

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

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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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TABLE OF CONTENTS

SUMMARY	IX
DECLARATION	XI
ACKNOWLEDGEMENTS	XII
ABBREVIATIONS USED IN THIS THESIS	XIII
PUBLICATIONS ARISING FROM THIS THESIS	XVII
PRESENTATIONS ARISING FROM THIS THESIS	XVIII
1 INTRODUCTION	1
1.1 CHROMOSOME INSTABILITY, GENOME DAMAGE AND DISEASE	1
1.1.1 <i>Determination of chromosomal damage using the CBMN Cytome assay</i>	1
1.1.1.1 Micronuclei	2
1.1.1.2 Nuclear Buds	2
1.1.1.3 Nucleoplasmic bridges	4
1.1.1.4 Breakage-fusion-bridge cycles	5
1.2 MICRONUTRIENTS AND CHROMOSOME INSTABILITY	6
1.2.1 <i>Folate insufficiency and disease risk</i>	8
1.2.2 <i>Folate: Recommended daily intake (RDI) and fortification of foods</i>	8
1.2.3 <i>Folate structure, one carbon metabolism and the folate pathway</i>	10
1.2.4 <i>Homocysteine</i>	11
1.2.5 <i>Genetic polymorphisms in the folate cycle</i>	12
1.2.6 <i>Folate insufficiency and chromosome instability</i>	14
1.2.7 <i>Folate insufficiency, uracil incorporation and telomeres</i>	15
1.3 TELOMERES	17
1.3.1 <i>Telomere attrition</i>	17
1.3.2 <i>Telomere structure and capping proteins</i>	20
1.3.3 <i>Maintenance of telomere length; telomerase and the ALT mechanism</i>	23
1.3.4 <i>Telomere length regulation and disease</i>	26
1.3.4.1 Telomere length in malignancies	27
1.3.4.2 Telomere length as a prognostic marker for malignancies	27
1.3.4.3 Telomere-associated genetic disorders	28
1.4 TELOMERES AND THE DNA DAMAGE RESPONSE (DDR)	30
1.4.1 <i>DNA damage checkpoint response</i>	30
1.4.2 <i>DNA damage repair</i>	31
1.4.3 Replicative senescence	32
1.4.4 <i>Proteins of the DDR and telomere homeostasis</i>	33
1.4.5 <i>The impact of telomere position effect (TPE) on DNA repair</i>	34
1.5 DYSFUNCTIONAL TELOMERES INITIATE CHROMOSOME INSTABILITY AND DISEASE	34

1.6	FOLATE DEFICIENCY, DYSFUNCTIONAL TELOMERES AND CHROMOSOME DAMAGE.....	36
1.7	KNOWLEDGE GAPS.....	38
2.	AIMS, HYPOTHESES AND MODELS.....	41
2.1	AIMS & HYPOTHESES	41
2.2	EXPERIMENTAL MODELS.....	41
3	MATERIALS AND METHODS.....	44
3.1	CELLS	44
3.1.1	<i>WIL2-NS human cell line</i>	44
3.1.2	<i>Fresh lymphocytes</i>	44
3.1.2.1	Blood collection.....	44
3.1.2.2	Isolation of mononuclear cells from whole blood	44
3.1.3	<i>1301 human cell line</i>	45
3.2	TISSUE CULTURE.....	45
3.2.1	<i>Media reagents and preparation</i>	45
3.2.1.1	Complete medium and tissue culture conditions for growth of the WIL2-NS cell line .	45
3.2.1.2	Complete medium and tissue culture conditions for fresh lymphocytes	45
3.2.1.3	Complete medium and tissue culture conditions for growth of the 1301 cell line...	46
3.2.1.4	Preparation of folic acid deficient medium.....	46
3.2.2	<i>Cell enumeration by Coulter Counter</i>	47
3.2.3	<i>Estimation of Cell viability by Trypan Blue exclusion</i>	47
3.2.4	<i>Long term cell storage in liquid nitrogen</i>	47
3.2.5	<i>Thawing cells from liquid nitrogen storage</i>	48
3.3	CYTOKINESIS-BLOCK MICRONUCLEUS (CBMN) CYTOME ASSAY.....	48
3.3.1	<i>Overview</i>	48
3.3.2	<i>Lymphocyte culture and addition of Cytochalasin-B</i>	49
3.3.2.1	Preparation of Cytochalasin-B stock and working solutions	50
3.3.2.2	Preparation of phytohaemagglutinin (PHA) stock solution	50
3.3.2.3	Establishment of WIL2-NS cultures for CBMN Cytome Assay	50
3.3.2.4	Establishment of fresh lymphocyte cultures for CBMN Cytome Assay	53
3.3.3	<i>Cell harvest and staining</i>	54
3.3.4	<i>Slide scoring method</i>	54
3.3.5	<i>Calculation of the nuclear division index (NDI)</i>	54
3.3.6	<i>Cell scoring criteria</i>	55
3.3.6.1	Criteria for scoring viable mono-, bi- and multinucleated cells.....	55
3.3.6.2	Criteria for scoring BN cells suitable for scoring markers of chromosomal damage	55
3.3.6.3	Criteria for scoring Apoptotic cells	55
3.3.6.4	Criteria for scoring Necrotic cells.....	56
3.3.6.5	Criteria for scoring Micronuclei (MNI)	56
3.3.6.6	Criteria for scoring Nucleoplasmic bridges (NPBs)	57

