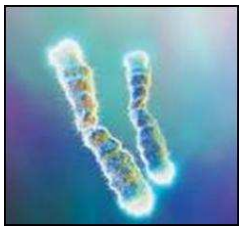

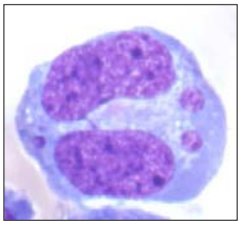

The impact of folate on telomere length and chromosome stability in human WIL2-NS cells and lymphocytes

Caroline Felicity Bull

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THE IMPACT OF FOLATE ON TELOMERE LENGTH AND CHROMOSOME STABILITY IN HUMAN WIL2-NS CELLS AND LYMPHOCYTES

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SUMMARY

Folate is an essential micronutrient required for one-carbon metabolism involved in regulating DNA synthesis, DNA repair and gene expression. Dietary deficiencies in folate result in an increased uracil:thymidine ratio and cytosine hypomethylation in the genome, as well as chromosomal aberrations, the latter being a validated biomarker of cancer risk. Telomeres, the regions of DNA that cap the ends of each chromosome, are critical for maintaining chromosome stability, however, the impact of folate deficiency on telomere structure and function had not previously been investigated. It was hypothesised that the high frequency of thymidine residues in the telomeric repeating hexamer, $(TTAGGG)_n$, may cause this region to be particularly vulnerable to damage caused by folate insufficiency, leading to accelerated telomere attrition if uracil was incorporated into DNA instead of thymidine.

In vitro studies were conducted to test this hypothesis using WIL2-NS cells (a p53 deficient B-lymphoblastoid cell line), cultured in medium containing low, medium or high concentrations of folic acid (FA). A flow cytometric method was used to measure telomere length (TL) at regular time points, and these data were correlated against biomarkers of chromosomal instability (CIN) scored in the cytokinesis-block micronucleus cytome (CBMN-Cyt) assay (micronuclei (MNi), nuclear buds (NBuds) and nucleoplasmic bridges (NPBs)), global hypomethylation and uracil incorporation into telomeric DNA sequences.

Findings in the WIL2-NS model showed a significant decline in TL over the longer term (> 14 days of culture), consistent with the hypothesis. In the short term (< 14 days of culture), however, a significant and rapid increase in TL was recorded in low FA cultures, in a dose-dependent manner. Furthermore, consistent with previous literature, all biomarkers of CIN increased significantly under low FA conditions. As such, the relationship between TL and CIN was found to be significant and positive in the short term, the opposite to that hypothesised, indicating that the generation of cells with longer telomeres by FA deficiency coincided in a greater degree of CIN during this period.

In exploring the mechanism underlying the rapid elongation of telomeres under low FA conditions, new evidence came to light which suggested that hypomethylation of the subtelomere may lead to increased TL. As folate is required for maintenance of DNA methylation, a new hypothesis was then proposed; that hypomethylation due to FA insufficiency results in telomere elongation. This new hypothesis was tested by culturing WIL2-NS cells in complete medium containing a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5azadC). Results showed a significant, rapid increase in TL with increasing

5azadC, verifying that hypomethylation was the likely cause of telomere elongation observed in this cell type and these events also coincided with large increases in CIN biomarkers.

Another novel finding arising from this project was a high frequency of cytokinesis-blocked, binucleated cells displaying multiple NPBs following culture either in low FA, or high 5azadC. New nuclear morphologies, possibly arising from the formation of multiple dicentric chromosomes, were then identified and scored as part of this study. As NPBs can be representative of fusions between chromosomes with compromised telomeres, the high frequencies of these nuclear morphologies suggest that maintenance methylation may play an important role in protecting telomere integrity.

Following on from the WIL2-NS studies, peripheral blood lymphocytes (PBLs) were cultured under FA deficient conditions. Results showed that FA concentration had no impact on TL in this cell type, however, significant increases in biomarkers of CIN were observed. Again, novel nuclear morphologies, possibly due to multiple dicentric chromosome formation, were identified in PBL cells cultured under FA deficient conditions. These findings further suggested that folate deficiency may result in enhanced chromosome fusigenic potential.

