Plasma micronutrient levels and telomere length in children

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Disclosure of Conflict of Interest: None identified

Abbreviations

BMI: Body mass index

TL: Telomere length

MTHFR: Methylenetetrahydrofolate reductase

RCF: Reduce folate carrier

MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase

MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase

XRCC1/3: X-ray repair cross-complementing protein 1/3

GST: glutathione S-transferase

NAD, Nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate.

ABSTRACT (250 words)

Objective

Telomeres are long hexamer (TTAGGG) repeats at the ends of chromosomes, and contribute to maintenance of chromosomal stability. Telomere shortening has been linked to cancers and other chronic diseases in adults, although evidence for causal associations is limited. Increasing evidence indicates that nutritional factors are associated with telomere length (TL) in adults, but there have been no studies of this association in children.

Research Methods & Procedures

We conducted a cross-sectional study of nutritional factors and TL in 450 children between 2009 and 2011. Healthy children aged 3, 6 and 9 years provided blood samples, and their parents completed a food frequency questionnaire and a telephone interview about relevant environmental exposures. TL and blood micronutrient levels were measured, and genotyping at 10 loci was undertaken. Associations between the micronutrients and other variables were assessed using linear regression.

Results

No significant main or interactive effects of age or sex were seen. After adjustment for age, sex, parental education and month of blood collection, TL was inversely associated with plasma zinc, and shorter in children with the homozygous mutant genotype of the RFC G80A (rs 1051266) polymorphism.

Conclusions

This is the first investigation of associations between telomere length and micronutrients in healthy children. ~~an inverse association with zinc was seen.~~ The reason for the inverse relationship of TL with zinc is unknown but could be due to an increase in telomere sequence

deletions caused by labile zinc-induced oxidative stress. These findings ~~are consistent with those of previous *in vitro* studies, but~~ should be corroborated in other studies before nutritional recommendations might be considered. ~~Our data on TL in healthy children are a useful reference for other studies in the field.~~

Keywords: DNA damage, nutrients, nutrition, pediatric, polymorphisms, zinc

INTRODUCTION

Telomeres are long hexamer (TTAGGG) repeats located at the ends of mammalian chromosomes that, together with the associated protein telosome structure, act to maintain chromosomal integrity and stability. However, telomeres shorten with age due to the incomplete replication of DNA at chromosome ends with each cell division and DNA strand breaks incurred by oxidative stress. Eventually telomeres shorten to the extent that they are unable to protect the ends of chromosomes from degradation, leading to accelerated cell senescence and death. Telomere degradation and/or dysfunction promote chromosomal instability via telomere end-fusion and the generation of breakage-fusion-bridge cycles within chromosomes [1, 2]. These threats to genomic stability may result in abnormal karyotypes, altered gene dosage, loss of heterozygosity or gene amplification, which in turn cause abnormal gene expression including activation of oncogenes – or inactivation of tumour suppressor genes – important initiating events in carcinogenesis. Telomere shortening has been linked to several types of cancer (including lung, breast, colon, prostate and certain leukemias); these associations have been the subject of two recent meta-analyses [3, 4]. While most studies have been cross-sectional or retrospective, some prospective studies have found telomere shortening to be predictive of risk of cancer [5-7], adverse cardio-vascular events [8, 9] and mortality [5, 6, 10, 11].

There is increasing evidence that nutritional factors are associated with telomere length (TL) in adults. A recent longitudinal study reported an inverse relationship between marine omega-3 fatty acid levels in the blood and the rate of telomere attrition [12]. Other cross-sectional studies have reported positive associations between TL and multivitamin use and dietary intake of vitamins C and E [13], folate [14, 15], vitamin D [16] and fibre [17]. *In vitro* studies have shown that age-related telomere shortening can be slowed by enrichment of

intracellular vitamins C [18] and E [19]. Shorter telomeres have been associated with higher intake of processed meat [20] and alcohol [21], and higher body mass index (BMI) in adults [22, 23]. There is also evidence that minerals involved in antioxidant response, such as zinc and selenium, may influence TL and telomere base damage, but the shape of the dose-response relationship within the physiological range has yet to be determined [24-26].