3.3.6.7	Criteria for scoring Nuclear buds (NBuds).....	57
3.4	MEASUREMENT OF TELOMERE LENGTH BY FLOW CYTOMETRY	61
3.4.1	Overview.....	61
3.4.2	Peptide nucleic acid (PNA) probe	64
3.4.3	1301 reference cells.....	64
3.4.4	Labelling of telomeres using the FITC-conjugated PNA probe.....	64
3.4.5	Data acquisition by flow cytometry	65
3.4.6	Estimation of relative telomere content of cells.....	66
3.5	MOLECULAR BIOLOGY	68
3.5.1	DNA isolation	68
3.5.2	Assessment of uracil incorporation into telomeric DNA by Quantitative Real-time PCR (qPCR).....	69
3.5.2.1	DNA digestion with uracil glycosylase	69
3.5.2.2	Amplification of telomeric DNA in digested and undigested samples.....	69
3.5.2.3	Analysis of uracil content in telomeric DNA	70
3.5.3	LINE1 assay for determining global hypomethylation	72
3.5.3.1	Overview	72
3.5.3.2	Sodium bisulphite pre-treatment.....	73
3.5.3.3	Standard curve preparation	73
3.5.3.4	Normalisation of samples using ribosomal DNA qPCR.....	73
3.5.3.5	LINE 1 Assay	74
3.5.4	Allelic discrimination: MTHFR and MTR.....	75
3.5.5	Analysis of gene expression	77
3.5.5.1	Isolation of RNA from WIL2-NS cells.....	77
3.5.5.2	cDNA Synthesis from Isolated RNA.....	77
3.5.5.3	Measurement of hTERT Gene Expression by qPCR.....	77
3.6	MICRONUTRIENT ANALYSES IN EXPERIMENTAL MEDIUM AND BLOOD SAMPLES	78
3.6.1	Measurement of L-homocysteine in spent medium and plasma.....	79
3.6.2	Measurement of folic acid in fresh medium, plasma and red blood cells.....	79
3.6.3	Quantification of Vitamin B ₁₂ in plasma.....	81
3.7	STATISTIC ANALYSES.....	81
3.7.1	Power analysis.....	81
3.7.2	Statistic analyses.....	82
3.7.3	Area under the curve abbreviations.....	83
4	THE IMPACT OF FOLIC ACID CONCENTRATION ON TELOMERE LENGTH AND CHROMOSOMAL DAMAGE IN WIL2-NS CELLS IN VITRO	86
4.1	INTRODUCTION	86
4.1.1	Aims	87
4.1.2	Hypotheses.....	87

