The effect of dietary micronutrients and micronutrient supplementation on telomere length

A thesis submitted for the degree of Doctor of Philosophy

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Table of contents

Abstract xi
Candidate declaration xiii
Acknowledgements xiv
List of figures xvi
List of abbreviations xxii
Standard international units of measure xxvi
Orders of magnitude xxvi

1 Introduction and literature review 1
1.1 Telomeres and telomere function 1
1.1.1 Telomere length 2
1.1.2 Telomerase enzyme and ALT mechanism 2
1.1.3 Telomere length and ageing 2
1.2 Telomere length epigenetics 4
1.2.1 DNA methylation and telomere length 4
1.2.1.1 Subtelomeric DNA methylation 5
1.2.1.2 TERRA 6
1.2.2 Chromatin and histone modifications 6
1.2.3 Post translational modifications to telomerase 7
1.2.4 MicroRNAs and telomere length 7
1.3 Disease and other influences on telomere length 8
1.3.1 Environmental and lifestyle influences on telomeres 8
1.3.2 Diet and telomere length 9
1.4 Folate and folic acid 11
1.4.1 Pathways and functions in the cell including importance for genome health 11
1.4.2 MTHFR 677 genotype can influence folate metabolism 13
1.4.3 Sources of folate; diet, fortified foods and supplementation 13
1.4.4 Australian folate RDI and actual consumption 15
1.4.5 Consequences of folate insufficiency 15
1.5 Micronutrients, DNA damage and disease 17
1.6 Folate and telomere length 19
1.7 Knowledge gaps and other challenges identified from the literature 21

2 Project synopsis, aims and hypotheses 23
2.1 Project synopsis 23
2.2 Aims 25
2.3 Hypotheses 26

3 Materials and methods........ 27
3.1 qPCR for absolute telomere length 27
3.1.1 Normalisation of qPCR template DNA 27
3.1.2 Positive and negative controls for qPCR 28
3.1.3 Telomere and *36B4* primers and oligonucleotide standards........ 28
3.1.4 qPCR mastermix 30
3.1.5 PCR cycle conditions........ 31
3.1.6 Absolute telomere length calculations 32
3.2 *Polypill* human study........ 33
3.2.1 Ethics, recruitment and reimbursement........ 33
3.2.2 Inclusion and exclusion criteria 33
3.2.3 Study design, randomisation and blinding 34
3.2.4 *Polypill* study power 35
3.2.5 Volunteer withdrawals, reasons for withdrawal and adverse events 36
3.2.6 *Polypill* formulation 37
3.2.7 Collection blood from volunteers........ 41
3.2.8 Blood processing for separation of plasma and PBMC 42
3.2.9 Plasma measurements 42
3.2.10 Isolation of DNA from PBMC 43
3.2.11 Statement of candidate's contribution as relates to data presented in Chapters 4, 5 and 6. 44
3.3 *In vitro* modelling 45
3.3.1 The WIL2-NS cell line 45
3.3.2 WIL2-NS cell challenge with FA, dUTP and SAM 45
3.3.3 Spent media analysis 48
3.3.4 Determination of WIL2-NS cell concentration and viability........ 48
3.3.5 Calculation of population doublings 48
3.3.6 Isolation of DNA from cultured WIL2-NS cells 48
3.3.7 Quantification of isolated DNA 49
3.3.8 USER™ digest of DNA for qPCR detection of UDG-sensitive lesions in telomeric sequence........ 50
3.3.9 Determination of global DNA methylation 53
3.4 Statistical analysis 55

4 Cross-sectional associations of telomere length with dietary micronutrients and other factors........ 57

4.1 Introduction 57
4.1.1 Telomeres 57
4.1.2 Dietary micronutrients, DNA and telomeres 57
4.1.2.1 In vitro studies 58
4.1.2.2 Caloric, protein and amino acid restriction 58
4.1.2.3 Cross-sectional studies: self-reported data 59
4.1.2.4 Cross-sectional studies: associations of telomere length with biochemical data 60
4.1.3 Aims and hypotheses 62
4.2 Methods 64
4.2.1 WHO definition of BMI categories 64
4.2.2 Socio-Economic index for areas 64
4.2.3 Statistical analyses 66
4.3 Results 68
4.4 Discussion 92
4.4.1 Recent supplementation use did not influence telomere length 92
4.4.2 Telomere length did not significantly differ with gender 94
4.4.3 BMI and obesity did not significantly affect telomere length 95
4.4.4 Telomere length was not correlated with age 96
4.4.5 Telomere length was associated with parental age 96
4.4.6 Telomere length was associated with plasma biochemical measures 99
4.4.6.1 Telomere length was negatively associated with homocysteine 100
4.4.6.2 Telomere length was positively associated with vitamin D 104
4.4.6.3 Appropriateness of plasma micronutrients as biomarkers of exposure 106
4.4.7 Additional covariates of telomere length identified in recent literature were unmeasured 107
4.4.8 There is evidence of telomere length heterogeneity across different chromosomes, chromosome arms, and tissue types 110
4.5 Conclusions 112
4.5.1 Significance 112
4.5.2 Strengths and weaknesses 112
4.5.3 Future studies 113

5 The impact of a micronutrient *Polypill* on telomere length *in vivo* over time 115
5.1 Introduction 115
5.1.1 Intervention studies or RCTs and telomere length 115
5.1.2 Longitudinal studies of telomere length 117
5.1.3 *Polypill* intervention study rationale 122
5.1.4 Aims and hypotheses 123
5.2 Methods 124
5.2.1 Randomisation to treatment groups 124
5.2.2 Missing samples 124
5.2.3 Definition of seasons 124
5.2.4 Longitudinal-specific calculations and analyses 125
5.2.5 Statistical analyses 125
5.3 Results 127
5.3.1 Description of the study population 127
5.3.2 Effect of *Polypill* supplementation on micronutrient levels 129
5.3.3 Effect of *Polypill* supplementation on telomere length 131
5.3.4 Analyses by telomere length trajectory 133
5.3.5 Partial and bivariate correlation 143
5.3.6 Linear regression 154
5.4 Discussion 166
5.4.1 The *Polypill* study was a successful double-blinded RCT 166
5.4.2 *Polypill* supplementation increased plasma micronutrients and decreased homocysteine 166
5.4.3 Telomere length change over time was used to determine telomere length trajectory 168
5.4.3.1 There is potential for regression toward the mean in telomere length trajectory analyses 171
5.4.4 Telomere length trajectory is associated with change in plasma zinc 173
5.4.5 Correlation of week 16 telomere length with covariates differed with treatment group 175
5.4.6 Telomere length at week 16 was correlated with change in plasma micronutrients 175
5.4.6.1 Increase in plasma calcium over time was negatively associated with telomere length at week 16 176
5.4.6.2 Increase in plasma magnesium over time was negatively associated with telomere length at week 16 177
5.4.7 Week 0 telomere length significantly explained week 16 telomere length in regression models 178
5.4.8 Changes in plasma zinc and niacin explained change in telomere length in regression models 178
5.4.9 Study limitations and power 179

5.5 Conclusion 181
5.5.1 Significance 181
5.5.2 Strengths and weaknesses 181
5.5.3 Future directions 182

6 A pilot study to investigate the impact of a modified micronutrient *Polypill* on telomere length *in vivo* over time 183
6.1 Introduction 183
6.1.1 Aims and hypotheses 183
6.2 Methods 184
6.2.1 Missing samples 186
6.2.2 Randomisation to treatment groups 186
6.2.3 Longitudinal-specific calculations and analyses 186
6.2.4 Statistical analyses 186
6.3 Results 187
6.3.1 Descriptives of the treatment population 187
6.3.2 Telomere length changes and trajectories over time 187
6.4 Discussion 198
6.4.1 There were no significant changes in telomere length within modified *Polypill* or placebo groups 198
6.4.2 Modified *Polypill* supplements differentially influenced telomere trajectories 198
6.4.3 Previous treatment allocation influenced telomere length trajectory with modified *Polypill* formulations 199
6.4.4 Those in the telomere shortened trajectory were most likely to be male, have lower BMI and longer telomeres at week 16 200
6.4.5 Limitations of this study design 201
6.5 Conclusions 202
6.5.1 Significance 202
6.5.2 Strengths and weaknesses 202
6.5.3 Future directions 202

7 A quantitative PCR method for the detection of uracil bases within the telomere 203
7.1 Introduction 203
7.1.1 Uracil in DNA 203
7.1.2 Uracil in DNA and folate 206
7.1.3 Uracil in the telomere 206
7.1.4 Measurement of base damage within the telomere 207
7.1.5 Aims and hypotheses 208
7.2 Methods 209
7.2.1 USER™ enzyme 209
7.2.2 Deoxyuridine oligonucleotide standards 209
7.2.3 A standard curve of telomere standards containing uracil 209
7.2.4 Sensitivity of the USER-qPCR method 211
7.2.5 WIL2-NS *in vitro* experimentation 211
7.2.6 Statistical analysis 211
7.3 Results 213
7.3.1 The USER assay with synthetic telomere oligonucleotides containing deoxyuridine displayed proof of concept 213
7.3.2 Verification with *in vitro* challenge 216
7.3.2.1 Live cell concentration was decreased with reduced folic acid concentration and increased dUTP concentration 216
7.3.2.2 There was a significant effect of dUTP on telomere length 216
7.3.2.3 There were no significant effects of folic acid or dUTP on uracil/kb telomere sequence 219
7.3.2.4 Telomere length was non-significantly correlated with uracil/kb telomere 219
7.4 Discussion 224
7.4.1 Pre-qPCR digestion with USER enzyme mix enables the detection of uracil in telomeric sequence 224
7.4.2 Both folate deficiency and dUTP supplementation *in vitro* impacted live cell concentration at day 7 225
7.4.3 Folic acid deficiency did not significantly alter telomere length 226
7.4.4 dUTP supplementation of 15 µM was associated with longer telomeres 226
7.4.5 Uracil/kb telomere length was not significantly altered with folic acid deficiency or dUTP supplementation 227
7.4.6 Telomere length may be influenced by the content of uracil in telomeric sequence 228
7.5 Conclusion 230
7.5.1 Significance 230
7.5.2 Future directions 230

8 The effects of folic acid, SAM and dUTP on telomere length, telomeric uracil and DNA methylation in WIL2-NS cells 231
8.1 Introduction 231
8.1.1 DNA methylation and folate 231
8.1.2 DNA methylation and nutrients... 232
8.1.3 Subtelomeric DNA methylation and telomere length 234
8.1.4 Aims and hypotheses 235
8.2 Methods 236
8.2.1 WIL2-NS *in vitro* experimentation 236
8.2.2 Telomere length and telomere uracil content 236
8.2.3 Global cytosine DNA methylation 236
8.2.4 Statistical graphs and analyses 237
8.3 Results 239
8.3.1 Experiment one: Folic acid, dUTP and SAM influenced WIL2-NS cell viability 239
8.3.2 Experiment one: Folic acid, SAM and dUTP influenced WIL2-NS telomere length 241
8.3.3 Experiment one: Folic acid and SAM influenced telomeric uracil content in WIL2-NS cells 243
8.3.4 Experiment one: Folic acid, SAM and dUTP influenced global 5-methylcytosine content in WIL2-NS cells 245
8.3.5 Experiment two: Folic acid, and SAM influenced WIL2-NS cell viability 247
8.3.6 Experiment two: Folic acid, dUTP and SAM influenced WIL2-NS telomere length 249
8.3.7 Experiment two: SAM, but not folic acid or dUTP influenced telomeric uracil content in WIL2-NS cells 251
8.3.8 Experiment two: Folic acid, dUTP and SAM concentration influenced global 5-methylcytosine in WIL2-NS cells 253
8.4 Discussion 255
8.4.1 FA, dUTP and SAM may affect cell viability alone and interactively 255
8.4.2 Telomere length.. 257
8.4.3 Telomeric uracil 257
8.4.4 Global 5-methylcytosine 259
8.5 Conclusion 261
8.5.1 Future directions.. 261

9 Discussion, conclusions and knowledge gaps 263
9.1 Discussion and conclusions 263
9.1.1 *In vivo*: cross-sectional associations from the *Polypill* study 263
9.1.2 *In vivo*: longitudinal observations from the *Polypill* study 264
9.1.3 *In vivo*: longitudinal observations from the pilot study of modified *Polypill* composition 266
9.1.4 *In vitro*: uracil within the telomere can be detected by a novel qPCR method 267
9.1.5 *In vitro*: folic acid may influence telomere integrity, functionality and length 268
9.2 Remaining knowledge gaps 269
9.2.1 What are the optimal micronutrient conditions for telomere length? 269
9.2.2 Are PBMCs the optimal tissue for measuring telomere length and how long should an intervention study examining telomere length be?. 270
9.2.3 Is telomere quality and functionality more important that telomere length? 272
9.3 Overview of main study findings and future directions 273
9.3.1 Diet 273
9.3.2 Base damage 273
9.3.3 Heritability 274
9.3.4 Future directions 274

10 Appendices 277
10.1 Supplementary data 277
10.2 Publications arising from this thesis 319
10.3 Presentations at scientific meetings arising from this thesis 333
10.3.1 Oral 333
10.3.2 Oral/poster 333
10.3.3 Poster 333

References 335

Abstract

Telomeres are nucleoprotein complexes, which cap the ends of linear chromosomes to prevent against chromosome end-to-end fusions in the cells. The underlying DNA sequence in humans is a TTAGGG hexamer that is repeated many times at each telomere. Due to the end-replication problem at DNA ends, the length of the telomere sequence shortens with each cycle of cell division. Telomere length has been associated with chronic disease, markers of oxidative stress and inflammation as well as diet patterns and individual nutrients. Evidence in the literature indicates that telomere length has both elements of heritability and may be modified by various environmental exposures including diet. However, the relationship of telomere length with dietary micronutrients in Australian populations has been largely unexplored. Additionally, the possibility that nutrients can influence telomere length in humans gives rise to the opportunity to modify telomere length through targeted dietary interventions. A double-blinded micronutrient intervention randomised controlled trial (RCT) – the *Polypill* study – provided the opportunity to assess changes in telomere length over time in a metropolitan Australian population.

Peripheral blood mononuclear cell (PBMC) telomere length in a healthy cohort of middle-aged South Australians was measured and cross-sectional associations with demographic and anthropometric variables were explored. Plasma levels of dietary micronutrients such as vitamin D and the metabolite homocysteine (associated with B vitamin deficiency) were correlated with telomere length. Vitamin D was found to be positively associated with PBMC telomere length while plasma homocysteine was inversely associated with PBMC telomere length.

The *Polypill* double-blinded RCT investigated changes in PBMC telomere length following 16 weeks of treatment with a micronutrient supplement containing folic acid, vitamin B_{12}, vitamin E, retinol, nicotinic acid and calcium (FBERNC) and compared this change to participants who received an inactive placebo. When groups were compared, there were no significant differences in the

mean changes in telomere length over time. Additionally, there was no significant difference in the proportions of individuals placed in telomere length trajectory groups defined as telomere sequence loss, maintenance or gain.

It was hypothesised that the integrity of the telomere sequence may be a more valuable biomarker than telomere length alone to assess responses to B vitamin supplementation. As uracil misincorporates in to the genome under low folate conditions, a qPCR method to detect uracil within the telomere sequence was conceived and optimised. The reproducible assay was developed with the use of synthetic uracil-containing primers and was then applied to DNA extracted from the WIL2-NS human lymphoblastoid cell line cultured *in vitro* under low folic acid and with supplemented dUTP. Next, *in vitro* modelling with various concentrations of folic acid, dUTP and additionally S-adenosyl methionine (SAM) were performed with the WIL2-NS cell line. There were no clear trends of either folic acid, dUTP or SAM on cell viability, telomere length, telomeric uracil content nor global methylation. Additionally, complex but statistically significant interactive effects for each of the experimental endpoints were noted indicating the possibility of strong homeostatic mechanisms regulating telomere integrity under conditions of folate deficiency and dUTP excess.

Candidate declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Carly Moores

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List of figures

Figure 1.1 – Schematic diagram of the lariat structure at the terminal telomere end 1
Figure 1.2 – Lifestyle, genetic and environmental factors which influence DNA, telomere welfare and risk of ageing-related diseases 10
Figure 1.3 – Chemical structure of folic acid 11
Figure 1.4 – Folate metabolism 12

Figure 3.1 – Schematic of study design 35
Figure 3.2 – Number of participants and dropouts by each study phase 36
Figure 3.3 – Concentrations of each of the three treatments used *in vitro* 45
Figure 3.4 – Staggered timeline of cell culture experiments 47

Figure 4.1 – Descriptions of the socio-economic index for areas measures 65
Figure 4.2 – Absolute telomere length against age, by gender 73
Figure 4.3 – Scatterplot matrix of bivariate correlation associations reported in Table 4.7 77
Figure 4.4 – Scatterplot of absolute telomere length against age, BMI, maternal age and paternal age at week 0 78
Figure 4.5 – Scatterplot matrix of bivariate correlation associations between telomere length and measured plasma micronutrients at week 0, as reported in Table 4.8 82
Figure 4.6 – Absolute telomere length against plasma homocysteine at week 0 83
Figure 4.7 – Absolute telomere length against plasma vitamin D at week 0 for all participants, and individually for females and males 86

Figure 5.1 – Timeline of study defining week 0 and week 16 124
Figure 5.2 – Graphical representation of the mean change in absolute telomere length over time and by treatment group 132
Figure 5.3 – Graphical representation of individual telomere length trajectory from week 0 to-, for all participants 134
Figure 5.4 – Graphical representation of individual telomere length trajectory from week 0 to 16, for all participants, by treatment group 135
Figure 5.5– Telomere length trajectory is associated with change in plasma zinc 142

Figure 6.1 – Schematic of study detailing the six modified *Polypill* active treatments used in the second intervention 185
Figure 6.2 – Graphical representation of change in telomere length from week 16 to week 32, by treatment group 191

Figure 7.1 – Incorporation of uracil in DNA..204
Figure 7.2 – Pyrimidine metabolism...205
Figure 7.3 – Products from complete USER digestion of the 4U-containing telomere oligomer..210
Figure 7.4 – Schematic representation of the experimental cultures 212
Figure 7.5 – Standard curve of telomere oligonucleotide containing 0, 1, 2 and 4U 214
Figure 7.6 – Standard curve of 0 – 100% 4U telomere oligonucleotide 215
Figure 7.7 – Live cell concentration following 7 day *in vitro* culture 217
Figure 7.8 – Telomere length 218
Figure 7.9 – Number of uracil bases/kb telomere sequence...........................220
Figure 7.10 – Correlation of telomere length with uracil bases/kb telomere sequence 221

Figure 8.1 – DNA methylation: chemical conversion of cytosine to 5-methylcytosine 231
Figure 8.2 – Schematic representation of the experimental cultures 238
Figure 8.3 – Experiment one: viable cells at day 7..240
Figure 8.4 – Experiment one: telomere length 242
Figure 8.5 – Experiment one: uracil/kb telomeric sequence 244
Figure 8.6 – Experiment one: percent global 5-methylcytosine.......................246
Figure 8.7 – Experiment two: viable cells at day 7..248
Figure 8.8 – Experiment two: telomere length 250
Figure 8.9 – Experiment two: uracil bases/kb telomere sequence..................252
Figure 8.10 – Experiment two: percent global 5-methylcytosine....................254

Figure 9.1 – Piecing together the main study findings to identify the remaining knowledge gaps..275

Figure 10.1 – Scatterplot matrix of bivariate correlation associations reported in Table 5.8 290
Figure 10.2 – Scatterplot matrix of bivariate correlation associations reported in Table 5.9 291
Figure 10.3 – Scatterplot matrix of bivariate correlation associations reported in Table 5.10 292
Figure 10.4 – Scatterplot matrix of bivariate correlation associations reported in Table 5.11 293