Virtually all previous studies of factors associated with TL and other forms of DNA damage have been conducted in adults, and the focus of the few studies conducted in children to date has been on the effects of environmental pollutants and socioeconomic status [27, 28] rather than nutritional factors. Given that diseases of adulthood may originate early in life [29] and that lifelong dietary habits are established in childhood [30], it is important to examine whether nutrition during childhood is associated with telomere shortening. In this paper, we present the results of the first comprehensive study of blood micronutrient levels and TL in healthy children.

MATERIALS AND METHODS

A cross-sectional study of nutritional factors and DNA damage in children was conducted 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. Almost all volunteers were accepted into the study; only children with asthma, diabetes, cancer, arthritis or epilepsy were not eligible to take part. 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 required to provide consent. The study was approved by the Department of Education and Training, and the University of Western Australia Human Research Ethics committee.

Parents provided information about the family's demographics, the child's health, and relevant exposures in a telephone interview. Parents were mailed instructions about giving the child a simple breakfast on the day of the scheduled blood collection, and recording exactly what was eaten; toast, milk, butter, margarine and/or jam and water were allowed. Parents were also mailed a tube of anaesthetic cream to apply to the child's arm an hour before the appointment time. Height was measured in centimetres to one decimal point using a portable stadiometer (Seca, Medical Scales and Measuring systems, UK) and weight was measured in grams (to 100g) using a digital bathroom scale (HD-327, Tanita Corporation, Japan).

The phlebotomist collected 18mL of blood on either one or two days. Blood samples were maintained at steady temperature (5-10°C) in Labtop® coolers and couriered to the laboratory in insulated boxes (DGP BioTherm 25, Singapore); temperature during transportation was tracked with a digital thermometer placed with the samples. 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 4°C and 3.8mL of the plasma collected were used to measure folate, vitamins B12, and D, α -tocopherol and carotenoids (lutein, retinol, lycopene, α -carotene and β -carotene), and minerals (selenium, calcium, magnesium and zinc) levels.

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 chemiluminescent assay after 2010 (both from Immuno Diagnostic Systems Ltd, Boldon, UK). Vitamin B3 (niacin number), was measured with a validated colorimetric enzymatic assay [31, 32]. 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). Peripheral

blood lymphocytes (PBL) were isolated using Ficoll-paqueTM Plus separation medium (Pharmacia Biotech, Uppsala, Sweden), prepared for cryopreservation in fetal bovine serum (FBS) plus 10% DMSO, frozen and stored at -80°C until required. DNA was isolated from peripheral whole blood mononuclear cells using a QIAGEN DNeasy Kit with minor modifications to prevent DNA oxidation, as described by Lu et al. [33]. Absolute TL was measured in duplicate samples by determining the number of TTAGGG hexamer repeats using quantitative real-time PCR (qPCR) as described by O'Callaghan et al. [34]. TL values in kb/diploid genome were calculated using a synthesised TTAGGG 84mer oligonucleotide to generate a standard curve; the number of diploid genome copies per reaction was determined using the single copy gene *36B4*. If the results for duplicate samples differed by more than one Cq value, the results were discarded and the assay repeated. Using our internal control (1301 cell line DNA) we estimated that the inter-experimental variability was $\leq 7\%$ (n=55) and the intra-experimental variability 1.1 % (n=34).

Genotyping was performed at the following loci according to standard protocols: MTHFR C677T (rs 1801133), MTHFR A1298C (rs 1801131), MTR A2756G (rs 1805087), MTRR A66G (rs 1801394), ~~OGG1 Ser326Cys (rs-1052134)~~, XRCC1 Arg399Gln (rs 25487), RFC G80A (rs 1051266), GST Mu, GST Theta, and XRCC3 Thr241Met (rs 861539). These loci were chosen because they had previously been shown to be associated with chromosome instability [35, 36], but only MTHFR C677T had been previously investigated in relation to TL (in adults) [14]. MTHFR, MTR, MTRR and RFC enzymes are required for bioavailability and metabolism of folate and vitamin B12, and the chosen polymorphisms are known to reduce the activity of these enzymes [37, 38]. Thus, genotype may impact the availability of folate for DNA synthesis/repair and SAM for maintenance of DNA methylation, which are essential for genome and telomere integrity [35, 39]. The MTHFR enzyme is required for the conversion of the 5,10 methylenetetrahydrofolate, the folate form required for thymidine

