



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

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**CHAPTER 7:
THE EFFECT OF FOLIC ACID ON TELOMERE
LENGTH AND CHROMOSOMAL DAMAGE IN
CULTURED HUMAN PERIPHERAL BLOOD
LYMPHOCYTES**

7 THE EFFECT OF FOLIC ACID ON TELOMERE LENGTH AND CHROMOSOMAL DAMAGE IN CULTURED HUMAN PERIPHERAL BLOOD LYMPHOCYTES (PBL)

7.1 INTRODUCTION

Genome instability and telomere shortening have been associated consistently with higher risk for important degenerative diseases including immune dysfunction³⁷⁵, cardiovascular disease³⁷⁶, neurodegenerative conditions⁴⁰ and cancers^{2,16,56,167,377}. Previous studies, both *in vivo* and *in vitro* have confirmed the importance of dietary micronutrients, including folic acid (FA), in maintaining genomic stability for disease prevention^{35,56}. In culture human peripheral blood lymphocytes (PBL) show an inverse, dose-dependent correlation between concentration of FA (within the physiological range) and increased chromosomal damage biomarkers (MNI, NBuds and NPBs)^{37,93,95}. Published studies used experimental concentrations of FA in medium ranged between 12nM and 240nM, and in all studies markers of genome damage were shown to be minimised at FA concentrations greater than 60nM, a level greater than that observed in plasma in the general population (<30nM)^{28,37,54,98,378}. While these *in vitro* studies using PBL have examined the relationship between FA and DNA damage biomarkers, its effect on telomeres has not previously been reported.

The effect of FA-deficiency on telomeric DNA has not previously been explored *in vitro* in PBL. Accordingly, the purpose of this study was to determine the effect of FA dose response on TL and chromosome stability in PBL, and to compare these effects to those observed in WIL2-NS cells in earlier experiments.

7.1.1 Aims

1. To determine the effect of folic acid (FA) concentration, within the physiological range, on telomere length (TL) in peripheral blood lymphocytes (PBL) over 21 days *in vitro*.
2. To determine whether FA insufficiency impacts on chromosomal instability (CIN), assessed using the CBMN Cytome assay, in PBL cultured *in vitro* over 21 days, and to explore whether a relationship exists between TL and CIN.

7.1.2 Hypotheses

1. FA insufficiency causes telomere elongation in the short term, followed by shortening in the longer term, in PBL.
2. FA insufficiency leads to increased CIN in PBL, in a dose dependent manner.
3. Increased levels of CIN are negatively associated with TL over 21 days of culture in folate deficient or replete conditions.

7.2 EXPERIMENTAL DESIGN

Freshly isolated peripheral blood lymphocytes (PBL) were grown in bulk cultures at different FA concentrations within the physiological range (20, 60 and 180nM). Multiple samples were collected for biomarker and cell count measurements at different time points (Figure 7.1).

117ml of blood was collected from a single volunteer in 13 x 9ml EDTA vacutainer collection tubes (Chapter 3.1.2.1), yielding 200×10^6 lymphocytes following isolation. The blood donor was a 44 year old Caucasian male. Day 0 measures of plasma folate, vitamin B12 (B12) and homocysteine (Hcy) were analysed as per Chapter 3.7. Plasma folate concentration was 33.9nmol/L (reference interval 6.5 – 45.0), and B12 was 359pmol/L (reference interval 100–700), indicating the donor was not deficient for these nutrients. Fasted plasma Hcy concentration was 6.2 μ mol/L, also in the normal reference range of 4.0 – 14.0 μ mol/L. The donor was genotyped by allelic discrimination for methylene tetrahydrofolate reductase *MTHFR C677T* polymorphism, and found to be heterozygous, CT.

Peripheral blood lymphocytes (PBL) were isolated from a fresh blood sample using Ficoll-paqueTM (Chapter 3.1.2.2). Isolated cells were immediately transferred into pre-warmed, complete medium containing 20, 60 or 180nM FA where they were maintained for 21 days. Folate deficient treatment medium, supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% L-Glutamine, was prepared as described in Chapter 3.2.1.4. Five percent interleukin-2 (IL2) was included in all media treatments to sustain lymphocyte growth, and 1.3 μ l/ml PHA (22.5mg/ml) was added on a single occasion, Day 0, to activate T cells (Chapter 3.2.2.2). All cultures were seeded at a concentration of 0.5×10^6 viable cells/ml and maintained at 40ml total volume in a 75cm² vented-cap culture flask. Pilot studies showed that a smaller culture surface area encouraged more robust growth, and as such all flasks were incubated in an upright position. Fresh media was replaced twice weekly, incorporating five percent conditioned medium (spent medium obtained from the previous flask following centrifugation), at each subculture.

Cells were sampled at days 0, 3, 7, 10, 14, 17 and 21 for cell growth and viability (Coulter Counter and Trypan blue exclusion, Chapters 3.2.2 and 3.2.3). Total growth in each condition was calculated by counting the numbers of viable cells in the culture at the time of harvesting and extrapolating to the numbers that would have been expected if all cells had been re-seeded at each split. Cells were sampled at days 0, 7, 14, 17 and 21 for telomere length (TL) measurement by flow cytometry (Chapter 3.4), and days 7, 14 and 21 for chromosomal damage markers (CBMN Cytome assay, Chapter 3.3). Spent medium was assessed for Hcy

concentration at Day 14 (Chapter 3.7.1). Cells were frozen at each sample point and stored in liquid nitrogen (Chapter 3.2.4). DNA was later isolated from thawed samples (Chapter 3.2.5) for uracil incorporation (Chapter 3.5.2) by qPCR.

It should be noted that the scoring criteria for the CBMN Cyt assay were amended and expanded during the scoring process for this experiment to incorporate new morphologies observed in these cells (see Chapter 7.3.3.1). As such, it was necessary to re-score all slides to accommodate the changed criteria. Due to time limitations the number of BN scored per slide was reduced from 1000 (as presented in Chapters 4 and 5) to 500 BN per slide. This number was selected as a compromise to achieve statistically meaningful data within the required time frame. Duplicate slides were scored for each treatment at each time point.

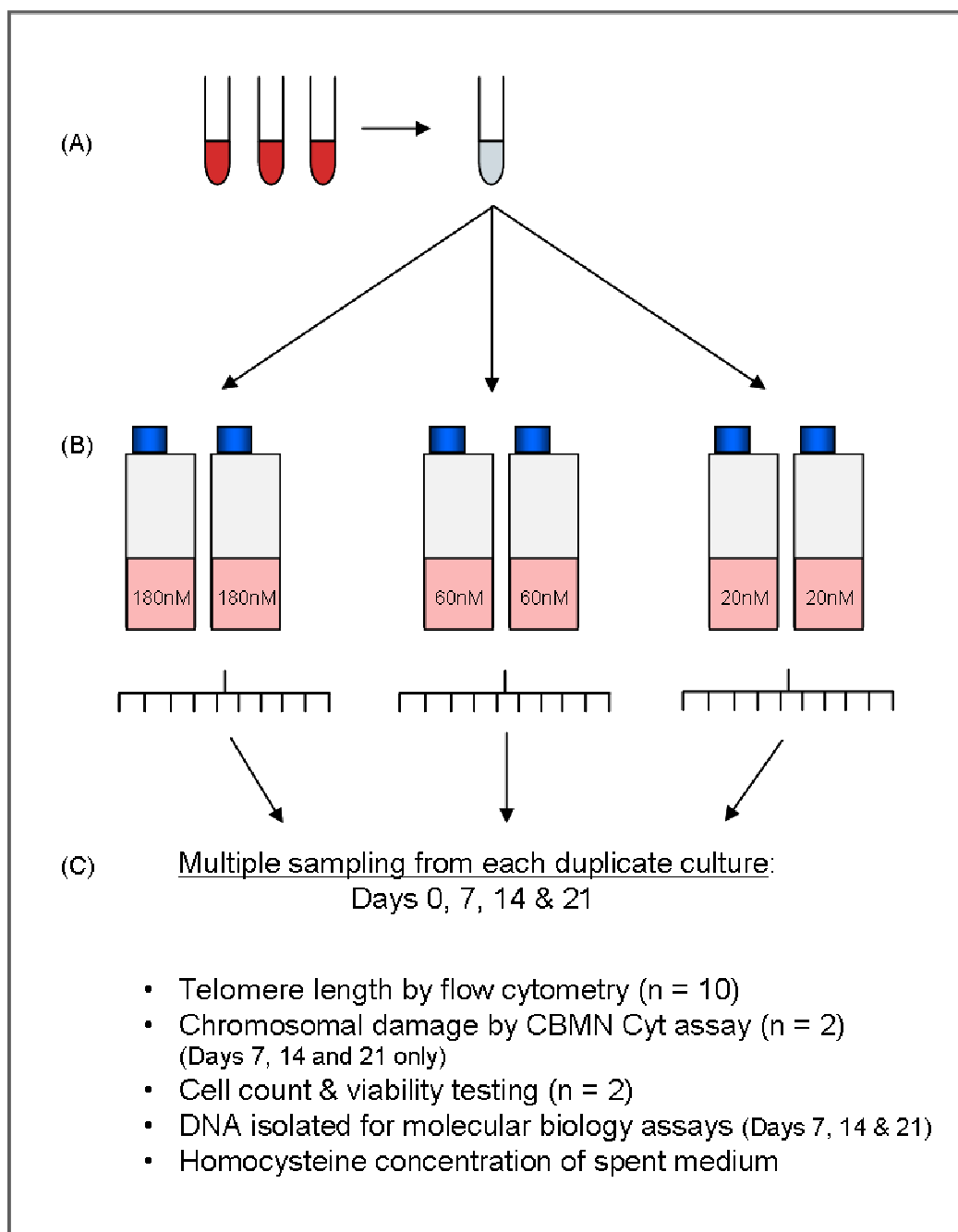


Figure 7.1 Overview of experimental design for FA dose response study in peripheral blood lymphocytes (PBL) *in vitro*. (A) A venous blood sample was obtained from an adult male. PBL were isolated from the fresh blood sample by Ficoll-paque™ gradient separation prior to (B) splitting into complete medium containing either 20, 60 or 180nM FA. Each treatment was cultured upright, in duplicate flasks. (C) Multiple samplings were conducted from each culture and assays conducted as specified.

7.3 RESULTS

7.3.1 Cell growth, viability, necrosis and nuclear division index (NDI)

The increase in cell numbers was strong in both the 180 and 60nM conditions up to day 14. Cells grown in the 60nM treatment did not increase beyond day 14. The rate of growth for cells cultured in the 20nM FA was significantly reduced beyond 7 days. FA treatment explained 16.6% of observed variance ($p < 0.0001$) and time explained 35.2% ($p < 0.0001$). 39.6% of variance was attributable to the interaction of both factors ($p < 0.0001$). Bonferroni post-test indicated significant differences in growth at day 17 between 180nM and 60nM compared with 20nM, and at day 21 TL in the 60nM and 20nM conditions differed significantly from those in 180nM FA (Figure 7.2A).

Cell viability above 80% was observed for all groups for the first 7 days, but beyond this point viability of cells in 20nM FA reduced steadily to 23% by Day 21. Cells cultured in 60nM FA maintained approximately 80% viability until day 21 where viability dropped to 64%, while the cells in the FA-replete (180nM) concentration showed strong, consistent viability (over 90%) for the duration of the experiment (Figure 7.2B). When analysed by two-way ANOVA, 31.7% of variance was found to be due to FA concentration ($p < 0.0001$). The impact of time was responsible for 31.6% of variance ($p < 0.0001$), and 34.9% of variance was attributable to interaction of FA concentration with time ($p < 0.0001$).

Nuclear division index (NDI) was also affected significantly by the interaction of FA concentration with time explaining 6.6% of the variance ($p < 0.0001$). FA concentration alone was responsible for 40.3% of variance ($p < 0.0001$) with a dose-dependent decline in NDI observed as FA concentration was reduced. Time alone explained 52.8% of the variance ($p < 0.0001$) (Figure 7.3A).

The percentage of cells with morphological evidence of necrosis showed an inverse relationship to FA concentration, with 25.8 of the variance due to interaction of FA and time ($p < 0.0001$). FA alone was responsible for 49.4% of variance ($p < 0.0001$), and time alone explained 24.5% of variance ($p < 0.0001$) (Figure 7.3B).

The percentage of apoptotic cells also showed an inverse relationship to FA concentration, with 25.8% of the variance due to interaction of FA and time ($p = 0.05$). FA alone was responsible for 21.7% of variance ($p = 0.02$), and time alone explained 36.5% of variance ($p = 0.005$) (Figure 7.3C).

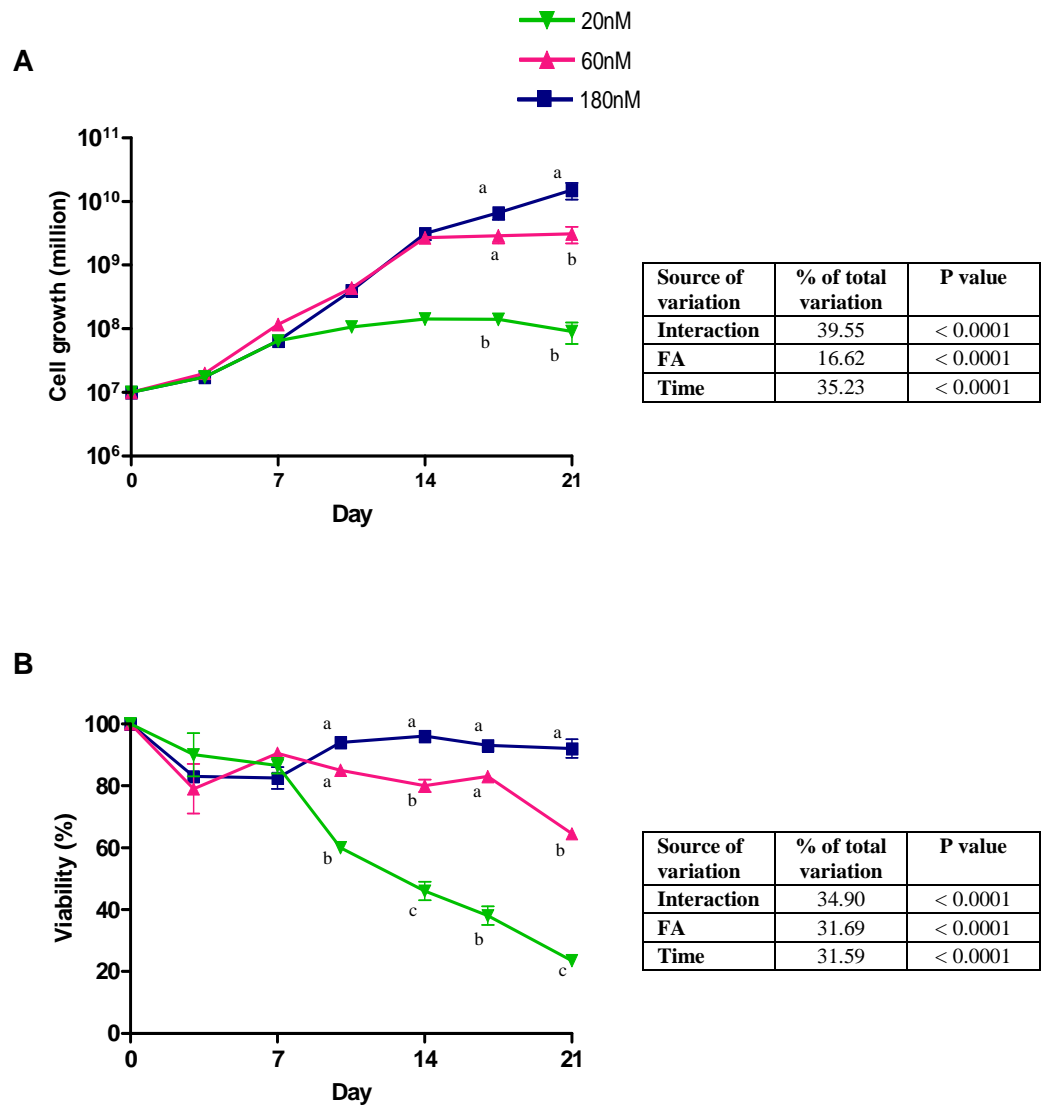


FIGURE 7.2 The effects of folic acid (FA) deficiency on cell growth and viability of PBL grown in medium containing 20, 60 or 180nM FA over 21 days. (A) Cell growth (calculated as described in 7.2; (B) Cell viability (%). (N = 2 for each treatment at each time point. Error bars indicate SD. Data tables represent two-way ANOVA analyses. Points not sharing the same letter at each time point differ significantly from each other, as measured by Bonferroni post test).