List of tables

Table 1.1 – Dietary sources of folate 14
Table 1.2 – Various dietary micronutrients and their function with DNA in the cell 18

Table 3.1 – Human telomere and *36B4* single copy gene forward and reverse primer sequences and oligonucleotide standards.. 29
Table 3.2 – Amount of telomere oligonucleotide standards and kb telomere sequence used in the qPCR assay 30
Table 3.3 – Amount of *36B4* oligonucleotide standards and kb telomere sequence used in the qPCR assay 30
Table 3.4 – Make up of telomere and 36B4 single copy gene qPCR reactions .. 31
Table 3.5 – PCR cycling conditions for telomere length and 36B4 single copy gene amplification.. 31
Table 3.6 – Micronutrients and doses in the *Polypill*... 38
Table 3.7 – EAR, RDI and UL for *Polypill* micronutrients.................................... 39
Table 3.8 – Formulation of folic acid and vitamin B_{12} tablet administered in the *Polypill* intervention study 40
Table 3.9 – Formulation of nicotinic acid and calcium carbonate tablet administered in the *Polypill* intervention study 40
Table 3.10 – Formulation of vitamin E and retinol capsule administered in the *Polypill* intervention study 41
Table 3.11 – Collection and distribution of blood samples for various analyses 41
Table 3.12 – Oligonucleotide sequences used in optimisation of the USER assay 52
Table 3.13 – Standard curve of DNA with 50% methylated cytosine used in global methylation assays 54

Table 4.1 – Characteristics of the study population 68
Table 4.2 – Distribution of body mass index categories of participants 69
Table 4.3 – Self-reported recent supplement use prior to study period............ 70
Table 4.4 – Measured micronutrient levels in study participants 71
Table 4.5 – Telomere length for all participants, by gender and obesity at week 0.. 72
Table 4.6 – Demographic and anthropometric descriptives of the study population at week 0, by self-reported recent supplementation status 74
Table 4.7 – Correlation matrix of telomere length with age, maternal age, paternal age and BMI at week 0 76
Table 4.8 – Bivariate correlation matrix of telomere length with measured plasma micronutrient status at week 0 80

Table 4.9 – Partial correlation matrix of telomere length with measured plasma micronutrient status at week 0, with adjustment for participant age, gender, maternal age, paternal age and BMI 84
Table 4.10 – Multiple linear regression model one: demographic factors, BMI and previous supplement use as predictors of telomere length at week 0 88
Table 4.11 – Multiple linear regression model two: measured plasma micronutrients as predictors of telomere length at week 0 90

Table 5.1 – Longitudinal telomere length investigations from the literature 118
Table 5.2 – Demographic and anthropometric descriptives of the study population at week 16....127
Table 5.3 – Demographic and anthropometric descriptives of the study population at week 16, by treatment group 128
Table 5.4 – Change in plasma micronutrient levels and homocysteine from week 0 to 16, by treatment group 130
Table 5.5 – Change in telomere length from week 0 to week 16, by treatment group 131
Table 5.6 – Demographic and anthropometric descriptives and telomere length of the study population, by treatment group and telomere length trajectory from week 0 to week 16....136
Table 5.7 – Change in plasma micronutrient levels of the study population, by treatment group and telomere length trajectory from week 0 to week 16....140
Table 5.8 – Bivariate correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, for *Polypill* group 144
Table 5.9 – Bivariate correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, for Placebo group 146
Table 5.10 – Bivariate correlation matrix of telomere length at week 16, change in telomere length from week 0 to week 16, participant age, maternal age, paternal age and BMI, for *Polypill* group 148
Table 5.11 – Bivariate correlation matrix of telomere length at week 16, change in telomere length from week 0 to week 16, participant age, maternal age, paternal age and BMI, for placebo group 149
Table 5.12 – Partial correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, with adjustment for participant age, gender, maternal age, paternal age and BMI, for *Polypill* group....150
Table 5.13 – Partial correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, with adjustment for participant age, gender, maternal age, paternal age and BMI, for placebo group....152
Table 5.14 – Multivariate linear regression to explain telomere length at week 16, for all participants 155
Table 5.15 – Multivariate linear regression to explain telomere length at week 16, for *Polypill* group 157

Table 5.16 – Multivariate linear regression to explain telomere length at week 16, for placebo group 158
Table 5.17 – Multivariate linear regression to explain telomere length at week 16 with change in micronutrients from week 0 to week 16, for *Polypill* group160
Table 5.18 – Multivariate linear regression to explain telomere length at week 16 with change in micronutrients from week 0 to week 16, for placebo group 162

Table 6.1 – Demographic and anthropometric descriptives and telomere length, by treatment group from week 16 to week 32 188
Table 6.2 – Change in telomere length from week 16 to week 32, by treatment group 190
Table 6.3 – Telomere length trajectory from week 16 to week 32, by treatment group 192
Table 6.4 – Week 16 to week 32 treatment allocation for week 16 and week 32 completers 194
Table 6.5 – Demographic and anthropometric descriptives and telomere length of the study population, by telomere length trajectory from week 16 to week 32........ 196

Table 7.1 – Partial correlation of individual WIL2-NS endpoint measures....... 222
Table 7.2 – Partial correlation of group WIL2-NS endpoint measures............ 223

Table 8.1 – Micronutrients which can influence DNA methylation................. 233

Table 10.1 – Volunteer withdrawals during the *Polypill* study: stage and reason for withdrawal, adverse events 278
Table 10.2 – Volunteer withdrawals during the *Polypill* study, by study phase and treatment group........ 279
Table 10.3 – Compliance data from week 0 to week 16 *Polypill* intervention, by treatment group........ 280
Table 10.4 – Compliance data from week 16 to week 32 *Polypill* intervention, by treatment group........ 281
Table 10.5 – Correlation of longitudinal telomere length measures for all participants, and separately for week 0 – week 16 groups............................. 282
Table 10.6 – Differences in plasma micronutrient levels and homocysteine at week 0 and 16 in *Polypill* supplement group........ 283
Table 10.7 – Differences in plasma micronutrient levels and homocysteine at week 0 and 16, by treatment group 284
Table 10.8 – Demographic and anthropometric descriptives and telomere length of the study population, by telomere length trajectory from week 0 to week 16 286
Table 10.9 – Change in micronutrient levels by telomere length trajectory.... 287

Table 10.10 – Bivariate correlation matrix of telomere length and change in telomere length with change in plasma micronutrient status from week 0 to week 16 288

Table 10.11 – Bivariate correlation matrix of telomere length at week 16, change in telomere length from week 0 to week 16, participant age, maternal age, paternal age and BMI 294

Table 10.12 – Partial correlation matrix of telomere length and change in telomere length with change in plasma micronutrient and homocysteine status from week 0 to week 16, with adjustment for participant age, gender, maternal age, paternal age and BMI 296

Table 10.13 – Micronutrient and homocysteine concentrations at weeks 0, 16 and 32 by season of blood sample collection 298

Table 10.14 – Micronutrient and homocysteine concentrations at week 0, 16 and 32 by season of blood collection, and treatment group 304

Table 10.15 – Supplementary data from Chapter 7: cell count data, population doublings and produced homocysteine 309

Table 10.16 – Supplementary data from Chapter 7: telomere length, USER ΔC_T and uracil per kb telomere sequence...310

Table 10.17 – Supplementary data from Chapter 8: cell count data, population doublings and produced homocysteine – experiment one 312

Table 10.18 – Supplementary data from Chapter 8: cell count data, population doublings and produced homocysteine – experiment two 315

List of abbreviations

5-MeTHF	5-methyltetrahydrofolate
5,10-MeTHF	5,10-methylenetetrahydrofolate
A	adenine
ALT	alternate lengthening of telomeres
ANOVA	analysis of variance
ATCC	American Type Culture Collection
AUD	Australian dollar
B_2	vitamin B_2/riboflavin
B_6	vitamin B_6/pyridoxine
B_{12}	vitamin B_{12}/cobalamin
B	unstandardised regression coefficient
B in FBERNC	vitamin B_{12} (*Polypill*)
β	standardised regression coefficient
BER	base excision repair
BFB	breakage-fusion-bridge
BHMT	betaine homocysteine methyltransferases
BMI	body mass index
bp	base pairs
C	cytosine
C in FBERNC	calcium (*Polypill*)
CBMN	cytokinesis-block micronucleus assay
CH_3	methyl group
CO_2	carbon dioxide
CpG	cytosine phosphate guanine
CRP	c-reactive protein
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	cycle threshold
CV	coefficient of variation
CVD	cardiovascular disease
Δ	change
Δ_{0-16}	change from week 0 to week 16
Δ_{16-32}	change from week 16 to week 32
ΔC_T	change in cycle threshold
DC	dyskeratosis congenita
DDR	DNA damage response
DHF	dihydrofolate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
dU	deoxyuridine

dUTP	deoxyuridine triphosphate
dUMP	deoxyuridine monophosphate
E	vitamin E/alpha-tocopherol (*Polypill*)
EAR	estimated average requirement
EDTA	ethylenediaminetetraacetic acid
ex.	excludes
F	folate (*Polypill*)
FA	folic acid
FBS	foetal bovine serum
FFQ	food frequency questionnaire
FISH	fluorescence in-situ hybridisation
FPG	formamidopyridine DNA glycosylase
G	guanine
H_2O	water
HBSS	hanks balanced salt solution
hOGG1	human 8-oxoguanine DNA N-glycosylase 1
HPLC	high performance liquid chromatography
HREC	human research ethics committee
hTERT	human telomerase reverse transcriptase
ICF	immunodeficiency, centromere instability and facial anomalies syndrome
IEO	index of education and occupation
IER	index of economic resources
IRSD	index of relative socio-economic disadvantage
IRSED	index of relative socio-economic advantage and disadvantage
IU	international unit
IQR	interquartile range
kb	kilobase
LDL	low density lipoprotein
m^5C	5-methyl cytosine
hm^5C	5-hydroxymethyl cytosine
MN	micronuclei
MTHFR	methylene tetrahydrofolate reductase
MTR	methionine reductase
MTRR	methionine synthase reductase
n	number
N	nicotinic acid (*Polypill*)
n-3	omega 3
n-6	omega 6
NAD	nicotinamide adenine dinucleotide

NADP	nicotinamide adenine dinucleotide phosphate
NaK	sodium-potassium
NER	nucleotide excision repair
NHANES	National Health and Nutrition Examination Survey
NHMRC	National Health and Medical Research Council
NO	nitric oxide
NTC	no template control
P	probability
PARP	poly ADP ribose polymerase
PASW	predictive analytical software
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acid
qPCR	quantitative polymerase chain reaction
qFISH	quantitative fluorescence in-situ hybridisation
R	Pearson correlation coefficient
R in FBERNC	retinol (*Polypill*)
R^2	coefficient of determination
RCT	randomised controlled trial
RDA	recommended daily allowance
RDI	recommended daily intake
RNA	ribonucleic acid
RP-HPLC	reverse phase high performance liquid chromatography
SAM	s-adenosyl methionine
SD	standard deviation
SDS	sequence detection software
SE	standard error
SEIFA	socio-economic index for areas
SEM	standard error of the mean
SHMT	serine hydroxymethyltransferase
SNP	single nucleotide polymorphism
T	thymine
TERRA	telomeric repeat-containing RNA
TERT	telomerase reverse transcriptase
TGA	Therapeutic Goods Administration
THF	tetrahydrofolate
TL	telomere length
TPE	telomere position effect
TRAP	telomere repeat amplification protocol
U	uracil
UDG	uracil DNA glycosylase
UL	safe upper level of intake

USER	uracil specific enzyme reagent
UTR	untranslated region
UV	ultra violet
VNTR	variable number tandem repeat

Standard international units of measure

°C	degrees Celsius
Gy	Gray
h	hour
L	litre
m	metre
mol	mole
M	molar
min	minute
s	second

Orders of magnitude

M	mega	($\times 10^{6}$)
k	kilo	($\times 10^{3}$)
m	milli	($\times 10^{-3}$)
µ	micro	($\times 10^{-6}$)
n	nano	($\times 10^{-9}$)
p	pico	($\times 10^{-12}$)
f	femto	($\times 10^{-15}$)
a	atto	($\times 10^{-18}$)

1 Introduction and literature review

1.1 Telomeres and telomere function

Telomeres are repeats of the hexamer sequence $(TTAGGG)_n$, which with the associated telosome (aka shelterin) protein complex, cap the end of all mammalian chromosomes. The terminal end of the telomere consists of single and double-stranded DNA which loops back upon itself (Griffith *et al.*, 1999). This lariat structure (Figure 1.1) contains a larger, double stranded telomere duplex loop (t-loop) into which the long 3' G-rich single strand overhang, the displacement loop (D-loop) is inserted (Greider, 1999, Griffith *et al.*, 1999, Luke-Glaser *et al.*, 2012). The D-loop-t-loop lariat, and associated proteins including the telosome/shelterin complex, serves to eliminate 'free ends' of DNA that may be processed as double or single stranded DNA breaks. The structure prevents the triggering of an apoptotic response within the cell which is otherwise induced at broken chromosome loci (Karlseder *et al.*, 1999).

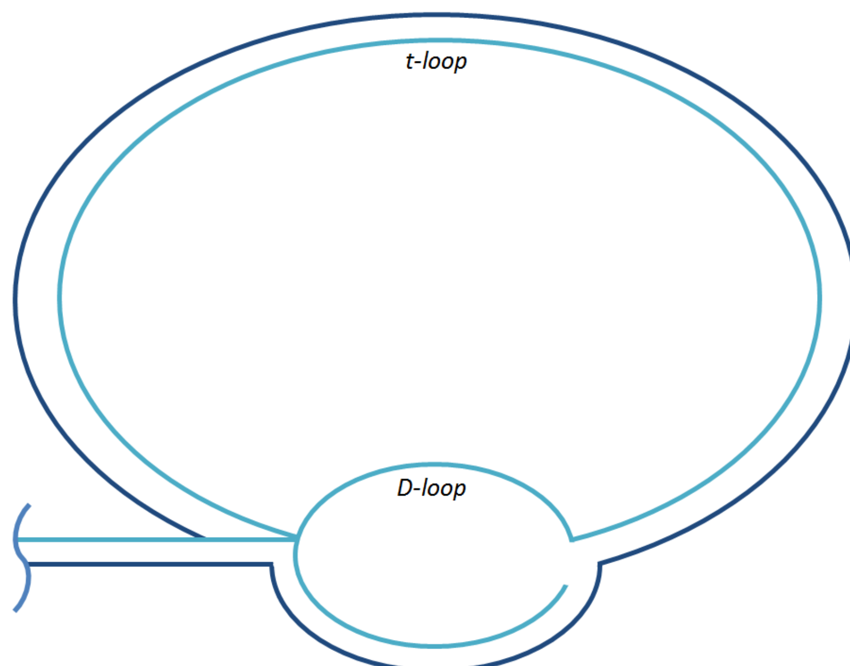

Figure 1.1 – Schematic diagram of the lariat structure at the terminal telomere end

Image modified from Shay (1999) and Greider (1999).

1.1.1 Telomere length

The ends of double helical DNA of linear chromosomes are unable to be entirely replicated during each cycle of nuclear division due to unidirectional DNA polymerases which work in a 5' to 3' fashion. To overcome this, telomeric DNA is sacrificed to ensure coding genetic sequence is not lost from the extremities of the chromosome. This limitation is known as the end-replication problem, DNA underreplication, or marginotomy (Olovnikov, 1971, Watson, 1972, Olovnikov, 1973, Lu *et al.*, 2013). As a consequence of the end-replication problem, somatic cell telomeres are abridged by an estimated 30 – 200 bp (Sitte *et al.*, 1998) to 50 – 150 bp (Muntoni and Reddel, 2005) with each cycle of cell division. Telomere length may be increased or maintained through two known methods; the enzyme telomerase or by an alternative (ALT) recombination-based method.

1.1.2 Telomerase enzyme and ALT mechanism

Human telomerase adds *de novo* TTAGGG to existing telomere sequences thus increasing or maintaining telomere length. Catalytically active telomerase enzyme is composed of two molecules each of telomerase reverse transcriptase (TERT), telomerase RNA (TERC), and dyskerin (DKC1) (Cohen *et al.*, 2007). Telomerase was long thought to be present and active exclusively in cancer cells as a method of escaping cellular senescence and cell death pathways (i.e. acquiring immortality), however normal human cells possess telomerase, which is very highly regulated (Blackburn, 2005). Approximately 85 – 90% of cancers utilise telomerase to grow indefinitely (Shay and Bacchetti, 1997, Shay *et al.*, 2012) while some cancers (the remaining 10 – 15%) have no detectable telomerase and appear to utilise another mechanism of telomere elongation (Bryan *et al.*, 1995, Bryan *et al.*, 1997, Shay and Bacchetti, 1997). The alternative lengthening of telomeres (ALT) mechanism is an homologous recombination-mediated mechanism which increases telomere length, however much molecular detail of the process remains unknown (Conomos *et al.*, 2013).

1.1.3 Telomere length and ageing

Telomere attrition occurs naturally during the ageing process (Lindsey *et al.*, 1991, Vaziri *et al.*, 1993, Lopez-Otin *et al.*, 2013) but is accelerated in certain premature ageing syndromes such as ataxia telangiectasia. The length of the telomeric

sequence declines with age until the telomere becomes critically short, typically signalling cellular senescence and resulting in programmed cell death (Lundblad and Szostak, 1989, Harley *et al.*, 1990, Allsopp *et al.*, 1992, Harley *et al.*, 1992). Telomere length is regarded as an indicator of the biological age of a cell or its ability to undergo additional mitotic divisions (Donate and Blasco, 2011). Since telomere length changes are also known to be induced by various environmental, physiological and psychological stressors (Epel *et al.*, 2004), such variations in telomere length may suggest that aside from the effect of the end-replication problem, other factors may contribute to telomere shortening.

1.2 Telomere length epigenetics

Epigenetics typically refers to heritable changes in DNA and chromatin structure which do not involve changes in the protein coding DNA sequence. Epigenetic mechanisms control gene expression through chromatin re-modelling events as a consequence of DNA methylation and histone modifications. More recently, microRNA (miRNA) was also considered as an alternative epigenetic mechanism for its ability to influence gene expression post-transcriptionally (Sato *et al.*, 2011).

1.2.1 DNA methylation and telomere length

Vertebrate DNA methylation is the addition of a methyl group (CH_3) to cytosine residues to produce the modified base 5-methylcytosine (m^5C). A family of DNA methyltransferase (*DNMT*) genes are responsible for catalysing methylation of cytosine bases in mammals. DNMT3A and DNMT3B enzymes catalyse primarily *de novo* methylation (Okano *et al.*, 1998) whilst DNMT1 is the principal enzyme involved in maintenance methylation of hemi-methylated DNA following semi-conservative DNA replication (Yoder *et al.*, 1997). It has been suggested that approximately 4% of cytosine bases are methylated in vertebrates (Iguchi-Ariga and Schaffner, 1989), with these modified residues largely occurring at CpG (cytosine-phosphate-guanine) dinucleotides located within promoter regions (Choi *et al.*, 2005b). As gene promoter regions are typically rich in these CpG dinucleotides, they are known as CpG islands. CpG islands are also in large abundance in human gene exons.