A final investigation was conducted to explore the *in vivo* relationship between TL of PBL with plasma folate (PF), vitamin B12 (B12) and homocysteine (Hcy) status, and whether any such relationship was dependent on age, gender, body mass index (BMI) and common polymorphisms in folate metabolism genes. Significant relationships were only observed in the older male subset of the cohort whereby plasma folate was found to be positively associated with shorter TL, while TL and plasma Hcy were inversely associated.

Overall, the findings of these studies demonstrate that FA deficiency *in vitro* impacts telomeres differentially, depending on cell type and cell culture duration, and that the hypomethylating effect of low folate may impact telomere integrity indirectly possibly via hypomethylation or other unexplored mechanisms. The findings that short-term folate deficiency and DNA hypomethylation may lead to telomere elongation, in parallel with a dramatic increase in CIN, and specifically multiple NPBs, has not previously been shown. *In vivo* findings, however, suggest that low folate, and high Hcy, may also have an adverse impact on telomere length, particularly in older males. Most importantly, results of this study show that TL, alone, is probably inadequate and inappropriate as a sole measure of chromosomal instability, and that biomarkers of telomere structure and dysfunction, and possibly subtelomeric DNA methylation, are likely to be of considerably greater value in this context and should be considered for validation in future studies.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Caroline Felicity Bull

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Thanks also to all my friends and family. To Mum and Dad – thank you for showing us that learning isn't about a finish line, but a life long journey - thank you for being interested, for trying really (really) hard to understand what I was talking about, for lots of thesis writing therapy, and for even reading some of it along the way!

Most importantly, though, love and thanks to Justin - for your belief in me, for your belief that it mattered, and for your patience of life being put on hold for a moment. But most of all thank you for your pride, and for being alongside every step throughout this very long journey. It means the most.

ABBREVIATIONS USED IN THIS THESIS

5azadC	5-aza-2'-deoxycytidine
ALT	Alternative lengthening of telomeres
ANOVA	Analysis of variance
APB	ALT-associated promyelocytic leukemia body
APE1	Apurinic/apyrimidinic endonuclease 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
AUC	Area under the curve
AUC LINE1 hypomethylation	AUC for LINE1 hypomethylation with time (days)
AUC MNi	AUC for total number of MNi per 1000 BN cells, with time (days)
AUC NBud	AUC for total number of NBuds per 1000 BN cells, with time (days)
AUC NPB	AUC for total number of NPB per 1000 BN cells, with time (days) (including only cells meeting standard CBMN Cyt scoring criteria)
AUC total NPB (incl CG)	AUC for total number of NPB per 1000 BN cells, with time (days) (including BN cells displaying 'chewing-gum' morphology)
AUC total DNA damage biomarkers	AUC for the total number of DNA dmage biomarkers scored for the CBMN Cyt assay (MNi, NPB, NBuds), per 1000 BN cells, with time (days)
AUC TL	AUC for telomere length with time (days)
B12	Vitamin B12
BER	Base excision repair
BFB	Breakage-fusion-bridge
BN	Binucleated cell
CBMN Cyt	Cytokinesis-block Micronucleus Cytome Assay
CIN	Chromosome instability
CO_2	Carbon dioxide
Cq	Cycle quantitation for quantitative real-time PCR
CVD	Cardiovascular disease
Cyto-B	Cytochalasin-B

DDR	DNA damage response
DHF	Dihydrofolate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dsDNA	Double stranded DNA
DSB	Double-stranded DNA break
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
EDTA	Ethylenediamine tetra-acetic acid
FA	Folic acid
FBS	Foetal bovine serum
FBS-HI	Foetal bovine serum, heat inactivated
FDM	Folate deficient culture medium
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
γH2AX	Histone H2A phosphorylated at serine 139
HBSS	Hank's balanced salt solution
Hcy	Homocysteine
HR	Homologous recombination
hTERT	Human telomerase reverse transcriptase
IL-2	Interleukin-2
Kb	Kilobase
LINE	Long interspersed nuclear element
MN	Micronucleus
MNed	Micronucleated cell
MNi	Micronuclei
mRNA	Messenger RNA
MRN complex	Mre11, RAD50 & NBS1