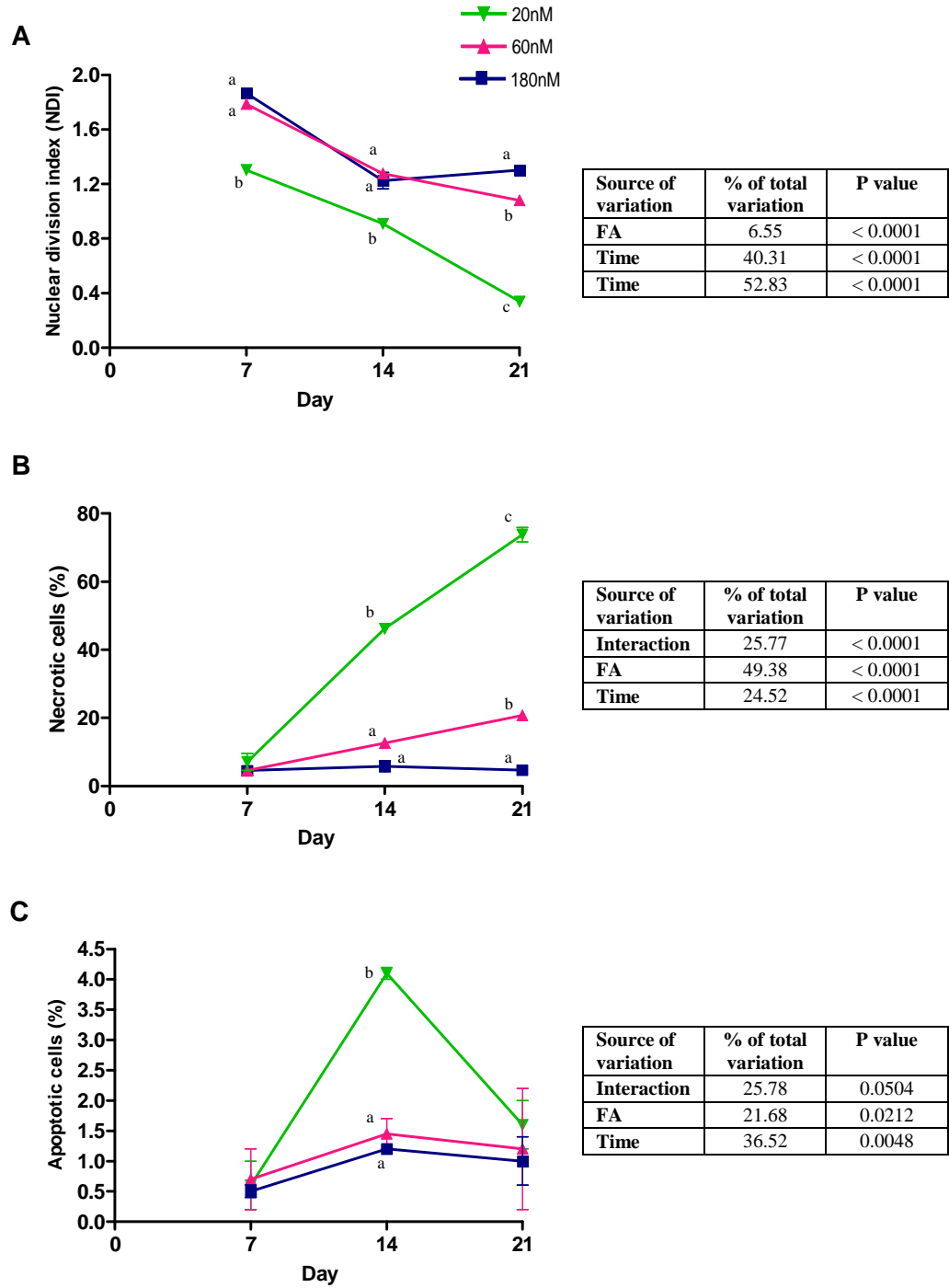


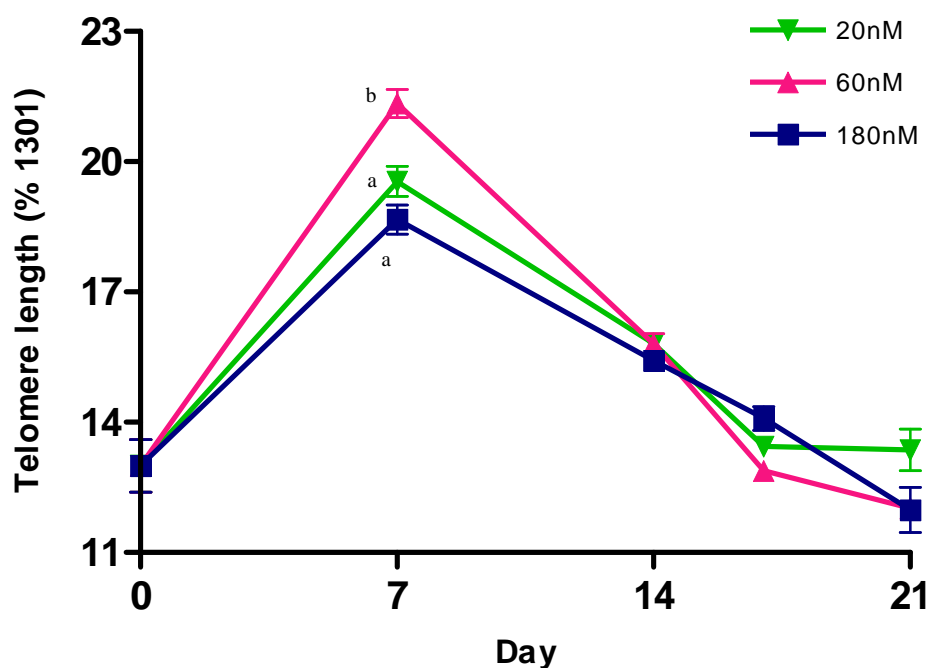
FIGURE 7.3 The effects of folic acid (FA) deficiency on nuclear division index (NDI), necrosis and apoptosis in PBL grown in medium containing 20, 60 or 180nM FA over 21 days. (A) Nuclear division index (NDI); (B) necrotic cells (%); (C) apoptotic cells (%). (N = 2 for each treatment at each time point. Error bars indicate SD. Data tables represent two-way ANOVA analyses. Points not sharing the same letter at each time point differ significantly different from each other, as measured by Bonferroni post test).

7.3.2 Assessment of telomere length by flow cytometry

Flow cytometry results showed an increase in telomere length for all 3 treatments from day 0 to day 7, followed by a reduction back to day 0 levels by day 17 (Figure 7.4). Two-way ANOVA analysis indicated FA concentration in medium was responsible for only 0.4% of variance ($p = 0.18$). 81.4% of variance was attributable to time ($p < 0.0001$), and interaction of time and FA concentration together explained 3.9% of variance ($p < 0.0001$).

At day 7, TL of cells cultured in 60nM FA (21.3 ± 1.0) was 13% greater than that of cells cultured in 180nM FA (18.7 ± 1.1) (Bonferroni post test; $p < 0.001$). The TL of these cells was also 9% greater than those cultured in 20nM FA (19.6 ± 1.1) ($p < 0.01$), while the TL of cells in 20nM was only 4.5% greater than cells in 180nM (not significant).

By day 21, following a sharp decline in TL in all conditions, TL in cells grown in 20nM FA (13.4 ± 1.5) was greater than that of cells grown in 60nM (12.0 ± 0.3) or 180nM FA (11.9 ± 1.6) (ANOVA $p = 0.04$). The area under the curve for telomere length with time (AUC TL) over the 21 day experimental time frame was greater in the 20nM (335.2) and 60nM FA (343.1) cultures relative to the 180nM (326.6) cultures. These results are shown in detail in Table 7.1.



Source of variation	% of total variation	P value
Interaction	3.88	< 0.0001
FA	0.37	0.1796
Time	81.38	< 0.0001

Figure 7.4 Telomere length (TL) measured by flow cytometry in PBL cultured in medium containing 20, 60 or 180nM FA over 21 days. (N = 20 at day 0, n = 10 for all time points thereafter. Error bars indicate SD. TL of sample was calculated relative to the TL of standard 1301 cell line. Data table represents analysis by two-way ANOVA. Points not sharing the same letter at each time point differ significantly, as measured by Bonferroni post test).

Table 7.1 Telomere length (TL) measured by flow cytometry in PBL cultured in medium containing 20, 60 or 180nM FA over 21 days. PBL were grown in medium containing 20, 60 or 180nM FA over 21 days. (N = 20 at day 0, n = 10 per treatment per time point thereafter). Data represents mean \pm SD. P values represent analysis by one-way ANOVA; points not sharing the same letter at each time point differ significantly, as measured by Tukey's post test. Also shown is area under the curve calculated for TL versus time for each treatment (AUC TL), over the 21-day course of the experiment.

<i>Day</i>	<i>20nM</i>	<i>60nM</i>	<i>180nM</i>	<i>One-way ANOVA P value</i>
<i>0</i>	13.0 \pm 1.9	13.0 \pm 1.9	13.0 \pm 1.9	-
<i>7</i>	19.55 \pm 1.1 ^a	21.3 \pm 1.0 ^b	18.7 \pm 1.1 ^a	< 0.0001
<i>14</i>	15.8 \pm 0.5	15.8 \pm 0.7	15.4 \pm 0.5	0.2829
<i>17</i>	13.4 \pm 0.5 ^{ab}	12.9 \pm 0.5 ^a	14.1 \pm 0.9 ^b	0.0009
<i>21</i>	13.4 \pm 1.5	12.0 \pm 0.3	11.9 \pm 1.6	0.0395
<i>AUC TL</i>	335.2	343.1	326.6	

7.3.3 Chromosome Damage

7.3.3.1 Frequency of BN cells displaying one or more DNA damage biomarker

The results in this section, and in Table 7.2, show the frequency of BN cells that display one or more of the biomarkers of DNA damage examined in the CBMN Cyt assay (MNi, NPB, NBuds), per 500 BN cells. Each BN cell containing a damage event, irrespective as to whether it contains one or multiple damage biomarkers, is recorded as a single event in this data. It should be noted that while newly defined DNA damage morphologies were identified in the cells scored in this study, the data presented in this, and the subsequent section (7.3.3.1 and 7.3.3.2) relate only to BN cells and biomarkers meeting the standard scoring criteria for the CBMN Cytome assay¹⁴. Data for the new morphologies are presented separately in Section 7.3.3.3.

Representative photomicrographs of the increasing levels of chromosomal aberrations, including new morphologies, over the 21 day time period are shown at Figure 7.9.

FA concentration in medium was shown to be responsible for 54.8% of the variance in the frequency of BN cells with one or more MNi ($p < 0.0001$). At day 21 the frequency in the 20nM treatment was 700% higher (mean \pm SD; 35 ± 8.5) than in cells cultured in 180nM FA at the same time point (5 ± 3.5). Cells grown in 60nM FA displayed 530% greater frequency (26.5 ± 0.7) compared with those grown in 180nM FA (Table 7.2A).

The frequency of BN cells displaying one or more NPB at day 21 was 33.5 ± 5 , 12.5 ± 1 and 5 ± 4.2 for the 20nM, 60nM and 180nM FA conditions, respectively. These values represent an increase of 670% in the 20nM culture, and an increase of 250% in the 60nM culture, compared with cells grown in the FA replete (180nM) condition at the same time point. Analysis by two-way ANOVA showed that 45.79% of variance in the frequency of BN with one or more NPB was attributable to FA concentration in medium ($p = 0.001$) (Table 7.2B).

The difference between treatments for the frequency of cells containing one or more NBud was also significant, with 21.6% of variance being attributable to FA concentration in culture medium ($p = 0.001$) (Table 7.2C). At day 21 the frequency of BN cells with one or more NBud was 66.5 ± 19 , 29 ± 8.5 and 5.5 ± 2 for the 20nM, 60nM and 180nM treatments, respectively (Table 7.2C). The difference in the frequency of NBuds at this time point between the low FA (20nM) and the FA replete medium (180nM) was 1200%.

At day 21 an 840% greater frequency was recorded for BN cells exhibiting one or more of any of the DNA damage biomarkers (MN or NPB or NBud) in the cells grown in 20nM FA, compared to those grown in 180nM FA. Analysis by two-way ANOVA indicated 38% of observed variance was attributable to FA treatment ($p < 0.0001$), 34.2% to time ($p < 0.0001$), and 23.2% of the variance was explained by the interaction of FA concentration with time ($p = 0.001$) (Table 7.2D).

TABLE 7.2 Frequency, in the binucleated (BN) subset of PBL, of cells displaying one or more of the chromosomal damage biomarkers scored in the CBMN Cyt assay following culture in medium containing 20, 60 or 180nM folic acid (FA) over 21 days. Frequency per 500 BN PBL of cells that contained (A) one or more micronuclei (MNi), (B) one or more nucleoplasmic bridge (NPB), (C) one or more nuclear bud (NBud), and (D) one or more of any damage biomarker (MN or NPB or NBud). Data is shown for each FA concentration and for each time point. (N = 2. Mean \pm SD. P values represent analysis by two-way ANOVA. Data not sharing the same superscript letter within each time point differ significantly from each other, as measured by the Bonferroni post test. NPB data relate only to those meeting standard CBMN Cyt scoring criteria).

(A)	Time	[FA] in medium (nmol/L)			
		20nM	60nM	180nM	
BN cells with one or more MNi	Day 7	21.5 \pm 2.1 ^a	5.0 \pm 2.8 ^b	8.5 \pm 0.7 ^b	Effect of time p = 0.0023
	Day 14	20.0 \pm 4.2 ^a	16.5 \pm 3.5 ^{ab}	6.0 \pm 1.4 ^b	
	Day 21	35.0 \pm 8.5 ^a	26.5 \pm 0.7 ^a	5.0 \pm 3.5 ^b	
			Effect of FA, p < 0.0001	Interaction, p = 0.01	
(B)	Time	[FA] in medium (nmol/L)			
		20nM	60nM	180nM	
BN cells with one or more NPB	Day 7	11.5 \pm 2.1	6.5 \pm 0.7	4.5 \pm 0.7	Effect of time p 0.0237
	Day 14	17.0 \pm 2.8	5.5 \pm 3.5	10.5 \pm 12.0	
	Day 21	33.5 \pm 4.9 ^a	12.5 \pm 0.7 ^b	5.0 \pm 4.2 ^b	
			Effect of FA, p = 0.0014	Interaction, p = 0.0485	
(C)	Time	[FA] in medium (nmol/L)			
		20nM	60nM	180nM	
BN cells with one or more NBud	Day 7	5.0 \pm 2.8	3.0 \pm 4.2	0.5 \pm 0.7	Effect of time p = 0.0001
	Day 14	9.5 \pm 3.5	6.0 \pm 4.2	5.0 \pm 2.8	
	Day 21	66.5 \pm 19.1 ^a	29.0 \pm 8.5 ^b	5.5 \pm 2.1 ^c	
			Effect of FA, p = 0.0014	Interaction, p = 0.0026	
(D)	Time	[FA] in medium (nmol/L)			
		20nM	60nM	180nM	
BN cells with one or more DNA damage biomarker	Day 7	38.0 \pm 2.8	14.5 \pm 6.4	13.5 \pm 0.7	Effect of time p < 0.0001
	Day 14	46.5 \pm 10.6	28.0 \pm 11.3	21.5 \pm 13.4	
	Day 21	135.0 \pm 22.6 ^a	68.0 \pm 8.5 ^b	16.0 \pm 9.9 ^c	
			Effect of FA, p < 0.0001	Interaction, p = 0.0013	

7.3.3.2 Total number of DNA damage biomarkers present per 500 BN cells

Data presented in 7.3.3.1 above represents the frequency of BN cells displaying one or more DNA damage biomarker. Many BN cells, however, display multiple DNA damage biomarkers, but these additional damage events are not reflected in the ‘frequency’ data. Accordingly, data in this section, and in Figure 7.5, represent the actual numbers of MNi, NBuds and NPBs present per 500 BN cells in each FA culture condition for each time point, individually and combined.

ANOVA analysis indicated that FA deficiency was responsible for 53.3% of variance in the total number of MNi per 500 BN cells ($p = 0.001$), 17.6% was due to time ($p = 0.03$), and 14% of observed variance was due to the interaction of FA with time ($p = 0.16$) (Figure 7.5A). The total number of NPB present (scored using the standard criteria) also increased in low FA conditions, with 46.6% of the overall variance due to FA concentration ($p = 0.002$), 14% being attributable to time ($p = 0.05$), and 24.8% to the interaction of both factors ($p = 0.04$) (Figure 7.5B).

Up to 14 days, the total number of NBuds per 500 BN cells did not differ significantly between FA conditions, however between days 14 and 21 the numbers increased considerably in cells cultured in 20nM and 60nM FA. ANOVA analysis showed that the concentration of FA in medium explained 21.6% of the overall variance observed ($p = 0.001$), while 43.8% was due to time ($p = 0.0001$), and 28.1% of the variance was attributable to the interaction of FA concentration with time (Figure 7.5C).

ANOVA analysis of all DNA damage events combined (MNi plus NPB plus NBuds) per 500 BN cells showed that 43.8% of variance was attributable to FA concentration ($p < 0.0001$), 28.6% to time ($p = 0.0003$) and 21.9% to the interaction of FA with time ($p = 0.003$) (Figure 7.5D).

The relationship between FA concentration in culture medium and the total number of DNA damage biomarkers per 500 BN cells was explored using the area under the curve obtained from Figure 7.5. Strong negative relationships were observed between FA concentration and AUC for each DNA damage biomarker with time; AUC MNi (pearson’s $r = -0.95$, $p = 0.003$), AUC NPB ($r = -0.65$, $p = 0.16$ (ns)), AUC NBuds ($r = -0.91$, $p = 0.01$) (Table 7.3A). FA

concentration correlated with AUC total DNA damage biomarkers combined (total MNi plus NBuds plus NPB) showed a pearson's r value of -0.86 ($p = 0.02$) (Table 7.3A, Figure 7.6).

The relationship between AUC DNA damage biomarkers (individually and collectively) was also examined with AUC TL (data obtained from Figure 7.4), however, no statistically significant associations were observed (Table 7.3B). AUC TL versus AUC total DNA damage biomarkers combined showed an r value of 0.3 ($p = 0.57$) (Table 7.3B).

Overall, these results indicated that MNi, NPB and NBuds increased in cells cultured in conditions of FA deficiency, however these biomarkers are not significantly related to TL in PBL.

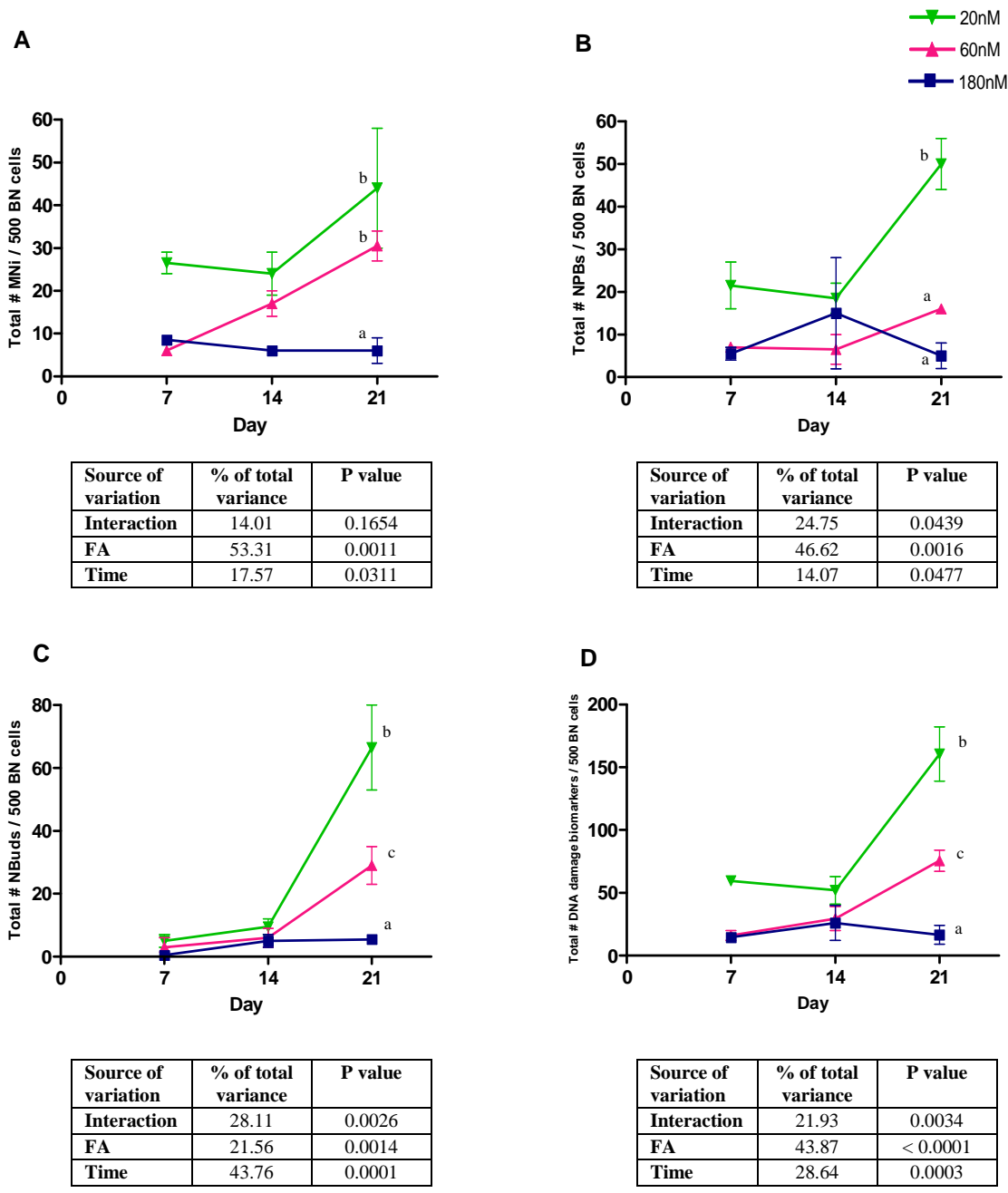


Figure 7.5 Chromosomal damage in PBL grown in medium containing either 20, 60 or 180nM folic acid (FA) for 21 days, measured by the CBMN Cyt assay. Data represent the total number of DNA damage biomarkers present per 500 BN cells. (A) Total micronuclei (MNi); (B) Total nucleoplasmic bridges (NPB); (C) Total nuclear buds (NBud); and (D) Total number of DNA damage biomarkers (MN plus NPB plus NBuds). (N = 2. Error bars indicate SD. Groups not sharing the same letter at each time point differ significantly from each other, as measured by the Bonferroni post-test).

Table 7.3 Correlations between biomarkers of DNA damage, FA concentration in medium and telomere length. Relationships between area under the curve (AUC) MNi, AUC NPB and AUC NBuds (individually and combined) per 500 BN PBL, and (A) FA concentration in medium; and (B) AUC telomere length with time (AUC TL). AUC data for DNA damage biomarkers were obtained from Figure 7.5. AUC TL data were obtained from Figure 7.4.