Throughout the course of ageing, there is an accumulation of genetic damage as well as an overall decrease in genomic DNA methylation levels (Vanyushin *et al.*, 1973a, Vanyushin *et al.*, 1973b). DNA methylation epigenetically controls gene expression by condensing chromatin structure (Keshet *et al.*, 1986, Buschhausen *et al.*, 1987, Cedar, 1988, Antequera *et al.*, 1990, Nguyen *et al.*, 2001) thereby hindering the binding of specific transcription factors to the DNA (Kovesdi *et al.*, 1987, Watt and Molloy, 1988, Iguchi-Ariga and Schaffner, 1989, Comb and Goodman, 1990, Deng *et al.*, 2004). Abnormal gene expression caused by local (gene specific) hyper- (Harada *et al.*, 2002, Wagner *et al.*, 2002, Yamamoto *et al.*, 2002) and hypomethylation (Cheah *et al.*, 1984, Nambu *et al.*, 1987, Tsukamoto *et al.*, 1992) has been shown to be prevalent in certain cancers (For reviews see Jones,

1986, Rountree *et al.*, 2001, Wajed *et al.*, 2001). In light of this, hypomethylation on a global (genome-wide) scale is recognised as a hallmark of cancer and ageing (Dunn, 2003). Both global (genome-wide) and local (gene-specific) DNA methylation patterns may be modified by deficiency and excess of micronutrients including folate, and these methylation patterns are often altered in cancer cells (Friso and Choi, 2002).

1.2.1.1 Subtelomeric DNA methylation

The subtelomeric region of the chromosome is located proximal to the telomeric hexamer repeats at the ends of the chromosome. Unlike the repeat unit of the telomere, which is highly conserved, the subtelomeric region of the chromosome is variable (Murray and Szostak, 1986, de Lange *et al.*, 1990). Whilst the hexamer repeat of the telomere is devoid of DNA methylation substrates, the subtelomeric region has been demonstrated to contain methylated CpG dinucleotides in human somatic cells (de Lange *et al.*, 1990, Brock *et al.*, 1999). Both maintenance (*DNMT1*) and *de novo* (*DNMT3A* and *DNMT3B*) DNA methyltransferases have been shown to negatively regulate telomere length (Gonzalo *et al.*, 2006). In the same study, loss of DNA methylation was shown to result in a higher frequency of telomeric sister chromatid exchanges (Gonzalo *et al.*, 2006). As mentioned previously, cancer cells may become immortal by acquiring telomerase activity or by recombination *via* ALT. Subtelomeric DNA methylation patterns were shown recently to have no correlation with the length of telomeric sequence in human cancer cell lines (Lee *et al.*, 2009a). Telomeres with subtelomeric regions that are less densely methylated have been hypothesised to perhaps shorten faster than those with greater subtelomeric methylation (Maeda *et al.*, 2009). Notably, subtelomeric regions are hotspots of interchromosomal recombination activity (Linardopoulou *et al.*, 2005).

Although a hypomethylated subtelomere appears to elicit characteristics observed in ALT-positive cells (Gonzalo *et al.*, 2006) telomerase-positive cells have hypermethylated subtelomeric regions (Ng *et al.*, 2009). Although the extent of typical subtelomeric CpG methylation is not known, it does appear that the epigenetic status of the subtelomere may be altered in both ALT- and telomerase-positive cells. That increase in telomere length – caused by *DNMT* knockdown leading to hypomethylated subtelomeres – occurred whilst other telomeric

heterochromatic marks such as histone methylation were unchanged suggests DNA methylation exerts higher-order control of telomere length (Gonzalo *et al.*, 2006). As the subtelomeric assembly of each chromosome arm is variable, with subtelomeric repeat sequences ranging from <10 kb to >300 kb (Riethman *et al.*, 2005), the number of methylation substrates (CpG) and hence the extent of subtelomeric methylation is likely to be different amongst human chromosomes.

1.2.1.2 TERRA

It was initially believed that telomeric sequences were transcriptionally silent, however telomeres transcribe telomeric repeat-containing RNA or TERRA (Azzalin *et al.*, 2007) known to localise at the telomere (Azzalin *et al.*, 2007, Schoeftner and Blasco, 2008) Non-coding TERRA RNA contains telomeric and subtelomeric sequence (Luke and Lingner, 2009) and recently, TERRA transcription was shown to be negatively regulated by cytosine methylation of its promoter, housed in the subtelomere (Nergadze *et al.*, 2009, Ng *et al.*, 2009). Increased methylation of the subtelomere, a notable feature of telomerase-expressing cells, results in silencing of subtelomeric/telomeric transcription of TERRA, hence suggesting telomerase is inhibited by TERRA, and that this transcriptional silencing may be selected for in cancer cells (Ng *et al.*, 2009). Whether the inhibition of telomerase by TERRA occurs primarily *in situ* or *in trans* is unknown (Ng *et al.*, 2009) and although TERRA transcription has been shown to be higher in ALT-positive cells than telomerase-positive cells, this may be due to increased TERRA signal by the generally longer telomeres characteristic of heterogeneous ALT cells (Ng *et al.*, 2009).

1.2.2 Chromatin and histone modifications

The human genetic code is organised within the cell as chromatin in the form of DNA packages of 146 bp wound around a nucleosome comprising pairs of each histone H2A, H2B, H3 and H4 (Geiman and Robertson, 2002). DNA methylation at CpG dinucleotides, along with histone acetylation, induces reversible changes on chromatin structure. Condensed and transcriptionally silent chromatin or heterochromatin largely contains methylated cytosine residues and histones that are deacetylated. Histone modifications typical of heterochromatic telomeres include increased tri-methylation of histones H3 (Lysine 9) and H4 (Lysine 20) and lowered acetylation of histones H3 and H4 (Yehezkel *et al.*, 2008). Other

modifications at H3 and H4 tails include phosphorylation, ubiquitination and methylation (Geiman and Robertson, 2002). The observation that induced changes in telomeric heterochromatin, via histone methyltransferase (HMTase) knockdown, imparted abnormal elongation of telomeric sequence, suggests involvement of histone modifications in telomere length control (Garcia-Cao *et al.*, 2004). Another exemplar of histone modification affecting telomere function includes heterochromatin protein 1 (HP1) which is involved in telomere capping and telomeric function (Fanti *et al.*, 1998). HP1 functions to cap the telomere by binding directly to the telomeric sequence, whilst telomere elongation and transcriptional regression of the telomere by HP1 can occur via interaction with histone H3 methylated at lysine 9 (Savitsky *et al.*, 2002, Perrini *et al.*, 2004).

1.2.3 Post translational modifications to telomerase

The telomerase enzyme is also suggested to be modified post-translationally, in particular through phosphorylation at specific serine/threonine or tyrosine residues (Aisner *et al.*, 2002), perhaps affecting telomerase activity.

1.2.4 MicroRNAs and telomere length

MicroRNAs (miRNAs) modulate gene expression by binding to target mRNAs and thereby negatively regulating the efficiency of translation and stability of translation products (Ambros, 2004). miRNAs have been shown to play important roles in numerous cellular processes such as apoptosis, proliferation and differentiation (Lee *et al.*, 1993, Grishok *et al.*, 2001, Chen and Stallings, 2007). Small interfering RNA molecules have been probed for indirect roles in telomere length homeostasis. Mutant mice deficient in Dicer1 caused reduced expression of DNMT enzymes with global DNA hypomethylation, increased recombination at telomeres and telomere length (Benetti *et al.*, 2008). miR-290 was observed to be downregulated in the Dicer1-null mice and was shown to directly regulate Rbl2-dependent DNMT expression to indirectly affect telomere-length homeostasis (Benetti *et al.*, 2008).

1.3 Disease and other influences on telomere length

As previously mentioned, short telomeres and/or accelerated telomere attrition are key features of premature ageing syndromes including the inherited autosomal recessive disease ataxia telangiectasia (Harnden, 1994). Yet, telomere length has also been associated with risks of acquired disease including coronary heart disease (Samani *et al.*, 2001, Brouilette *et al.*, 2007, Mainous *et al.*, 2010, Willeit *et al.*, 2010a), various cancers (Willeit *et al.*, 2010b, Willeit *et al.*, 2011), type II diabetes (Shen *et al.*, 2012, Zhao *et al.*, 2013) and obesity (Zannolli *et al.*, 2008, Buxton *et al.*, 2011). In addition to disease, biomarkers of inflammation, such as C-reactive protein (CRP), have been negatively associated with telomere length (Willeit *et al.*, 2010a). CRP is an acute-phase protein in blood, which can be utilised as an index of inflammation associated with acute and chronic inflammatory conditions (Gabay and Kushner, 1999). CRP has been negatively associated with telomere length, in that high levels of CRP are noted in those with shorter telomeres (Fitzpatrick *et al.*, 2007, Richards *et al.*, 2007, Richards *et al.*, 2008). In addition to CRP, other markers of oxidative stress and inflammation, such as IL-6, uric acid, oxidised LDL and fibrinogen, have been negatively associated with telomere length (Bekaert *et al.*, 2007, Willeit *et al.*, 2010a). Although telomere length is associated with cardiovascular disease (Fitzpatrick *et al.*, 2007) and its clinical markers, the direction of association (causality) remains to be established i.e. whether short telomere length is a cause or consequence of CVD (Fuster and Andres, 2006).

With its large ratio of guanine, the telomeric hexamer repeat is particularly prone to oxidative lesions (Retel *et al.*, 1993) largely caused by oxidative stress, which is known to increase with ageing parallel to the decline in an organism's antioxidant defence (Sohal and Weindruch, 1996). Moreover, oxidative stress may inhibit telomerase (Kurz *et al.*, 2004), potentially perturbing telomere maintenance and recovery efforts.

1.3.1 Environmental and lifestyle influences on telomeres

Although there exists strong evidence for heritability of telomere length (Broer *et al.*, 2013), it also appears that telomere length can be affected by lifestyle and environmental factors including diet and as such is potentially modifiable (Figure 1.2). A comprehensive change in lifestyle behaviours including diet, exercise, stress

management and relaxation has been shown to increase telomerase expression in males with low risk prostate cancer (Ornish *et al.*, 2008). However, there is individual evidence of benefit for these lifestyle changes with leukocyte telomere length and PBMC telomerase observed to be higher in those with a higher Mediterranean diet score (Boccardi *et al.*, 2013). Independently, physical activity (Cherkas *et al.*, 2008) and weight loss achieved through a calorie-restricted diet (O'Callaghan *et al.*, 2009) have individually been associated with longer telomere length.

1.3.2 Diet and telomere length

It is generally recognised firsthand that those who live a healthy lifestyle appear to live longer. Since an *in vitro* study showed vitamin C enrichment slows telomere attrition in a human endothelial cell line (Furumoto *et al.*, 1998), various *in vivo* studies have investigated the effect of dietary components on telomere length in humans. These associative studies have shown that vitamin D (Richards *et al.*, 2007), vitamin E (Tanaka *et al.*, 2007), multivitamin use (Xu *et al.*, 2009), dietary fibre consumption (Cassidy *et al.*, 2010) and marine omega-3 fatty acid (Farzaneh-Far *et al.*, 2010b) were correlated with longer telomeres, whereas processed meat consumption (Nettleton *et al.*, 2008), increased alcohol intake (Pavanello *et al.*, 2011) and low fruit and vegetable and increased meat intake (Diaz *et al.*, 2010) dietary patterns were negatively associated with telomere length. A comprehensive review of the potential effects of additional nutrients on telomere mechanics has also recently been published (Paul, 2011). That the collective effect of genetic factors, diet, disease, environmental and psychological stressors on an individuals' cell can be measured using telomere length ('biological age') makes it valuable as a biomarker of biological ageing (Olovnikov, 1996).

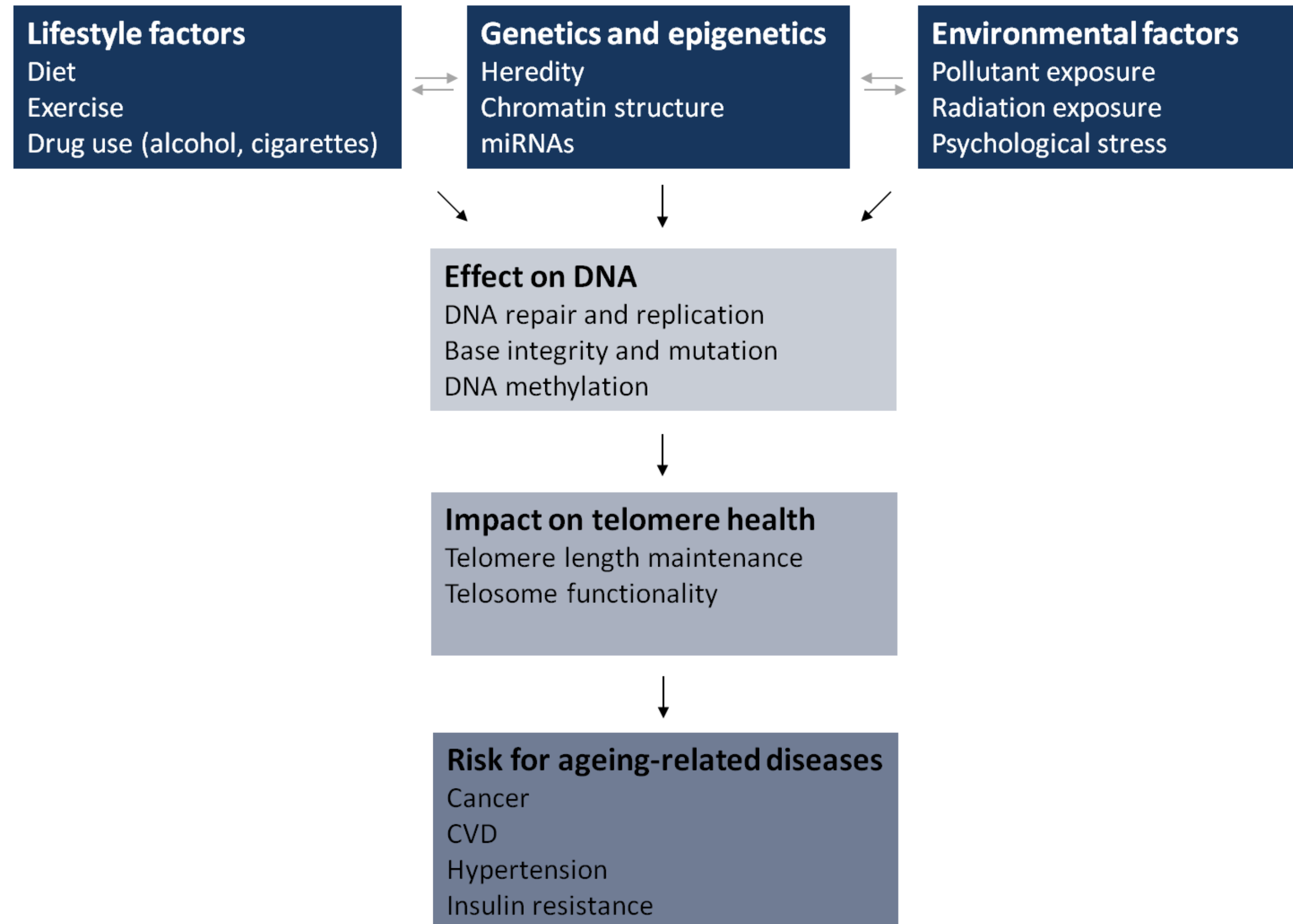

Figure 1.2 – Lifestyle, genetic and environmental factors which influence DNA, telomere welfare and risk of ageing-related diseases

Lifestyle, genetics, epigenetics and environmental factors are each hypothesised to – independently and in association – affect DNA and telomere welfare. Undesirable lifestyle and environmental conditions can hence increase the risk of ageing related diseases.

1.4 Folate and folic acid

Folate is a water-soluble B complex vitamin (B_9) that can exist in multiple chemical forms. Of these, folic acid (Figure 1.3) is the most stable and is the form which is utilised in dietary supplementation, food fortification and *in vitro* cell culture.

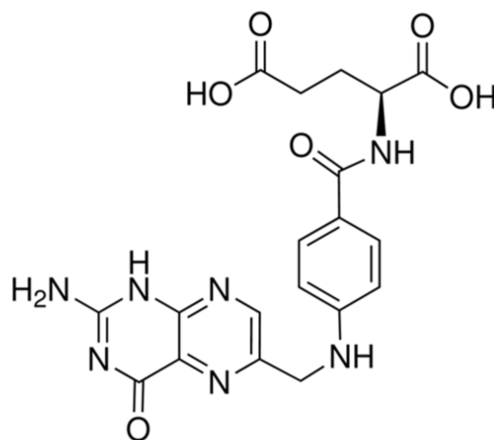

Figure 1.3 – Chemical structure of folic acid

Formula: $C_{19}H_{19}N_7O_6$; molecular weight: 441.3975; N-[p[[(2-amino-4-hydroxy-6-pteridinyl)methyl]-amino]benzoyl]-L-glutamic acid (Source: Chemspider; PubChem Compound, NCBI).

1.4.1 Pathways and functions in the cell including importance for genome health

Folate, along with choline, methionine, cobalamin (vitamin B_{12}), pyridoxine (B_6) and riboflavin (B_2), are involved in several essential metabolic processes within the cell, in particular DNA synthesis and repair. Such essential cellular pathways are affected by total dietary intake of folate and its aforementioned metabolic cofactors, as well as some genetic variants (Bailey and Gregory, 1999). Further, some metabolic forms of folate (Figure 1.4) e.g. 5-methyltetrahydrofolate (5-MeTHF) and 5, 10-methylenetetrahydrofolate (5, 10-MeTHF) can donate methyl groups to other molecules including nucleotides such as uridine. Folate is notably essential as a methyl donor (Bottiglieri, 2013, Glier *et al.*, 2013) in the conversion of dUMP to dTTP and hence also the maintenance of dUMP: dTTP ratios within the cell (Benesh and Carl, 1978, Appling, 1991).