5-MeC	5-methyl cytosine
5-MeTHF	5-metyl tetrahydrofolate
5,10-MeTHF	5,10-methylene tetrahydrofolate
MTHFR	Methylene tetrahydrofolate reductase
MTR	Methionine synthase
NBud	Nuclear bud
NDI	Nuclear division index
NHEJ	Non-homologous end joining
NPB	Nucleoplasmic bridge
NTD	Neural tube defect
OF	Older female cohort
OM	Older male cohort
PARP	Poly(ADP-ribose) polymerase
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative Real-time PCR
PF	Plasma folate
PHA	Phytohaemagglutinin
PI	Propidium iodide
PML	Promyelocytic leukaemia
PNA	Peptide nucleic acid
POT-1	Protection of Telomeres-1
RAP-1	Repressor/activator Protein
RBC	Red blood cell
RCF	Red cell folate
RDI	Recommended daily intake
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature

SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SCE	Sister chromatid exchange
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
ssDNA	Single stranded DNA
TA	Telomerase activity
TANK	Tankyrase
TelRNA	Telomeric repeat-containing-RNA
THF	Tetrahydrofolate
TI	Telomerase inhibitor
TIF	Telomere-damage induced DNA foci
TIN2	TRF1-interacting protein-2
TL	Telomere length
TPP1	Telosome protein previously referred to as TINT1, PTOP or PIP1
TRF	Telomere restriction fragment, indicating telomere length as measured using the Southern blot method
TRF1 / TRF2	Telomere repeat binding factor 1/2
T-SCE	Sister chromatid exchange involving telomeres
UV	Ultraviolet
YF	Younger female cohort
YM	Younger male cohort

PUBLICATIONS ARISING FROM THIS THESIS

Bull, C & Fenech, M. (2008)
Genome-health nutrigenomics and nutrigenetics: nutritional requirements of 'nutriomes' for chromosomal stability and telomere maintenance at the individual level.
Proceedings of the Nutrition Society, **67,** 146-156.
Impact Factor[1]: 3.981

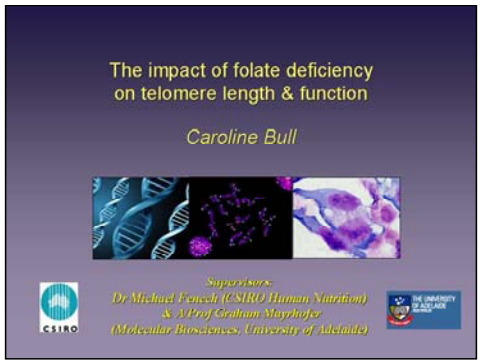
Bull, CF, O'Callaghan, NJ, Mayrhofer, G & Fenech, MF (2009)
Telomere length in lymphocytes of older South Australian men may be inversely associated with plasma homocysteine.
Rejuvenation Research, **12(5),** in press
Impact Factor[1]: 5.008

[1]*Journal impact factors from 2008 ISI Journal Citation Reports*

PRESENTATIONS ARISING FROM THIS THESIS

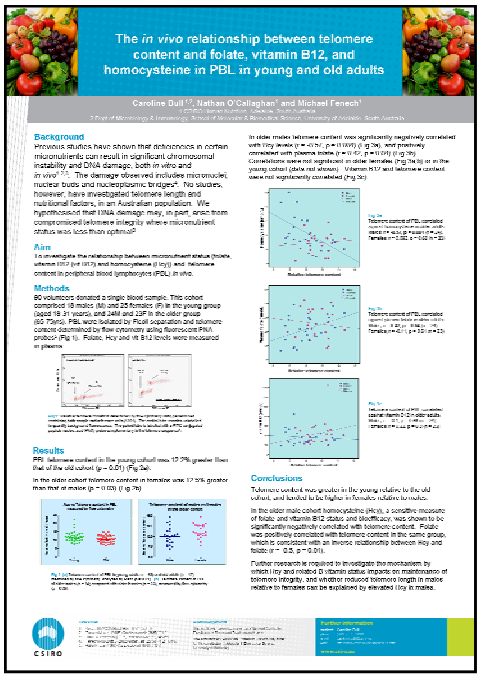
Do micronutrient deficiencies determine the rate of telomere shortening in human cells?