(A) [FA] versus:	Pearson's r	P value
AUC MNi	-0.9535	0.0032
AUC NPB	-0.6523	0.1604
AUC NBud	-0.9117	0.0114
AUC total DNA damage biomarkers	-0.8630	0.0269

(B) AUC TL versus:	Pearson's r	P value
AUC MNi	0.4996	0.3130
AUC NPB	-0.0311	0.9534
AUC NBud	0.3946	0.4388
AUC total DNA damage biomarkers	0.2950	0.5703

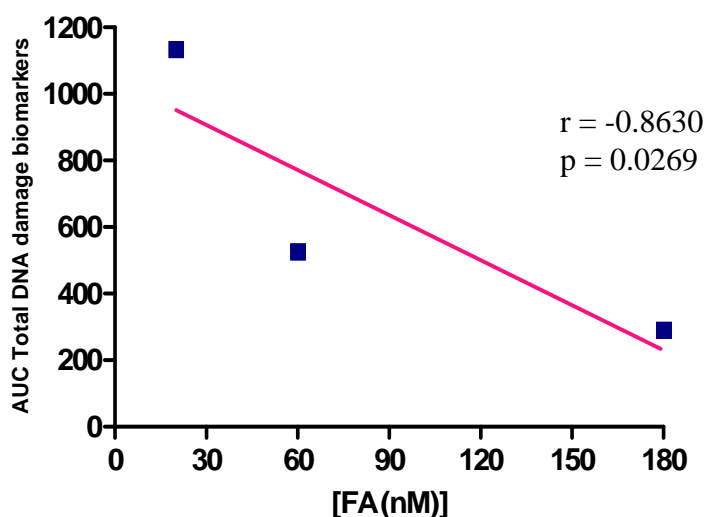


Figure 7.6 Correlation between FA concentration in medium and AUC total DNA damage biomarkers, in PBL cultured over 21 days. Area for total DNA damage biomarkers was obtained from Figure 7.5D.

7.3.3.3 BN cells with multiple NPB and/or unusual nuclear morphologies

Data presented in Sections 7.3.3.1 and 7.3.3.2 above include NPBs scored using the standard criteria for the CBMN Cytome assay. There were, however, a number of cells, most notably in the lower (20 and 60nM) FA treatments, which exhibited ‘chewing-gum’-like NPB patterns (see Chapter 5.3.3.3), as well as other, not previously reported, morphologies which did not meet the standard scoring criteria. As such these cells were not included in the main data set, and are presented, separately, in this section.

The newly-identified abnormal morphologies in BN PBL recorded in this study appear to have arisen from the formation of multiple dicentric chromosomes, resulting in NPBs following failure to separate at anaphase. Dicentric chromosomes are formed following misrepair of DS breaks, or from chromosome fusion events, such as may occur with recombination and/or BFB cycling. A separate set of scoring criteria was formulated to cater for cells with these unusual morphologies. In combination with existing criteria for individual, clearly identifiable bridges, the following NPB scoring criteria were prepared. The scoring criteria are described below, noting that (i) and (ii) are the standard NPB criteria, while (iii) – (vi) represent the abnormal morphologies that may arise from multiple NPB. These are presented graphically at Figure 7.7.

- (i) single NPB, not exceeding $1/4^{\text{th}}$ of the nuclear diameter;
- (ii) multiple (2, 3 or 4), clearly discernible NPBs, allowing accurate scoring of the total number of bridges;
- (iii) ‘chewing-gum’ NPBs where multiple bridges are present. Visually the NPBs appear as strands, but are too numerous or too close together to be accurately counted for scoring purposes. These may also appear as a single, very wide NPB (greater than $1/4^{\text{th}}$ the nuclear diameter) arising from multiple chromosome bridges in close proximity. ‘Chewing-gum’ cells are scored as containing 5 NPB, if included in the total NPB score;
- (iv) ‘horseshoe’ nucleus; arising from multiple NPBs in close proximity located to one side of the replicating nucleus;
- (v) ‘donut’ nuclear morphology; arising from multiple NPBs at the extremities of the nucleus, causing a hole to be formed as the daughter chromosome are pulled to the separate poles at anaphase, and the nuclear membrane is formed;
- (vi) Another class of chromosome damage scored was micronuclei in mononucleated cells. These may arise in dividing cells that experience mitotic failure, which may occur in BN cells with multiple NPBs. Mitotic failure or slippage occurs when replicated sister chromatids within a nucleus fail to separate, resulting in a 4N (or 8N or 16N)

mononucleated cell. In the current context, it is proposed that mitotic slippage has occurred due to mechanical failure caused by a very high number of telomere fusion events. These mononucleated cells may also contain micronuclei (MNi) arising from chromosome breakage or loss (see example in Figure 7.9).

The new criteria evolved over the course of the scoring (and re-scoring) process and as such have not been formally tested or validated. In this context, the following data is provided in an attempt to validate these biomarkers as indicators of chromosomal instability caused by FA deficiency. Data presented in Table 7.4 below shows the frequency of BN cells which meet the criteria for each of these six categories, at days 7, 14 and 21, for each FA condition. Data presented in Figure 7.8 compares the frequencies in each category at day 7.

Frequencies of BN cells exhibiting NPBs which were clearly defined for scoring purposes (categories (i) and (ii)) increased at each time point in the lower FA treatments, with the highest frequencies observed at day 21. For analysis purposes category (ii) (BN with multiple, clearly defined NPB) was split into (a) BN cells with only 2 NPB, and (b) BN cells with 3 or 4 NPB (Table 7.4(ii)). Analysis of data at day 21 by one-way ANOVA and Tukey's post-test identified significantly higher frequencies in 20nM FA of BN cells with only 1 ($p = 0.015$), only 2 ($p = 0.02$), or 3 or 4 ($p = 0.04$) NPBs (Table 7.4).

The frequency of mononucleated cells containing one or more MNi (category (vi), possibly indicating damage resulting in mitotic failure) in the 20nM FA condition was significantly greater than in either the 60 or 180nM cultures at days 7 (Figure 7.8A), and day 21 ($p = 0.04$ and 0.01 , respectively) (Table 7.4(vi), Figure 7.8A).

The frequencies of BN cells displaying 'chewing gum', 'horseshoe' or 'donut'-shaped nuclei (categories iii, iv and v) were all greatest at day 7 in 20nM FA (Figure 7.8B), and reduced thereafter (Table 7.4). At day 7 the frequency of BN cells with 'chewing gum' morphology in the 20nM condition (241.5 ± 77) was significantly higher than in the 60nM (10 ± 1.4) and 180nM (6 ± 5.7) conditions (one-way ANOVA, $p = 0.02$). An opposite effect was observed in the 60nM treatment, with the frequency increasing at each time point from 10 ± 1.4 at day 7 to 22.5 ± 7.8 at day 14 and 82.5 ± 61.5 by day 21 (Table 7.4(iii)).

The frequency of BN with 'horseshoe' nuclei differed significantly at day 7, with 44 ± 8.5 in the 20nM condition, compared with 5 ± 5.7 in the 60nM, and 2 ± 2.8 in the 180nM treatments ($p = 0.01$) (Table 7.4(iv), Figure 7.8B). 'Donut' nuclear morphologies were also observed in

significantly greater frequencies in the 20nM culture at day 7 (86.5 ± 12), compared with the 60nM (6 ± 0) and the 180nM (4.5 ± 0.7) conditions ($p = 0.002$) (Table 7.4(v), Figure 7.8B). Both of these morphologies declined in the 20nM treatment after day 7.

When comparing the absolute frequency levels of these FA-sensitive biomarkers in 20nM FA cultures it is evident that the 'chewing-gum' NPB was by far the most sensitive biomarker both in terms of fold-increase relative to the level in 180nM FA culture, and in absolute terms. The frequency of 'chewing gum' cells at day 7 in 20nM FA (241 ± 77) showed a 40-fold increase compared with the frequency in 180nM FA cultures at the same time point. The frequency of BN cells displaying a single NPB in 20nM FA culture, on the other hand, was 22.5 ± 3.5 at day 21 compared with 4 ± 2.8 in 180nM FA, a 5.5 fold increase in the FA deficient culture.

Figure 7.7 (following page)

Proposed models of cellular morphology arising from misrepaired breaks, or from chromosome fusion events, as observed in PBL grown in complete medium containing 20, 60 or 180nM FA *in vitro*. Using the CBMN Cytome assay, wherein cells are blocked at the binucleated (BN) stage, it is possible to visualise nucleoplasmic bridge (NPB) formation following damage/fusion events. Under normal mitotic conditions, in the absence of mitotic failure, daughter cells would be cleaved at anaphase causing the NPBs to break unevenly, resulting in exposure of uncapped ends and potential for further end-to-end fusions, thus perpetuating the breakage-fusion-bridge (BFB) cycle.

- (A) End fusion and misrepair of double strand breaks can lead to the formation of dicentric chromosomes, the initiating factor for NPB formation; (i) individual chromosomes; (ii) end fusions arising from compromised telomeric ends form a dicentric chromosome; (iii) the fused chromosome is replicated during mitosis resulting in an increased gene copy number and an increase in NPBs when the centromeres are pulled to separate poles.
- (B) Different nuclear morphologies arising from NPB formation.
- (i) single NPB, not exceeding 1/4th of the nuclear diameter;
 - (ii) multiple (2, 3 or 4), clearly discernible NPBs, allowing accurate scoring of total number of bridges;
 - (iii) 'chewing-gum' NPBs where multiple bridges are present. Visually the NPBs may appear as strands, but are too numerous or too close together to be accurately counted for scoring purposes. These may also appear as a single, very wide NPB (greater than 1/4th the nuclear diameter) arising from multiple chromosome bridges in close proximity. 'Chewing-gum' cells are scored as containing '5' NPB, if included in the total NPB score;
 - (iv) 'horseshoe' nucleus; arising from multiple NPBs in close proximity located to one side of the replicating nucleus;
 - (v) 'donut' nuclear morphology; arising from multiple NPBs at the extremities of the daughter nuclei, causing a hole to be formed as the daughter chromosome are pulled to the separate poles at anaphase, and the nuclear membrane is formed;
 - (vi) Another class of chromosome damage scored was micronuclei in mononucleated cells. These may arise in dividing cells that experience mitotic failure, which may occur in BN cells with multiple NPBs. Mitotic failure or slippage occurs when replicated sister chromatids within a nucleus fail to separate, resulting in a 4N mononucleated cell. In the current context, it is proposed that mitotic slippage has occurred due to mechanical failure caused by a very high number of telomere fusion events. These mononucleated cells may also contain micronuclei (MNI) arising from chromosome breakage or loss (see example in Figure 7.9)

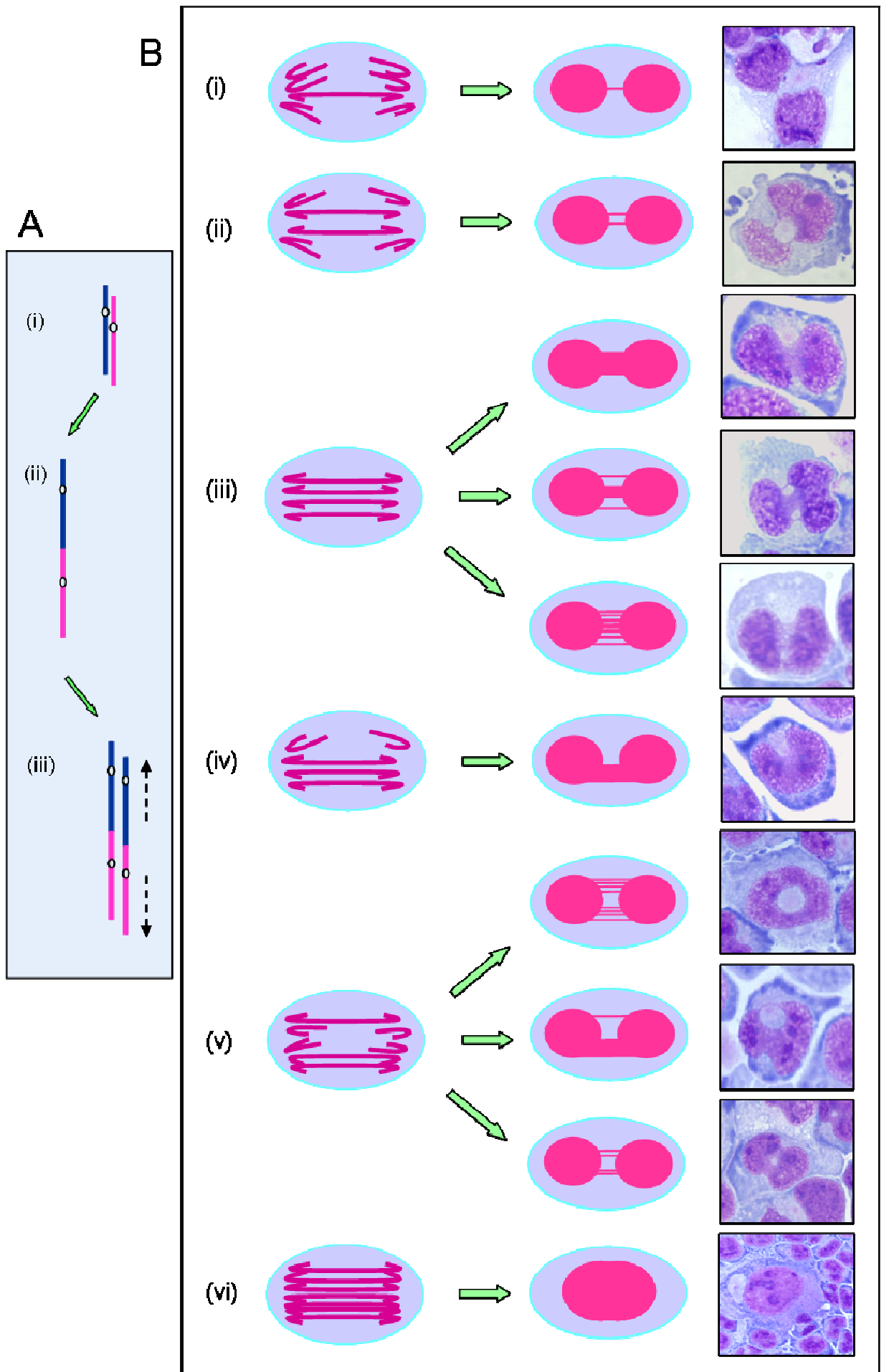


Table 7.4 Frequency of new cellular morphologies possibly arising from chromosome fusion events or misrepaired breaks, as observed in PBL grown in complete medium containing 20, 60 or 180nM FA *in vitro*. (Data represents mean frequency \pm SD per 500 BN cells for each treatment at each time point. N = 2 slides, 500 BN scored per slide. Scoring category represents the groupings as specified at Figure 7.6 (above). Category (ii) has been split into two, namely BN cells with only 2 NPB, and BN cells with 3 or 4 NPB. P values represent one-way ANOVA analysis (*denotes statistically significant differences between conditions). Data not sharing the same superscript letter within each time point differ significantly from each other, as measured by Tukey's post test).

Category	Time	[FA] in medium (nmol/L)			One-way ANOVA p value
		20nM	60nM	180nM	
(i) BN cells with only 1 NPB	Day 7	5.5 \pm 2.1	6.0 \pm 1.4	3.5 \pm 0.7	0.3536
	Day 14	9.5 \pm 9.2	4.5 \pm 2.1	8.0 \pm 8.5	0.7967
	Day 21	22.5 \pm 3.5 ^a	11.0 \pm 1.4 ^b	4.0 \pm 2.8 ^b	0.0149*
(ii) BN cells with only 2 NPB	Day 7	2.5 \pm 2.1	0.5 \pm 0.7	1.0 \pm 1.4	0.4854
	Day 14	0.0 \pm 0.0	1.0 \pm 1.4	1.0 \pm 1.4	0.6495
	Day 21	6.0 \pm 0.0 ^a	2.0 \pm 1.4 ^b	0.5 \pm 0.7 ^b	0.0192*
(iib) BN cells with 3 or 4 NPB	Day 7	3.5 \pm 2.1	0.0 \pm 0.0	0.0 \pm 0.0	0.1004
	Day 14	0.5 \pm 0.7	0.0 \pm 0.0	1.5 \pm 2.1	0.5630
	Day 21	5.0 \pm 1.4 ^a	1.5 \pm 0.7 ^{ab}	0.5 \pm 0.7 ^b	0.0408*
(iii) BN cells with 'chewing gum' morphology	Day 7	241.5 \pm 77.1 ^a	10 \pm 1.4 ^b	6.0 \pm 5.7 ^b	0.0209*
	Day 14	21.0 \pm 8.5	22.5 \pm 7.8	44.5 \pm 4.9	0.0795
	Day 21	46.5 \pm 14.8	82.5 \pm 61.5	13.0 \pm 5.7	0.3071
(iv) BN cells with 'horseshoe' morphology	Day 7	44 \pm 8.5 ^a	5.0 \pm 5.7 ^b	2.0 \pm 2.8 ^b	0.0107*
	Day 14	3.0 \pm 0.0	13.0 \pm 5.7	5.0 \pm 7.1	0.2748
	Day 21	1.0 \pm 1.4	3.5 \pm 2.1	1.0 \pm 1.4	0.3588
(v) BN cells with 'donut' morphology	Day 7	86.5 \pm 12.0 ^a	6.0 \pm 0.0 ^b	4.5 \pm 0.7 ^b	0.0021*
	Day 14	12.0 \pm 9.9	12.5 \pm 4.9	7.0 \pm 0.0	0.6731
	Day 21	6.0 \pm 0.0	7.5 \pm 3.5	7.5 \pm 6.4	0.9207
(vi) Mononucleated cells with a MN	Day 7	5.5 \pm 2.1 ^a	1.0 \pm 0.0 ^{ab}	0.0 \pm 0.0 ^b	0.0394*
	Day 14	1.0 \pm 1.4	2.0 \pm 1.4	1.5 \pm 2.1	0.8463
	Day 21	2.5 \pm 0.7 ^a	5.5 \pm 0.7 ^b	0.5 \pm 0.7 ^a	0.0132*