synthesis, to 5-methyltetrahydrofolate, the folate form required for conversion of homocysteine to methionine from which the common methyl donor S-adenosyl methionine (SAM) is synthesised[40]. Synthesis of methionine is catalysed by MTR, which requires vitamin B12 as cofactor in its reduced form which is maintained in this state by the activity of the MTRR enzyme [41]. In addition entry of 5-methyltetrahydrofolate from plasma and interstitial fluids into cells occurs through the activity of RFC, the reduced folate carrier [42]. XRCC1 and XRCC3 are required for the repair of DNA base lesions and DNA single and double strand breaks caused by oxidative stress which lead to telomere dysfunction and attrition [36, 43]. GST Mu and GST Theta are enzymes required for detoxification of genotoxic chemicals by catalysing conjugation with reduced glutathione [39, 44]. The polymorphisms we studied for the XRCC and GST genes involve mutations that reduce the activity of their protein products [39, 43, 44].

Statistical analysis

The following variables were considered as possible predictors of TL: blood micronutrient levels, supplement use, child's BMI z-score, parents' ages at the child's birth, socioeconomic status (parental education, household income), ethnicity, blood cortisol and cotinine levels, environmental exposures (x-rays, immunizations, pesticide exposure, history of fevers, medication use, sunburn, parental smoking), and genotype. We aimed to identify the smallest subset of these variables that still had good predictive ability for TL using multivariate linear regression. Analyses were conducted in SPSS 22 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

As TL is potentially related to age, sex and month of blood collection, these variables were entered into all models. All nutrient, demographic, environmental and genotype variables were then added one at a time to assess the relationship with TL. Nutrient variables with a univariate P -value ≤ 0.10 were assessed for inclusion in a combined model, and for

interactions with genotypes. Genotype main effects or interactions that indicated larger or smaller effect sizes for heterozygotes than both wild-type and variant homozygotes were excluded. All variables or interactions of interest were then added to a combined model and retained if the variable block caused a significant change in R-squared, or (for ordinal variables and bi-allelic genotypes), had a significant trend *P*-value of the coefficient.

RESULTS

Data on blood micronutrient levels and TL were available for 464 children. The age and sex distribution of these children is shown in Table 1, along with other anthropometric and demographic measures of interest. Most children were in the normal height, weight and healthy BMI ranges for age. The distribution of household income and parental education was higher than in the general Australian population; 53% of study children lived in households with an annual income >\$100,000, compared with 13% of Australian households with children [45]; and 71% of children had at least one parent with a tertiary education, compared with 43% of Australian adults [46].

Table 1: Descriptive statistics of participants

Age group	3-year olds	6-year olds	9-year olds	All
N	155	155	154	464
N with TL data	146	147	144	437
N (%) boys	75 (48.4)	86 (55.5)	73 (47.4)	234 (50.0)
Weight: Mean (SD) (kg)	16.1 (2.0)	23.2 (3.7)	33.2 (6.9)	24.2 (8.4)
Height: Mean (SD) (cm)	99.9 (4.3)	121.2 (5.2)	137.9 (8.0)	119.7 (16.7)