4.2	EXPERIMENTAL DESIGN	88
4.2.1	<i>Folic acid concentrations and cell growth estimates</i>	89
4.3	RESULTS	91
4.3.1	<i>Cell growth & viability, necrosis and nuclear division index (NDI)</i>	91
4.3.1.1	Short term (21-day) study	91
4.3.1.2	Long term (42 day) study	93
4.3.2	<i>Assessment of telomere length by flow cytometry</i>	95
4.3.2.1	Short term (21 day) study	95
4.3.2.2	Long term (42 day) study	96
4.3.3	<i>Chromosomal damage</i>	101
4.3.3.1	Frequency of BN cells displaying one or more DNA damage biomarker (21 day study).....	101
4.3.3.2	Total number of DNA damage biomarkers per 1000 BN cells (21 day study).....	103
4.3.3.3	Frequency of BN cells displaying one or more DNA damage biomarker (42 day study).....	107
4.3.3.4	Total number of DNA damage biomarkers per 1000 BN cells (42 day study).....	110
4.3.4	<i>Homocysteine in spent medium</i>	114
4.3.5	<i>Effects of FA on levels of expression of telomerase in WIL2-NS cells</i>	116
4.3.5.1	Telomerase Inhibitor study	116
4.3.5.2	hTERT Expression analysis.....	119
4.4	DISCUSSION	121
4.4.1	<i>Impact of FA depletion on telomere length</i>	121
4.4.2	<i>FA depletion and chromosome instability</i>	128
4.5	CONCLUSIONS.....	130
5	THE EFFECT OF DNA METHYLTRANSFERASE INHIBITOR 5-AZA-2'-DEOXYCYTIDINE ON TELOMERE LENGTH AND CHROMOSOMAL DAMAGE IN WIL2-NS CELLS IN VITRO	132
5.1	INTRODUCTION	132
5.1.1	<i>Epigenetics</i>	132
5.1.2	<i>DNA methylation</i>	132
5.1.3	<i>Aberrant DNA methylation and disease</i>	134
5.1.4	<i>Epigenetics and the telomere</i>	135
5.1.5	<i>5-aza-2'-deoxycytidine (5azadC)</i>	136
5.1.6	<i>Aims</i>	138
5.1.7	<i>Hypotheses</i>	138
5.2	EXPERIMENTAL DESIGN	140
5.3	RESULTS	143
5.3.1	<i>Cell growth, viability, nuclear division index (NDI) & necrosis</i>	143
5.3.2	<i>Assessment of telomere length by flow cytometry</i>	145

5.3.3	<i>Chromosome damage</i>	148
5.3.3.1	Frequency of BN cells displaying one or more DNA damage biomarker	148
5.3.3.2	Total number of DNA damage biomarkers per 1000 BN cells.....	150
5.3.3.3	Cells exhibiting multiple NPB (“Chewing-gum” cells).....	155
5.4	DISCUSSION	157
5.4.1	<i>Impact of 5azadC treatment on telomere length</i>	157
5.4.2	<i>5azadC treatment and chromosome instability</i>	158
5.5	CONCLUSIONS.....	161
5.6	FUTURE DIRECTIONS	161
6	THE RELATIONSHIP BETWEEN TELOMERE LENGTH, GLOBAL DNA METHYLATION, URACIL INCORPORATION IN THE TELOMERE AND NUCLEOPLASMIC BRIDGE FORMATION.....	163
6.1	INTRODUCTION	163
6.1.1	<i>Aims</i>	165
6.1.2	<i>Hypotheses</i>	165
6.2	METHODS.....	166
6.2.1	<i>Assessment of global hypomethylation</i>	166
6.2.2	<i>Quantification of uracil in telomeric sequences</i>	167
6.2.3	<i>Frequency of binucleated cells containing multiple NPB</i>	167
6.3	RESULTS	169
6.3.1	<i>Global DNA Methylation</i>	169
6.3.1.1	Long term (42 day) folic acid study.....	169
6.3.1.2	5azadC study.....	169
6.3.2	<i>Uracil incorporation into telomeric DNA</i>	172
6.3.2.1	Long term (42 day) folic acid study.....	172
6.3.2.2	5azadC study.....	172
6.3.3	<i>Frequency of BN cells with multiple NPBs</i>	175
6.3.3.1	Long term (42 day) folic acid study.....	177
6.3.3.2	5azadC study.....	182
6.4	DISCUSSION	187
6.4.1	<i>Global (LINE1) DNA Methylation Status</i>	187
6.4.2	<i>Uracil incorporation into telomeres</i>	188
6.4.3	<i>Frequency of nucleoplasmic bridges (NPBs)</i>	190
6.4.3.1	Altered chromatin & compromised capping may lead to fusions of intact telomeres.....	194
6.4.3.2	Do shortened telomeres have increased fusigenic potential?.....	195
6.4.3.3	Hypomethylation leads to recombination and potential BFB cycling	196
6.4.3.4	Could disruption of the cohesin pathway at telomeres lead to multiple NPBs and BN cells with ‘chewing-gum’ morphology?	197