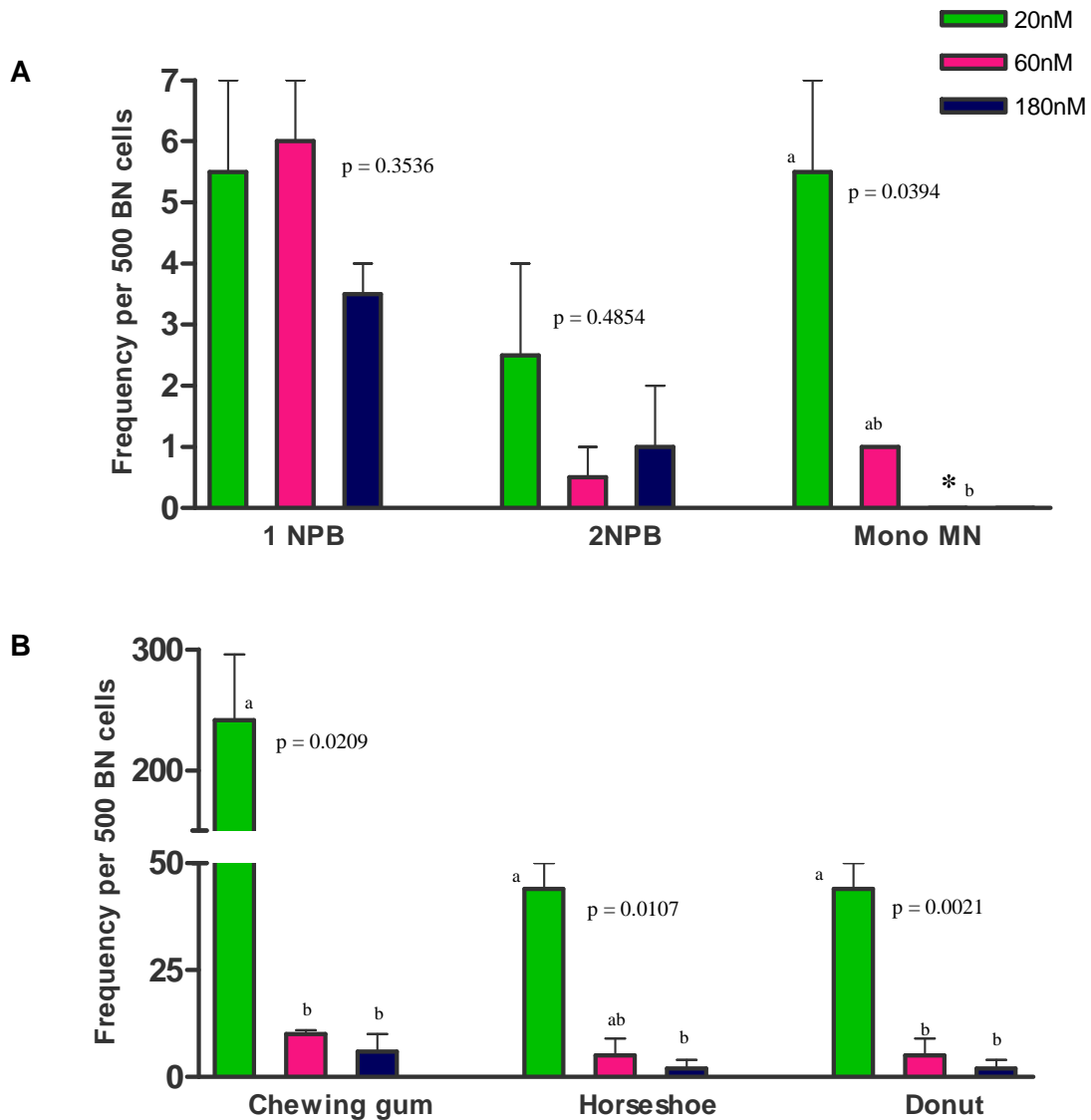
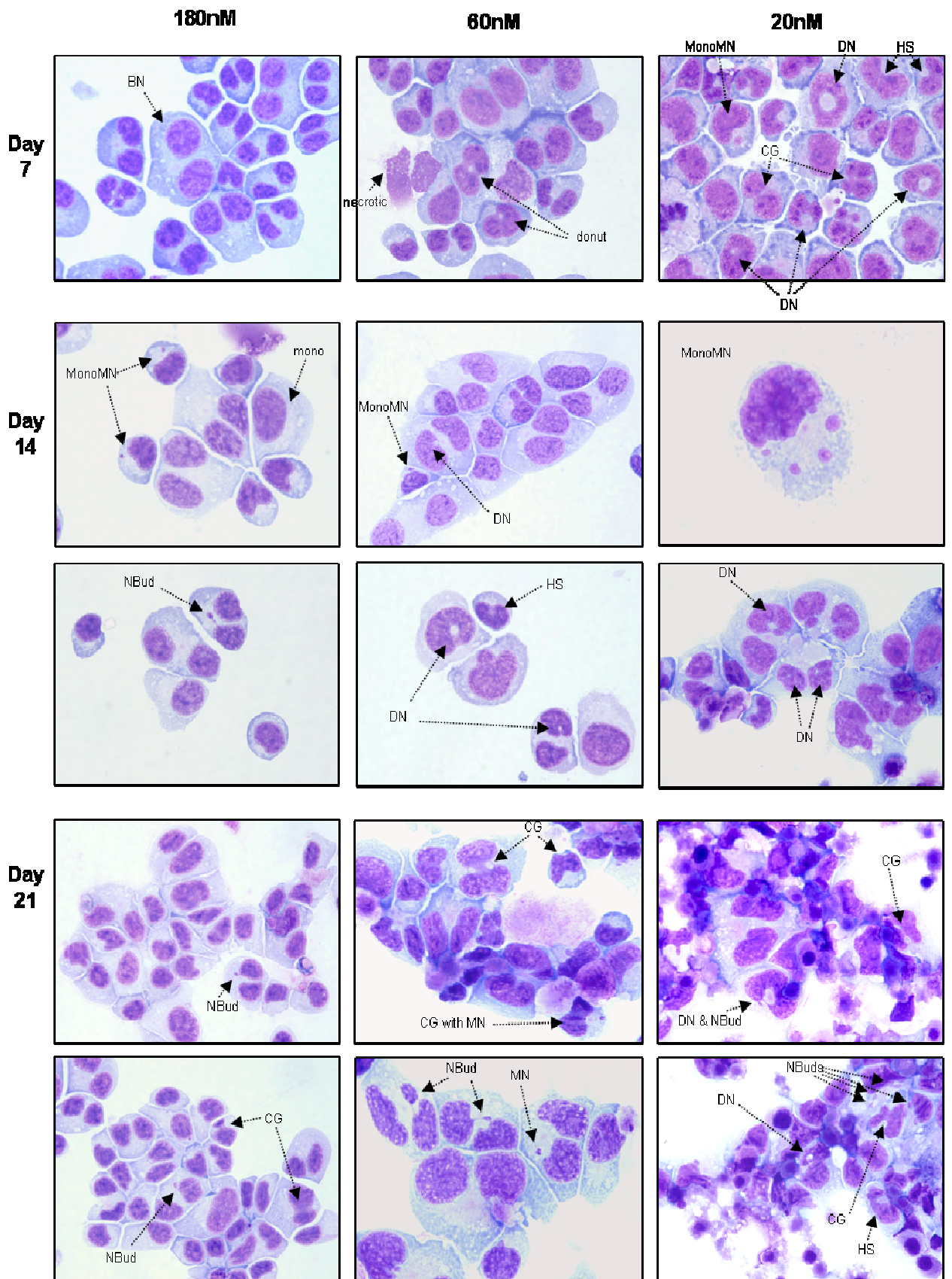


FIGURE 7.8: Frequency of BN PBL displaying 1 NPB, 2 NPB, or newly defined nuclear morphologies, after 7 days grown in culture medium containing 20, 60 or 180nM FA. The frequency, at day 7, per 500 PBL of (A) BN with 1 NPB, BN with 2 NPB, Mononucleated cells with a MN (*indicates zero events); (B) BN PBL containing ‘chewing-gum’ nuclear morphology, ‘horseshoe’ nuclei, or ‘donut’ nuclei. (N = 2. Error bars indicate SD. P values represent results of analysis by one-way ANOVA. Bars not sharing the same letter differ significantly from each other, as measured by Tukeys post-test).

Figure 7.9 (Following page)

Representative photomicrographs indicating the progressive increase in chromosomal damage in PBL grown in medium containing 20, 60 or 180nM FA over 21 days (1000x magnification). Arrows indicate damage scored as part of the CBMN Cytome assay, including new criteria for scoring unusual nuclear morphologies, possibly arising from chromosome fusions and nucleoplasmic bridges. (BN, binucleated cell; Multi, multinucleated cell; MN, micronucleus; NBud, nuclear bud; NPB, nucleoplasmic bridge; DN, 'donut'-shaped nucleus; HS, 'horseshoe'-shaped nucleus; CG, 'chewing-gum'; Mono MN, mononucleated cell with MN).



7.3.4 Homocysteine in spent medium

Homocysteine (Hcy) measurements were performed on spent medium collected at day 14. Viable cell counts were performed on the contents of each flask, so that Hcy concentrations ($\mu\text{mol/L}$) could be calculated per million viable cells. To ensure accurate comparison between treatments, the Hcy concentration was then converted, using the formula weight for Hcy and the volume of each flask, to μg Hcy produced per million viable cells.

Cells grown in 20nM FA produced a significantly greater amount of Hcy per 1×10^6 viable cells (1.4 μg), compared with cells cultured in 60 (0.6 μg) and 180nM (0.8 μg) FA. Analysis by one way ANOVA showed significant differences in the amounts of Hcy produced between all three conditions ($p < 0.0001$) (not shown).

7.4 DISCUSSION

7.4.1 *Telomere content in PBL cultured under FA deficient conditions*

The impact of low folate on DNA metabolism in culture medium takes effect after approximately four days, at which time intracellular stores become depleted³⁷⁹. Folate insufficiency beyond this time then causes cells to accumulate in S phase due to nucleotide imbalance and slow DNA synthesis^{37,91,93,379}. Findings from the current 21-day study were in agreement with previous observations^{37,91,93,95,379} whereby cells cultured in medium for 9 and 10 days in low folate showed significant, dose-dependent, reductions in growth rates, cell viability and NDI, while the percentage of necrotic and apoptotic cells increased in the low FA cultures.

FA treatment did not, however, impact significantly on TL in PBL. A sharp increase was observed in all three treatments over the first 7 days, followed by a subsequent reduction back to day 0 levels by days 17 and 21, in all treatments. The greatest difference in TL between conditions occurred at day 7, whereby cells cultured in 60nM FA had TL 13% greater than that of cells grown in 180nM FA. Area under the curve for TL with time tended to be very slightly higher in the lower FA cultures relative to the replete, 180nM FA, culture. This result differs markedly from that observed in the WIL2-NS cell line (Chapter 4) in which a significant, FA dose-dependent effect on TL was observed.

There are several possible reasons for the short term TL increase of approximately 150% from day 0 to day 7 observed in PBL in all three FA conditions. This may represent a stress response as cells adapt from an *in vivo* to and *in vitro* environment, or it may represent a shift in cell type ratios in the early days of culture. The inclusion of PHA at day 0, and IL2 in medium throughout the experiment, has been shown previously to maintain PBL for several weeks, with the percentage of cells expressing CD3, the marker for T cell lineage, increasing from 50% at day 3 to >95% by day 6³⁷⁹. TL is known to differ between subpopulations of lymphocytes, including between CD4+ and CD8+ cells^{288,380,381}, and this may be of relevance in the current context. In their recent work Lin *et al* (2009) have shown that of the PBMC fraction, B cells have the longest telomeres and highest telomerase activity, while CD4+ T cells have slightly higher enzymic activity than CD8+ T cells, but TL in the two subpopulations are comparable. Furthermore, results from a 10 day *in vitro* study demonstrated that low folate impacts the CD4+/CD8+ T cell ratio due to a significant decrease in replication of CD8+ cells³⁷⁹. While ratios were not measured in the present study, it is plausible that levels were altered by the culture conditions, and impacted on mean TL values.

The short term increase in TL may also be due to a transient, PHA-induced, upregulation of telomerase which has been shown to occur in both B and T lymphocytes^{382,383}. T cells activate telomerase to allow rapid, large scale replication for an immune response while maintaining telomere length³⁸²⁻³⁸⁴. The telomerase response differs, however, between sub-populations of T cells; CD4+ T cells upregulate this enzyme following repeated stimulation *in vitro*, while CD8+ T cells show a decline in telomerase activation over progressive stimulations³⁸². The latter effect is possibly regulated by expression of the cell surface marker CD28, as telomerase upregulation in CD4/CD8 T cells is positively correlated with surface CD28 expression³⁸⁵. This is consistent with the recent work from Lin *et al* (2009) which showed that telomerase activity levels are strongly correlated between CD4+ and CD8+CD28+ T cells from the same donor, and that CD8+CD28- T cells are replicatively senescent and have the shortest telomeres, and the lowest telomerase activity, of these subsets³⁸⁰. According to Lin *et al* (2009) telomerase activity is a dynamic and modifiable factor which can be mediated by environmental and lifestyle changes³⁸⁰. Accordingly, it is plausible that the rapid increase, followed by rapid decrease in TL in the present study may suggest a transient upregulation of telomerase in response to PHA stimulation.

With respect to the TL shortening observed from days 7 to 21, this may represent an ageing effect, as has been seen with approaching senescence in long term lymphocyte cultures¹⁵². The latter findings were, however, from cells which had been stimulated on multiple occasions over 27 weeks¹⁵², a much longer timeframe to that of the present study. While each stimulation was followed by a brief, transitory increase in TL, overall TL declined over time¹⁵². Another key finding of this long term study was a reduction in expression of proteins of the shelterin/telosome complex with time, resulting in a progressive loss of telomere structure integrity¹⁵². Telomere uncapping was associated with an increase in γ -H2AX, 53BP1 and telomere-damage induced foci (TIFs), leading to an increasingly dysfunctional telomeres¹⁵². While the PBL in the present study may be many weeks from reaching senescence, the progressive TL shortening may be due to a reduction in capping proteins over the experimental time frame of 21 days. This theory is consistent also with the progressive increase in DNA damage recorded in these cells with time.

It is possible that the different degree of TL response between the B-lymphoblastoid cell line, WIL2-NS, and PBL in the present study may be attributable to altered folate metabolism arising from polymorphisms in the gene coding for *MTHFR C677T*, phenotypic differences between B and T lymphocytes, or from differences in the genetic background of the two

models. The possibility of a gender effect was excluded due to the fact that both cell types came from male donors.

Methylene tetrahydrofolate reductase (MTHFR) is a key enzyme that can affect the folate/homocysteine balance in cells. When folate is limiting, common polymorphisms, such as *MTHFR C677T* which affect the rate of enzyme activity, have been shown to alter the rate of thymidine synthesis and/or DNA methylation^{91,93,323}. As such, both the WIL2-NS cell line and the PBL used in the present study were genotyped for this polymorphism to determine whether this may be impacting on the utilisation of folate within the cell. Allelic discrimination analysis showed that the WIL2-NS cell line is homozygous for the more frequent C allele, while the PBL are heterozygous (CT). Homozygosity for the T allele results in >50% reduction in enzyme activity, leading to an increase in the cellular concentration of 5,10-MeTHF and reduced levels of uracil incorporation into DNA, at the expense of DNA methylation^{37,91,93}. The effect of heterozygosity (CT), however, only reduces the enzyme activity by approximately 10%. Accordingly, it is unlikely that this polymorphism has impacted on folate metabolism in the PBL or on TL results observed in this study.

A further possible reason for the differences in WIL2-NS cells and PBL is that the former is a B lymphoblastoid cell line, while PBL are T lymphocytes stimulated in culture with PHA. In previous chapters of this thesis (Chapters 4, 5 & 6) it was proposed that the significant TL elongation observed in the WIL2-NS was likely due to recombination caused by hypomethylation of the subtelomere under low FA conditions. Unfortunately, methylation data was not available at the time of writing and thus, comparisons are not able to be made between the two studies, and cell types, under FA deficient conditions. The lack of effect in PBL may suggest that the WIL2-NS are more actively recombinogenic. It could be speculated that the components required for achieving immunological diversity in B and T cells, specifically V(D)J recombination, may be expressed at higher levels in the WIL2-NS, compared with PBL. V(D)J recombination refers to the 'variable', 'diversity' and 'joining' segments of the immunoglobulin (Ig) and T-cell receptor (TCR) genes, all of which are flanked by recombination signal sequences specific to the generation of an adaptive immune response. A means of exploring this theory would be an experimental model whereby cells were cultured in the presence of 5azadC to hypomethylate both cell types, followed by a comparison of SCE events involving telomere sequences.

It may also be possible that 21 days is an insufficient time frame in which to see an effect of low FA on telomeres in PBL, or that the TL effect observed herein may be unique to the single

donor used for this study. Further study is required, possibly over a longer time frame, to examine the effects of FA on TL in a broader range of cell lines, and PBL from multiple donors.

7.4.2 FA deficiency and biomarkers of chromosome damage

Frequencies of binucleated (BN) cells displaying biomarkers of chromosome damage (MNI, NPB and NBuds) increased over 21 days in cells cultured in low FA medium. The effect of FA concentration in medium was shown to be highly significant for each biomarker. These findings are consistent with those of previous *in vitro* studies whereby human and animal cells show an inverse, dose-dependent relationship between concentration of folate in the physiological range and increased markers of DNA damage, suggesting that chromosome breakage and gene amplification are induced by FA deficiency^{28,93,95,364}.

Both MNI and NBuds showed significant increases over 21 days in low FA. Mechanistically, evidence has shown that these biomarkers arise from different forms of DNA damage¹⁵. In normal lymphocytes grown under FA replete conditions 43% of NBuds contained interstitial DNA without centromere or telomere label, compared with only 13% of MNI. Sixty-two percent of MNI, on the other hand, contained a telomere signal, and 22% both a telomere and centromere signal, indicating that MNI derive from lagging chromosomes and terminal acentric fragments¹⁵. Characterisation of the contents of 894 NBuds and 1392 MNI using FISH in normal and folate-deprived 9-day cultures of human lymphocytes demonstrated that, while the frequency of BN cells containing NBuds and MNI increased with folate depletion, the proportion of MNI containing telomeric DNA remained unchanged (87%) and NBuds with telomeric DNA increased from 56% in 120nM to 63% in 12nM FA¹⁵. These findings suggest that FA depletion causes increased breakage of terminal fragments, evidenced by increased frequencies of MNI with telomeric DNA, as well as amplification of telomeric DNA, indicated by the increase in the frequency of NBuds containing telomeric DNA¹⁵. These authors also propose a model whereby MNI and NBuds may originate from the breakage of NPBs at telophase. They suggest that MNI may arise where two breaks occur in a bridge, while NBuds may form if a bridge is only broken at a single point¹⁵.

Surprisingly, while low FA was shown to be responsible for the significant increase in NPBs in the present study, the negative correlation observed between bridges and FA concentration did not reach statistical significance. This is not consistent with previous findings in PBL which have shown low folate to be associated with an increase in NPBs^{37,93}. This result may be due to the fact that NPBs in the 60nM treatment remained low until day 21, or it may be due to the

fact that many BN cells exhibited nuclear morphologies which appeared to arise from the formation of multiple NPB but did not meet the standard definition for NPB scoring. As such, additional, separate scoring criteria were developed to compare the frequencies of these morphologies between FA treatments. The new scoring criteria for NPBs incorporated the standard criteria for one or more clearly discernible bridges as well as for 'chewing-gum' NPB formation (discussed in detail in Chapter 6 of this thesis). In addition, high frequencies of cells were observed with 'horseshoe' or 'donut' shaped nuclei, and cells with larger nuclei suggestive of polyploidy, with or without MNi present.

The frequency of BN cells exhibiting only one, two, or 3 or 4 NPB were shown to be significantly increased in cells grown in 20nM FA, compared with 60 and 180nM FA, over 21 days. Cells exhibiting unusual nuclear morphologies such as 'chewing-gum', 'horseshoe', and 'donut'-shaped nuclei, as well as mononucleated cells with one or more MNi, were significantly increased at day 7 in the 20nM FA cultures. The frequencies recorded beyond this time point in the 20nM cultures, were, however considerably lower. It is likely that these morphologies are indicative of a degree of chromosomal instability that renders the cells non-viable, or results in mitotic arrest. Indeed, a peak in apoptosis was observed in the 20nM cultures at day 14, followed by a reduction by day 21, while the percentage of necrotic cells increased rapidly from 46% at day 14 to 74% by day 21. Furthermore, while the frequency of BN with 'horseshoe' or 'donut' nuclei peaked at day 7 in the 20nM FA cultures, the peak in these morphologies in the 60nM cultures was recorded at day 14, suggesting a delayed deterioration, with the reduction in frequency by day 21 possibly due to cell death.

NPBs can be caused by misrepair of DNA breaks, fusions between abnormally shortened, dysfunctional or uncapped telomeres^{172,276,277,331}, or persistent chromatid cohesion^{322,386}. Authors of a study which examined the formation of end-to-end fusions in non-transformed human breast epithelial cells concluded that shortened telomeres are an initiating factor for post replicative rejoining of sister chromatids³³¹. It is proposed that different types of fusions occur in a chronological order with sister chromatid fusions occurring initially, followed by interchromosome fusions, leading to conventional dicentric rearrangements, and eventually to BFB cycling and the generation of high levels of genetic diversity³³¹. The authors proposed that when only one chromosome in a cell is unprotected, the most likely fusion is with a sister chromatid. As a cell ages and telomeres shorten through natural attrition, the overall reduction in capping may cause many more chromosome ends to become fusogenic, and non-sister fusions will occur³³¹.

