	Referent	Referent	Referent
	$20,001-$50,000	-0.20 (0.54)	-0.12 (0.72)	-0.72 (0.17)	0.04 (0.56)	0.02 (0.93)	1.46 (0.49)
	$50,001-$100,000	-0.14 (0.63)	-0.04 (0.89)	-0.68 (0.17)	0.05 (0.49)	0.003 (0.99)	1.81 (0.35)
	$100,001-$150,000	0.01 (0.96)	-0.15 (0.63)	-0.60 (0.22)	0.04 (0.54)	0.10 (0.69)	-0.64 (0.74)
	>$150,001	-0.08 (0.80)	-0.10 (0.75)	-0.17 (0.74)	0.06 (0.36)	0.04 (0.87)	0.40 (0.84)
Ethnicity[b]	European	Referent	Referent	Referent	Referent	Referent	Referent
	50% European	-0.23 (0.14)	-0.06 (0.70)	0.04 (0.88)	0.01 (0.82)	-0.10 (0.44)	-0.91 (0.38)
	50% non-European	-0.18 (0.28)	0.12 (0.48)	0.18 (0.53)	-0.01 (0.89)	-0.07 (0.62)	0.03 (0.98)
	indeterminate	-0.34 (0.31)	-0.32 (0.35)	0.19 (0.74)	-0.12 (0.11)	-0.48 (0.09)	-2.20 (0.33)
Child had X-ray[c]	Yes	-0.10 (0.58)	-0.17 (0.37)	-0.35 (0.25)	-0.02 (0.64)	0.05 (0.77)	-0.43 (0.73)
Child had fever[c]	Yes	-0.27 (0.01)	-0.05 (0.64)	-0.18 (0.32)	0.01 (0.74)	-0.10 (0.27)	-0.11 (0.87)
Child had immunization[c]	Yes	0.07 (0.72)	-0.09 (0.62)	0.09 (0.77)	0.01 (0.86)	-0.36 (0.02)	-1.26 (0.29)
Child had sunburn[c]	Yes	0.06 (0.72)	-0.06 (0.73)	0.30 (0.31)	-0.01 (0.84)	0.22 (0.14)	0.37 (0.75)
Child had medicines[c]	Yes	-0.10 (0.40)	0.03 (0.82)	-0.11 (0.59)	<0.000 (0.99)	0.04 (0.68)	-0.14 (0.86)
Professional pest control in house[c]	Yes	0.47 (0.01)	0.11 (0.53)	-0.01 (0.97)	0.04 (0.26)	0.13 (0.35)	-0.49 (0.66)
Self-use pesticides in house[c]		0.07 (0.50)	0.07 (0.50)	-0.21 (0.24)	0.001 (0.98)	0.10 (0.27)	1.21 (0.09)
UV 16 weeks from blood test[d]		0.001 (0.53)	-0.003 (0.27)	0.01 (0.001)	<0.000 (0.85)	0.01 (0.01)	-0.01 (0.66)
UV 15 weeks from blood test		0.001 (0.69)	-0.003 (0.18)	0.01 (0.01)	<0.000 (0.78)	0.01 (0.001)	-0.001 (0.96)
UV 14 weeks from blood test		0.001 (0.56)	-0.003 (0.20)	0.01 (0.03)	<0.000 (0.82)	0.01 (0.001)	-0.001 (0.94)
UV 13 weeks from blood test		0.002 (0.46)	-0.003 (0.18)	0.01 (0.05)	<0.000 (0.56)	0.01 (0.002)	-0.003 (0.85)
UV 12 weeks from blood test		0.002 (0.43)	-0.004 (0.15)	0.01 (0.06)	<0.000 (0.75)	0.01 (<0.001)	0.001 (0.97)
UV 11 weeks from blood test		0.003 (0.24)	-0.004 (0.10)	0.01 (0.16)	<0.000 (0.56)	0.01 (0.003)	0.01 (0.47)
UV 10 weeks from blood test		0.004 (0.13)	-0.004 (0.14)	0.01 (0.22)	<0.000 (0.96)	0.01 (0.001)	0.02 (0.31)

UV 9 weeks from blood test		0.003 (0.26)	-0.005 (0.07)	0.004 (0.35)	<0.000 (0.70)	0.01 (0.003)	0.005 (0.77)
UV 8 weeks from blood test		0.003 (0.24)	-0.01 (0.02)	0.003 (0.49)	<0.000 (0.76)	0.01 (0.03)	-0.001 (0.95)
UV 7 weeks from blood test		0.003 (0.36)	-0.01 (0.02)	0.003 (0.51)	<0.000 (0.67)	0.01 (0.03)	-0.004 (0.82)
UV 6 weeks from blood test		0.002 (0.59)	-0.01 (0.02)	0.001 (0.81)	<0.000 (0.63)	0.01 (0.06)	-0.001 (0.96)
UV 5 weeks from blood test		0.001 (0.80)	-0.01 (0.18)	0.01 (0.10)	<0.000 (0.94)	0.01 (0.07)	-0.02 (0.50)
UV 4 weeks from blood test		0.002 (0.48)	-0.001 (0.73)	0.01 (0.02)	<0.000 (0.72)	0.01 (0.05)	-0.02 (0.38)
UV 3 weeks from blood test		0.003 (0.38)	-0.001 (0.69)	0.003 (0.57)	0.001 (0.29)	0.01 (0.03)	-0.01 (0.67)
UV 2 weeks from blood test		0.002 (0.60)	-0.002 (0.64)	0.004 (0.54)	0.001 (0.22)	0.01 (0.07)	-0.01 (0.83)
UV 1 weeks from blood test		0.001 (0.78)	-0.002 (0.56)	-0.001 (0.83)	0.001 (0.06)	0.01 (0.03)	-0.01 (0.84)

MNi, Micronucleus frequency in binucleated cells; NPBs, Number of binucleated cells with nucleoplasmic bridges; NBuds, Number of binucleated cells with nuclear buds; NDI, nuclear division index.

[a]Preliminary associations; each coefficient adjusted only for child's age, sex and season of blood collection.

[b]Ethnic groups: European, at least 3 European grandparents; 50% European, at least 2 European grandparents and other 2 unknown; 50% non-European, at least 2 non-European grandparents and other 2 unknown; Indeterminate, no 2 grandparents of same ethnicity or all unknown.

[c]In the 4 months before the interview.

[d]UV erythema level (standard erythemal dose) 16 to 1 weeks before blood collection.