Oral Presentation
5th June, 2005
University of Adelaide, Molecular & Biomedical Sciences Seminar Series
Adelaide, South Australia



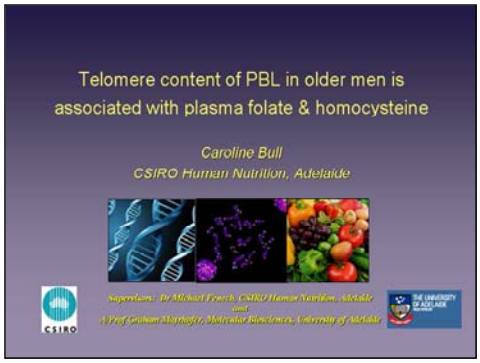
The impact of folate deficiency on telomere length & function

Oral Presentation
2nd June, 2008
Adelaide University, Molecular & Biomedical Sciences Seminar Series
Adelaide, South Australia



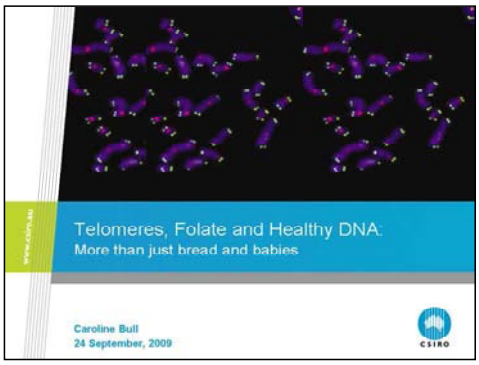
Caroline Bull, Nathan O'Callaghan & Michael Fenech
The in vivo relationship between telomere content and folate, vitamin B12, and homocysteine in PBL in young and old adults

Poster Presentation
15-19th September, 2008
EMBO Conference Series:
"Telomeres and the DNA Damage Response"
Villars-sur-Ollon, Switzerland



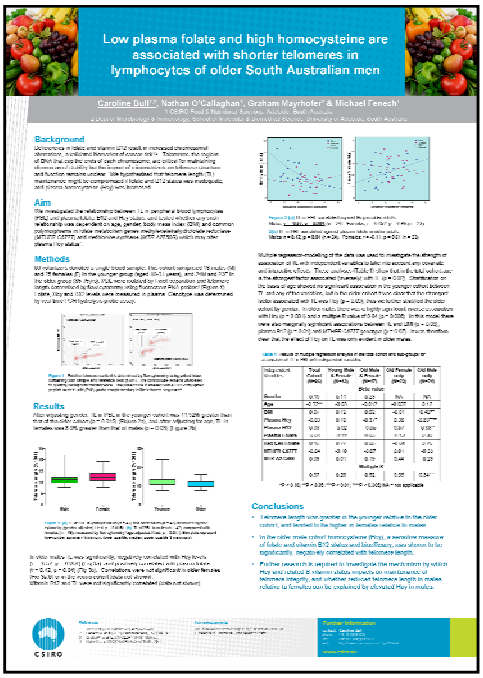
Telomere content of PBL in older men is associated with plasma folate & homocysteine

Oral Presentation
17th October, 2008
Australian Telomere Workshop IV,
Sydney, NSW



Telomeres, folate and healthy DNA: More than just bread and babies

Oral Presentation
24th September, 2009
CSIRO Food & Nutritional Sciences
Adelaide, South Australia



Caroline Bull, Nathan O'Callaghan,
Graham Mayrhofer & Michael Fenech
Low plasma folate and high homocysteine are associated with shorter telomeres in lymphocytes of older South Australian men.

Poster Presentation
9-14th October, 2009
Keystone Symposia:
"Telomere Biology & DNA Repair"
Gold Coast, Queensland, Australia