With regard to the mononucleated cells with large, possibly polyploid, nuclei, with or without MNi, these may be explained by a phenomenon known as mitotic slippage. High levels of maturation promoting factor (MPF) (cyclin B1 associated with p34cdc2 kinase), are critical for blocking the metaphase to anaphase transition^{387,388}. For the cell to progress through mitosis to interphase precise timing for degradation of the cyclin B1 component is required³⁸⁹. Instances have been shown, however, whereby MPF undergoes spontaneous inactivation, leading to mitotic slippage³⁸⁸, a state characterised by a single, large, polyploid (4N or greater) nucleus, and unsegregated sister chromatids³⁸⁷. *In vitro* lymphocyte studies have shown that frequencies of mononucleated cells with MNi (Mono MN) increased significantly following exposure to aneugenic compounds{Elhajouji, 1998 #179. The frequency of Mono MN observed in the present study, while low, was found to differ significantly between FA treatments, suggesting that the effects of low FA may, in some instances, lead to disruption of mitotic segregation of chromatids. More accurate determination of whether these cell morphologies have arisen due to mitotic slippage could be achieved if nuclear DNA content and area were measured to verify that they are actually polyploid and not simply diploid cells in G₂ phase.

As discussed in detail in Chapter 6, FA depletion may also lead to shortened or compromised telomeres due to uracil incorporation, and/or reduced structural integrity of heterochromatin caused by hypomethylation; each of which may cause an increase in fusion events. The large number of 'traditional' NPBs observed in the present study, combined with the unusual nuclear morphologies, highlights the possibility that FA deficiency may lead to increased formation of dicentric chromosomes. This may be due to telomere end fusions or misrepair of DNA breaks, both of which may lead to significant increases in CIN.

7.4.3 The relationship between FA and homocysteine

The concentration of homocysteine (Hcy), a biomarker known to relate inversely to FA, was measured in spent culture medium. Results from *in vivo* studies have found low plasma folate to be strongly correlated with elevated plasma Hcy, which in turn has been shown to be a predictor of mortality in older people{Dangour, 2008 #284}. A study in endothelial cells exposed to Hcy *in vitro* showed increased loss of telomere sequence per population doubling, accelerated senescence and up-regulation of surface molecules linked to vascular disease⁸⁷. Interestingly, a more recent study explored the *in vivo* relationship between plasma Hcy and telomere length of leukocytes obtained from a large, predominantly female cohort³⁹⁰. The findings of this latter study showed that individuals in the lowest tertile for Hcy and the highest

tertile for plasma folate had the longest telomeres, while the shortest telomeres were observed in participants whose plasma Hcy and folate concentrations fell into the highest and the lowest tertiles respectively³⁹⁰.

Consistent both with *in vitro* and *in vivo* findings, after 14 days in culture, results of the current study showed that Hcy in the spent 20nM culture medium was significantly higher than that of the spent medium from the 60 and 180nM FA cultures. The elevated Hcy in medium did not, however, translate to accelerated telomere shortening in these cells, as previous studies may suggest. This may be due to the low Hcy concentrations observed in the current study (concentrations prior to adjustment ranged between 3.3-6.9 μ mol/L), while the *in vitro* study showed an effect on TL in cells exposed to 50-750 μ M Hcy over 25 days⁸⁷, and the *in vivo* study associated Hcy of $11.8 \pm 4.2\mu$ M with telomere shortening.

7.4.4 Uracil incorporation into telomeric DNA

Uracil incorporation into genomic DNA is a well documented feature of folate insufficiency^{74,91-93,99}. The qPCR assay to determine incorporation of uracil into telomeric DNA was conducted as part of the current study, however, the results showed no changes from day 0 in any of the samples, except in the cells cultured in 60nM FA at Day 14, which showed an increase in uracil content. Previous studies have reported significant changes in uracil in genomic DNA over shorter time periods (9 days culture), in similar FA concentrations (12-120nM^{91,93} and 0-3000nM⁹⁹). These studies measured whole of genome uracil incorporation using a gas chromatography-mass spectrometry method, whereas the current study used a novel qPCR technique developed specifically for the purposes of quantifying the presence of uracil in telomeric sequences. As stated, this method had not been fully optimised at the time of writing, and as such it is anticipated that refinements currently being tested will lead to improved sensitivity. Accordingly, samples from the present study may yet yield significant data.

7.5 CONCLUSIONS

- Telomere elongation was seen in the short term in PBL, as hypothesised, however, this effect occurred in all three FA treatments and was likely due to a mitogenic response to PHA and a transient up-regulation of telomerase. Although there was a weak trend for longer TL in lower FA conditions no clear dose-dependent effect was observed, thus effectively negating the hypothesis.
- Consistent with previous, shorter term findings, FA insufficiency resulted in significant increases in biomarkers of CIN. This effect was observed in a dose-dependent manner.
- FA deficiency, in PBL from this donor, resulted in unusual nuclear morphologies which may have arisen from the formation of multiple dicentric chromosomes, nucleoplasmic bridges, BFB cycling and possibly disruption to chromatid segregation. It appears from these results that frequency of BN cells with ‘chewing-gum’, ‘horseshoe’ and ‘donut’ morphologies are relevant CIN biomarkers in the CBMN Cytome assay that maybe more sensitive to FA deficiency than the standard NPB score.
- TL and CIN were not significantly associated, contrary to the hypothesis that a negative relationship would be observed.

7.6 FUTURE DIRECTIONS

- This study should be repeated to confirm the findings, and to broaden the scope to verify that these observations are applicable to lymphocytes from other donors, and with different genetic backgrounds with regard to folate metabolism.
- Telomerase expression should be explored, particularly over the initial 14 days, to assess whether PHA stimulation affected the TL findings.
- Ratios of CD4+ and CD8+ T cell subpopulations should be determined, in addition to examine whether TL in these subpopulations is impacted differently by FA depletion and to eliminate potential confounding effects by changing ratios of T cell sub-types during culture.
- The effect of 5azadC should be tested in this model to provide a comparison of the specific effect of hypomethylation on TL and damage biomarkers in PBL. The concentrations used for the 4-day WIL2-NS study may need to be reduced to allow for a longer term study, 9 days if possible, to allow for closer comparison with previous FA depletion studies.
- Fluorescence *in situ* hybridisation (FISH) studies would be beneficial to assess whether telomeric DNA was present in MNi, NBuds and NPBs, as well as in cells displaying the unusual nuclear morphologies that were likely caused by multiple NPB formation (i.e. 'chewing-gum', 'horseshoe' and 'donut' BN cells). In addition FISH and cytogenetic methods would be useful to examine TRF1/2 binding at telomeres, cell ploidy and the presence of ALT-associated promyelocytic bodies (APBs).

CHAPTER 8:
**THE RELATIONSHIP OF TELOMERE LENGTH IN
PERIPHERAL BLOOD LYMPHOCYTES WITH AGE,
GENDER, BMI, FOLATE, VITAMIN B12 AND
HOMOCYSTEINE STATUS *IN VIVO***

8 THE RELATIONSHIP OF TELOMERE LENGTH IN PERIPHERAL BLOOD LYMPHOCYTES WITH AGE, GENDER, BMI, FOLATE, VITAMIN B12 AND HOMOCYSTEINE STATUS *IN VIVO*

8.1 INTRODUCTION

The integrity of the telomere structure and its DNA hexamer (TTAGGG)_n repeat sequence is critical for the protection of the ends of chromosomes from degradation and in maintaining overall genomic stability^{103,104}. The number of DNA hexamer (TTAGGG)_n repeats is reduced during each cell division in differentiated cells, and as a consequence telomere length (TL) decreases in most differentiated cells throughout the lifespan of the organism¹⁰⁴. Shortening of telomeres can result in telomere end fusions and an increased level of chromosome instability (CIN), which is in turn a key initiating event in numerous cancers (including lung, breast, colon and prostate cancers, as well as certain leukemias)^{6,106-110}. It has been shown that telomere shortening can be accelerated by environmental factors such as psychological and physiological stress, cigarette smoking and obesity^{102,134,135,138}. Efficiency of TL maintenance is also affected by gender. While males and females start life with similar TLs in leukocytes, the rate of attrition is more rapid in males^{129,130}. The mechanism underlying this gender effect is unknown, but it has been proposed that genetic factors may be responsible, and that the same factors may contribute to the difference in life expectancy observed between males and females^{129,132}.

Folate is essential for chromosome stability, due to its role both in the conversion of dUMP to dTTP for incorporation into DNA and in maintaining DNA methylation, the latter being achieved in concert with vitamin B12 (B12) via the synthesis of methionine and the common methyl donor S-adenosyl methionine⁷⁶. Previous studies have reported that moderate folate deficiency resulted in levels of chromosomal damage comparable to those caused by carcinogenic doses of ionising radiation³¹⁸, and was associated with both an increase in plasma homocysteine (Hcy) levels and an increase in nucleoplasmic bridges (NPB) in peripheral blood lymphocytes (PBL), possibly arising from telomere end fusions^{13,37,93}. Plasma Hcy is a sensitive metabolic marker of folate and B12 status and bioavailability⁷⁶ and has been implicated in some degenerative disease states, most notably cardiovascular disease and its

sequelae^{78,79}, as well as cognitive deficit (in the elderly)⁸², bipolar disorder and depression^{83,84}. A recent *in vivo* study has shown that TL in leukocytes is related inversely to plasma Hcy, possibly reflecting underlying oxidative and inflammatory stresses³⁹⁰, while endothelial cells exposed to Hcy *in vitro* show increased loss of telomere sequence per population doubling, accelerated senescence and up-regulation of surface molecules that are linked to vascular disease⁸⁷. Long term *in vivo* studies show that elevated plasma Hcy is a predictor of mortality in older people⁸⁶.

In vitro findings presented in Chapters 4 and 7 of this thesis showed that folate depletion caused an increase in chromosome instability (CIN) in both WIL2-NS cells and PBL, however, the impact on TL varied considerably between cell types. In WIL2-NS cells TL was significantly, increased in the short term by folate depletion, whereas in PBL TL was not significantly affected by folate status. Accordingly, the present study was established to further explore the relationship, in an *in vivo* context, between folate status and TL, in association with age and gender, and other key nutrients involved in the folate metabolism pathway.

8.1.1 Aims

1. To examine the *in vivo* relationship between TL in PBL and plasma folate, vitamin B12, plasma Hcy and red cell folate status.
2. To investigate whether any significant emerging associations were also dependent on age, gender, BMI or common polymorphisms in the folate metabolism genes *methylenetetrahydrofolate reductase (MTHFR C677T)* and *methionine synthase (MTR A2756G)*.

8.1.2 Hypotheses

1. TL is positively associated with folate, and negatively associated with plasma homocysteine (Hcy) concentration *in vivo*.
2. The relationship between plasma Hcy and TL in lymphocytes is modulated by the presence of common polymorphisms that alter activity of the MTHFR and MTR enzymes.
3. TL is negatively associated with age and BMI, and modified by gender.

8.2 EXPERIMENTAL DESIGN

A single fasted venous blood sample was obtained from 43 younger and 47 older adults. Samples were analysed for red cell folate (RCF), plasma folate (PF), homocysteine (Hcy) and vitamin B12 (B12) concentrations. Telomere length (TL) was measured in isolated lymphocytes by flow cytometry. Volunteers were genotyped for common polymorphisms in the folate metabolism genes *methylenetetrahydrofolate reductase (MTHFR C677T)* and *methionine synthase (MTR A2756G)*, which may affect Hcy status^{90,93}. The relationships between TL, PF, B12, Hcy and RCF status were explored. Furthermore, investigations were made as to whether any significant associations were dependent on age, gender, BMI, or MTHFR or MTR status. The experimental design is summarised in Figure 8.1.

8.2.1 Study population, blood samples and ethical review

Ninety healthy (not on medication for life-threatening illness) non-smoking volunteers were recruited in two distinct age groups (18-32 years and 65-83 years). The younger cohort consisted of 18 males (M) and 25 females (F) (mean age \pm SD: 23.9 \pm 3.7 years) and the older group included 24M and 23F (mean age \pm SD: 69.7 \pm 3.7 years). Body mass index (BMI) (Table 8.1) was calculated by the standard formula of weight (kg) divided by height (m)² measured during their visit to the CSIRO Human Nutrition Clinic. Fasting venous blood samples were also collected at this single Clinic visit. The study was approved by the Human Research Ethics Committee of CSIRO Human Nutrition, Adelaide, Australia. Informed consent was obtained from all participants.

8.2.2 Telomere length measurement.

Peripheral blood lymphocytes (PBL) were isolated using Ficoll-paqueTM Plus separation (as detailed in Chapter 3.1.2.2), prepared for cryopreservation in fetal bovine serum (FBS) plus 10% DMSO, frozen and stored at -80°C until required (Chapter 3.2.4). Cells were then thawed (as per Chapter 3.2.5) and TL was measured by flow cytometry (full detail provided in Chapter 3.4). In brief, cells were labelled with an 18mer FITC-conjugated peptide nucleic acid (PNA) probe complementary to the telomere repeat sequence, using Kit K5327, and counterstained with propidium iodide (PI) to measure DNA content and identify cell cycle stage. Cells with high TL (cell line 1301), which are also tetraploid, were included in all tubes as reference cells. These reference cells were used to calculate the relative TL of cells in the test samples. Each sample was prepared in paired tubes. For each individual TL was measured in duplicate

samples. For the purpose of quantifying background fluorescence of both the sample and reference cells one tube was incubated in hybridisation mixture with the PNA probe, while the paired tube was incubated in hybridisation mixture only. TL and DNA content measurements were then acquired using a FACSCalibur flow cytometer and analysed using BD CellQuest™ Pro software (v5.2). A mean FITC fluorescence value was obtained for labelled and unlabelled sample and reference cell populations by gating at G₀₋₁ phase of the cell cycle. TL relative to that of 1301 cells was then calculated using the following equation, which incorporates correction for ploidy (DNA index) of the different cell populations:

$$\text{TL (\%1301)} = \left[\frac{(\text{mean FL1 of test cells with probe}) - (\text{mean FL1 of test cells without probe}) \times \text{DNA index of 1301 cells}}{(\text{mean FL1 of 1301 cells with probe}) - (\text{mean FL1 of 1301 cells without probe}) \times \text{DNA index of test cells}} \right] \times 100$$

where FL1 = fluorescence detected in FL1 (green fluorescence) channel, DNA index of 1301 reference cells = 4 and DNA index of lymphocytes (test cells) = 2.

The coefficient of variation (mean ± SE) of duplicate measurements was 9.4 ± 1.0%.

8.2.3 *Micronutrient analyses*

Concentrations of RCF, plasma folate, Hcy and B12 were measured using an Architect® analyser at the Department of Chemical Pathology certified diagnostic laboratory of the Institute of Medical and Veterinary Services (IMVS) (Adelaide, South Australia) (detail provided in Chapter 3.7.1, 3.7.2 and 3.7.3). The coefficient of variation of duplicate measurements did not exceed 5%.

8.2.4 *DNA isolation, MTHFR and MTR genotyping*

DNA was isolated from each sample using the silica-gel membrane based DNEasy blood and tissue kit, following the manufacturer's instructions (Chapter 3.5.1). Samples were then genotyped for the MTHFR C677T and MTR A2756G polymorphisms by Real-Time PCR hydrolysis probe assays (Chapter 3.5.5). Approximately 20ng of genomic DNA was used as template per 20.0µl reaction. PCR amplification and genotype detection was performed using TaqMan® Universal PCR Master mix, without AmpErase® UNG, on an Applied Biosystems 7300 Real Time PCR System and ABI 7300 Sequence Detection System with the SDS Ver. 1.9 software. Each reaction was performed in duplicate.

8.2.5 *Statistical analyses*

Parametric statistical analyses were used, as all variables reported here were normally distributed. Student's unpaired t-test was used when comparing two groups, with or without age or gender adjustment. One-way ANOVA was used to compare TL, BMI, and micronutrient levels between more than two groups. Pearson's correlation coefficient was used to study the associations between TL, BMI and micronutrient concentrations. To account for the possibility of finding a significant difference at $p < 0.05$ purely by chance alone due to multiple testing when comparing effects in four subgroups (younger males, younger females, older males, older females) only accepted p values less than 0.0125 were accepted as being significant. The 0.0125 significance cut off value was determined using a Bonferroni correction for multiple comparisons. Multivariate regression analyses, adjusting for age, gender and age/gender interaction were used to explore the relationship of TL with micronutrients, MTHFR and MTR genotypes.

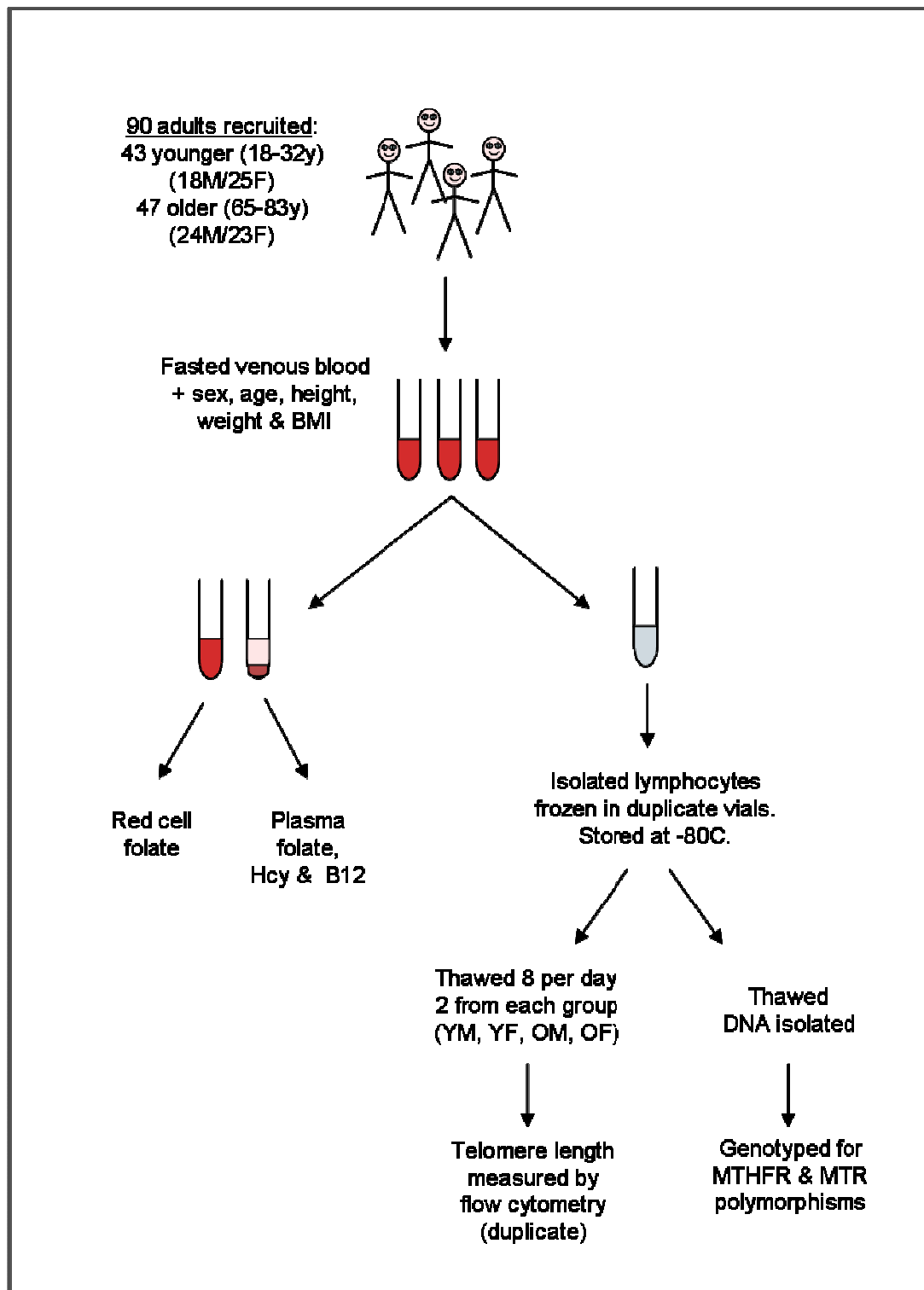


Figure 8.1 Experimental design 90 adults were recruited in older and younger age cohorts, 43 aged 18-32 and 47 aged 65-83 years. A single fasted blood sample was collected for RCF, PF, Hcy & B12 analysis. Lymphocytes were isolated, stored frozen in duplicate vials and thawed in batches for (i) telomere length measurement by flow cytometry, and (ii) genotype analyses. (Y, years; YM, younger male; YF, younger female; OM, older male, OF, older female).