BMI: Mean (SD) (kg/m ²)	16.1 (1.2)	15.7 (1.7)	17.4 (3.7)	16.41 (2.6)
BMI z-score: Mean (SD)	-0.39 (0.73)	-0.29 (1.14)	-0.17 (1.27)	-0.28 (1.07)
Cotinine Mean (SD) (ng/ml)	0.81 (0.99)	0.59 (0.75)	0.64 (0.44)	0.68 (0.76)
Cortisol Mean (SD) (ug/dl)	0.38 (0.22)	0.34 (0.20)	0.33 (0.18)	0.35 (0.20)
N(%) child ethnicity European	120 (77.4)	120 (77.4)	118 (76.6)	358 (77.2)
N(%) child ethnicity at least				
50% European	18 (11.6)	15 (9.7)	19 (12.3)	52 (11.2)
N(%) child ethnicity at least				
50% non-European	13 (8.4)	18 (11.6)	13 (8.4)	44 (9.5)
N(%) child ethnicity				
indeterminate	4 (2.6)	2 (1.3)	4 (2.6)	10 (2.2)
N % Parental education some				
high school	6 (3.9)	8 (5.2)	9 (5.8)	23 (5.0)
N (%) Parental education				
complete high school	5 (3.2)	13 (8.4)	17 (11.0)	35 (7.5)
N (%) Parental education trade				
or technical qualification	22 (14.2)	31 (20.0)	25 (16.2)	78 (16.8)
N (%) Parental education				
university/college	122 (78.7)	103 (66.5)	103 (66.9)	328 (70.7)
N(%) household income up to				
\$20,000pa	2 (1.3)	7 (4.5)	5 (3.2)	14 (3.0)
N(%) household income	13 (8.4)	19 (12.3)	22 (14.3)	54 (11.7)

\$20,001 to \$50,000 pa				
N(%) household income				
\$50,001 to \$100,000 pa	52 (33.8)	51 (32.9)	48 (31.2)	151 (32.6)
N(%) household income				
\$100,001 to \$150,000pa	54 (35.1)	43 (27.7)	38 (24.7)	135 (29.2)
N(%) household income over				
\$150,000	33 (21.4)	35 (22.6)	41 (26.6)	109 (23.5)
N(%) Child had X-ray ^a	9 (5.8)	10 (6.5)	17 (11.0)	36 (7.8)
N(%) Child had medications ^a	123 (79.4)	122 (78.7)	111 (72.1)	356 (76.7)
N(%) Professional pesticide				
control in the house ^a	10 (6.5)	17 (11.0)	18 (11.7)	45 (9.7)
N(%) Child had immunization ^a	30 (19.4)	8 (5.2)	4 (2.6)	42 (9.1)
N(%) Child had a fever ^a	67 (43.2)	47 (30.3)	44 (28.6)	158 (34.1)
N(%) Child got sunburnt ^a	3 (2.0)	8 (5.1)	14 (9.1%)	25 (5.4)
Mean (SD) mothers age	32.2 (4.9)	31.1 (5.0)	30.7 (5.5)	31.3 (5.1)
Mean (SD) fathers age	34.9 (5.9)	33.2 (6.0)	33.4 (6.7)	33.8 (6.3)
N(%) Mother smoked ^a	11 (7.1)	13 (8.4)	16 (10.4)	40 (8.6)
N(%) Father smoked ^a	20 (13.3)	20 (13.2)	29 (19.0)	69 (15.2)

^aIn the 4 months before the interview.

Mean values for each of the 16 blood micronutrients investigated varied little by sex or age group (Table 2). Likewise, raw mean values for TL were similar across sexes and age groups.