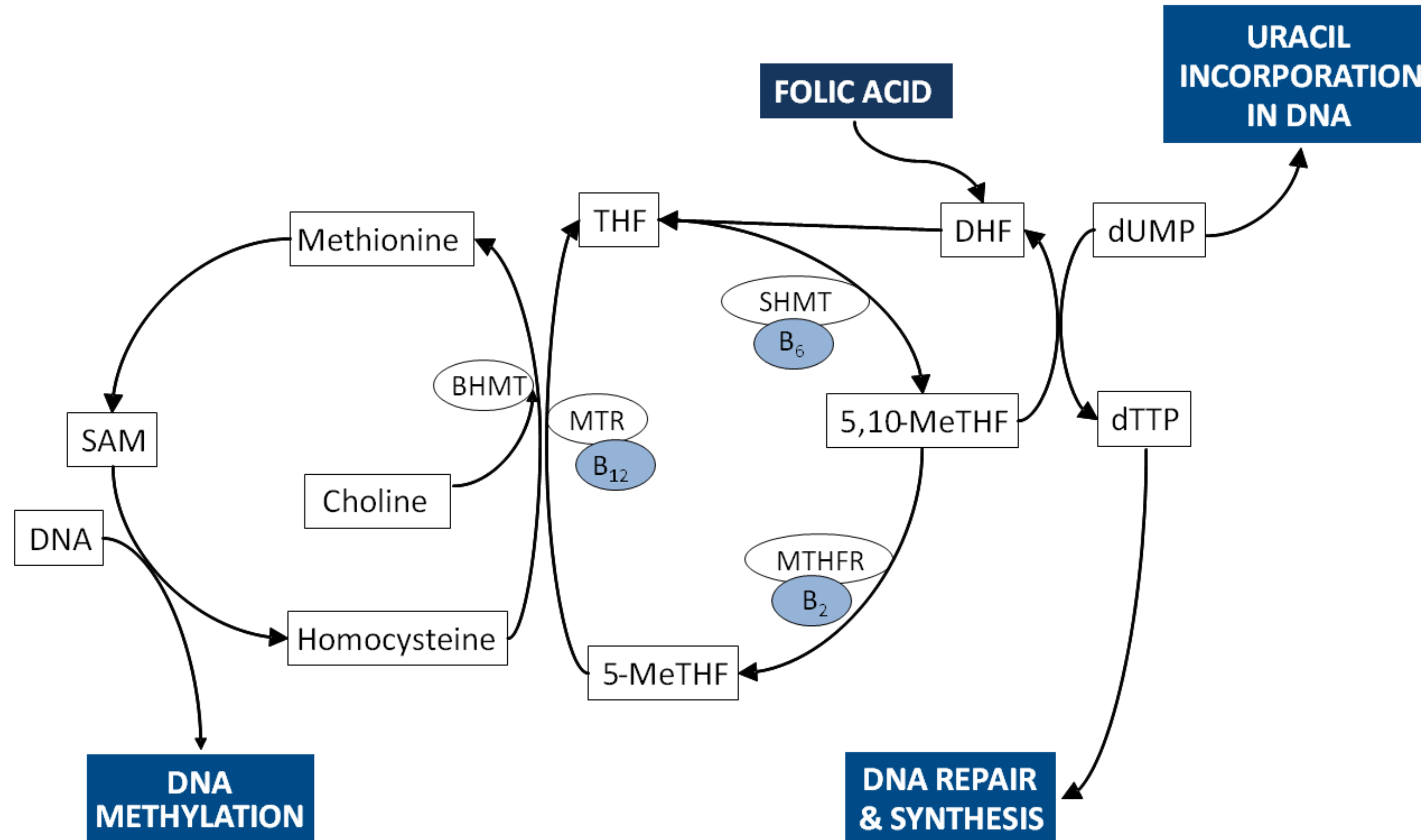

Figure 1.4 – Folate metabolism

Abbreviations: 5-MeTHF, 5-methyltetrahydrofolate; 5, 10-MeTHF, 5, 10-methylenetetrahydrofolate; B_2*, riboflavin;* B_6*, pyridoxine;* B_{12}*, cobalamin; BHMT, betaine-homocysteine S- methyltransferase; DHF, dihydrofolate; DNA, deoxyribonucleic acid; dUMP, deoxyuridine monophosphate; dTTP, deoxythymidine triphosphate; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine reductase; SAM, S-adenosyl methionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; Image modified from Kimura et al. (2004).*

The enzyme 7, 8-dihydrofolate reductase (aka tetrahydrofolate dehydrogenase) reduces folic acid and dihydrofolic acid substrates to the fully reduced tetrahydrofolic acid which can be utilised in the folate and nucleotide synthesis pathways. High levels of 7, 8-dihydrofolate reductase activity are found in the liver, kidney and rapidly dividing cells such as those in tumours.

1.4.2 MTHFR 677 genotype can influence folate metabolism

The folate pathway is influenced by genetic variations within the genes that govern folate utilisation. For instance, methylenetetrahydrofolate reductase (MTHFR) is involved in the catalysis of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Figure 1.4). The common *MTHFR* 677C→T transition polymorphism results in a reduced activity of MTHFR and consequently a decline in 5-methyltetrahydrofolate available for methylation of homocysteine to form methionine (Kang *et al.*, 1988a, Kang *et al.*, 1988b). As such, plasma folate is notably higher in *MTHFR* 677 CC homozygotes than in CT heterozygotes and subsequently is lowest in TT individuals; contrary to plasma homocysteine, which is highest in TT homozygotes followed by CT and CC homozygotes (Paul *et al.*, 2009). Additional single nucleotide polymorphisms (SNPs) in folate transport and metabolism genes may also influence the concentration of folate in plasma (Shaw *et al.*, 2009 identify 118 SNPs in 14 folate-related genes).

1.4.3 Sources of folate; diet, fortified foods and supplementation

Rich dietary sources of folate include green leafy vegetables (asparagus, broccoli, lettuce, and spinach), lentils, peanuts and offal (liver and kidney; Table 1.1).

Table 1.1 – Dietary sources of folate

FOOD	AMOUNT OF FOLATE/100 g	NOTES
Asparagus	149 µg	Cooked; boiled, drained
Broccoli	108 µg	Cooked; boiled, drained
Chickpeas	172 µg	Cooked; boiled
Kidney	83 µg	Beef kidney Cooked; simmered
Lettuce	136 µg	Cos or romaine Raw
Liver	560 µg	Chicken liver* Cooked; pan-fried
Peanuts	145 µg	Cooked; dry roasted
Spinach	146 µg	Cooked; boiled, drained

** there is greater folate in goose and turkey liver*
Source: USDA National Nutrient Database Food Search for Windows Version 1.0, Database Version SR25, United States Department of Agriculture and Agricultural Research Centre

Australians also consume folate from dietary supplements and folic acid-fortified breads and cereals. In the United States and Canada, mandatory fortification of all cereal products with folic acid has been in effect from January 1998. As a result, foods from cereal grain such as breads, flour, pasta, breakfast cereals and rice are fortified with 140 µg/kg folic acid. Voluntary folic acid fortification of some foods (including breakfast cereals, bread, and juice to 50% RDI per reference quantity) has been permitted in Australia from 1995 (NHMRC Standard A9). Under Food Standards Australia New Zealand (FSANZ) Standard 2.1.1, mandatory fortification of bread making flour has been enacted in Australia since September 2009 however New Zealand has deferred enforcement of the Standard such that fortification is indefinitely voluntary. As a result of this mandatory fortification Standard in Australia, wheat flour is required to be fortified with no less than 2 mg/kg and no

more than 3 mg/kg of folic acid; corresponding to 120 µg folic acid per 100 g of bread or approximately 40 µg per slice.

1.4.4 Australian folate RDI and actual consumption

The estimated average requirement (EAR) for Australian and New Zealand adult men and women is 320 µg dietary folate equivalents per day (National Health and Medical Research Council (Australia) *et al.*, 2006). The recommended daily intake (RDI) is 400 µg/d, however a greater intake is suggested for pregnant and lactating women. The upper level of intake (UL) of folic acid from supplements and fortified foods is 1000 µg/d for all adults.

The estimated folic acid consumption for male and female adults assessed in the 1995 Australian National Nutrition Survey was 127 µg/d (n = 1163) (Dugbaza and Cunningham, 2012). With current folic acid fortification legislation, this estimated folic acid consumption increases by 147% to 314 µg/d (Dugbaza and Cunningham, 2012). The National Nutrition Survey was a comprehensive study of dietary intakes in ≈14000 Australians of which 1163 provided two 24-hour food recalls from non-consecutive days. As this survey was conducted nearly two decades ago, the need for updated information is vital in order to reliably reflect the changing eating habits, food availability and preferences.

1.4.5 Consequences of folate insufficiency

Homocysteine accumulates in response to folate and vitamin B_{12}-deficient conditions as there is insufficient 5-MeTHF to provide methyl groups to re-form methionine. A high plasma level of homocysteine has been shown to increase risk of adverse cardiovascular events (Shammas *et al.*, 2008) and is associated with increased chromosome damage (Picerno *et al.*, 2007). Periconceptional exposure to elevated folate has long been recognised to prevent neural tube defects in the developing foetus (Smithells *et al.*, 1981, MRC Vitamin Study Research Group, 1991) and use of a folate supplement before and during early pregnancy is now best practice in the Western world (Bhutta and Hasan, 2002). Additionally, suboptimal folate status has been associated with an increased risk of many cancer types such as colorectal cancer, adenoma, oesophageal and gastric cancer (Ames and Wakimoto, 2002) as well as cardiovascular disease (Jang *et al.*, 2005).

In a folate-deficient state, a cell is limited in 5,10-MeTHF required for conversion of dUMP to dTTP by methylation and hence the ratio of dUMP: dTTP increases, leading to inadvertent incorporation of uracil in the DNA of dividing cells (Blount *et al.*, 1997). 5-MeTHF is required to synthesise methionine from homocysteine and this reaction requires vitamin B_{12} as a cofactor and zinc at the catalytic site to activate homocysteine (Matthews and Goulding, 1997, Koutmos *et al.*, 2008). Methionine is then converted to S-adenosyl methionine, the universal methyl donor employed for methylation of histones and cytosine in mammalian DNA (Paul *et al.*, 2009) (refer Figure 1.4). When folate is deficient, the maintenance of methylation at histones and cytosine may be inadequate (Smith *et al.*, 2008). Additionally, folate deficiency is known to induce the extensive incorporation of uracil into DNA, on a scale of up to 4 million uracils/human cell (Blount *et al.*, 1997) which can lead to DNA damage.

1.5 Micronutrients, DNA damage and disease

DNA damage is the collective name given to a number of modifications to the bases of DNA or to strand breaks in DNA. DNA damage may be caused by endogenous factors such as reactive oxygen species or through errors in DNA replication and/or repair within the cell. Exogenous or external agents which are known to cause DNA damage include radiation and environmental pollutants.

The deficiency of selected micronutrients including folic acid, vitamins B_6, B_{12}, C, and E, iron, zinc, niacin and selenium are known to induce types of DNA damage akin to those provoked by radiation, including base oxidation, DNA strand and chromosome breaks and perturbations in DNA repair (Ames, 1999). Table 1.2 shows the biological involvement of some essential micronutrients. The inadequate intake of some essential micronutrients, including folate and vitamins B_6 and C, are suggested to cause cancer and degenerative disease (Ames, 2001).

Table 1.2 – Various dietary micronutrients and their function with DNA in the cell

MICRONUTRIENT	BIOLOGICAL INVOLVEMENT WITH DNA
α-tocopherol	Antioxidant; prevents lipid peroxidation and lipid peroxide DNA adducts
Vitamin B_{12}	Coenzyme for methionine synthesis and folate forms essential for S-adenosyl methionine (methylation) and nucleotide synthesis
Calcium	Chromosome segregation, induces apoptosis and cell differentiation
Vitamin D	DNA stability, involved in cell proliferation, differentiation and apoptosis
Folate	Chromosome stability, methyl donor for DNA methylation, dUMP → dTTP
Homocysteine*	Important risk factor for genetic instability and DNA hypomethylation
Magnesium	DNA stability, protects against double strand breaks
Nicotinic acid	NAD precursor → PARP cofactor → DNA repair, telomere maintenance
Retinol	Binds to retinoic acid receptors, interact with gene transcription factors to activate or inhibit gene expression
Selenium	Induces apoptosis, DNA repair, some selenoproteins have antioxidant activity, DNA methylation
Zinc	DNA replication, transcription and translation, various metalloenzymes including DNA repair enzymes (e.g. hOGG1)

** Homocysteine is a metabolite of the amino acid methionine and is elevated when folate and B_{12} are low.*
Abbreviations: NAD, nicotinamide adenine dinucleotide; PARP, Poly (ADP-ribose) polymerase; Adapted from Fenech and Ferguson (2001) and Fenech (2003).

1.6 Folate and telomere length

MTHFR 677T homozygosity, along with below median plasma folate in a group of middle-aged men, was weakly associated ($P = 0.065$) with increased telomere length in comparison to wild type 677C homozygotes and CT heterozygotes (Paul *et al.*, 2009). However it is unclear whether adjustment for the false discovery rate of SNPs was made in this study as previously described (Benjamini *et al.*, 2001) and performed (Liu *et al.*, 2013a). Taken together, these data suggest folate may modulate telomere length through epigenetic regulation by DNA methylation, or through its influence on DNA integrity (Paul *et al.*, 2009). As folate is involved in methylation maintenance, DNA repair and synthesis, and in particular thymine synthesis, it may be more beneficial to the telomere than some other micronutrients.

Plasma homocysteine concentration – which increases when folate and B_{12} are deficient – has been shown to be inversely associated with telomere length in human cross-sectional studies comprising up to 1319 subjects (Richards *et al.*, 2008, Bull *et al.*, 2009, Panayiotou *et al.*, 2010). The attrition of telomeric sequence induced by elevated homocysteine is possibly mediated by increased oxidative stress or inflammation (Richards *et al.*, 2008, Bull *et al.*, 2009), or the increased demand for proliferation of certain cell types, in these cases haematopoietic stem cells (Richards *et al.*, 2008). This negative effect of homocysteine on telomere length is purported to also be the effect of folate deficiency on the telomere, as the amino acid and vitamin are typically inversely correlated, and as such it is suggested the effect would be mitigated by increased folate intake (Richards *et al.*, 2008, Bull *et al.*, 2009). To date, constituents of the folate pathway have not been adequately studied for their effect on telomere length in humans while B_{12} has been reported to have no effect on telomere length (Bull *et al.*, 2009, Paul *et al.*, 2009) even though plasma B_{12} is considered an important factor in folate and homocysteine metabolism and should be considered in statistical analyses involving these measures. The effect of folate on telomere length appears to be complex from the few studies that have explored the relationship. In one study, a low level of plasma folate was correlated with shorter telomere length in the older male cohort though

no such effect was observed in corresponding older female participants or in younger adults (Bull *et al.*, 2009).

Perplexingly, in another study plasma folate was inversely correlated with longer telomere length when folate levels were below the cohort median yet once plasma levels were above this level, the relationship with telomere length was positive (Paul *et al.*, 2009). In a recent review it has been suggested that folate deficiency might induce telomere attrition and/or dysfunction by molecular mechanisms including (1) the excision of increased uracil in the telomeric hexamer repeat which is known to generate abasic sites and DNA breaks; (2) aberrant epigenetic state of the subtelomeric DNA; and (3) inefficient binding of the telosome proteins to the telomeric DNA due to reduced affinity to uracil and/or abasic sites, resulting from excision repair of uracil, in the telomere sequence (Bull and Fenech, 2008). Under conditions of folate deficiency, incorporation of uracil instead of thymine in DNA is increased (Blount *et al.*, 1997, Duthie and Hawdon, 1998). Uracil may also arise in the telomeric hexanucleotide repeat due to spontaneous deamination of cytosine. Excision repair of uracil in DNA has a range of molecular consequences, including the generation of abasic sites through base-excision repair pathways, which can cause single or double strand DNA breaks and chromosomal aberrations (Blount *et al.*, 1997, Ahmad *et al.*, 1998, Toussaint *et al.*, 2005). A lowered synthesis of dTTP from dUMP has been suggested to accelerate telomere shortening (Toussaint *et al.*, 2005) while successive uracil misincorporation within the telomere could result in shorter telomeres as single-strand breaks of the G-rich strand may not be repairable, or cause degradation of the complementary C-rich strand (Toussaint *et al.*, 2005). However, continued investigation, both *in vitro* and *in vivo* is required in order to validate or repudiate these plausible mechanisms and to understand the biological impact of folate deficiency on telomere function.

1.7 Knowledge gaps and other challenges identified from the literature

In the evolving field of telomere biology, there appears to be an absence of large cohort longitudinal studies to investigate the association of dietary micronutrients with telomere length and telomere function. The current literature is limited to cross-sectional studies, which have fewer cost limitations and compliance considerations than an intervention or randomised controlled trial (RCT), but provide a lower level of evidence because a cause and effect relationship cannot be unequivocally ascertained. Some of these cross-sectional studies are restricted to self-reported dietary intake information, e.g. that from food frequency questionnaires, which is subject to bias and not necessarily reflective of exposure.

At present there are a number of assays which are able to quantify varied aspects of telomere integrity, including but not limited to measurement of absolute telomere length (O'Callaghan *et al.*, 2008, O'Callaghan and Fenech, 2011), chromosome-arm specific telomere length (Baird *et al.*, 2003, Britt-Compton *et al.*, 2006), subtelomeric methylation (Lee *et al.*, 2009a), telomeric dysfunction (Gisselsson *et al.*, 2002, Jiang *et al.*, 2008), measurement of critically short telomeres (Vera and Blasco, 2012), and those which provide the confirmation of telomerase or ALT action. Critical assessment of the available tools to measure telomere length has been provided by Aubert *et al.* (2012). Recent protocols involving digestion of DNA by DNA repair enzymes prior to qPCR amplification of telomeric sequence has allowed for the quantification of base damage solely within the telomere (O'Callaghan *et al.*, 2011, Vallabhaneni *et al.*, 2013). It has been shown that DNA damage repair within the telomeric region is less efficacious than repair to regions of coding sequence (Kruk *et al.*, 1995, Petersen *et al.*, 1998, von Zglinicki *et al.*, 2000) with both the rate and degree of telomeric repair declining with age (Kruk *et al.*, 1995). Thus, while relative changes in telomere length are compelling – and can be associated with ALT mechanisms, telomerase activity or subtelomeric methylation patterns – the integrity and state of the underlying DNA sequence may be more appropriate as a marker of potential telomeric decline and instability.

The need to probe further the dynamic relationship between telomere length and telomere base damage as well as the influence of inter-individual variations in

nutrition and genotype is unmistakable. Whilst telomere length comparisons are often drawn from measurements on a single occasion, it may be more appropriate to measure changes in telomere length dynamics over time in order to obtain a more comprehensive picture of variation in telomere maintenance depending on environment, diet and lifestyle conditions.

2 Project synopsis, aims and hypotheses

2.1 Project synopsis

At present, there is little information about the effects of micronutrients and homocysteine on telomere length in the Australian population. The study, "A *Polypill* to prevent genome damage", provided an opportunity to investigate these questions cross-sectionally. The study also permitted the investigation of whether a micronutrient supplement (*Polypill* containing folic acid, vitamin B_{12}, α-tocopherol, retinol, nicotinic acid and calcium) could affect telomere length in otherwise apparently healthy South Australians.

Given the impending mandatory fortification of folic acid in Australian flour, it was additionally of interest to test whether folate deficiency causes uracil misincorporation in the telomere sequence. For this reason, it was important to first develop an assay to measure telomeric uracil content and then to validate this method in an *in vitro* model of folic acid deficiency and dUTP excess.

The research in this thesis can be separated into three parts:

1) an *in vivo* double-blinded randomised placebo-controlled intervention trial to assess the influence of a micronutrient *Polypill* on telomere length over time, where each phase of the study is described as a separate chapter detailing

a) cross-sectional associations of telomere length with demographics and micronutrients at baseline (Chapter 4);

b) differences in telomere length change and trajectory over time between the *Polypill* intervention group and the placebo control (Chapter 5); and

c) the differences in telomere length change and trajectory over time in subgroups within the *Polypill* study cohort who consumed a modified micronutrient supplement after the original intervention was completed (Chapter 6)

2) the conception, validation and application of a method to measure uracil content within the telomere hexamer repeat (Chapter 7)

3) the application of telomeric uracil method to assess the influence of folate, S-adenosyl methionine and dUTP on telomeric uracil content and telomere length *in vitro* in WIL2-NS lymphoblastoid cells (Chapter 8).

2.2 Aims

The specific aims of this doctoral research project are:

1) to explore which micronutrients and demographic factors are cross-sectionally associated with telomere length in a healthy, middle-aged cohort of South Australian adults

2) to assess the impact that a *Polypill* supplement containing six micronutrients (folic acid, vitamin B_{12}, α-tocopherol, retinol, nicotinic acid and calcium as previously associated with reduced chromosomal DNA damage) has on mean longitudinal changes in telomere length and telomere length trajectories over a 16-week randomised controlled trial (RCT) in a healthy, middle-aged cohort of South Australian adults

3) to preliminarily investigate the impact of removing one micronutrient from the *Polypill* supplement on mean longitudinal changes in telomere length and telomere length trajectories in a 16-week RCT pilot phase

4) to develop a technique capable of measuring uracil specifically within telomeric sequence

5) to determine whether uracil in the telomere corresponds with telomere length *in vitro*

6) to investigate if folic acid, dUTP and S-adenosyl methionine *in vitro* can influence the extent of uracil within the telomere.