8.3 RESULTS

8.3.1 *PF, B12, Hcy, RCF, and BMI of cohort*

Mean concentrations of PF, B12, Hcy and RCF were within the healthy reference range defined by the diagnostic laboratory for Australian cohorts of clinically normal subjects of ages approximating those of the study group (Table 8.1)³⁹¹. Mean PF and RCF concentrations were higher in the older cohort compared to the younger cohort and this difference was significant for both measures of folate status ($p < 0.0001$ for both). Furthermore, mean plasma Hcy concentration was greater in males compared to females in both the younger ($p = 0.0012$) and older cohorts ($p = 0.017$) (Table 8.1). However, no differences were observed in mean concentrations of B12 between age groups, or in relation to gender (Table 8.1). Mean BMI was greater in the older group than in the younger group ($p < 0.0001$), and in the younger cohort was higher in males than in females ($p = 0.014$) (Table 8.1). BMI of older and younger males did not differ significantly, however, BMI of older females was significantly higher than that of younger females ($p < 0.0001$) (Table 8.1).

8.3.2 *Relationship between PF, B12, Hcy and RCF status*

There was a significant negative correlation between the concentrations of PF and Hcy in the younger male ($r = -0.49$, $p = 0.04$), the older male ($r = -0.5$, $p = 0.02$) and younger female cohorts ($r = -0.4$, $p = 0.05$). There was a robust positive association between PF and RCF in the younger male ($r = 0.54$, $p = 0.02$), older male ($r = 0.75$, $p = 0.001$) and older female ($r = 0.62$, $p = 0.002$) cohorts. No significant relationship between RCF and plasma Hcy was observed in any of the groups. In younger females, B12 concentration correlated positively with both PF ($r = 0.41$, $p = 0.05$) and RCF ($r = 0.55$, $p = 0.007$), while in the older female cohort plasma B12 correlated negatively with plasma Hcy ($r = -0.57$, $p = 0.004$). In older males, B12 correlated positively with both PF ($r = 0.43$, $p = 0.04$) and RCF ($r = 0.6$, $p = 0.002$).

8.3.3 *The relationship between TL, age, gender and BMI*

Our preliminary analyses of the data showed that age and gender were the strongest factors affecting TL both in univariate and multivariate regression modeling. Consequently the data was stratified by age and by gender prior to investigating effects of folate, Hcy and folate metabolism genotype. The highest mean TL (\pm SD) was observed in the younger female group (12.1 ± 2.7), with the older males having the shortest telomeres (10 ± 2) (Table 8.1). When telomeres in PBL from the younger and older groups were analysed without separating

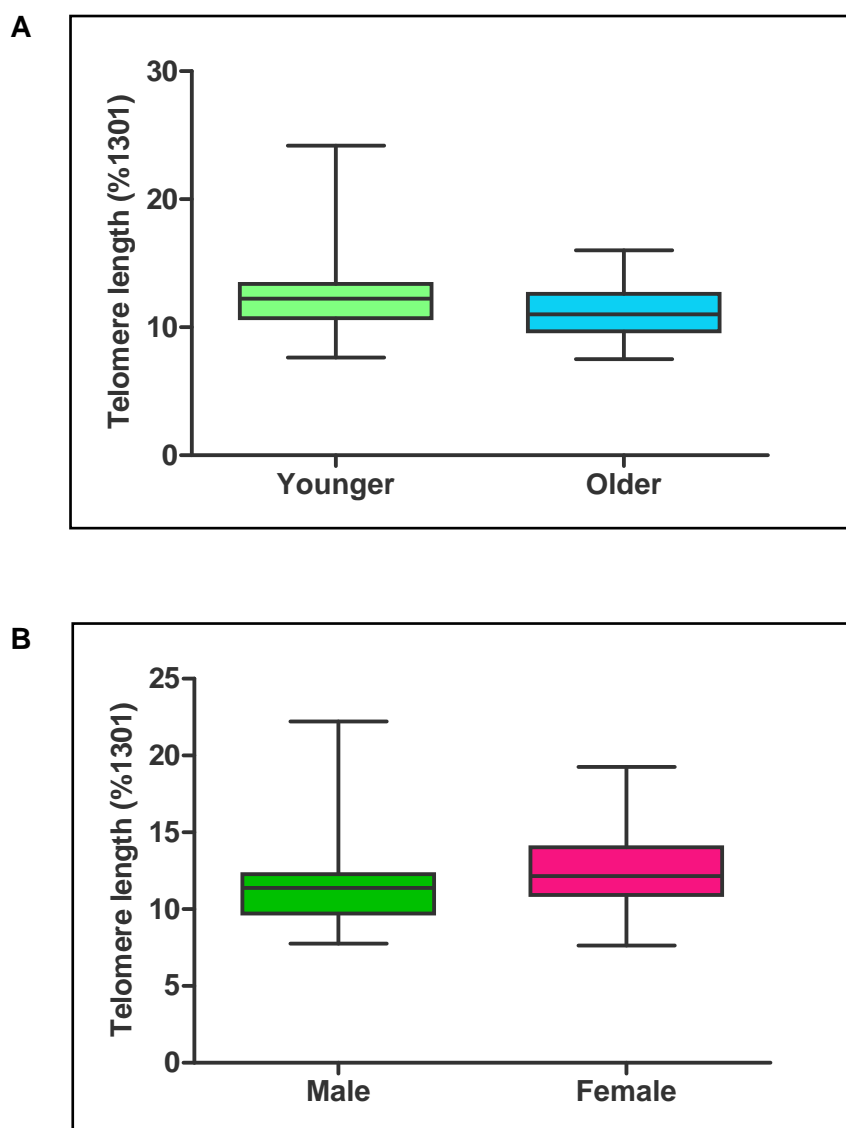


Figure 8.2 Telomere length (TL) of PBL from adult donors measured by flow cytometry (A) TL of PBL in younger adults (n = 43) compared to older adults (n = 47), adjusted for gender differences and analysed by t-test ($p = 0.015$); (B) TL of PBL in male (n = 42) compared to female (n = 48) adults, adjusted for age differences and analysed by t-test ($p = 0.077$). The TL measures are expressed as a percentage (%) relative to the 1301 cell line standard. Data are shown as box plots, which represent five-number summary of the data (the minimum, lower quartile, median, upper quartile, and maximum).

Table 8.1 Age, BMI, TL, PF, plasma B12, plasma Hcy and RCF in younger males (YM), younger females (YF), older males (OM) and older females (OF)

	<i>YM</i>	<i>YF</i>	<i>OM</i>	<i>OF</i>	<i>ANOVA</i> <i>P</i>
<i>N</i>	18	25	24	23	
<i>Age</i> (<i>years</i>)	24.28 ± 3.94 ^a (19, 32)	23.56 ± 3.60 ^a (18, 32)	69.92 ± 4.01 ^b (65, 83)	69.43 ± 3.64 ^b (65, 78)	<0.0001
<i>BMI</i>	25.46 ± 3.37 ^{ab} (21.1, 33.4)	22.82 ± 3.31 ^d (18.26, 31.21)	26.89 ± 2.40 ^{ac} (22.45, 30.36)	26.72 ± 3.12 ^{bc} (20.89, 31.74)	<0.0001
<i>Telomere</i> <i>length*</i>	11.72 ± 2.91 ^{abc} (8.33, 22.21)	12.12 ± 2.71 ^{bc} (7.63, 19.3)	10.04 ± 2.09 ^{ad} (6.91, 14.69)	11.29 ± 1.66 ^{cde} (7.75, 14.73)	0.0195
<i>PF</i> (<i>nmol/L</i>)	10.64 ± 6.54 ^a (4.3, 28.9)	16.92 ± 9.38 ^{ab} (5.9, 45.3)	21.68 ± 9.54 ^c (6.3, 39.3)	25.69 ± 10.34 ^{bc} (8.2, 42.3)	<0.0001
<i>B12</i> (<i>pmol/L</i>)	341.5 ± 137.5 ^{abc} (191, 726)	392.0 ± 194.0 ^{ade} (197, 1000)	364.0 ± 156.6 ^{bdf} (185, 846)	431.1 ± 258.7 ^{cef} (186, 1450)	0.4798
<i>Hcy</i> (<i>μmol/L</i>)	10.35 ± 3.75 ^{ab} (7.0, 22.9)	7.413 ± 1.52 ^c (4.9, 11)	10.31 ± 2.36 ^{ad} (7, 16)	8.891 ± 1.45 ^{bcd} (6.4, 11.8)	<0.0001
<i>RCF</i> (<i>nmol/L</i>)	377.9 ± 147.6 ^a (175, 715)	446.9 ± 194.2 ^{ab} (217, 1116)	690.1 ± 375.0 ^c (236, 1800)	638.6 ± 256.4 ^{bc} (230, 1174)	0.0004

BMI, body mass index (calculated as weight (kg) divided by height (m²); PF, plasma folate; Hcy, plasma homocysteine; B12, plasma vitamin B12; RCF, red cell folate. Normal ranges for PF, RCF, plasma B12 and plasma Hcy are 5-45nmol/L, 180-900nmol/L, 100-700pmol/L and 4-14μmol/L respectively. P value represents comparisons between groups using one-way ANOVA and Tukey's multiple comparison test; groups within the same row not sharing a common letter are significantly different. The results shown are mean ± SD. Results in brackets refer to minimum and maximum values. * The telomere length measurements are expressed as a percentage (%) relative to the 1301 cell line standard.

male and female volunteers, the mean TL was greater by 12.2% in the younger cohort than in the older cohort ($p = 0.01$). In the older cohort, TL in females was 12.5% greater than in males ($p = 0.03$). After adjusting for the effect of gender, the mean TL in the combined male and female younger cohort was greater than that of the combined male and female older cohort by 11.52% ($p = 0.02$) (Figure 8.2A). After adjusting for effect of age, TL in PBL of combined younger and older females was 8.0% greater than that of combined younger and older males ($p = 0.08$) (Figure 8.2B). There was no significant association between BMI and TL in any of the cohorts (younger male, older male, younger female, older female) (Table 8.2).

8.3.4 Correlation of TL with PF, B12, Hcy and RCF status

In older males, there was a significant negative correlation between mean TL and plasma Hcy ($r = -0.57$, $p = 0.004$) (Figure 8.3A), and a positive correlation between TL and PF ($r = 0.42$, $p = 0.04$) (Table 8.2, Figure 8.3B). However, no significant correlation was observed between mean TL and either RCF ($r = 0.3$, $p = 0.15$) or plasma B12 ($r = -0.1$, $p = 0.64$) in the older male cohort. There were no significant associations in older females between mean TL and either levels of plasma Hcy, PF (Figures 8.3C&D) or B12 (not shown), or with RCF (not shown) (Table 8.2). Similarly mean TL did not significantly correlate with any of these nutrient measurements in either younger males or younger females (Table 8.2).

8.3.5 Impact of *MTHFR* (C677T) and *MTR* (A2756G) genotypes on TL in PBL

Volunteers were genotyped for the *MTHFR* C677T and *MTR* A2756G polymorphisms. *MTHFR* C677T genotype frequencies were CC (0.43), CT (0.49) and TT (0.08); *MTR* A2756G genotype frequencies were AA (0.66), AG (0.29) and GG (0.05). These genotype frequencies did not differ significantly from Hardy-Weinberg equilibrium and are in agreement with previously published data³⁹². To be able to fully examine the relationship between TL and these genotypes TL data was adjusted for age and gender. When age and gender-adjusted differences in TL were compared between carriers of the rarer T allele (TT, CT genotypes) of the *MTHFR* C677T polymorphism and homozygotes for the C allele (CC genotype), no significant differences were found between the groups. Furthermore, there was no difference in mean TL (age- and gender-adjusted) between carriers of the rarer G allele (AG, GG genotypes) compared with homozygotes bearing the A allele of the *MTR* A2756G polymorphism. There was no significant effect of *MTHFR* C677T or *MTR* A2756G genotype on Hcy concentration.

Table 8.2 The relationships between telomere length (TL) and PF, plasma B12, plasma Hcy and RCF in younger males (YM), younger females (YF), older males (OM) and older females (OF)

	<i>YM</i>	<i>YF</i>	<i>OM</i>	<i>OF</i>
<i>N</i>	18	25	24	23
<i>PF</i> (<i>nmol/L</i>)	-0.19 (0.046)	0.16 (0.448)	0.42 (0.043)	-0.11 (0.610)
<i>B12</i> (<i>pmol/L</i>)	0.06 (0.812)	0.10 (0.632)	-0.10 (0.643)	0.23 (0.290)
<i>Hcy</i> (<i>μmol/L</i>)	0.24 (0.344)	-0.32 (0.123)	-0.57 (0.0004)	0.09 (0.677)
<i>RCF</i> (<i>nmol/L</i>)	-0.13 (0.613)	0.37 (0.081)	0.30 (0.154)	-0.11 (0.619)
<i>BMI</i>	-0.08 (0.751)	0.12 (0.575)	0.16 (0.444)	-0.10 (0.645)

Data represents Pearson's *r* values for TL (measured by flow cytometry, expressed as a percentage relative to the 1301 cell line standard) correlated against each micronutrient, for each cohort. Results in brackets represent significance (*p* values). PF, plasma folate; B12, plasma vitamin B12; Hcy, plasma homocysteine; RCF, red cell folate; BMI, body mass index (calculated as weight (kg) divided by height (m²)).

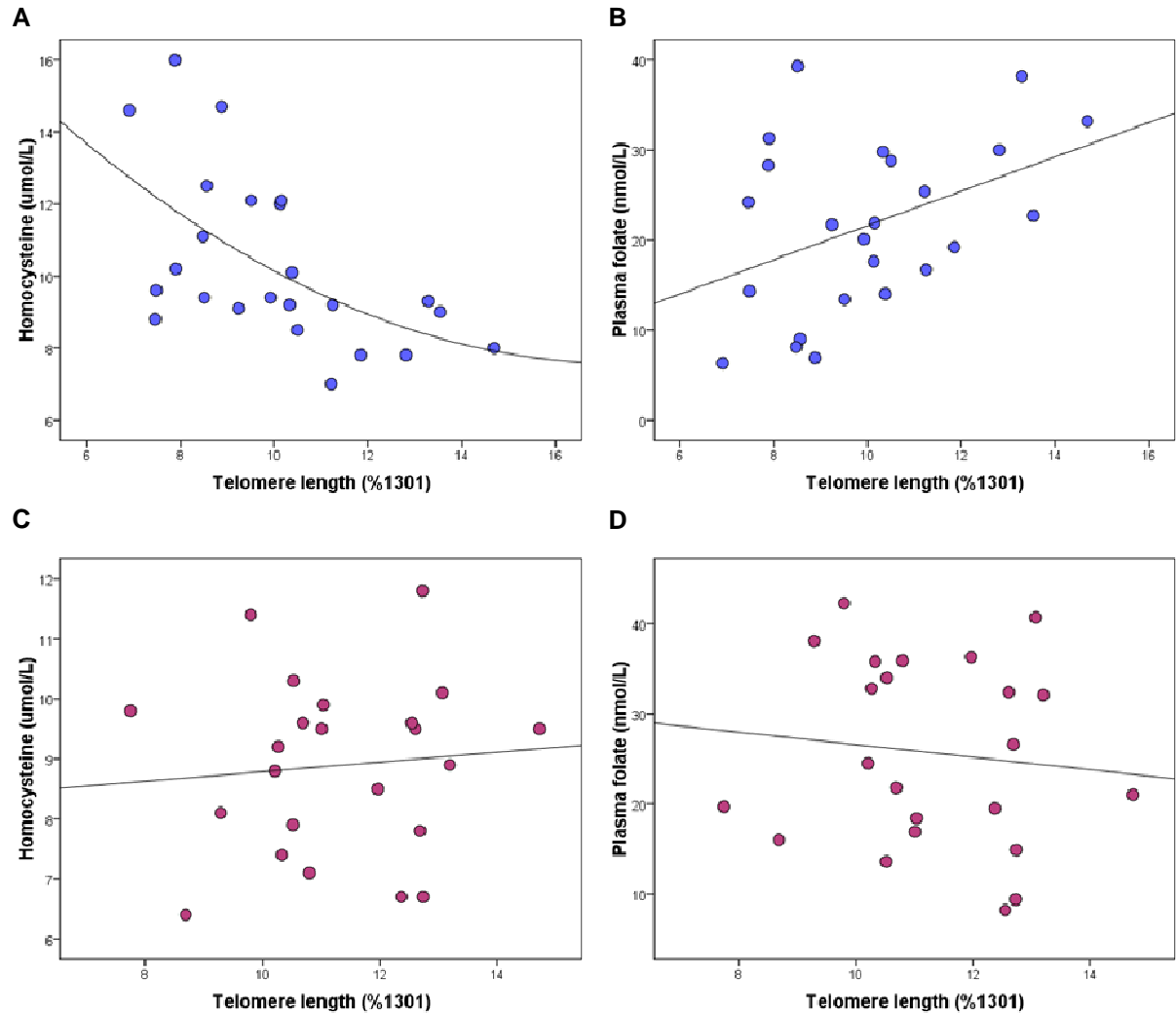


Figure 8.3 The relationship between telomere length (TL), plasma homocysteine (Hcy) and plasma folate (PF) concentrations in older males and females. Correlation of TL and plasma Hcy in (A) older males ($r = -0.57$, $p = 0.004$, $n = 24$), and (C) older females ($r = 0.092$, $p = 0.68$, $n = 23$). Correlation of TL and PF in (B) older males ($r = 0.42$, $p = 0.04$, $n = 24$) and (D) older females ($r = -0.11$, $p = 0.61$, $n = 23$). The TL measures are expressed as a percentage (%) relative to the 1301 cell line standard.

8.3.6 The relationship of TL with independent variables using multiple regression analysis

Multiple regression modelling of the data was also used to investigate the strength of association of TL with independent variables to take into account for any covariate and interactive effects. These analyses (results in Table 8.3) show that in the total cohort age is the strongest factor associated (inversely) with TL (beta = -0.32, $p = 0.02$). We therefore stratified the cohort on the basis of age and showed by multiple regression analysis that there was no significant association in the younger cohort between TL and any of the variables, but in the older cohort it was clear that the strongest factor associated with TL was Hcy (beta = -0.37, $p = 0.09$). To determine whether the association with Hcy was gender specific the older cohort was stratified by gender. Multiple regression analysis showed a null effect of TL with any of the independent variables in the older female group but in older males there was a highly significant inverse association with Hcy (beta = -0.83, $p = 0.001$) and the regression model had a multiple R value of 0.84 ($p = 0.006$). In this model there were also marginally significant associations between TL and BMI (beta = 0.40, $p = 0.03$), plasma B12 (beta = -0.64, $p = 0.01$) and MTHFR C677T genotype (beta = -0.33, $p = 0.07$). It was therefore clear that even with multiple regression modelling the effect of Hcy on TL was only evident in older males.