6.5	CONCLUSIONS.....	201
6.6	FUTURE DIRECTIONS.....	202
7	THE EFFECT OF FOLIC ACID DOSE RESPONSE ON TELOMERE LENGTH AND CHROMOSOMAL DAMAGE IN PERIPHERAL BLOOD LYMPHOCYTES (PBL) <i>IN VITRO</i>	204
7.1	INTRODUCTION.....	204
7.1.1	<i>Aims</i>	205
7.1.2	<i>Hypotheses</i>	205
7.2	EXPERIMENTAL DESIGN.....	206
7.3	RESULTS.....	209
7.3.1	<i>Cell growth, viability, necrosis and nuclear division index (NDI)</i>	209
7.3.2	<i>Assessment of telomere length by flow cytometry</i>	212
7.3.3	<i>Chromosome Damage</i>	215
7.3.3.1	Frequency of BN cells displaying one or more DNA damage biomarker.....	215
7.3.3.2	Total number of DNA damage biomarkers present per 500 BN cells.....	218
7.3.3.3	BN cells with multiple NPB and/or unusual nuclear morphologies.....	222
7.3.4	<i>Homocysteine in spent medium</i>	231
7.4	DISCUSSION.....	232
7.4.1	<i>Telomere content in PBL cultured under FA deficient conditions</i>	232
7.4.2	<i>FA deficiency and biomarkers of chromosome damage</i>	235
7.4.3	<i>The relationship between FA and homocysteine</i>	237
7.4.4	<i>Uracil incorporation into telomeric DNA</i>	238
7.5	CONCLUSIONS.....	239
7.6	FUTURE DIRECTIONS.....	240
8	RELATIONSHIP OF TELOMERE LENGTH IN PERIPHERAL BLOOD LYMPHOCYTES (PBL) WITH AGE, GENDER, BMI, FOLATE, VITAMIN B12 AND HOMOCYSTEINE STATUS <i>IN VIVO</i>	242
8.1	INTRODUCTION.....	242
8.1.1	<i>Aims</i>	243
8.1.2	<i>Hypotheses</i>	243
8.2	EXPERIMENTAL DESIGN.....	244
8.2.1	<i>Study population, blood samples and ethical review</i>	244
8.2.2	<i>Telomere length measurement</i>	244
8.2.3	<i>Micronutrient analyses</i>	245
8.2.4	<i>DNA isolation, MTHFR and MTR genotyping</i>	245
8.2.5	<i>Statistical analyses</i>	246
8.3	RESULTS.....	248
8.3.1	<i>PF, B12, Hcy, RCF, and BMI of cohort</i>	248
8.3.2	<i>Relationship between PF, B12, Hcy and RCF status</i>	248

8.3.3	<i>The relationship between TL, age, gender and BMI</i>	248
8.3.4	<i>Correlation of TL with PF, B12, Hcy and RCF status</i>	251
8.3.5	<i>Impact of MTHFR (C677T) and MTR (A2756G) genotypes on TL in PBL</i>	251
8.3.6	<i>The relationship of TL with independent variables using multiple regression analysis</i> ..	254
8.4	DISCUSSION	256
8.5	CONCLUSIONS.....	260
8.6	FUTURE DIRECTIONS	260
9	GENERAL DISCUSSION, CONCLUSIONS & FUTURE DIRECTIONS	262
9.1	THE IMPACT OF FA DEPLETION ON TELOMERE LENGTH.....	262
9.2	THE RELATIONSHIP BETWEEN FOLATE DEPLETION, TL AND CIN	269
9.3	THE <i>IN VIVO</i> RELATIONSHIP BETWEEN PLASMA FOLATE AND TL	272
9.4	CONCLUSIONS & NEW KNOWLEDGE GENERATED BY THIS THESIS	274
9.5	SIGNIFICANCE.....	275
	REFERENCES	276
	APPENDICES: PAPER REPRINTS	303

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 fusogenic 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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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/aprimidinic 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 damage 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 <i>in situ</i> hybridisation
FITC	Fluorescein isothiocyanate
γ H2AX	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-methyl 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 Factor¹: 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 Factor¹: 5.008