2.3 Hypotheses

The corresponding hypotheses are:

1) plasma micronutrients are significantly associated with telomere length, after controlling for age, gender and BMI

2) telomere length is longer in humans supplemented with micronutrients important for genome maintenance than in those given a placebo

3) telomere length change and trajectory varies depending on the composition of a *Polypill* supplement due to the differential roles of micronutrients on genome maintenance

4) uracil within the telomere can be quantitated by modified qPCR

5) uracil increases telomere attrition and is negatively associated with telomere length

6) *in vitro* folic acid, dUTP and SAM concentration affects telomere length and telomeric uracil content.

3 Materials and methods

3.1 qPCR for absolute telomere length

Telomere length was previously thought to be unmeasurable by PCR due to the repeat nature of the sequence and subsequent primer design issues. However primer pairs in a qPCR protocol to measure telomere length have been designed to overcome undesirable primer-dimer product formation to give a measure of telomere length relative to a single copy gene (Cawthon, 2002). By also incorporating individual standard curves for synthetic telomere and single copy gene sequence (*36B4*) sequence, this qPCR method can be used to generate a measure of absolute telomere length (kb/diploid genome) (O'Callaghan *et al.*, 2008).

Terminal restriction fragment (TRF) analysis is the current gold standard for telomere length measurement; however this Southern blot-based technique is laborious and additionally requires a large amount of sample DNA (Wang *et al.*, 2013a). In addition, the qPCR method for absolute telomere length can provide a more accurate measure of mean telomere length than TRF, which overestimates telomere length by including undigested subtelomeric sequence in the measurement (Allshire *et al.*, 1989, Brown *et al.*, 1990, O'Sullivan *et al.*, 2004). Strong correlations between telomere length measured by TRF analysis and the qPCR method for absolute telomere length have been independently demonstrated ($R = 0.88$, $P < 0.0001$, O'Callaghan *et al.* (2008); $R = 0.73$, $P < 0.001$, Fick *et al.* (2012)).

3.1.1 Normalisation of qPCR template DNA

Isolated DNA from volunteer PBMC or WIL2-NS cells is normalised in order to minimise pipetting error and variation when template DNA is added to the PCR mastermix. Each DNA is diluted in UltraPure water (PCR grade; Fisher Biotec, Wembley, WA, Australia) to a final concentration of 5 ng/µL such that when 4 µL of each sample is added to each PCR reaction, there is a total amount of 20 ng template DNA.

3.1.2 Positive and negative controls for qPCR

1301 is a T-cell lymphoblastic leukaemia cell line which was derived from parent line CCRF-CEM (Hultdin *et al.*, 1998). The 1301 cell line is tetraploid and has unusual and extremely long telomeres (Hultdin *et al.*, 1998) due to a lack of telomere shortening as the line overexpresses telomerase hence contributing to a consistently long telomere length (Rufer *et al.*, 1999, Morla *et al.*, 2006). 1301 telomere length has been estimated at 23.5 kb (Derradji *et al.*, 2005) to 80 kb (Jeyapalan *et al.*, 2004). The characteristically long telomeres of the 1301 cell line makes this line a suitable positive control or reference in methods of telomere length determination, such as in this qPCR assay for absolute telomere length. The 1301 cell line was obtained from the European Collection of Cell Cultures (ECACC catalogue number 01051619). 1301 cells were cultured in complete RPMI1640 medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma Aldrich, St Louis, MO, USA) and 10% (v/v) FBS (Bovogen Biologicals, Melbourne, VIC, Australia) at 37°C in a humidified incubator with 5% CO_2. The cells were harvested following centrifugation of the culture and disposal of the supernatant. DNA was extracted from the cells described in section 3.3.6. The negative or no template control (NTC) in each assay is the PCR grade H_2O used in the preparation of the PCR reaction.

3.1.3 Telomere and *36B4* primers and oligonucleotide standards

The following oligonucleotide primers and standards (Table 3.1) were purchased from a local supplier (Geneworks, Hindmarsh SA, Australia). Where the length of the oligonucleotide was > 50 bases, as is the case with both the telomere and *36B4* standards, RP-HPLC-purified sequences were ordered to ensure ≥97% of complete sequence. The amounts of telomere and 36B4 oligonucleotide standards for constructing the standard curves are shown in Table 3.2 and Table 3.3, respectively.

Table 3.1 – Human telomere and *36B4* single copy gene forward and reverse primer sequences and oligonucleotide standards

PRIMER OR STANDARD	SEQUENCE (5' – 3')	OLIGO LENGTH	MW	PRODUCT SIZE
Telo forward primer	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	39	12237	≥76
Telo reverse primer	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	39	11652	
36B4 forward primer	CAGCAAGTGGGAAGGTGTAATCC	23	7138	75
36B4 reverse primer	CCCATTCTATCATCAACGGGTACAA	25	7570	
Telo standard	$(TTAGGG)_{14}$	84	26667	84
36B4 standard	CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCAGG ACTCGTTTGTACCCGTTGATGATAGAATGGG	75	23268	75

Table 3.2 – Amount of telomere oligonucleotide standards and kb telomere sequence used in the qPCR assay

VOL IN PCR	CONCENTRATION	AMOUNT OF OLIGO IN PCR	TELOMERE SEQUENCE
4 µL	15 pg/µL	60 pg	1.18×10^8 kb
4 µL	1.5 pg/µL	6 pg	1.18×10^7 kb
4 µL	0.15 pg/µL	0.6 pg	1.18×10^6 kb
4 µL	0.015 pg/µL	0.06 pg	1.18×10^5 kb
4 µL	0.0015 pg/µL	0.006 pg	1.18×10^4 kb
4 µL	0.00015 pg/µL	0.0006 pg	1.18×10^3 kb

Table 3.3 – Amount of *36B4* oligonucleotide standards and kb telomere sequence used in the qPCR assay

VOL IN PCR	CONCENTRATION	AMOUNT OF OLIGO IN PCR	*36B4* SEQUENCE
4 µL	50 pg/µL	200 pg	5.26×10^9 kb
4 µL	5 pg/µL	20 pg	5.26×10^8 kb
4 µL	0.5 pg/µL	2 pg	5.26×10^7 kb
4 µL	0.05 pg/µL	0.2 pg	5.26×10^6 kb
4 µL	0.005 pg/µL	0.02 pg	5.26×10^5 kb
4 µL	0.0005 pg/µL	0.002 pg	5.26×10^4 kb

3.1.4 qPCR mastermix

Power SYBR® Green Master Mix (Applied Biosystems, Life Technologies, Warrington, Cheshire, UK) was used for all single copy gene (*36B4*) and telomere qPCRs and contains AmpliTaq Gold® DNA polymerase, dNTPs, SYBR® I Green Dye and ROX™ passive reference dye. Each reaction also contained PCR grade ultra pure water and an excess of reverse and forward primers.

Table 3.4 – Make up of telomere and 36B4 single copy gene qPCR reactions

	COMPONENT	VOLUME	FINAL CONCENTRATION /AMOUNT
	2 × *Power* SYBR® Green PCR Master Mix	10 µL	1×
	Forward primer (10 µM)	1 µL	0.5 µM
	Reverse primer (10 µM)	1 µL	0.5 µM
	H_2O	4 µL	-
And one of	Oligonucleotide standard	4 µL	Various: telomere: 60 pg – 600 ag; 36B4: 200 pg – 2 fg
	Template genomic DNA	4 µL	20 ng
	1301 DNA (positive control)	4 µL	20 ng
	H_2O (NTC)	4 µL	-
	Total volume	**20 µL**	

3.1.5 PCR cycle conditions

The following PCR program (Table 3.5) was run on the Applied Biosystems 7300 Sequence Detection System (SDS) with SDS Version 1.3 software (Applied Biosystems, Foster City, CA, USA).

Table 3.5 – PCR cycling conditions for telomere length and 36B4 single copy gene amplification

STAGE	TEMPERATURE	TIME	CYCLES
Initiation and Ampli*Taq* Gold® DNA polymerase activation (hot start)	50°C	2 min	1
	95°C	10 min	1
Target region amplification	95°C	15 s	40
	60°C	1 min	
Dissociation stage/ melt curve	95°C	15 s	1
	60°C	30 s	1

3.1.6 Absolute telomere length calculations

In using the telomere standard curve generated by the PCR results, the length of telomeric sequence was calculated for each sample (kb/reaction). Correspondingly, the *36B4* single copy gene PCR standard curve was used to determine the number of genome copies in each sample amplified by PCR (genome copies/reaction). Using these two values, the length of telomeric sequence per diploid genome was calculated (kb/diploid genome). Telomere length as determined by this method is expressed as kb/diploid genome in this thesis, which corresponds to an average absolute telomere length per diploid genome of each sample. This value can be further divided by a factor of 92, which is the number of telomeres in a diploid human genome, to give an average measure of telomere length per chromosome in a single diploid genome (O'Callaghan and Fenech, 2011).

3.2 *Polypill* human study

The *Polypill* human intervention study is an Australian National Health and Medical Research Council (NHMRC) funded collaborative research study "A *Polypill* to prevent genome damage" (Reference number: 464895). The aim of this research project was to determine whether daily intake of a pill containing certain micronutrients (folic acid, vitamin B_{12}, vitamin E, niacin, retinol and calcium) causes a reduction in DNA damage in blood cells. The human study was conducted at CSIRO Human Nutrition, now CSIRO Animal, Food and Health Sciences in Adelaide, South Australia and was completed in the 2008 calendar year.

3.2.1 Ethics, recruitment and reimbursement

Ethics approval for the study was granted by the CSIRO Human Nutrition Human Research Ethics Committee (Reference number 07/01) in accordance with the National Statement on Ethical Conduct in Human Research (National Health and Medical Research Council Australia *et al.*, 2007). Ethics approval was also granted from the Flinders University and Medical Centre Human Research Ethics Committee in 2009. The trial is registered with the Australian New Zealand Clinical Trials Registry (ACTRN12607000651482).

Participants were recruited through advertisements in local newspapers and flyers on noticeboards at workplaces in the Adelaide Central Business District (CBD) with large numbers of employees, for example at Government departments. Written, informed consent was obtained from each volunteer before his or her enrolment in the study.

In recognition of disruption to each volunteer's daily routine and efforts in complying with the study instructions as well as to reimburse travel costs to and from the CBD-located onsite clinic, participants were given a $20 (AUD) Coles Group and Myer gift card on each visit to the clinic.

3.2.2 Inclusion and exclusion criteria

The aim was to recruit 250 middle-aged, healthy volunteers in the study. Participants were selected for inclusion in the study if they met the following inclusion criteria;

- Male or female;
- Aged 25-50 years;
- Willing to refrain from using any dietary supplements throughout the study period other than those provided in the study.

Conversely, participants were excluded from participating in the study if they met any of the following exclusion criteria;

- Cigarette smoker currently or within the past six months;
- Receiving medical treatment for life-threatening diseases including anti-folate drugs either currently or within the past six months;
- Planning a pregnancy, or currently pregnant;
- Current daily vitamin supplement intake exceeds 50% of the recommended daily allowance (RDA) of any nutrients identified for the study.

3.2.3 Study design, randomisation and blinding

Participants were randomised in strata of age and sex to receive either the *Polypill* micronutrient supplement or the inactive placebo control. As the study was double-blinded, clinic staff, research staff and participants themselves were blinded to their treatment group. Bottles containing the tablets and capsules used in the intervention were coded by the manufacturer, Blackmores® (Warriewood NSW, Australia), who also retained the code.

At each of three visits to the clinic, participants returned a FFQ for the previous 16-week period (Figure 3.1). Volunteers also supplied a fasted blood sample at each of these visits, were weighed and were given their study supplements for the proceeding 16-week period.

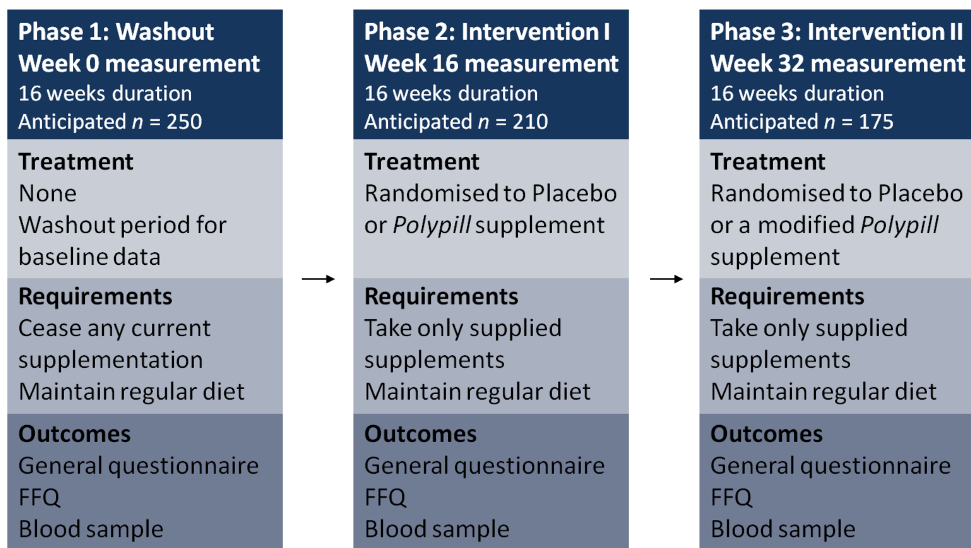

Figure 3.1 – Schematic of study design

3.2.4 *Polypill* study power

The primary aim of this study was to determine whether a micronutrient *Polypill* could reduce biomarkers of genomic DNA damage measured by the cytokinesis-block micronucleus assay (CBMN). As reducing these markers of DNA damage was the primary outcome, the study power calculation was based on the expected variation in micronuclei (MN) frequency in *ex vivo* cultured lymphocytes. Briefly, historical data for 65 subjects close to the age group for the *Polypill* study (24 – 45 years of age) shows a mean of 8.7 MN per 1000 bi-nucleated cells and a SD of 5.6 MN. The expected sample size of 210 subjects provided 80% power to detect an absolute reduction in MN frequency from 8.7 (assumed placebo group mean) to 6.5 (≈25%) between the two intervention groups with P = 0.05 (two-sided). An absolute reduction of 2.2 in average MN frequency is biologically significant being equivalent to the genome damage induced by 5 cGy of X-rays (25 times the annual allowed limit for radiation exposure for the general public) and approximately equivalent to the difference in genome damage prevalence observed with a chronological age difference of 5 years in age groups between 35 and 75 years (references). A 21% drop-out rate would still allow detection of an absolute reduction of 2.5 (29%) at the same power (assumes 80 subjects per treatment group).

3.2.5 Volunteer withdrawals, reasons for withdrawal and adverse events

There were 266 volunteers recruited to the study, with dropouts between each study milestone illustrated in Figure 3.2. Of the 234 individuals who commenced the first phase of the study, 90% completed phase 1, 87% completed phase two and 83% completed phase three (Appendix Table 10.2).

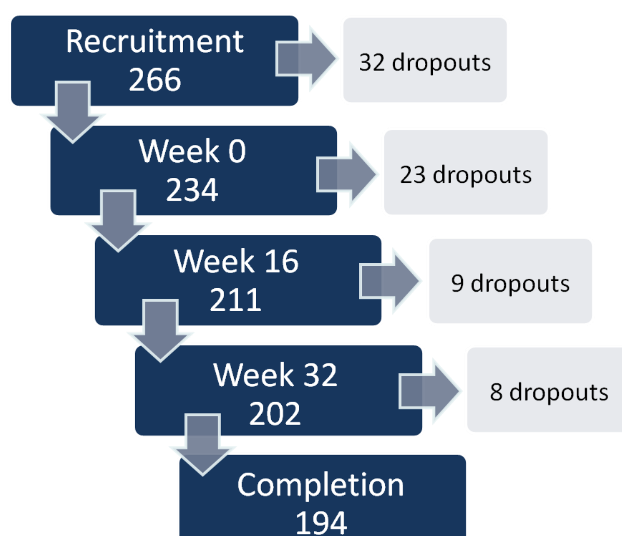

Figure 3.2 – Number of participants and dropouts by each study phase

Common reasons for withdrawal classified as personal include change of mind, modified working hours or other time-commitments and travel (Appendix Table 10.1). Adverse events reported throughout the course of the study included constipation, episodes of dizziness, diverticulitis and angina from a pre-existing conditions and bladder and kidney infection. The study physician addressed all reported events and where necessary, participants were referred to their general practitioner. Volunteers withdrew from the study at their desire. One participant was withdrawn from the study following a severe adverse reaction during incomplete collection of the first blood sample. Strategies employed to improve study retention included providing participants with a study newsletter, appointment reminders by preferred media and the offer of a $AUD20 gift voucher upon each presentation to the clinic site as reimbursement and recognition of disruption to routine. As observed in Appendix Table 10.2, there was an overall dropout rate of 27% of all recruited volunteers (*n* = 72 of 266). There were 40

individuals who withdrew from the study following commencement of phase one, as such this study observed a dropout rate of 17% of those who were enrolled in the study and attended the clinic (n = 40 of 234).

The number of withdrawals by study treatment group is described in Appendix Table 10.2. Overall, there was acceptable retention of participants during the two intervention periods

3.2.6 *Polypill* formulation

The dosages selected in the *Polypill* were intended to obtain a combined intake level with dietary intake from foods that was well within the range associated with DNA damage prevention in previous studies. In some cases the doses in the *Polypill* were limited because of concerns of increased risk of cancer growth (folic acid) or because the safe upper limit would be exceeded (calcium). In the case of vitamin B_{12}, it was acceptable to increase the dose to the higher range for efficacy because there was no safety risk even at doses that exceeded the RDA eight-fold. The combined doses estimated from dietary intake and the FBERNC *Polypill* were within the range associated for decreased DNA damage as explained in Table 3.6 and Table 3.7. The *Polypill* supplements were manufactured and generously provided by Blackmores® (Warriewood NSW, Australia) to the requirements shown below.

Table 3.6 – Micronutrients and doses in the *Polypill*

MICRONUTRIENT (units)	RDA[a]	UL[a]	MEAN DIETARY INTAKE LEVELS[b]	DAILY INTAKE RANGE ASSOCIATED WITH LOWEST MN FREQUENCY[c]	DESIRED AMOUNT IN *POLYPILL*
Folate (μg)	400	1000	240	277-700	200
Vitamin B_{12} (μg)	2.4	ND	4	7-20	20
Vitamin E (mg)	15	1000	10	11-50	15
Retinol (μg)	800	3000	512	458-2708	800
Niacin (mg)	16	35	23	26-49	10
Calcium (mg)	1000	2500	1098	1250-2313	500

[a] RDA, recommended dietary allowance; UL, safe upper intake limit (Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes., 1997, Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. et al., 1998, Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000); [b] estimated daily dietary intake data from CSIRO study in South Australia (8); [c] based on diet and/or supplement studies (Gaziev et al., 1996, Fenech et al., 1998, Ames and Wakimoto, 2002, Ames, 2004, Kimura et al., 2004, Fenech, 2005, Fenech et al., 2005). ND, not determined yet.