Table 8.3 Results of multiple regression analysis in the total cohort and sub-groups for association of telomere length in peripheral blood lymphocytes with independent variables.

Independent Variables	Total Cohort (N=86)	Younger Male & Female (N=43)	Older Male & Female (N=47)	Older Female only (N=23)	Older Male only (N=24)
	Beta value				
Gender	0.16	0.11	0.23	NA	NA
Age	-0.32**	-0.03	-0.012	-0.037	0.12
BMI	0.07	0.12	0.02	-0.01	0.40**
Plasma Hcy	-0.03	0.10	-0.37*	0.38	-0.83****
Plasma B12	0.03	-0.02	-0.05	0.37	-0.63**
PF	-0.04	-0.12	0.03	-0.13	0.30
RCF	0.16	0.27	0.06	-0.04	0.26
MTHFR C677T	-0.04	-0.10	-0.07	0.01	-0.33
MTR A2756G	0.08	0.01	0.19	0.44	-0.23
Multiple R					
	0.37	0.28	0.52	0.55	0.84****

* P<0.10; **P<0.05; ***P<0.01; ****P<0.005; NA=not applicable.

8.4 DISCUSSION

The novel finding of this study is that lower mean TL in PBL of older South Australian males is associated with higher concentrations of plasma Hcy. These data support the hypothesis that the mean length of telomeres in lymphocytes of older men is affected by availability of folate, and the capacity to metabolise Hcy. Plausible mechanisms by which folate deficiency could cause telomere shortening or dysfunction include; (i) DNA strand breaks and abasic sites within the telomere sequence as a result of excision of excessive uracil incorporation into DNA; (ii) sub-optimal binding of telomere-associated proteins such as TRF1 and TRF2 due to damage to the telomere sequence leading to breaks in telomeric DNA; and (iii) hypomethylation of subtelomeric DNA that could result in altered telosome structure or stability¹³.

Numerous population studies have reported correlations between *in vivo* plasma Hcy levels and poor health prognoses, including those related to the consequences of vascular disease⁸²⁻⁸⁴, but only two studies have focused specifically on the direct *in vivo* relationship between TL and plasma Hcy^{130,390}. Richards *et al* measured TL in blood leukocytes obtained from a large, predominantly female, cohort using the telomere restriction fragment (TRF) method³⁹⁰. Their findings, which are consistent with those of the present study, showed that individuals in the lowest tertile for plasma Hcy ($6.0 \pm 0.85 \mu\text{mol/L}$) and the highest tertile for PF (folate levels not specified) had the longest TL, while the shortest TL measurements were observed in participants whose plasma Hcy and PF concentrations fell into the highest ($11.8 \pm 4.2 \mu\text{mol/L}$) and the lowest tertiles respectively³⁹⁰. A difference in the current study is that plasma Hcy and TL were significantly associated only in the older male cohort, with no correlation observed in either female cohort or the younger male cohort. Richards *et al* (2008) analysed their data as a single cohort, with statistical adjustment for age, gender and lifestyle factors, such as exercise and smoking, and their measurements were performed in leukocytes (a combination of all white blood cells) not only in lymphocytes. As such, it is not possible to compare results of the two studies directly. However, the mean BMIs (kg/m^2) in the two studies are similar, with 25.4 ± 3.5 and 25.6 ± 4.6 (mean \pm SD) in the present study and the Richards *et al* study, respectively³⁹⁰. A possible reason that a significant association between plasma Hcy and TL was not detected in the current study in the older female cohort may have been the lesser power of the study. Another plausible reason may have been that the range of plasma Hcy concentration ($6.4 - 11.8 \mu\text{mol/L}$) in the older female cohort was small, relative to that for the older men ($7.0 - 16.0 \text{mol/L}$). It is evident from the multiple regression analysis that age was

the major variable affecting TL in the total cohort and that the effect size of the B vitamin-related variables was small by comparison. The possibility can not be excluded that the sample might have been too small to detect subtle effects of B-vitamin variables on TL in both the younger groups and the older females relative to the effects observed in older men for Hcy and PF.

A related study by Bekaert *et al* which did differentiate between males and females for markers of cardiovascular disease (CVD) and TL, was conducted on a Belgian cohort of 2509 volunteers, aged 35-55 (mean 46 ± 6) years¹³⁰. The cohort contained equal numbers of men and women and a condition for enrolment was that the volunteers were free of clinical signs of CVD. Interestingly, no associations were found between TL and conventional CVD predictors such as cholesterol, triglycerides, blood pressure or plasma Hcy. The authors speculated that the lack of an association between TL and CVD risk factors might have been due to the relative youth of the cohort. This finding is consistent with those of the present study, which found a significant relationship between TL and plasma Hcy only in the older male cohort (mean age of 69.9 ± 3.6 years), not in the younger male cohort (mean age of 24.3 ± 3.9 years). Another observation provided by Bekaert *et al* was that while individual life-style factors failed to correlate significantly with TL, a combined unhealthy lifestyle index (defined as high alcohol consumption, large waist circumference, increased smoking, with low physical activity and low fruit and vegetable intake), correlated negatively with TL in men ($p = 0.033$), but not in women ($p = 0.2$)¹³⁰. These authors concluded that a pro-ageing effect of an unhealthy lifestyle impacted on TL and that this was most obvious in males¹³⁰. These findings support the notion that TL may reflect the interactions of multiple factors, whose individual contributions might only be resolved by larger studies in well-defined populations to achieve higher statistical power. The tendency for a higher BMI in men in the present cohort suggests that other dietary and life-style factors may also contribute to modify TL. The effect of BMI on TL did not achieve statistical significance in the whole cohort using multiple regression analysis and, contrary to the results of Bekaert *et al* (2007), TL in older men in the south Australian cohort correlated positively with TL, although this effect was smaller than that of plasma Hcy which correlated negatively with TL.

The combined effect of folate deficiency and oxidative stress is a plausible explanation for the relative reduction in TL that was observed in older males compared to older females, because oxidative damage associated with raised plasma Hcy levels, or inflammation, may contribute more to telomere loss than the effect of incomplete end-replication alone¹⁰². Furthermore, oxidative stress mechanisms have been implicated strongly in the vascular damage that is

observed in individuals with hyper-homocysteinemia of genetic or nutritional origin³⁹³. Due to its high guanine content, telomeric DNA is potentially more sensitive to accumulation of 8-oxoguanine than DNA elsewhere in the genome¹⁰² and results from both *in vitro* and *in vivo* studies support the hypothesis that oxidative stress causes accelerated telomere shortening^{130,394} and premature senescence³⁹⁵. Only one study has reported the effect of Hcy on TL *in vitro*, and this showed that TL was reduced in endothelial cells exposed to Hcy⁸⁷. This telomere shortening effect could be attenuated in a dose dependent manner by the addition of a peroxide scavenger (catalase), suggesting that the effect of Hcy is probably mediated through a redox-dependent pathway⁸⁷. The observation that sensitivity to ionising radiation-induced chromosome damage is enhanced when folate is deficient also supports the possibility of an interactive effect between folate status and oxidative stress on exacerbating telomere shortening, possibly arising from strand breaks in the telomere sequence^{95,396}. This hypothesis can only be properly tested once reliable methods for measuring uracil, 8-oxoguanine and DNA strand breaks within the telomere sequence are developed and validated. It is also plausible that simultaneous excision of uracil and 8-oxoguanine on opposite strands of the telomere sequence could result in double strand breaks and telomere shortening^{13,74}, but further studies are needed to shed light on this possibility.

The ability of cells to utilise folate and metabolise Hcy is dependent on the activity of enzymes in the folate/methionine cycle. Two key enzymes that can affect Hcy concentration in cells are methylene tetrahydrofolate reductase (MTHFR) and methionine synthase (MTR)^{90,93}. Common polymorphisms in these genes (*MTHFR C677T*, *MTR A2756G*) have been shown to affect activity of these enzymes and alter plasma Hcy concentration when folate is limiting. In addition evidence has accumulated that different genotypes of these polymorphisms, have different rates of chromosomal instability, and the methylation status of DNA could be affected by these polymorphisms⁹⁰. In the current study no significant impact of these polymorphisms upon TL was observed, possibly because the effect size for results between the various genotypes was small and the study was insufficiently powered to detect the likely miniscule differences. Based on the current studies the standard deviation for TL in the younger and older cohorts was 2.77 and 1.97 respectively, which would have allowed a difference of 1.69% TL (younger cohort) and 1.15% TL (older cohort) to be detected with 80% power at $p < 0.05$, assuming $n = 45$ in each group. Future studies will need to be appropriately powered to detect not only statistically significant effects on TL, but also biologically meaningful differences such as a TL difference that is equivalent to that observed for a chronological age difference of approximately 10 years. Although this study detected the anticipated effect of age on TL (*ie.* significant shortening with age) the effect size observed in

TL relative to the difference in ages was small (11.52% difference in TL compared with 292% difference in age). This reduction in TL represents approximately 2.6% shortening per decade, assuming a linear decline with age. Notably, the impact of plasma Hcy on TL in older men appears to be greater than that of ageing itself given that, based on the Hcy vs TL regression line, older men with plasma Hcy of 8 μ mol/L have an estimated average TL (%1301) of 15 as compared to older men with plasma Hcy of 14 μ mol/L who have a TL (%1301) of 6, a relative reduction of 60%.

Viewed in the wider context of published evidence, results presented in this chapter indicate that older males may be susceptible to abnormally high acceleration in telomere shortening and that this could be due to increased Hcy possibly caused by insufficient dietary folate, despite mean PF and RCF falling within the accepted “normal” range. Increased vitamin B12 intake is often associated with reduced Hcy^{57,397} but multiple regression analysis of data for older men suggested a negative correlation of plasma B12 with TL and B12 was not correlated significantly with Hcy. These results raise some doubt about the benefits of vitamin B12 or common dietary sources of B12 (eg red meat) with respect to telomere maintenance. TL in this cohort of older men is compromised at plasma Hcy concentrations greater than 8 μ mol/L, above which a sharp decrease in TL is observed. This observation is consistent with the results of studies on micronucleus frequency in lymphocytes of older men, which show that this biomarker of chromosome damage is minimised when plasma Hcy concentration is less than 7.5 μ mol/L³³³. Accordingly, it is proposed that dietary insufficiency of folate, which is likely to result in elevated plasma Hcy, is a potential cause of telomere shortening. As a result dietary intake levels of folate may need to be adjusted to ensure optimal maintenance of telomeres in older age, particularly in males.

8.5 CONCLUSIONS

- TL is positively associated with plasma folate and negatively associated with plasma Hcy, in support of the original hypothesis. However, this effect was only observed, in the older male cohort.
- TL is negatively associated with age, and TL of females was shown to be longer than that of males. These observations supported the hypothesis and were in agreement with previous findings.
- These findings provide evidence suggesting that a high level of plasma Hcy is associated with short TL in lymphocytes of older men. This needs to be confirmed in a larger cohort.

8.6 FUTURE DIRECTIONS

- Further studies to confirm and extend these preliminary findings is warranted, including nutritional and lifestyle interventions that focus on reducing physiological stressors that may harm telomere integrity, in particular plasma levels of Hcy and reactive oxygen species. Knowledge gained from these studies will help to define the most effective dietary strategies for optimal telomere maintenance and to reduce the incidence of age-related neoplastic and degenerative pathologies, caused by accelerated telomere shortening.
- Given plasma and red blood cell micronutrient concentrations may fluctuate on testing days depending on dietary intake, consideration should be given to testing on multiple occasions over a longer time period, more effectively capturing the life-span of the long-lived T lymphocytes which would represent the majority of PBL cultured in these assays. Such an approach may provide a more accurate indication of micronutrient status over the lifespan.

**CHAPTER 9:
GENERAL DISCUSSION, CONCLUSIONS
& FUTURE DIRECTIONS**

9 GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

The experiments and investigations presented in this thesis aimed to test the following hypotheses:

1. Folic acid (FA) deficiency causes telomere shortening in human cells cultured *in vitro*.
2. Chromosomal instability (CIN) arising from FA deficiency (as determined by frequency of biomarkers MN, NPBs and NBuds) is negatively associated with telomere length in human cells cultured *in vitro*.
3. Lower folate status *in vivo* is associated with shorter telomeres in PBL of healthy adults.

Findings for each are discussed below.

9.1 THE IMPACT OF FA DEPLETION ON TELOMERE LENGTH

The first hypothesis was tested in the WIL2-NS cell line, by culturing the cells in medium that contained low, medium or high concentrations of FA. Telomere length (TL) was measured using a flow cytometric method. Although there was initial lengthening of the telomeres in FA-deficient medium, the hypothesis was supported over the longer term, with significant shortening of telomeres recorded between day 14 and day 42 of culture in the lowest concentration of FA. As predicted, the content of uracil in telomeric DNA increased during culture under these conditions. Together, these findings suggest that incorporation of uracil into telomeres under conditions of FA insufficiency may result in accelerated attrition at telomeres. Two mechanisms are likely to be responsible for this rapid reversal in telomere length. In the first, the incorporation of uracil within telomeres, followed by excision, increases the frequency of close proximity abasic sites and formation of double strand breaks (DSB). Secondly, the presence of uracil, and uracil-induced BER intermediates, may disrupt binding kinetics of the telomere-binding proteins TRF1 and TRF2, resulting in compromised telomere capping. Previous studies have demonstrated that disruption to TRF1 and TRF2 binding results in the formation of telomere-induced DNA damage foci (TIFs), whereby γ H2AX signaling recruits members of the DNA repair pathway^{153,172}. It was suggested that disruption of capping leads to targeting of intact telomeres in the same manner as DSBs, potentially leading to end fusions and initiation of breakage-fusion-bridge (BFB) cycling²⁷. BFB cycling is known to result in DNA amplification, as well as large terminal deletions, thus altering the gene (or telomere) dosage in the subsequent daughter cells²⁷.

The finding that, in the longer term, culture in FA-deficient medium leads to telomere shortening provides new knowledge regarding the potential impact of folate insufficiency on TL and integrity. However, unanswered questions remain regarding the relationship between uracil incorporation into telomeres and telomere capping. Further studies are needed to examine the effects of even longer-term cultures (i.e. >42 days) in FA-deficient medium. This is necessary to verify whether the observed downward trend in TL continues under conditions that are sustainable with respect to cell viability. Future studies are also required to explore whether there is a causal link between uracil incorporation, abasic sites, TRF1/2 binding kinetics and the presence of TIFs. One means that may yield definitive data would be to use the approach published by Opresko *et al* (2005), whereby TRF1/2 bindings assays were conducted using specific oligonucleotides synthesised with known base substitutions, to explore TRF1/2 binding under FA deficient conditions¹³⁹.

A surprising finding from this study was that the short-term effect of culture of WIL2-NS cells in FA-deficient medium was a transient increase in TL. This effect was reproducible and it occurred in a dose dependent manner. As it was not in agreement with the hypothesis, this result prompted further exploration of the literature and attempts to determine the mechanism. A major focus of telomere research has been to discover whether there is a relationship between TL and malignancy with a view that TL might prove to be a useful indicator of malignancy and prognosis for individual tumours. A significant proportion of studies have indicated that shortening of telomeres is an early event in neoplastic change (reviewed by Svenson and Roos (2009)²). However, there is increasing evidence that long telomeres in diseased tissues (relative to benign tissue or PBL) can also be associated with poor prognostic outcome^{2,220-223,233,239,398,399}. In the majority of these reports, increased telomerase activity appears to be the mechanism underlying telomere elongation^{220-222,233,239,399}. Furthermore, increased telomerase activity is significantly associated with tumour aggressiveness and poor prognosis^{220-222,233,239,399}.

For this reason, an increase in telomerase activity was explored as a plausible mechanism for the acute lengthening of TL observed when WIL2-NS cells are transferred to FA-deficient medium. Results of a 42-day pilot study using telomerase inhibition (TI) conducted in the early stages of this project were revisited. These results had shown no effect on TL, suggesting that in WIL2-NS this enzyme may not be expressed, or may be expressed at negligible concentrations. It was then considered that reduction in DNA methylation might have induced up-regulation of hTERT, the active component of telomerase. This was also excluded, however, as no significant change in hTERT RNA expression was found in

WIL2-NS over 42 days in low, medium or high FA culture. Taken together, these results suggested that the increase in TL was unlikely to be due to telomerase activity. Whether FA treatment induced changes in the chromatin structure at telomeres, allowing enhanced access by telomerase to its substrate, remains an unanswered question.

A polymorphism in the gene encoding methylene tetrahydrofolate reductase (MTHFR) that altered the activity of the enzyme was considered as another potential factor that could contribute to the acute telomere elongation observed under low FA conditions. WIL2-NS cells were tested for common polymorphisms in this gene. It was confirmed that the cells are homozygous for the more frequent C allele, indicating MTHFR enzyme activity in this cell type was not abnormal, and thus folate utilisation unlikely to be impaired.

Another plausible theory for the increased TL came to light in newly published evidence demonstrating that changes in methylation at the subtelomere impacted on TL, with murine cells deficient for DNA-methyltransferase (DNMT) enzymes exhibiting significant telomere elongation^{148,328}. This effect was associated both with a reduction in methylation at the subtelomere (normally heavily methylated) and an increase in recombination and SCE, possibly due to activation of ALT^{148,328}. The telomeric DNA sequence, TTAGGG, lacks CpGs, the substrate for DNMT enzymes. As such, the hypomethylating effect of FA insufficiency had not been considered as part of the original hypotheses for this thesis. These new reports, however, raised the question as to whether the telomere elongation observed in the present study may be due to hypomethylation, not at the telomere, but at the subtelomeric DNA region.

To test the new hypothesis, that hypomethylation due to FA insufficiency resulted in telomere elongation, a further study was conducted whereby the DNMT1 inhibitor, 5-aza-2'-deoxycytidine (5azadC), was selected as a means of specifically exploring the effect of DNA hypomethylation, without the concomitant incorporation of uracil into DNA that accompanies FA deprivation. Results showed that DNMT inhibition caused a rapid, sustained increase in TL. Analysis by qPCR confirmed a significant increase in global hypomethylation of LINE1 sequences in cells cultured with 5azadC. Furthermore, a significant increase in hypomethylation was also confirmed in the cells cultured in low FA cultures. Together, these findings verified hypomethylation as a likely mechanism underlying the short term TL elongation observed under FA deficient conditions. However, the extent to which LINE1 hypomethylation correlates with subtelomere methylation needs to be tested, and ideally, future studies will involve direct measurement of subtelomere methylation. The correlation of

LINE1 methylation with subtelomere methylation should be investigated under different hypomethylating conditions (eg. FA deficiency, 5azadC treatment and DNMT mutants).

Further work is required to elucidate the specific mechanism underlying hypomethylation and increased TL. Evidence of increased SCE and presence of APBs is strongly suggestive of ALT activation³²⁸. The ALT phenotype is characterised by TL heterogeneity, increased T-SCE, t-circles (possibly arising from recombination-dependent misprocessing of t-loops), and movement of sequences between telomeres^{197,199,400,401}. The factors responsible for recombination in ALT+ cells are Mre11, RAD50 and NBS1; together known as the MRN complex^{197,199,401}. One theory proposed is that loosened chromatin arising from hypomethylation may allow increased access by MRN to the telomere¹⁴⁸, while others suggest that methylation directly inhibits recombination, and as such the effect of hypomethylation is one of de-repression of these factors^{199,400}. Recent reports exploring these associations have found contradictory results; one concluding that no correlation exists between TL and epigenetic changes at the subtelomere⁴⁰², and the other showing that hypomethylation, T-SCE and the ALT phenotype were negatively correlated in some cell types³⁵⁹.