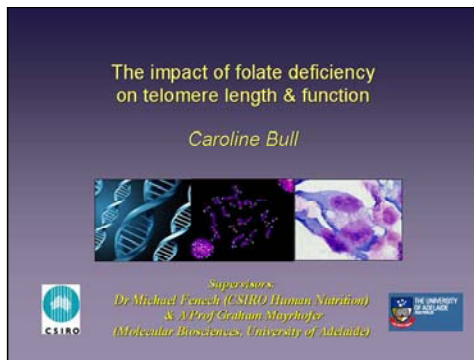
¹Journal impact factors from 2008 ISI Journal Citation Reports

PRESENTATIONS ARISING FROM THIS THESIS



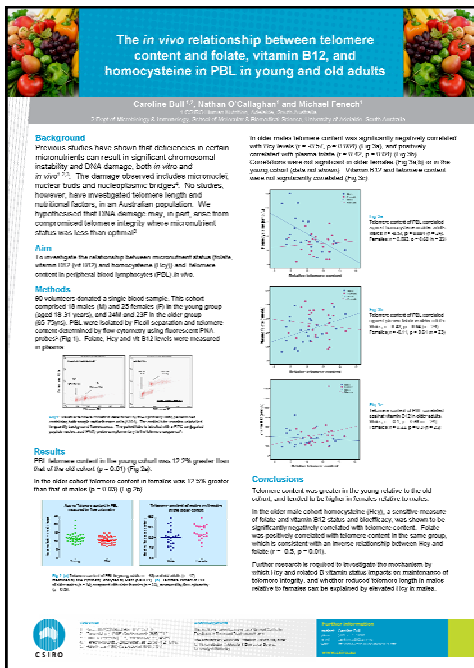
Do micronutrient deficiencies determine the rate of telomere shortening in human cells?

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



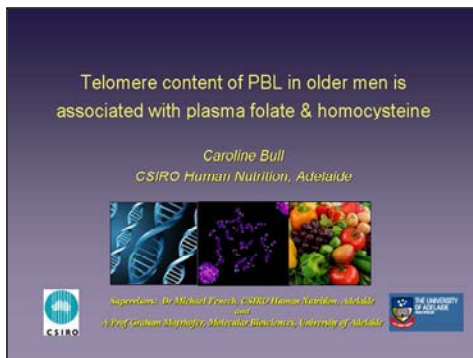
The impact of folate deficiency on telomere length & function

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



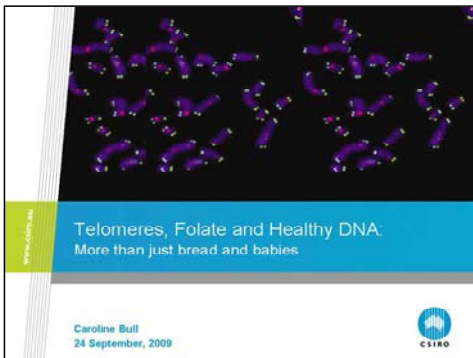
Caroline Bull, Nathan O'Callaghan & Michael Fenech
The in vivo relationship between telomere content and folate, vitamin B12, and homocysteine in PBL in young and old adults

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



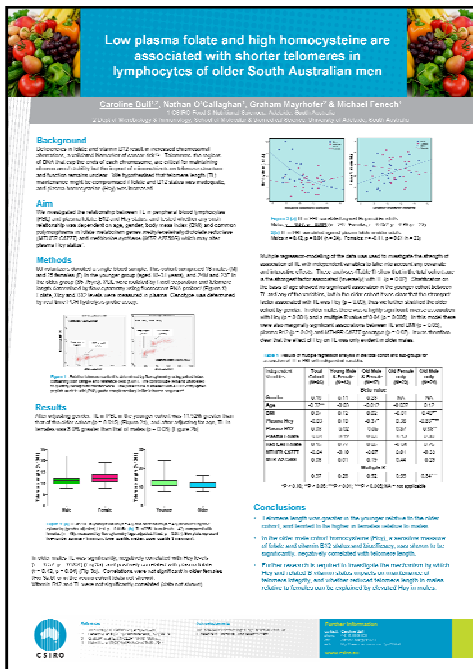
Telomere content of PBL in older men is associated with plasma folate & homocysteine

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