Table 3.7 – EAR, RDI and UL for *Polypill* micronutrients

MICRONUTRIENT (UNITS) [AMOUNT IN *POLYPILL*]		EAR	RDI	AI	UL	NOTES
Folate (µg)	♀	320	400	-	1000	UL of folate as folic acid
[200]	♂	320	400	-	1000	
Vitamin B_{12} (µg)	♀	2.0	2.4	-	-	Insufficient data to allow setting of UL
[20]	♂	2.0	2.4	-	-	
Vitamin E (mg)	♀	-	-	7	300	AI based on median intakes in Australia and New Zealand from National Nutrition Surveys
[8.25]*	♂	-	-	10	300	
Retinol (µg)	♀	500	700	-	3000	
[800]	♂	625	900	-	3000	
Niacin (mg)	♀	11	14	-	35	UL of niacin as nicotinic acid (nicotinamide 900 mg/d)
[10]	♂	12	16	-	35	
Calcium (mg)	♀	840	1000	-	2500	Higher EAR and RDI for women >51 y and men >70 y (1100 mg and 1300 mg, respectively)
[500]	♂	840	1000	-	2500	

**Vit E 8.25mg α-tocopherol equivalent where mg γ-tocopherol = 0.1 α-tocopherol equivalence. EAR, Estimated Average Requirement; RDI, Recommended Dietary Intake; AI, Adequate Intake (used when an RDI cannot be determined); UL, Upper Level of Intake (National Health and Medical Research Council (Australia) et al., 2006).*

Due to the ingredients and the amount of these individual micronutrients, the desired amount as in Table 3.6 was achieved using three individual supplements; two tablets and one capsule as detailed below in Table 3.8, Table 3.9 and Table 3.10. Active and placebo supplements were matched for weight, colour and shape.

Table 3.8 – Formulation of folic acid and vitamin B_{12} tablet administered in the *Polypill* intervention study

COMPONENT	PLACEBO	*POLYPILL*
Folic Acid 200 µg + moisture compensation + 15% overage	00.00 mg	0.25 mg
Vitamin B_{12}, 20 µg as 0.1% WS + 10% overage	00.00 mg	22.00 mg
Pregelatinised maize starch	204.25 mg	193.125 mg
Microcrystalline cellulose	204.25 mg	193.125 mg
Sodium starch glycolate	20.00 mg	20.00 mg
Magnesium stearate	1.50 mg	1.50 mg
Total weight	430.00 mg	430.00 mg

Table 3.9 – Formulation of nicotinic acid and calcium carbonate tablet administered in the *Polypill* intervention study

COMPONENT	PLACEBO	*POLYPILL*
Nicotinic Acid 10 mg + 10% overage	00.00 mg	11.00 mg
Calcium carbonate heavy DC 500 mg	00.00 mg	1388.90 mg
Pregelatinised maize starch	437.50 mg	
Microcrystalline cellulose	437.50 mg	85.10 mg
Sodium starch glycolate	36.80 mg	25.00 mg
Anhydrous silica-colloidal	5.20 mg	8.00 mg
Magnesium stearate	3.00 mg	15.00 mg
Total weight	920.00 mg	1533.00 mg

Table 3.10 – Formulation of vitamin E and retinol capsule administered in the *Polypill* intervention study

COMPONENT	PLACEBO	*POLYPILL*
Alpha-tocopherol 7.5 mg as vitamin E preparation USP	00.00 mg	8.61 mg
Gamma-tocopherol 7.5 mg as vitamin E Covi-Ox T-70	00.00 mg	18.00 mg
Retinol 800 µg as vitamin A Palmitate + 10% overage	00.00 mg	1.62 mg
Tocopherols concentrate as mixed (low alpha)	00.00 mg	1.50 mg
Soya oil	290.00 mg	260.27 mg
Total weight	290.00 mg	290.00mg

3.2.7 Collection blood from volunteers

Approximately 46 mL of venous blood was collected from overnight-fasted volunteers by a trained phlebotomist in 6 various Vacuette® blood collection tubes (all Greiner Bio-One, Kremsmünster, UA, Austria) from fasted volunteers on three separate occasions spaced 16 weeks apart.

Table 3.11 – Collection and distribution of blood samples for various analyses

AMOUNT	BLOOD COLLECTION TUBE	STORAGE CONDITIONS	ANALYSES
3× 8 mL	Vacuette® Lithium Heparin	Room temperature	Plasma minerals PBMC, CBMN
1× 9 mL	Vacuette® EDTA	On ice, in the dark	NAD/NADP, retinol, alpha-tocopherol
1× 9 mL	Vacuette® Gel Serum	Room temperature for 20 min, then ice	Folate, vitamin B_{12}, vitamin D
1× 4 mL	Vacuette® EDTA/NaF	Ice	Homocysteine

3.2.8 Blood processing for separation of plasma and PBMC

Blood was processed in a biological safety cabinet as follows. Collected fasted venous blood from the Vacuette® Lithium Heparin tubes was centrifuged at 1500 ×g for 20 min at room temperature. At least 6 mL of plasma was isolated and aliquotted in four 1500 µL volumes for individual trace element analysis. Plasma samples were temporarily stored at -20°C (for <24 h prior to micronutrient analysis) and there was approximately 3 mL of remaining plasma which was aliquotted and stored at -80°C.

Following removal of plasma from the samples, blood was reconstituted with HyClone Hanks' balanced salt solution (HBSS) (Thermo Scientific, Waltham, MA, USA) to replace the volume of plasma removed, and then the blood was mixed 1:1 with HBSS. This diluted blood was then gently layered on to 10 mL of Ficoll-Paque™ (Amersham Biosciences, Uppsala, UC, Sweden) in a sterile 50 mL tube. The blood was centrifuged for 30 min at 400 ×g at 18 – 20°C to isolate PBMC using Ficoll density gradient. The PBMC layer was carefully removed with a glass Pasteur pipette and placed in a new sterile 50 mL tube, where 3× the volume of HBSS was added. The cell suspension was centrifuged at 180 ×g for 10 min. The supernatant was discarded and the PBMC cell pellet was resuspended in 90% (v/v) HyClone dialysed foetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 10% (v/v) dimethyl sulphoxide Hybri-Max™ (Sigma Aldrich, St Louis, MO, USA). These isolated PBMC were stored at -80°C.

3.2.9 Plasma measurements

Plasma concentrations of the micronutrients included in the *Polypill* were measured to give an indication of participant compliance to the study protocol and also to assess the efficacy or bioavailability of supplementation. In addition, plasma vitamin D, zinc, magnesium, selenium and homocysteine – the latter of which is a sensitive metabolic marker of folate and vitamin B_{12} status (Refsum *et al.*, 1989, Ueland and Refsum, 1989, Allen *et al.*, 1990) – were also measured.

Retinol, niacin number and α-tocopherol were measured by high performance liquid chromatography in-house (CSIRO Animal Food and Health Sciences, Adelaide, SA, Australia). The folate, vitamin B_{12} and homocysteine and 25-OH vitamin D

measurements were outsourced to a local accredited clinical laboratory (Institute of Medical and Veterinary Sciences, Adelaide, SA, Australia) where they were measured by immunoassay. Concentrations of trace elements calcium, magnesium, selenium and zinc were measured by a separate laboratory (Waite Analytical Services, Glen Osmond, SA, Australia). Calcium, magnesium and selenium were measured by inductively coupled plasma mass spectrometry (ICPMS) while zinc was quantified by inductively coupled plasma optical emission spectrometry (ICPOES).

3.2.10 Isolation of DNA from PBMC

DNA was extracted from isolated PBMC using a commercially available kit (Qiagen® DNeasy® Blood and Tissue Kit) with minor modifications. Isolated PBMC were thawed overnight in the refrigerator at 4°C from deep freeze storage at -80°C. The isolated PBMC in FBS/DMSO solution were centrifuged at high speed (16000 ×g) for 5 min to pellet the PBMC. The supernatant (FBS/DMSO) was removed by pipette and discarded. The procedure then follows from that described for the extraction of DNA from WIL2-NS cells in Section 3.3.6. Isolated PBMC DNA was eluted to a final volume of 200 µL with two consecutive steps of adding 100 µL AE buffer, incubating at room temperature and centrifugation at 6000 ×g for 1 min. DNA was stored in microcentrifuge tubes at 4°C for the short term, prior to quantification. Spectrophotometric quantification of isolated PBMC DNA is as described in Section 3.3.7.

Isolated PBMC DNA was required to determine telomere length using the method described in Section 3.1. Using the 1301 cell line positive control, the inter-assay variability for the *36B4* single copy gene PCR C_T was 1.7% (n = 22) and the mean intra-assay variability was 0.4% (0 – 0.9%; n = 66). The inter-assay variability for the telomere repeat PCR C_T was 1.5% (n = 28) and the average intra-assay variability was 2.3% (0.6 – 6.7%; n = 84).

3.2.11 Statement of candidate's contribution as relates to data presented in Chapters 4, 5 and 6.

The "A *Polypill* to prevent genome damage" study was conceived and designed by Michael Fenech, Bruce Armstrong and Peta Forder in 2006. The project grant was funded by the Australian National Health and Medical Research Council in 2007.

The *Polypill* study, was a large undertaking with numerous people involved and duly acknowledged here. The study commenced in 2007 at CSIRO Animal Food and Health Sciences (CAFHS) with volunteer samples collected in the 2008 calendar year from 4th February to 16th December. The CAFHS Clinical Trial Unit co-ordinated the operational aspects of the study (recruitment, eligibility screening, visit co-ordination, etc), collected the venous blood samples and other relevant information from volunteers. CAFHS Nutrigenomics laboratory staff processed and stored blood samples accordingly. The candidate performed the extraction of DNA from the frozen PBMC samples, telomere length assays on these samples along with the associated data analysis and interpretation.

3.3 *In vitro* modelling

3.3.1 The WIL2-NS cell line

The WIL2-NS cell line is a line of human B-lymphoblast origin (Levy *et al.*, 1968). The cell line possesses mutated p53 (G: A transition at codon 237) which results in decreased levels of apoptosis and hence a reduced removal of cells with DNA damage (Zhen *et al.*, 1995, Greenwood *et al.*, 1998). WIL2-NS cells were sourced from the American Type Culture Collection (ATCC catalogue number CRL-8155™; Manassas, VA, USA) and maintained in RPMI1640 media supplemented with 50 U/mL Penicillin, 50 µg/mL streptomycin, 2 mM L-Glutamine (all Sigma Aldrich, St Louis, MO, USA) and 5% (v/v) FBS (Bovogen Biologicals, Melbourne, VIC, Australia).

3.3.2 WIL2-NS cell challenge with FA, dUTP and SAM

In vitro challenge of WIL2-NS cells with varying concentrations of FA, dUTP and SAM were conducted.

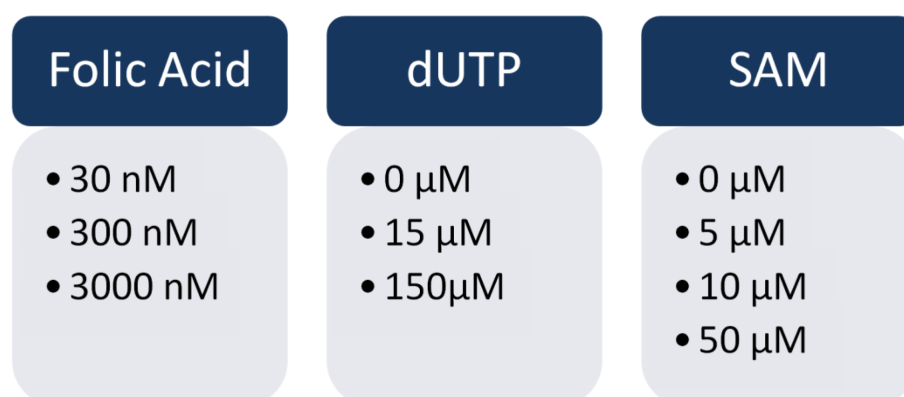

Figure 3.3 – Concentrations of each of the three treatments used *in vitro*

Individual wells of 24 well cell culture plates were seeded with 5×10^4 cells in 1 mL of RPMI1640 with either low (≈30 nM), medium (≈300 nM) or high (≈3000 nM) folic acid concentrations based on previous studies showing that chromosome instability is increased within this concentration range as folic acid concentration decreases (Beetstra *et al.*, 2005). These media were prepared to also contain 50 U/mL Penicillin, 50 µg/mL streptomycin, 2 mM L-Glutamine (all Sigma Aldrich, St Louis, MO, USA), 5% (v/v) HyClone dialysed foetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 2 mg/mL sodium bicarbonate (Sigma Aldrich, St Louis, MO, USA). Sodium bicarbonate was dissolved in sterile Milli-Q H_2O and filter-sterilised through a 0.20 µm pore filter (Merck Millipore, Billerica, MA, USA).

S-(5′-Adenosyl)-L-methionine chloride dihydrochloride ($C_{15}H_{23}ClN_6O_5S \cdot 2\ HCl$; SAM) of yeast origin (L-Methionine enriched) powder (Sigma Aldrich, St Louis, MO, USA) was prepared in sterile Milli-Q H_2O and filter-sterilised through a 0.45 µm pore filter (Merck Millipore, Billerica, MA, USA). The percentage of L-SAM in this commercial product is unknown (Sigma Aldrich, personal communication 2013). 2´-Deoxyuridine, 5´-Triphosphate (dUTP; Promega, Madison, WI, USA) in H_2O (pH 7.5) and prepared SAM solutions were added directly to the cultures once plated. Cells were cultured at 37°C with 5% CO_2 in a humidified incubator for 7 d, total and live cell concentration was assessed immediately prior to harvest (day 7). Due to the large number of cultures – 72 per folic acid concentration – the experiments were staggered for handling and logistical reasons (Figure 3.4).

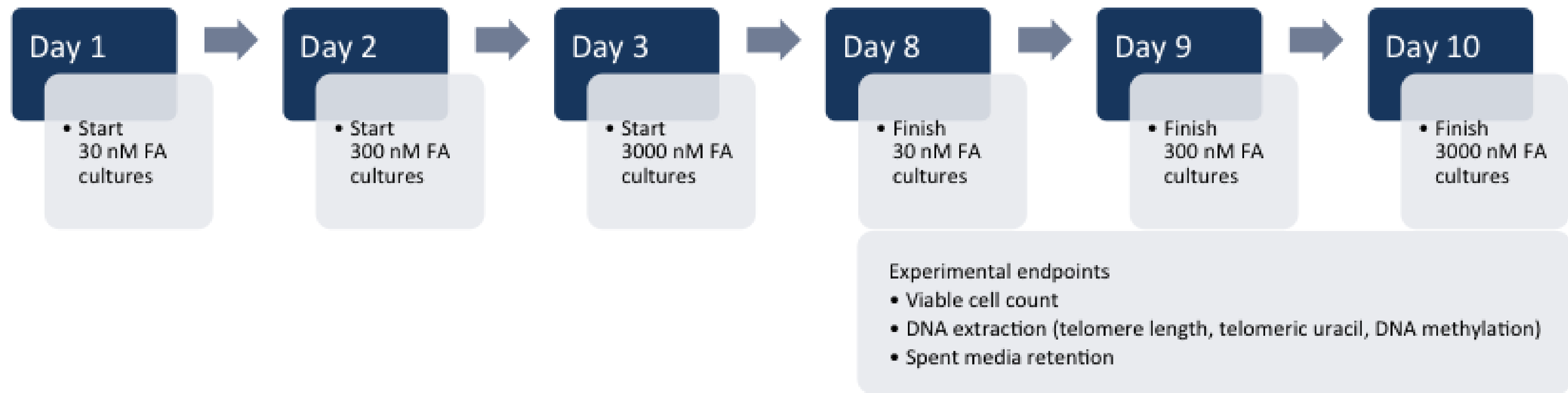

Figure 3.4 – Staggered timeline of cell culture experiments

3.3.3 Spent media analysis

Folic acid and homocysteine in complete media stocks and in spent media was measured by immunoassay offsite at a local NATA-accredited laboratory (Institute of Medical and Veterinary Sciences, Adelaide, SA, Australia). The concentration of homocysteine in spent media was used to determine the amount of homocysteine produced by the cultured WIL2-NS cells. The concentration of homocysteine generated *in vitro* is expressed as µmol of homocysteine per million total cells at day 7 (µmol/10^6 total cells).

3.3.4 Determination of WIL2-NS cell concentration and viability

To determine total and viable cell concentrations, the trypan blue exclusion assay was used. Cell culture was agitated to release semi-adherent cells before sampling and was mixed 1:1 with trypan blue solution (0.4% trypan blue, 0.81% sodium chloride, 0.06% potassium phosphate, dibasic; Sigma Aldrich, St Louis, MO, USA). For each culture, a minimum of 200 cells was counted in a haemocytometer chamber under a light microscope. In large experiments, the TC10™ automated cell counter (Bio-Rad, Gladesville, NSW, Australia) was used to rapidly quantify live and total cell numbers. Comparison of the manual and automated counting methods was performed on 30 separate cell culture samples (Pearson $R = 0.929$, $P = 1.30 \times 10^{-13}$). To ensure accuracy of cell counts, the TC10™ system verification kit containing circles simulating live and dead cells was regularly scored. As there were $n = 6$ samples for each treatment combination, cell counts were completed for half of the replicates in an effort to reduce the time and cost of cell counts; $n = 3$ counts per treatment.

3.3.5 Calculation of population doublings

The number of cell population doublings during the period of cell culture was calculated as previously described (Bode-Boger *et al.*, 2005, Khan *et al.*, 2010) with the formula:

$$Log_2(total\ cell\ count\ at\ day\ 7) - Log_2(cell\ number\ inoculated\ at\ day\ 0)$$

3.3.6 Isolation of DNA from cultured WIL2-NS cells

DNA was isolated from cultured WIL2-NS cells using a commercially available kit (Qiagen® DNeasy® Blood and Tissue Kit) with minor protocol modifications.

Twenty-four well culture plates containing cultured WIL2-NS cells were spun in a plate centrifuge at 180 ×g for 10 min. The supernatant (spent media) was removed by pipette and either discarded or retained; stored in individual microcentrifuge tubes (1 tube per well). The WIL2-NS cell pellet was lysed with a mixture of supplied lysis buffer (Qiagen® DNeasy® AL) and proteinase K enzyme (Qiagen® DNeasy®) in 1× phosphate buffered saline (PBS) in accordance to the Qiagen® recommended protocol. The lysed cell pellet was incubated at 37 C for ≥2 h. Following incubation, 200 µL absolute ethanol was added to the lysis mixture to precipitate the DNA. At this point each individual DNA extraction mixture was transferred to separate manufacturer-supplied spin columns. The spin columns were centrifuged at 6000 ×g for 1 min before consecutive washes with 500 µL Qiagen® DNeasy® AW1 and AW2 buffer solutions at 6000 ×g for 1 min and 16000 ×g for 3 min, respectively with the flow-through discarded after each centrifugation step. After the two washes, the spin column was placed into clean 2 mL collection tubes. 100 µL of supplied elution butter (Qiagen® DNeasy® AE) supplemented with 50 µM of free radical scavenger phenyl-*tert*-butyl nitrone (Atamna *et al.*, 2000) was added directly to the column. The columns were then incubated at room temperature for 3-5 min before centrifugation at 6000 ×g for 1 min. This final step was repeated with an additional 100 µL solution added to the column to give a final volume of ≈200 µL isolated WIL2-NS DNA. These volumes of AE buffer were reduced where cell concentration was low on the day of harvest, to 100 µL or at times ≤50 µL.DNA was stored in microcentrifuge tubes at 4°C for the short term, prior to concentration determination.

3.3.7 Quantification of isolated DNA

Concentration of DNA from each WIL2-NS culture was determined by NanoDrop™ spectrophotometric analysis (NanoDrop™ ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA) where an optical density at 260 nm (OD_{260}) of 1 corresponds to approximately 50 ng/µL of double stranded DNA (Sambrook *et al.*, 1989). Qiagen® DNeasy® AE buffer supplemented with 50 µM phenyl-*tert*-butyl nitrone was used as the DNA sample blank on the ND-1000 spectrophotometer. DNA purity was assessed using the absorbance ratios 260: 280 nm and 260: 230 nm, which estimates purity of the sample against contamination with protein, and organic

compounds such as phenolate and thiocyanate, respectively. A DNA sample with a 260: 280 nm of ≥1.8 and a 260: 230 nm of ≥2.0 was regarded as of sufficient quality and purity to use in further downstream applications.