Compromised telomere capping, particularly reduced TRF2, has also been associated with activation of the ALT phenotype^{199,400,401}. An interesting new report has demonstrated a linkage between methylation and telomere capping integrity⁴⁰³. These researchers have shown that disruption to methylation of arginine residues on the N-terminal domain of TRF2 resulted in dysfunctional telomeres, induction of TIFs, and cell senescence⁴⁰³. The possibility that WIL2-NS cells are ALT+, and/or that FA deficiency induces an ALT+ phenotype is speculation at this stage. If not ALT+, the p53 deficient nature of the WIL2-NS would suggest that this cell type may be predisposed to the ALT phenotype under stress conditions^{401,404,405}. P53 is known to function at telomeres, and to be involved in inhibiting recombination, and as such p53 mutations are associated with the occurrence of ALT in human tumours^{401,404,405}. It is possible that cells with compromised p53 function may be increasingly vulnerable to effects of nutritional deficiencies, such as low folate which is, in fact, the case for WIL2-NS cells which exhibit genome instability at higher FA concentration than normal lymphocytes⁹⁵. Further analyses are required to explore these questions.

From the *in vitro* studies conducted in WIL2-NS it was concluded that hypomethylation (possibly subtelomeric), arising either from FA insufficiency or from exposure to 5azadC, results in rapid, transient telomere elongation. This may be due to interstitial telomeric DNA (i.e. telomere sequences located between the telomere and centromere) arising from

recombination. Low FA was also associated with increased uracil in telomeric DNA; a likely cause of the longer term rapid telomere shortening observed in the lowest FA cultures. Further study is required to confirm the mechanisms underlying these observations, most specifically changes in TRF1/2 binding, the interstitial chromosomal location of telomeric DNA, and whether the ALT phenotype is present in these cells.

Following on from the WIL2-NS studies, the effect of low FA on TL was then explored in PBL *in vitro*. The observations in WIL2-NS cells did not translate to the PBL model, with FA dose response showing no effect on TL. A rapid increase in TL was observed in all treatments at day 7, possibly arising from a mitogenic response to PHA stimulation, and a transient up-regulation of telomerase. The TL increase was followed by a steady decline in all treatments through to the completion of the experiment at day 21. Clearly the different lineages and genetic backgrounds of these cells resulted in a different effect at telomeres. The primary differences between PBL and the WIL2-NS cell line are that the latter are a transformed B lymphoblastoid cell line with a defective p53, whereas PBL are T cells from a healthy donor. While these findings relate only to the effect in a single donor, they suggest that the effects of FA depletion vary depending on cell type, cell lineage and genetic background. In future studies the effect on lymphocytes from multiple donors should be investigated and specifically those that differ in their genetic make-up with respect to folate metabolism, DNA methylation and telomere maintenance genes.

In summary, the first hypothesis, that telomeres would shorten *in vitro* in cells cultured under FA deficient conditions was supported in the long term in WIL2-NS, but was disproved in the short term with telomere elongation occurring under FA deficiency conditions. The hypothesis was also disproved in fresh PBL where FA deficiency had no effect on TL *in vitro* up to 21 days of culture. Possible mechanisms underlying the observed changes in TL under FA-deficient conditions are summarised diagrammatically in Figure 9.1 below.

These findings have generated several new hypotheses requiring further examination:

- ***Uracil incorporation in telomeric DNA causes reduced binding kinetics of TRF1 and TRF2, resulting in compromised capping and formation of TIFs.***

Further study is required to explore causal linkages between the presence of uracil, abasic sites arising from BER, TRF1/2 binding kinetics, and the presence of TIFs. Fluorescent *in situ* hybridisation (FISH), chromatin immunoprecipitation (ChIP) or pulldown methods may provide comparative data between different FA treatments. More definitive data,

however, could also be obtained with methodology, such as that published by Opresko *et al*, whereby TRF1/2 binding assays could be conducted using oligonucleotides synthesised with specific, known frequencies of uracil¹³⁹.

- ***Elevated telomere content observed in WIL2-NS under hypomethylating conditions is due to interstitial telomere sequences arising from BFB cycle-induced amplification, recombination and T-SCE?***

This question needs to be explored using both FISH and cytogenetic methodologies to (i) determine the intrachromosomal location of telomere signal, and (ii) compare levels of SCE and T-SCE occurring in cells cultured either in low FA, and/or a hypomethylating agent, such as 5azadC. Measuring T-SCE at different time points within a dose response study may provide important evidence of a compounding effect of recombination events on CIN. These results should then be correlated against methylation status data (globally, and specifically at the subtelomere). From these studies it may be possible to extrapolate a level of folate that is protective for telomere integrity *in vitro*, and in the longer term, *in vivo*.

- ***WIL2-NS cells are positive for the ALT phenotype.***
FISH should be used to determine the presence of ALT-associated PML bodies (APBs) and whether these structures contain telomeric DNA.
- ***Cells that are negative for telomerase, and either deficient or proficient for p53 will respond differently at telomeres to hypomethylation.***

A p53 proficient cell line could be used for p53 knockdown studies to explore interactive effects of p53, hypomethylation and telomerase inhibition on TL and integrity. Such studies would ideally explore location of telomeric sequences, activation of ALT phenotype, capping integrity, recombination, T-SCE and CIN.

Following page, Figure 9.1:

Possible pathways by which folate insufficiency may affect telomeres and CIN

Low folate results in increased uracil in telomeric DNA and reduced DNA methylation (globally, and possibly at the subtelomere). Depending on cell type and genetic background, these factors are likely to have differing impacts at telomeres and on chromosome instability (CIN). Coloured text represents components of the pathway that were examined as part of this thesis. (ALT, alternative lengthening of telomeres; BER, base excision repair; BFB, breakage-fusion-bridge; CIN, chromosomal instability; FA, folic acid; hTERT, human telomerase reverse transcriptase; MRN complex, Mre11, RAD50, NBS1 recombination factors; MNI, micronuclei; MTHFR, methylene tetrahydrofolate reductase; NBud, nuclear bud; NPB, nucleoplasmic bridge; SAM, S-adenosyl methionine, the universal methyl donor; TRF, telomere repeat binding factor).

9.2 THE RELATIONSHIP BETWEEN FOLATE DEPLETION, TL AND CIN

Previous studies have demonstrated strong associations between shortened telomeres and high levels of chromosomal instability (CIN)^{2,6,106-110}. As such, the second hypothesis for this project was that CIN arising from FA deficiency (as determined by frequency of biomarkers MN, NBuds and NPBs) would be negatively associated with TL in human cells cultured *in vitro*. To test this hypothesis CIN was determined by the frequency of damage biomarkers present in cells cytokinesis-blocked at the binucleate (BN) stage, and this data was then correlated against TL data. In PBL no significant correlation was recorded between chromosomal damage biomarkers (MNI, NPB, NBud) and TL. Surprisingly, however, in WIL2-NS the relationship between TL and damage biomarkers, individually and collectively, was both significant and positive. This indicates that generation of cells with longer telomeres by FA deficiency also resulted in a greater degree of CIN, thus disproving the hypothesis, in this cell type. These findings provide important new evidence associating increased telomere content with heightened CIN. Furthermore, they demonstrate that telomere length alone is probably inadequate and inappropriate as a marker for genome instability.

As discussed in Chapter 9.1 above, a number of cancer studies have positively associated longer telomeres (in blood cells or in tumour tissue) with increased telomerase activity and poor prognosis^{2,220-223,233,239,398,399}. Rudolph *et al* (2001) proposed that dysfunctional telomeres, and not telomerase activity, may be the key early initiating factor in cancer²²⁶. CIN arising from dysfunctional telomeres may then lead to activation of telomerase, as a secondary effect²²⁶. Once activated, telomerase is then able to elongate and stabilise short telomeres, providing a means of immortality for preneoplastic cells²²⁶. Results of the current study are consistent with this concept, showing significant increases in CIN in association with increased TL, however, no increase in telomerase activity was found. Accordingly, it is proposed that, when CIN is initiated in WIL2-NS, the p53-deficient background predisposes them to the ALT phenotype, and not activation of telomerase⁴⁰⁴. The hypothesis generated is that in these cells ALT results in increased telomere content due to amplification, recombination and T-SCE.

Strong, significant increases in biomarkers of CIN were attributed to FA deficiency both in WIL2-NS and PBL. The theory underlying the original hypothesis of TL shortening under FA deficient conditions was that excision of uracil would lead to DS breakage within telomere sequences. MNI are a validated biomarker of chromosome breakage, and as such, were of interest in the context of this study. As predicted, significant increases in MNI were recorded in the low FA cultures in both WIL2-NS and PBL. Strong, negative correlations were

observed between total numbers of MNi and FA concentration in medium in both cell types. In WIL2-NS a significant, positive association was found between TL and total MNi. Lindberg *et al* (2007) demonstrated that FA deficiency resulted in increased incidence of telomeric DNA in MNi¹⁵, however, further work is required to explore whether this observation correlates with increased telomeric uracil, and with TL shortening. Currently, evidence linking these factors is coincident, not causative. In combination with results from uracil/TRF1/2 binding studies, solid evidence connecting uracil with telomeric deletions may be generated.

The frequency of nucleoplasmic bridges (NPBs) was also of particular interest in the current study due to previous reports that compromised telomeres resulted in increased chromosomal end-to-end fusions^{19,32,172,276-279,284,329-331}. Accordingly, detailed scoring of BN with multiple NPBs was added to the standard CBMN scoring criteria. In WIL2-NS the total number of NPBs observed was strongly, negatively correlated with FA concentration in medium, and positively associated with TL, however, the latter was not significant. In PBL a negative relationship was also recorded between total NPBs and FA concentration, however this did not reach statistical significance, although previous studies in the same laboratory observed significant increases with FA deficiency under similar conditions^{37,93}.

A new observation reported in this thesis was the number of both WIL2-NS cells and PBL containing such high numbers of NPBs that they were not individually discernible for scoring purposes. Due to their appearance these were termed ‘chewing gum’ cells, and were recorded in high numbers in the lowest FA treatment, and in cells exposed to 5azadC, suggesting a possible association with methylation status. Adding weight to this hypothesis, global LINE1 hypomethylation was correlated strongly and positively with total NPBs in both the FA and 5azadC studies in WIL2-NS. Neither the relationship between methylation and frequency of NPBs, nor the observation of ‘chewing gum’ nuclear morphologies (and related ‘horseshoe’ and ‘donut’ morphologies in PBL) have been reported previously.

New knowledge was also generated by examining TL and CIN effects by culturing PBL *in vitro* for 21 days in FA deplete medium. Previous studies exploring the effects of FA deficiency on CIN have been conducted over 9-10 days^{37,91,93} and the genomic impacts in primary lymphocytes beyond this time-frame were unknown. Data at day 7 of the current study, in low FA cultures, show comparable damage rates to the 9-10 day data reported previously. New data presented in this thesis, however, provides the levels of damage at days 14 and 21, demonstrating that CIN continues to increase at a significant rate, with a three-fold increase in total damage recorded from day 7 to day 21. Additionally, new nuclear morphologies were observed in PBL cultured in low FA. It was postulated that these

morphologies had arisen from end fusions, resulting in dicentric chromosomes and NPB formation. A new set of NPB scoring criteria was developed to ensure these unusual morphologies were included in the CIN data for this study. Cells cultured in low FA medium displayed significantly higher frequencies for each of these new categories. These morphologies have not previously been reported or documented.

Together with TL, uracil and methylation status data, new insights were generated regarding the potential mechanisms underlying NPB formation in WIL2-NS cells treated either in low FA medium, or in medium containing the hypomethylating agent, 5azadC. From these data a new hypothesis was generated that low FA may lead to fusions and NPB formation due to altered telomere length and structure dynamics (Figure 9.1).

In summary, the hypothesis that TL and CIN are negatively associated in cells cultured in FA deficient medium was not supported. While CIN increased significantly in low FA treatments, the increase in TL observed in the same low FA cultures resulted in a positive relationship between these two factors. New hypotheses generated by these findings are:

- ***Telomeric DNA will be increasingly detected in MNi and NBuds, over time, in cells cultured in FA-deficient medium, and these events will be positively correlated with both uracil in telomeric sequences, and subtelomere hypomethylation.***
- ***The presence of telomeric sequences in NPBs will be heterogeneous, reflecting the varied mechanisms underlying potential NPB formation.***

Fluorescence *in situ* hybridisation (FISH) studies are required to assess whether MNi, NBuds and NPBs contain telomeric DNA. This is of particular interest in cells with unusual nuclear morphologies and may assist in determining the underlying mechanism for NPBs, *ie.* fusions of intact but dysfunctional telomeres, fusions of shortened or deleted telomeres, interstitial telomere signal arising from recombination.

9.3 THE *IN VIVO* RELATIONSHIP BETWEEN PLASMA FOLATE AND TL

The final hypothesis for this thesis stated that lower folate status in healthy adults would be associated with shorter TL in PBL *in vivo*. This was tested in a cohort of 90 adults, recruited specifically in younger and older age groups, with an even split for gender in each cohort. Consistent with previous literature TL in the younger cohort was significantly higher than that of the older cohort, and females had longer TL than males. PF was found to be significantly, positively associated with TL in the older male cohort, in support of the hypothesis but this was only evident in univariate analysis. Furthermore, TL in this cohort was negatively associated with plasma Hcy concentration in both univariate and multivariate analysis. In the young female, young male, and older female cohorts no significant associations were observed between folate status and TL in PBL.

Two new, related reports have been published since the present study was completed, one of which, by Xu *et al* (2009), explored the relationship between TL and multivitamin use⁴⁰⁶. This study was conducted in a “risk-enriched” cohort of 586 women, aged 35-74, all of whom were sisters of a breast cancer patient⁴⁰⁶. TL in leukocytes was measured by quantitative PCR (qPCR), and data was adjusted for several parameters, including age, smoking status and BMI⁴⁰⁶. Compared with non-users of multivitamins, daily users had TL, on average, 5.1% longer than non-users. Folate status was significantly higher in supplement users, and regular consumers of ‘stress’/B-complex tablets (4-6 days per week) also had significantly longer TL than less frequent or daily users⁴⁰⁶. These findings are in agreement with those of the present study with respect to higher folate intake equating to increased TL, however, this relationship was only significant in the older male cohort of the present study, whereas Xu *et al* (2009) observed this effect in an all female cohort⁴⁰⁶. Interestingly, the study published by Richards *et al* (2008) was also undertaken in women; 1319 female twins with a mean age of 49 years, and here too, TL was positively correlated with plasma folate concentration, and negatively with plasma Hcy³⁹⁰.

A further new report, from Paul *et al* (2009) has examined the association of plasma folate status and the 677C>T polymorphism of the *MTHFR* gene with TL of leukocytes⁴⁰⁷. These authors also determined TL by qPCR, however, the cohort for this study was all males; 195 healthy men aged 40-68 years, an age group directly in between that of the younger and older cohorts recruited in the current study⁴⁰⁷. Results showed a non-linear association with PF concentration above the mean (11.6nmol/L) being positively correlated with TL, while PF

below the mean was negatively associated with TL⁴⁰⁷. The mean PF in the older male cohort for the current study was significantly higher than this level, at 21.68 ± 9.54 . Mean plasma Hcy concentrations, on the other hand, were similar between the two studies; $9.8 \pm 5.4 \mu\text{mol/L}$ recorded by Paul *et al* (2009), and 10.31 ± 2.36 in the present study. Interestingly, however, Paul *et al* found the highest TL in the quartiles with both the lowest PF ($6.74 \pm 1.32 \text{nmol/L}$, $n = 48$), and the highest plasma Hcy (16.2 ± 7.6 , $n = 49$). These are surprising findings given the observations of the current study. The authors propose that the long TL in the low PF cohort is due to DNA damage response and hypomethylation, the latter resulting in a loosened chromatin structure, and either increased telomerase access or increased recombination⁴⁰⁷. Furthermore, Paul *et al* show a weak association between longer TL and homozygosity for the T allele of the 677C>T polymorphism of the *MTHFR* gene, which the authors propose may be due to reduced methylation⁴⁰⁷.

From the findings reported in this thesis, taken together with these new reports, it is clear that further work is required to determine the relationship between folate status and TL *in vivo*. The relationship appears to differ depending on age, gender, plasma Hcy concentration, and possibly between *MTHFR* genotype and methylation status. Larger studies are required, powered appropriately to allow examination of the impact of age, gender and genotype on TL under folate deplete or replete conditions, to unravel the effect of interactions between these factors. As the recent findings of Epel *et al* have revealed that psychological factors are important variables in TL aetiology, perceived stress levels should be examined in association with broader dietary and lifestyle factors^{134,140}. Biomarkers of telomere integrity (such as TRF binding), subtelomere methylation status, telomerase activity, TIFs, APBs, SCE and CIN would also provide valuable complementary information in this context to inform mechanism of effect.

9.4 CONCLUSIONS & NEW KNOWLEDGE GENERATED BY THIS THESIS

- The impact of FA deficiency on telomere length differs depending on cell type.
- Folic acid insufficiency results in increased uracil in telomeric DNA in WIL2-NS cells.
- Increased uracil content in telomeres may be associated with telomere shortening and reduced telomere integrity.
- Hypomethylation arising either from folic acid insufficiency, or treatment with 5azadC, causes significant increases in telomere length/content, in a dose dependent manner, in WIL2-NS cells.
- Increased telomere length/content arising from hypomethylation is associated with increased chromosome instability in WIL2-NS cells.
- Global hypomethylation is positively associated with increased CIN, as evidenced by increased frequencies of NPBs, MNi and NBuds in WIL2-NS cells.
- Hypomethylation arising from 5azadC treatment is positively associated with increased CIN, in particular chromosome end fusions and multiple NPBs.
- FA deficiency in PBL *in vitro* is associated with unusual nuclear morphologies (ie 'chewing gum', 'horseshoe', 'donut' binucleated cells), possibly arising from compromised telomeres and extensive chromosome fusions.
- In older males short telomeres are positively associated with low plasma folate status and elevated plasma Hcy *in vivo*.
- Telomeric structure and function and subtelomeric DNA methylation may be detrimentally impacted by nutritional deficiencies, such as low folate status, potentially resulting in compromised telomeres and increased CIN.

9.5 SIGNIFICANCE

Data presented in this thesis has demonstrated the direct impact of folic acid deficiency on telomeres *in vitro* in human lymphocytes. A key finding of the research presented in this thesis is that TL, alone, is probably inadequate as a sole measure of chromosomal instability, and that long telomeres (or increased telomere content) can be associated with extremely high levels of chromosome instability. As such, these findings demonstrate that biomarkers of telomere structure and dysfunction, and possibly subtelomeric methylation, are likely to be of considerably greater value in this context, and should be considered for validation in future studies.

These findings also demonstrate that folate insufficiency has a detrimental impact on telomeres, and that this effect is mediated through multiple biochemical pathways initiated both by increased uracil incorporation and reduced DNA methylation status. The significant impact of low folate-induced hypomethylation on increasing telomere length in the short term was an unexpected finding, with potentially wide-reaching implications for optimal management of telomere maintenance, nutritionally or pharmacologically. Chromosome fusions have the capacity to generate rapid, severe genomic instability and damage. Due to the extraordinary increase in NPBs observed under hypomethylating conditions, this effect should, perhaps, be considered of even greater concern than uracil incorporation where folate is limiting. This observation may be of particular significance for individuals whose genetic makeup further compromises DNA methylation under low folate conditions, such as those who are homozygous for the T allele of *MTHFR*, or individuals with the ICF syndrome deficient in DNA methyltransferases.

The striking difference in the effect of FA depletion on TL between WIL2-NS cells and PBL demonstrated that cell type, cell lineage and genetic background are crucial factors determining cellular response to micronutrient deficiency.

The findings presented in this thesis may contribute to the development of a comprehensive panel of biomarkers to assess telomere integrity, which is implied in Figure 9.1. This may provide a means by which early, pre-symptomatic, information can be provided for individuals with an increased susceptibility for chromosome instability. Prophylactic measures based on dietary, or dietary supplement, interventions can then be developed that may prevent, stop, or even reverse potentially neoplastic cellular changes. Such early, informed, evidence-based interventions may contribute to a reduction in morbidities, and hopefully to a reduction in the considerable costs, both personally and for communities, of CIN-related diseases.