Table 2: Mean (SD) telomere length and blood micronutrient levels by sex and age

Micronutrient	boys		girls		3-year olds		6-year olds		9-year olds		Overall	
Telomere length (kb/diploid genome)	292.9	(89.6)	294.8	(84.0)	299.8	(90.8)	289.1	(83.4)	292.5	(86.3)	293.8	(86.8)
Zinc (μmol/L)	14.4	(1.9)	14.7	(1.7)	13.9	(1.7)	14.8	(1.9)	15.0	(1.6)	14.6	(1.8)
Calcium, total (mmol/L)	2.3	(0.2)	2.3	(0.1)	2.3	(0.2)	2.3	(0.1)	2.3	(0.1)	2.3	(0.1)
Magnesium (mmol/L)	0.8	(0.1)	0.8	(0.1)	0.8	(0.1)	0.8	(0.1)	0.8	(0.1)	0.8	(0.1)
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)	534.7	(208.8)	554.6	(226.7)	585.8	(258.6)	548.6	(197.2)	499.6	(183.2)	544.6	(217.9)
Plasma Folate (nmol/L)	36.7	(6.8)	36.6	(7.9)	38.7	(6.0)	36.7	(7.4)	34.4	(7.8)	36.6	(7.3)
Red Cell Folate (nmol/L)	830.1	(267.3)	777.7	(294.5)	828.2	(276.3)	827.3	(293.4)	756.9	(272.6)	803.8	(282.2)
Vitamin D (nmol/L)	91.4	(29.1)	85.5	(34.4)	82.1	(25.5)	92.4	(35.6)	90.8	(33.1)	88.5	(32.0)
Selenium (μmol/L)	1.1	(0.1)	1.1	(0.1)	1.1	(0.1)	1.1	(0.1)	1.1	(0.1)	1.1	(0.1)
Niacin Number (NAD/NADP*100)	173.1	(27.2)	181.8	(30.9)	182.6	(28.0)	175.7	(30.4)	173.5	(29.6)	177.6	(29.5)
Lutein (μmol/L)	0.4	(0.2)	0.5	(0.3)	0.4	(0.2)	0.5	(0.2)	0.5	(0.3)	0.4	(0.3)
Retinol (μmol/L)	1.1	(0.2)	1.2	(0.2)	1.2	(0.2)	1.1	(0.3)	1.2	(0.2)	1.2	(0.2)
α-tocopherol (μmol/L)	23.1	(11.8)	24.2	(12.6)	19.9	(9.4)	25.1	(12.5)	25.9	(13.5)	23.7	(12.2)

Lycopene (μmol/L)	0.5	(0.3)	0.5	(0.3)	0.4	(0.2)	0.6	(0.3)	0.6	(0.4)	0.5	(0.3)
α-Carotene (μmol/L)	193.8	(155.5)	198.1	(164.3)	163.8	(129.6)	210.2	(180.0)	213.6	(161.8)	195.9	(159.8)
β-Carotene (μmol/L)	0.5	(0.3)	0.6	(0.4)	0.5	(0.3)	0.6	(0.4)	0.6	(0.4)	0.6	(0.4)

Genotype frequencies for the group as a whole are shown in Supplemental Table 1.

The coefficients of all variables tested for inclusion in the model (with only child's age, sex and month of blood collection also in the model) are shown in Supplemental Table 2. TL was found to be best modelled, in terms of both fit and prediction accuracy, by a linear function of the variables shown in Table 3. Shorter TL was associated with higher plasma zinc (Figure 1), higher parental education and the AA genotype of the RFC G80A polymorphism. A one-standard deviation increase in blood zinc levels was associated with a 11 kb/diploid genome decrease in TL, while the RFC 80AA genotype was associated with a 26 kb/diploid genome decrease relative to RFC 80GG genotype. No significant interactions between zinc and child's age or sex were seen.

Table 3: Regression coefficients for prediction of telomere length

Term:	Estimate	95%CI	<i>P</i> value
Male	0		
Female	1.72	-15.13, 18.56	0.84
Age 3	0		
Age 6	-5.59	-26.75, 15.57	0.60
Age 9	-1.91	-23.44, 19.61	0.86
Month of blood collection	-1.75	-5.08, 1.59	0.30
Plasma zinc (μmol/L)	-6.07	-1.11, -1.03	0.02
Highest parental education some high school	0		
Highest parental education complete high school	-11.93	-60.16, 36.30	0.63
Highest parental education trade certificate	-37.12	-79.29, 5.06	0.08
Highest parental education university/college	-35.93	-74.46, 2.60	0.07

<i>RFC 80GG</i>	0		
<i>RFC 80GA</i>	-13.76	-32.04, 4.51	0.14
<i>RFC 80AA</i>	-25.70	-50.67, -0.74	0.04

Figure 1: Scatterplot of telomere length by plasma zinc level.