3.3.8 USER™ digest of DNA for qPCR detection of UDG-sensitive lesions in telomeric sequence

Uracil-Specific Excision Reagent (USER™) enzyme is a commercially prepared mix of uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase Endonuclease VIII which generates a single nucleotide gap at the location of uracil residues (New England Biolabs, Ipswich, MA, USA). The excision of uracil from DNA, catalysed by the UDG enzyme, forms an apyrimidinic site without cleaving the phosphodiester backbone (Lindahl *et al.*, 1977) however Endonuclease VIII contains both N-glycosylase and AP lyase activities, and subsequently cleaves the phosphodiester backbone 3' and 5' to the abasic site (Melamede *et al.*, 1994). UDG and Endonuclease VIII enzymes present in the USER™ preparation were separately purified from *Escherichia coli* K-12 strains (New England Biolabs, Ipswich, MA, USA).

200 ng of sample DNA or oligonucleotide sequence was digested with 1U of USER™ enzyme (New England Biolabs, Ipswich, MA, USA) overnight (≥16 h) in a total reaction volume of 20 µL with 1× T4 DNA ligase reaction buffer (New England Biolabs, Ipswich, MA, USA) containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM Dithiothreitol and 1 mM ATP. Mock-digested (control) DNA and/or oligonucleotide sequences were incubated under identical conditions, however with additional TE buffer in place of the volume of the USER™ enzyme. Following overnight incubation, all the mixes were heated to 95°C for 10 min to completely inactivate the Endonuclease VIII and to heat kill 95% of the UDG enzyme in the USER™ preparation.

A series of oligonucleotide standards containing deoxyuridine (dU) were designed and used in the optimisation and verification of the USER™ digest assay (Table 3.12). These oligonucleotides were purchased locally and HPLC-purified (Geneworks, Hindmarsh SA, Australia). The telomere qPCR ΔC_T values of digested and undigested oligonucleotide sequence containing 0, 1, 2 and 4 deoxyuridine residues per 84 bases were used to generate a standard curve of uracil per kb telomere sequence. Additionally, the telomere qPCR ΔC_T between digested and

undigested 4U standard oligonucleotide sequence was used as a positive control when WIL2-NS cell DNA was assayed for telomeric uracil content.

Digestion of DNA with USER™ involves excision of uracil from the sequence by UDG, creating an apyrimidinic site. Then, N-glycosylase and AP lyase activities of Endonuclease VIII generate a gap in the sequence. This nick or single nucleotide gap in the DNA strand impairs the kinetics of the telomere length qPCR, resulting in an increased C_T. This increase in C_T can be compared to the C_T of control DNA not treated with USER™ (ΔC_T). Uracil residues were placed within different repeats of the telomere oligonucleotide (Table 3.12) to determine the impact of increasing telomeric uracil on the telomere qPCR kinetics.

Table 3.12 – Oligonucleotide sequences used in optimisation of the USER assay

NAME	NUMBER OF DEOXYURIDINE RESIDUES/84 B	NUMBER OF DEOXYURIDINE RESIDUES/KB	SEQUENCE (5’ – 3’)	MW
Telo 0U	0	0	TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA GGGTTAGGGTTAGGGTTAGGGTTAGGG	26667
Telo 1U	1	11.9	TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG**U**TAGGGTTAGGGTTAGGGTTA GGGTTAGGGTTAGGGTTAGGGTTAGGG	26653
Telo 2U	2	23.8	TTAGGGTTAGGG**U**TAGGGTTAGGGTTAGGGTTAGGG**U**TAGGGTTAGGGTTAGGGTTA GGGTTAGGGTTAGGGTTAGGGTTAGGG	26639
Telo 4U	4	47.6	TTAGGGTTAGGG**U**TAGGGTTAGGGTTAGGGTTAGGG**U**TAGGGTTAGGGTTAGGGT**U**A GGGTTAGGGT**U**AGGGTTAGGGTTAGGG	26611

3.3.9 Determination of global DNA methylation

A commercial colourimetric assay kit for the detection of 5-methylcytosine was used to determine global DNA methylation (Epigentek MethylFlash™ Methylated DNA Quantification Kit; Epigentek, Farmingdale, NY, USA). Although Epigentek suggest duplicate measurement of samples and controls, each were instead assayed in triplicate to improve the quantification of 5-methylcytosine. DNA from within treatment groups with $n = 6$ replicates was pooled in order to achieve $n = 3$ samples per treatment combination and greatly reduce cost of analysis.

The manufacturer's protocol was followed with minor modifications. Briefly, 100 ng of input DNA was bound to the supplied strip wells in the presence of 80 µL ME2 binding solution. The supplied positive (50% 5-methylcytosine) and negative (unmethylated) control DNA were also bound to the assay wells and the plate incubated at 37°C for 90 min. A standard curve of positive 5-methylcytosine was run at every assay though was modified from the suggested serial 1:2 dilutions from 10 ng/µL – 0.5 ng/µL to 2.5 ng/µL – 0.04 ng/µL as seen in Table 3.13.

Following 90 min incubation of sample and control DNA, binding solution was discarded and the wells were washed 3 times with 150 µL 1× ME1 wash buffer. Capture of 5-methylcytosine signal was performed using 50 µL of 1 µg/mL ME5 capture antibody to each well, and incubation at room temperature for 60 min. Proceeding incubation, the ME5 solution was removed and the wells were washed 3 times with 150 µL 1× ME1 wash buffer. A second antibody, 50 µL of 0.2 µg/mL ME6 detection antibody was then added to each well and the plate incubated at room temperature for 30 min. After incubation, the ME6 solution was removed and the wells were washed 4 times with 150 µL 1× ME1 wash buffer. An enhancer was then added to the wells, specifically 50 µL ME7 enhancer solution diluted by a factor of 5000, prior to 30 min incubation at room temperature. Following incubation, the ME7 solution was discarded and the wells were washed 5 times with 150 µL 1× ME1 wash buffer. Detection of the 5-methylcytosine signal was performed with 100 µL neat ME8 developer solution. The plate was incubated at room temperature and the development of a blue colour in the samples and standards was monitored. Once a medium blue shade was observed in the most concentrated standard, after approximately 7 min, 100 µL neat ME9 stop solution was added to each well. The

colour changed from blue to yellow with the addition of ME9 solution and absorbance at 450 nm was detected on a SpectraMAX 250 version 1.03 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and PC with SOFTmax® Pro version 3.1.2 (Molecular Devices, Sunnyvale, CA, USA). Due to the light sensitivity of the reagents in this colourimetric assay, all solutions were kept covered and the above protocol was performed in an area with the lights off under ambient light. During all incubation steps, the plate was sealed and wrapped in aluminium foil.

Table 3.13 – Standard curve of DNA with 50% methylated cytosine used in global methylation assays

VOL OF ME4 POSITIVE CONTROL	CONCENTRATION OF ME4 POSITIVE CONTROL	AMOUNT OF ME4 POSITIVE CONTROL	AMOUNT OF METHYLATED CYTOSINE
1 µL	2.5 ng/µL	2.5 ng	1.25 ng
1 µL	1.25 ng/µL	1.25 ng	0.625 ng
1 µL	0.625 ng/µL	0.625 ng	0.3125 ng
1 µL	0.3125 ng/µL	0.3125 ng	0.15625 ng
1 µL	0.15625 ng/µL	0.15625 ng	0.078125 ng
1 µL	0.078125 ng/µL	0.078125 ng	0.0390625 ng
1 µL	0.0390625 ng/µL	0.0390625 ng	0.01953125 ng

3.4 Statistical analysis

Results presented are typically reported as mean ± standard deviation, range (minimum observation – maximum observation), number [% cases] or median (IQR; 25th – 75th percentile). Statistical tests were performed in IBM SPSS Statistics version 20.0 (International Business Machines Corp, Armonk, New York, USA) where the threshold for statistical significance was set at $P < 0.05$ and these significant associations are highlighted in bold typeface throughout. In most cases parametric statistics were used if biological variables were observed to follow a Gaussian normal distribution. If data were not normally distributed, they were either subsequently log-transformed to meet this assumption or when data were observed to be non-parametric, median based testing was performed. For single categorical outcome variables, chi-squared tests of goodness of fit for even proportions were performed and for two categorical variables, chi-squared tests of independence were conducted.

Relationships between measured variables were assessed by bivariate and partial Pearson correlation analyses. The Pearson product-moment correlation coefficient was squared in order to give R^2, the coefficient of determination. This value was then multiplied by 100 to yield a percentage which characterises the amount of shared variability between two correlated values. In order to correct for multiple correlation analyses, Bonferroni-adjusted *P* thresholds for statistical significance were computed by dividing the alpha value for type I error (0.05) by the total number of comparisons conducted in any correlation matrix. These adjusted *P* thresholds for statistical significance are provided as a footnote below correlation matrices, although reported *P* values < 0.05 are highlighted in bold typeface.

Standard multiple linear regression was performed with multiple predictor variables expected to influence telomere length. The accuracy of the model was assessed by the conformity of the measured dependent variables, where 99% of standardised residuals within ± 3.0 was acceptable.

In regression modelling, dichotomous variables were coded as 0 or 1 with gender modelled as male (0) or female (1) and active *Polypill* treatment modelled as no (0) or yes (1) and previous supplement use modelled as no (0) or yes (1).

Generalised linear modelling in the form of repeated measures analysis of variance was conducted with time as the repeated-measure factor and telomere length as the dependent quantitative variable. Advanced longitudinal modelling was performed in Stata version 12 (StataCorp, College Station, Texas, USA).

Power analyses were conducted in G*Power version 3.1.5 (Faul *et al.*, 2007, Faul *et al.*, 2009) and Stata version 12 (StataCorp, College Station, Texas, USA).

4 Cross-sectional associations of telomere length with dietary micronutrients and other factors

4.1 Introduction

4.1.1 Telomeres

Telomeres are nucleoprotein complexes found at the end of linear chromosomes, such as those in the human genome. The protein complex of the telomere, shelterin, functions to prevent chromosome end-to-end fusions within the cell and to avert the chromosome termini being mistaken for DNA double-stranded breaks. A small fraction of the underlying telomeric hexamer repeat sequence of TTAGGG is sacrificed during DNA replication which prevents the loss of coding DNA from chromosome ends. Telomeres shorten with every cycle of cell division as the unidirectional action of DNA polymerase moving in 5’ to 3’ direction restricts complete replication of DNA. And so with each cycle of replication, between 30 and 200 bp of telomeric sequence at the 5' end of the lagging strand is lost (Sitte *et al.*, 1998).

4.1.2 Dietary micronutrients, DNA and telomeres

Dietary micronutrients have been demonstrated to be involved in chromosome and genome stability (Table 1.2). Various micronutrients can impact upon DNA and chromosome stability through their involvement in DNA synthesis, repair and DNA methylation. It is proposed that these dietary micronutrients may also reduce damage to the telomeric DNA sequence, subsequently affecting telomere attrition or telomere stability and function. Whilst the human TTAGGG telomere repeat sequence is devoid of CpG dinucleotide (the substrate for DNA methylation of cytosine) the subtelomeric sequence immediately proximal to each telomere is CpG rich. The methylation status in this region has been shown to correspond with telomere length (Maeda *et al.*, 2009), and it is possible that through dietary methyl donor micronutrients, e.g. folate and vitamin B_{12}, the methylation status may be modified, along with telomere length.

4.1.2.1 In vitro studies

An early *in vitro* study found that age-dependent telomere shortening could be decelerated by intracellular ascorbic acid (vitamin C) (Furumoto *et al.*, 1998). In this study, vitamin C was found to suppress both intracellular oxidative stress and age-dependent decreases in telomerase, which they called telomerase retention, identifying two mechanisms by which vitamin C could slow telomere shortening (Furumoto *et al.*, 1998). Following this, *in vitro* experimentation with homocysteine was shown to increase the rate of senescence and increase telomere shortening in endothelial cells (Xu *et al.*, 2000). The induced effects of homocysteine (a toxic amino acid which increases with folate or vitamin B_{12} deficiency) on senescence and telomere shortening were significantly attenuated by the enzyme catalase, a peroxide scavenger known to mitigate the burden of cellular oxidative stress (Xu *et al.*, 2000).

In vitro administration of phosphorylated α-tocopherol has been shown to cause a reduction of intracellular reactive oxygen species (Tanaka *et al.*, 2007). In this same study, age-dependent telomere shortening was reduced, and telomerase activity retained in the cells that were treated with phosphorylated α-tocopherol. This retention of telomeric DNA sequence and telomerase activity was correlated with increased cellular longevity, which may have been a result of reduced oxidative stress in the cell (Tanaka *et al.*, 2007).

As cellular homocysteine is known to be modifiable with folate and vitamin B_{12} intake or supplementation, and as vitamins C and E can also be increased through diet and supplementation, these *in vitro* studies indicate that nutritional factors could affect or be utilised to modify telomere length.

4.1.2.2 Caloric, protein and amino acid restriction

Caloric restriction is known to reduce oxidative stress and oxidative damage, resulting in extension of average and expected lifespan (Sohal and Weindruch, 1996, Barja, 2002). Moreover, caloric restriction appears to reduce DNA damage by enhancing DNA repair through various pathways including nucleotide excision repair, base excision repair and double-strand break repair (Heydari *et al.*, 2007). However it is not known which specific dietary components are mechanistically responsible for the reduction in oxidative damage and increase in longevity

observed with general caloric restriction. Restriction of carbohydrates and lipids has previously been demonstrated to not increase longevity in rats (Iwasaki *et al.*, 1988, Khorakova *et al.*, 1990, Shimokawa *et al.*, 1996), while targeted restriction of up to 85% protein in various rodent studies has shown an increase in maximum lifespan of up to 43%, with an average increase of 20% (various studies reviewed in Pamplona and Barja, 2006). Additional studies in rodents have found specific restriction of methionine increases lifespan (Miller *et al.*, 2005) while restriction of other amino acids can reduce oxidative damage (Tanrikulu-Kucuk and Ademoglu, 2012).

4.1.2.3 Cross-sectional studies: self-reported data

The association of diet patterns and components with telomere length in humans has been largely probed in cross-sectional associations of self-reported frequencies of food consumption. An early study of 840 adults of African American, Hispanic and white ethnicity found a significant inverse association between reported intake of processed meat and leukocyte telomere length (Nettleton *et al.*, 2008). More recently, lymphocyte telomere length was significantly positively associated with intake of vitamins A, C, E, B_9 (folate) and β-carotene as measured by FFQ for dietary intake over the preceding 12 months (Marcon *et al.*, 2012). In general, a higher consumption of fruits and vegetables was significantly associated with longer telomeres (Marcon *et al.*, 2012). In further support of dietary patterns associating with telomere length, a higher Mediterranean diet score (as a measure of adherence (Trichopoulou *et al.*, 2003)) in 385 Southern Italian men and women was positively associated with both leukocyte telomere length and PBMC telomerase expression (Boccardi *et al.*, 2013).

Presence of coronary artery calcium (CAC) in 318 men and women was significantly associated with leukocyte telomere length and meat consumption which increased the odds ratio of CAC ($P = 0.04$), and leukocyte telomere length and fruit and vegetable consumption which decreased the odds ratio of CAC ($P = 0.02$) (Diaz *et al.*, 2010). These results suggest that healthy diet may attenuate the association of shorter telomere length with increased coronary atherosclerosis (Diaz *et al.*, 2010).

Associations of telomere length with dietary intake data appear to differ by gender in the published literature. In one study, consumption of Chinese tea was

significantly positively associated leukocyte telomere length in men but not women, while consumption of fats and oils for cooking was negatively associated with leukocyte telomere length in women but not men (Chan *et al.*, 2010). Additionally, in elderly Finnish women, leukocyte telomere length was positively associated with vegetable consumption, while in males of the same age group, leukocyte telomere length was associated negatively with total fat, saturated fatty acid and butter, whilst positively associated with fruits (berries and juice inclusive) (Tiainen *et al.*, 2012). These differences between men and women may reflect variances in their habitual dietary intakes, lifestyle or underlying biology.

Additional observations of diet with telomere length come from historical study cohorts which were restricted to women. In American women from the Nurses' Health Study, leukocyte telomere length has been associated positively with dietary intakes of fibre and cereal fibre (Cassidy *et al.*, 2010). A generally healthy lifestyle was associated with longer leukocyte telomere length in 5863 women also part of the Nurses' Health Study cohort (Sun *et al.*, 2012). The definition of healthy lifestyle was developed using five components; smoking, physical activity, adiposity, alcohol consumption and diet. Finally, in a study of multivitamin type, frequency of use and telomere length, there was generally longer telomere length in women who reported use of any multivitamins, once-a-day type multivitamins and antioxidant combinations (Xu *et al.*, 2009).

4.1.2.4 Cross-sectional studies: associations of telomere length with biochemical data

Cross-sectional studies utilising biochemical data as markers of specific micronutrient status have shown associations between some dietary micronutrients and telomere length. High plasma homocysteine was negatively correlated with leukocyte telomere length in 1319 participants who were mainly women (>90%) (Richards *et al.*, 2008). This correlation was weak in strength (Pearson R = -0.15), but highly statistically significant ($P < 0.0001$). Additionally, within tertiles based on plasma homocysteine levels, those with higher folate were seen to have longer leukocyte telomere length. When stratified by tertiles of plasma homocysteine, levels of CRP were higher in those with shorter leukocyte telomere length. These results collectively suggest that leukocyte telomere length is affected by plasma

homocysteine, which could be modified by serum folate or C-reactive protein (CRP) (Richards *et al.*, 2008).

An independent study with a much smaller cohort showed a significant inverse correlation of plasma homocysteine with peripheral blood lymphocyte telomere length in older men aged 65 to 83 y ($R = -0.57$, $P = 0.004$) but no significant association was observed in older women, younger men or women, or in the cohort as a whole (Bull *et al.*, 2009). In this same study, a significant positive association between plasma folate and telomere length was noted again in older men, ($R = 0.42$, $P = 0.04$), however not in the other subgroups or in the complete cohort of 90. This association between homocysteine and telomere length is much stronger in effect than that seen in the previous Richards study, and due to the small number of observations ($n = 24$), needs further replication within the population studied to verify the strength of association.

Another study looking at folate and telomere length, this time in peripheral blood mononuclear cells, found a non-linear association of plasma folate with telomere length in 195 Italian men (Paul *et al.*, 2009). In this study, plasma folate above the population median was positively associated with telomere length, while in quartiles of plasma folate below the median there was a negative association with telomere length. When folate levels were below the median, the *MTHFR* 677C→T polymorphism was weakly associated with longer telomere length ($P = 0.065$). The common 677C→T transition polymorphism results in a reduced activity and consequently a decline in 5-methyltetrahydrofolate available for methylation of homocysteine to form methionine (Kang *et al.*, 1988a, Kang *et al.*, 1988b). *MTHFR* 677C→T is also suggested to affect genomic DNA methylation (Friso *et al.*, 2002). Plasma folate is highest in *MTHFR* 677 CC homozygotes than in CT and lowest TT individuals, while the opposite is true for homocysteine, which is lowest in CC homozygotes, higher in CT heterozygotes and highest in TT homozygotes (Paul *et al.*, 2009). That *MTHFR* 677 C→T polymorphism was associated with longer telomere length at below median folate status is suggested to be the result of increased DNA hypomethylation observed with TT homozygosity (Paul *et al.*, 2009).