DISCUSSION

We have presented results for average blood micronutrient levels and TL in lymphocytes among healthy children aged 3, 6 and 9 years. The results of our multivariable analyses indicated that TL in children was inversely associated with plasma zinc, and shorter in those with the AA genotype for the RFC G80A polymorphism.

Although the association between zinc and TL has been little explored, alteration in zinc homeostasis has been associated with diseases in which telomere shortening is a key characteristic [47-49]. Several studies suggest that zinc deficiency and excess may both be associated with adverse health outcomes [26, 50].

Two recent *in vitro* studies, in human lymphoblastoid and keratinocyte cells showed that biomarkers of chromosomal damage and DNA strand breaks tended to be lowest between 4 and 16 $\mu\text{mol/L}$ zinc, and significantly increased when zinc was deficient; further analysis of TL and base damage in the telomere sequence in this study showed that TL was shorter as zinc concentration increased and that base damage within the telomere was minimised at 4 $\mu\text{mol/L}$ zinc concentration [25] (and unpublished observations). The mechanisms by which increases in zinc concentration may cause telomere shortening are unknown, but could include terminal deletions of chromosomes caused as a result of DNA strand breaks induced in the subtelomere or telomere sequence by reactive oxygen species generated by free labile zinc [26, 51, 52].

The observation that TL was shorter in those with the AA (compared to GG) genotype of the RFC G80A (rs 1051266) polymorphism is of interest because carriage of the A allele is associated with reduced uptake of 5-methyltetrahydrofolate that potentially could render cells prone to folate deficiency (39, 40). Our *in vitro* studies showed that chronic low folate conditions over several weeks can result in telomere attrition and increased frequency of chromosomal instability biomarkers such as micronuclei, an indicator of chromosome breakage or loss [53].

In our study, TL was shorter among children of parents with higher education; the reason for this is not clear. The only other study of this association we identified was conducted in the US and involved 70 white and black US children aged 7-13; it showed that children with at least one college-educated parent had longer telomeres than those whose parents never attended college [28]. This association and inconsistencies between studies warrants further investigation.

Our study is the first to report TL, and to investigate the association between nutrition and TL, among healthy young children. While our findings are consistent with associations seen in adult studies and with known biological pathways, they remain speculative and need to be corroborated. It is possible, for example, that the association we observed between TL and plasma zinc concentration is due to confounding by an unmeasured factor.

The study has strengths and limitations. Blood samples were collected and transported to the laboratory under tightly controlled conditions, and TL was measured using state of the art methods; between-person agreement of TL was high. Biochemical measures of a wide range of micronutrients were available, which precluded the need for reliance on parent recall of children's nutritional intake. The measurement of multiple micronutrients and other factors, and their comprehensive assessment for inclusion in our regression models, enabled us to take account of the possible contributions of a range of individual factors and their interactions.

This novel study was cross-sectional and exploratory in nature, and prospective follow-up of participants would be required to establish causal associations. The recruited families were of relatively high socioeconomic status, and their nutritional status was generally good, so our findings may not be generalizable to the wider population. ~~However, there is no evidence to suggest that the associations we observed would not also occur in children from lower socioeconomic status backgrounds.~~

Investigation into possible associations between TL and various disease outcomes is ongoing, and it is highly probable that factors involved in TL maintenance and other relevant processes make an additional contribution to the determination of disease risk. Thus, although nutritional factors may have an effect on TL, their ultimate impact on disease outcomes through these pathways are currently speculative. Nonetheless, the association we observed between plasma zinc and TL in children provides further insight into the important role that nutritional factors play in telomere maintenance and genome integrity. Our findings are novel and require corroboration in other studies before any nutritional recommendations might be considered.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the study coordinators: Meg McHugh, Sandy Costanzo and Wendy Chan She Ping-Delfos. Theodora Almond and Carolyn Salisbury (both CSIRO) are acknowledged for assisting with blood sample fractionation and storage for micronutrient analyses. Vanessa Russell is thanked and acknowledged for her role in performing the plasma carotenoid analyses

Grant Support

This work was funded by the Australian National Health and Medical Research Council (NH&MRC, Project Grant # 572623).

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