Although folate and homocysteine have been associated with telomere length, and the two are intimately associated with vitamin B_{12}, there is an unclear association of

B_{12} on telomere length. On one hand, users of B_{12} supplements were shown to have longer telomere lengths when compared to non-users (Xu *et al.*, 2009), however in biochemical cross-sectional studies of plasma vitamin B_{12}, there has been no association (Paul *et al.*, 2009), or a negative association of B_{12} on telomere length in older men (Bull *et al.*, 2009).

The single largest study investigating plasma dietary micronutrient status and telomere length exists for vitamin D (Richards *et al.*, 2007). In this study of 2160 women with a mean age of 49 y (ranging from 18–79 y of age), there was a significant, positive association of vitamin D with telomere length which persisted after adjustment for known covariates including age and physical activity. The correlation was again weak in magnitude, however highly statistically significant ($R = 0.09$, $P < 0.0001$). The large cross-sectional studies by Richards and colleagues (2007, 2008) show highly significant correlations of telomere length independently with vitamin D (positively correlated) and homocysteine (negatively correlated), illustrating that the associations of micronutrients or their associated metabolites with telomere length may be very small in magnitude, requiring a large number of individuals to accurately observe the relationships.

As these described studies are cross-sectional, conclusions regarding causality are unable to be drawn. It is possible that the dietary intakes or micronutrient concentrations which are significantly associated with telomere length may be serving as surrogate indicators of additional diet or lifestyle behaviours not measured or otherwise identified which themselves influence telomere length.

4.1.3 Aims and hypotheses

The specific aim of the cross-sectional study in this thesis was to explore which plasma dietary micronutrients and demographic measures may be associated with telomere length in a healthy, middle-aged cohort of South Australian adults.

It was hypothesised that individual plasma dietary micronutrients may be positively or negatively associated with telomere length in this cross-sectional study cohort, depending on the micronutrient and its biological functions. Demographic factors (e.g. age, gender) were hypothesised to be positively or negatively associated with

telomere length in this cross-sectional study cohort as they have been in many other cross-sectional studies reported in the literature.

4.2 Methods

Details of the study methods, including ethics approval and blood sample processing, are as described in Section 3.2. Briefly, participants were recruited to the study and asked to commence a 16-week period where they refrained from using any dietary supplements or ceased any previous patterns of use. A blood sample was taken after this 16-week washout period (week 0) for isolation of peripheral blood mononuclear cells and blood plasma. These blood samples were collected from volunteers at the onsite clinic at CSIRO Animal, Food and Health Sciences in Adelaide, South Australia from 4th February to 13th May 2008. Micronutrients were measured in blood plasma, while telomere length was measured in DNA from isolated PBMC cells. Volunteers completed a brief health status questionnaire and a validated food frequency questionnaire (Hodge *et al.*, 2000) in the clinic, where their height and weight was also measured and recorded.

4.2.1 WHO definition of BMI categories

Height and weight measurements were used to determine body mass index (BMI) using the formula: $BMI\ (kg/m^2) = \frac{weight}{height^2}$

The accepted international World Health Organisation (WHO) classification and cut points of BMI defining adult underweight, normal weight, overweight and obesity were used (World Health Organisation, 2006).

4.2.2 Socio-Economic index for areas

Residential postcodes were used to determine four social economic indexes for area (SEIFA) as compiled by the Australian Government based on 2006 census data (Australian Bureau of Statistics, 2008). These SEIFA measures are relative and summary measures which represent the average of people and households in a given postal area. The four SEIFA indexes were each used; The Index of Relative Socio-economic Advantage and Disadvantage (IRESD), The Index of Relative Socio-economic Disadvantage (IRSD), The Index of Economic Resources (IER) and The Index of Education and Occupation (IEO). Brief descriptions of these measures and what measures were used are described on the following page (Figure 4.1).

Figure 4.1 – Descriptions of the socio-economic index for areas measures

Source: Australian Bureau of Statistics (2008)

4.2.3 Statistical analyses

Results presented are typically reported as mean ± standard deviation, range (minimum observation – maximum observation), number [% cases] or median (IQR; 25th – 75th percentile). The threshold for statistical significance was set at $P < 0.05$ and these significant associations are highlighted in bold typeface throughout.

Relationships between measured variables were assessed by bivariate and partial Pearson correlation analyses. The Pearson product-moment correlation coefficient (R) was squared in order to give R^2, the coefficient of determination. This R^2 value was then multiplied by 100 to yield a percentage which characterises the amount of shared variability between two correlated values. In order to correct for multiple correlation analyses, Bonferroni-adjusted P thresholds for statistical significance were computed by dividing the alpha value for type I error (0.05) by the total number of comparisons conducted in any correlation matrix. However, Bonferroni-adjusted thresholds for statistical significance can be restrictive, especially when a number of correlation analyses are conducted. Although the adjustment greatly reduces the likelihood of type I error (incorrect rejection of the null hypothesis), this simultaneously gives rise to an increased likelihood of type II error (failure to reject a false null hypothesis, concluding no association when there is an association). As such, these P values for statistical significance should be interpreted with caution while further considering that the reported correlation between any two variables does not infer causation. In the correlation of two variables, it is intrinsic that there may be an additional variable or variables which may affect the correlation coefficient. Moreover, a given correlation coefficient is devoid of indicating the direction of relationship in two correlated variables, i.e. which variable is causing the change in the other variable. These Bonferroni-adjusted P thresholds for statistical significance are provided as a footnote below correlation matrices, although reported P values < 0.05 are highlighted in bold typeface.

Standard multiple linear regression was performed with multiple predictor variables expected to influence telomere length. The accuracy of the model was assessed by the conformity of the measured dependent variables, where 99% of standardised residuals within ± 3.0 was acceptable. In regression modelling, dichotomous

variables were coded as 0 or 1 with gender modelled as male (0) or female (1) and previous supplement use modelled as no (0) or yes (1).

Supplementary data is contained in the Appendix.

4.3 Results

Table 4.1 summarizes the demographic and anthropometric descriptives of the 212 volunteers comprising this cross-sectional study cohort. The average age of volunteers was 44.5 y and is representative of the desired middle-aged population. It was noted that more women (58%) than men (42%) volunteered for participation in the study. Recruited volunteers had a range of socio-economic backgrounds, based on their SEIFA deciles. The mean BMI was 27 kg/m^2 and 58 of 212 (27%) volunteers were classified as obese I, obese II or morbidly obese (Table 4.2).

Table 4.1 – Characteristics of the study population

VARIABLE	*n*	VALUE	RANGE (MIN – MAX)
Age (y)	212	44.5 ± 8.2	26 – 61
Gender males [%]	212	88 [41.5]	-
BMI (kg/m^2)	212	26.8 ± 5.2	17.6 – 44.1
Obese [%]	212	58 [27.4]	-
Maternal age[a] (y)	211	27.7 ± 5.7	17 – 45
Paternal age[b] (y)	209	30.7 ± 7.2	17 – 53
IRSED decile Median (IQR)	212	7 (5 – 9)	1 – 10
IRSD decile Median (IQR)	212	7 (4 – 9)	1 – 10
IER decile Median (IQR)	212	5 (2 – 8)	1 – 10
IEO decile Median (IQR)	212	8 (5 – 9)	1 – 10

Values supplied are mean ± standard deviation, number of cases [% cohort] or median (IQR).

[a] one volunteer was unable to provide maternal age at their birth

[b] three volunteers were unable to provide paternal age at their birth

Table 4.2 – Distribution of body mass index categories of participants

	BMI CATEGORY AND DEFINITION	*n* [%]	TOTAL *n* [%]
Non-obese	Underweight *BMI ≤ 18.49 kg/m^2*	3 [1.4]	154 [72.6%]
	Normal *BMI 18.5-24.99 kg/m^2*	88 [41.5]	
	Overweight *BMI 25-29.99 kg/m^2*	63 [29.7]	
Obese	Obese I *BMI 30-34.99 kg/m^2*	43 [20.3]	58 [27.4%]
	Obese II *BMI 35-39.99 kg/m^2*	11 [5.2]	
	Obese III (Morbidly obese) *BMI ≥ 40 kg/m^2*	4 [1.9]	

n = 212

Slightly fewer than half of volunteers in the study reported recent supplement use (47%). Those who did report recent supplement use indicated they took one or more supplements which were classed as vitamin, mineral, oil, herbal or other (Table 4.3). The most commonly reported supplement use consisted of single or multiple vitamin supplements (52% of users or 24% of the cohort) and fish oil or omega 3 preparations (39% of users, or 18% of the cohort). Other common supplements included individual minerals or multiple mineral supplements (18% of users, 8% of cohort) and glucosamine and/or chondroitin (17% of users, 8% of cohort). In order to assess blood plasma micronutrient status from dietary sources alone, participants were instructed to cease any current dietary supplementation and refrain from (re)commencing supplement use. Blood plasma micronutrients were measured following this 16-week "washout" period (Table 4.4).

Table 4.3 – Self-reported recent supplement use prior to study period

VARIABLE	NUMBER [% USERS]	% COHORT
No reported recent usage	113 [0]	53.3
Single vitamin supplement	13 [13.1]	6.1
Multiple vitamin supplements or multivitamin	38 [38.4]	17.9
Single mineral supplement	14 [14.1]	6.6
Multiple mineral supplements or multimineral	4 [4.0]	1.9
Combination multivitamin/multimineral	7 [7.0]	3.3
Fish oil and/or omega 3	39 [39.4]	18.4
Glucosamine and/or chondroitin	17 [17.1]	8.0
Other	23 [23.2]	10.8

Total n = 212; n users = 99
Other includes calorie control, cod liver oil, coenzyme Q10, evening primrose oil, fibre, flaxseed oil, garlic, ginko biloba, ginseng, horseradish, lysine, milkthistle, protein, seaweed kelp and valeriana

Table 4.4 – Measured micronutrient levels in study participants

VARIABLE	*n*	MEAN ± SD	RANGE (MIN – MAX)	REFERENCE RANGE (MIN – MAX)
Folate (nmol/L)	211	23.6 ± 8.8	(4.7 – 41.7)	5 – 45 nmol/L
Homocysteine (µmol/L)	211	8.2 ± 2.2	(4.3 – 21.5)	5 – 15 µmol/L
Vitamin B_{12} (pmol/L)	211	299.1 ± 150.8	(72 – 1450)	100 – 700 pmol/L
α-tocopherol (µmol/L)	203	29.7 ± 6.7	(14.8 – 55.6)	15 – 50 µmol/L
Retinol (µmol/L)	203	2.3 ± 0.6	(1.0 – 4.5)	1.1 – 2.8 µmol/L
Niacin number $\frac{\text{NAD}}{\text{NAD} + \text{NADP}} \times 100$	203	157.2 ± 27.6	(89.0 – 250.8)	84 – 236
Calcium (mmol/L)	212	2.3 ± 0.1	(2.0 – 2.5)	2.2 – 2.6 mmol/L
Vitamin D (nmol/L)	211	78.9 ± 24.5	(30 – 190)	25 – 135 nmol/L
Magnesium (µmol/L)	212	782.7 ± 55.4	(588.2 – 929.2)	650 – 1500 µmol/L
Selenium (µmol/L	212	1.4 ± 0.2	(0.9 – 2.0)	0.9 – 1.7 µmol/L
Zinc (µmol/L)	212	13.4 ± 1.6	(9.0 – 19.6)	10 – 22 µmol/L

n = 212;
Some plasma biomarkers have < 212 values due to insufficient plasma from ≤9 participant samples to conduct all micronutrient analyses

Telomere length in isolated PBMC from the supplied blood sample was measured for all individuals. The average telomere length for all individuals was 114.0 kb/diploid genome, with mean measures of 117.7 kb/diploid genome for women and 108.8 kb/diploid genome for men, however this difference was not statistically significant (*P* = 0.15, Table 4.5). Telomere length for obese and non-obese individuals was compared, however there was also no significant difference, 112.2 kb/diploid genome and 114.7 kb/diploid genome, respectively (*P* = 0.51). Additionally, there was no difference in telomere length between BMI categories (ANOVA *P* = 0.31, data not shown).

Table 4.5 – Telomere length for all participants, by gender and obesity at week 0

	GROUP	MEAN ± SD	RANGE (MIN – MAX)	*P* (ANOVA)
Telomere length at week 0	All [*n* = 212]	114.0 ± 59.0	20.5 – 487.5	-
	Females [*n* = 124]	117.7 ± 59.3	29.9 – 487.5	0.15
	Males [*n* = 88]	108.8 ± 58.4	20.5 – 445.3	
	Non-obese [*n* = 154]	114.7 ± 55.3	20.5 – 445.3	0.51
	Obese [*n* = 58]	112.2 ± 68.3	29.9 – 487.5	

Units for telomere length are kb/diploid genome

Figure 4.2 shows telomere length against age, stratified by gender. Telomere attrition (bp/diploid genome) per year (y) of ageing was calculated by linear regression and determined to be 11.46 bp/diploid genome/y (95% CI; -16.16 to +11.94 bp/diploid genome/y, n = 212) for all participants (P = 0.39; trendline not shown). Attrition in males was 11.15 bp/diploid genome/y (95% CI; -19.92 to +15.30 bp/diploid genome/y) and in females was 11.70 bp/diploid genome/y (95% CI; -18.44 to +12.85 bp/diploid genome/y).

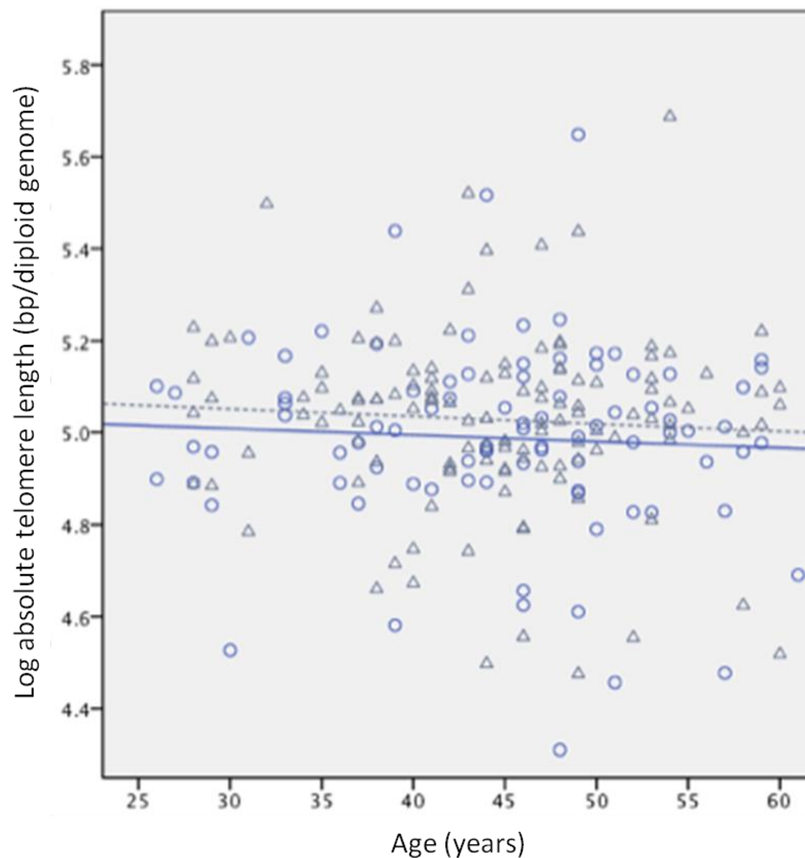

Figure 4.2 – Absolute telomere length against age, by gender

Log absolute telomere length at week 0 for females (Δ, --, R^2 = -0.003, n = 124, P = 0.44) and males (○, –, R^2 = 0.004, n = 88, P = 0.67)

To assess whether there were differences between those who reported recent use of dietary supplements and those who did not, demographic descriptives across the two groups were compared (Table 4.6). There were no significant differences noted between those who reported recent supplementation and non-supplementers.

Table 4.6 – Demographic and anthropometric descriptives of the study population at week 0, by self-reported recent supplementation status

VARIABLE	NON RECENT SUPPLEMENTERS (*n* = 113, 53.3%)	RECENT SUPPLEMENTERS (*n* = 99, 46.7%)	*P* (ANOVA, χ^2 OR MANN-WHITNEY)
Age (y)	43.8 ± 8.5 (26 – 61)	45.2 ± 7.7 (26 – 60)	0.18
Gender males [%]	48 [42.5]	40 [40.4]	0.43
BMI (kg/m^2)	27.1 ± 5.3 (17.6 – 44.1)	26.5 ± 5.0 (18.3 – 44.1)	0.44
Obese [%]	36 [31.9]	22 [22.2]	0.078
Maternal age (y)	27.7 ± 5.7 (17 – 42)[a]	27.8 ± 5.7 (17 – 45)	0.86
Paternal age (y)	30.8 ± 7.7 (18 – 53)[b]	30.6 ± 6.7 (17 – 52)	0.99
IRSED decile Median (IQR)	7 (4 – 9)	8 (5 – 9)	0.34
IRSD decile Median (IQR)	7 (4 – 9)	7 (4 – 9)	0.39
IER decile Median (IQR)	5 (2 – 7)	5 (2 – 8)	0.59
IEO decile Median (IQR)	7 (5 – 9)	8 (5 – 9)	0.11
TL at week 0 (kb)	117.6 ± 68.1 (20.5 – 487.5)	109.9 ± 46.4 (28.6 – 322.2)	0.51

[a] n = 112, [b] n = 110 due to missing data on parental ages

There appeared to be no significant difference in telomere length across quintiles of weekly alcoholic beverage intake, where mean consumption ranged from 0 mL to 2836 mL of alcoholic beverages per week (data not shown; ANOVA *P* = 0.45).

Bivariate correlation of telomere length against age, maternal age, paternal age and BMI to assess the associations with each of these continuous, demographic measures (represented as a correlation matrix in Table 4.6 and as a scatterplot matrix in Figure 4.3). Although not significant, age was negatively associated with telomere length over time. An increased maternal age was associated with an

increased telomere length, however this did not reach the α-level of statistical significance ($P = 0.06$). Paternal age was significantly positively associated with telomere length where it was observed those who had fathers who were older at birth, had longer telomeres. Maternal and paternal age were strongly correlated with each other (Pearson $R = 0.832$, $P = 7.8 \times 10^{-55}$) however, their individual relationships with telomere length weakened with strength and significance when whilst controlling for each other (maternal age, with adjustment for paternal age Pearson $R = 0.026$, $P = 0.706$; paternal age with adjustment for maternal age Pearson $R = 0.063$, $P = 0.4$). Body mass index was negatively correlated with telomere length, as previously noted by ANOVA, but again this was not a statistically significant observation.

Table 4.7 – Correlation matrix of telomere length with age, maternal age, paternal age and BMI at week 0

GROUP		TL week 0	AGE	MAT AGE	PAT AGE	BMI
TL at week 0	*R*	1	-0.059	0.128	0.151	-0.079
	P	-	0.390	0.063	**0.029**	0.253
	n	212	212	211	209	212
Age	*R*		1	0.104	0.130	0.246
	P	-	-	0.132	0.060	**<0.001**[a]
	n		212	211	209	199
Maternal age	*R*			1	0.832	-0.089
	P	-	-	-	**<0.0001**[b]	0.200
	n			211	209	211
Paternal age	*R*				1	-0.136
	P	-	-	-	-	**0.050**
	n				209	209
BMI	*R*					1
	P	-	-	-	-	-
	n					212

R, Pearson correlation coefficient

[a] *$P = 2.9 \times 10^{-4}$*

[b] *$P = 7.8 \times 10^{-55}$*

To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.005

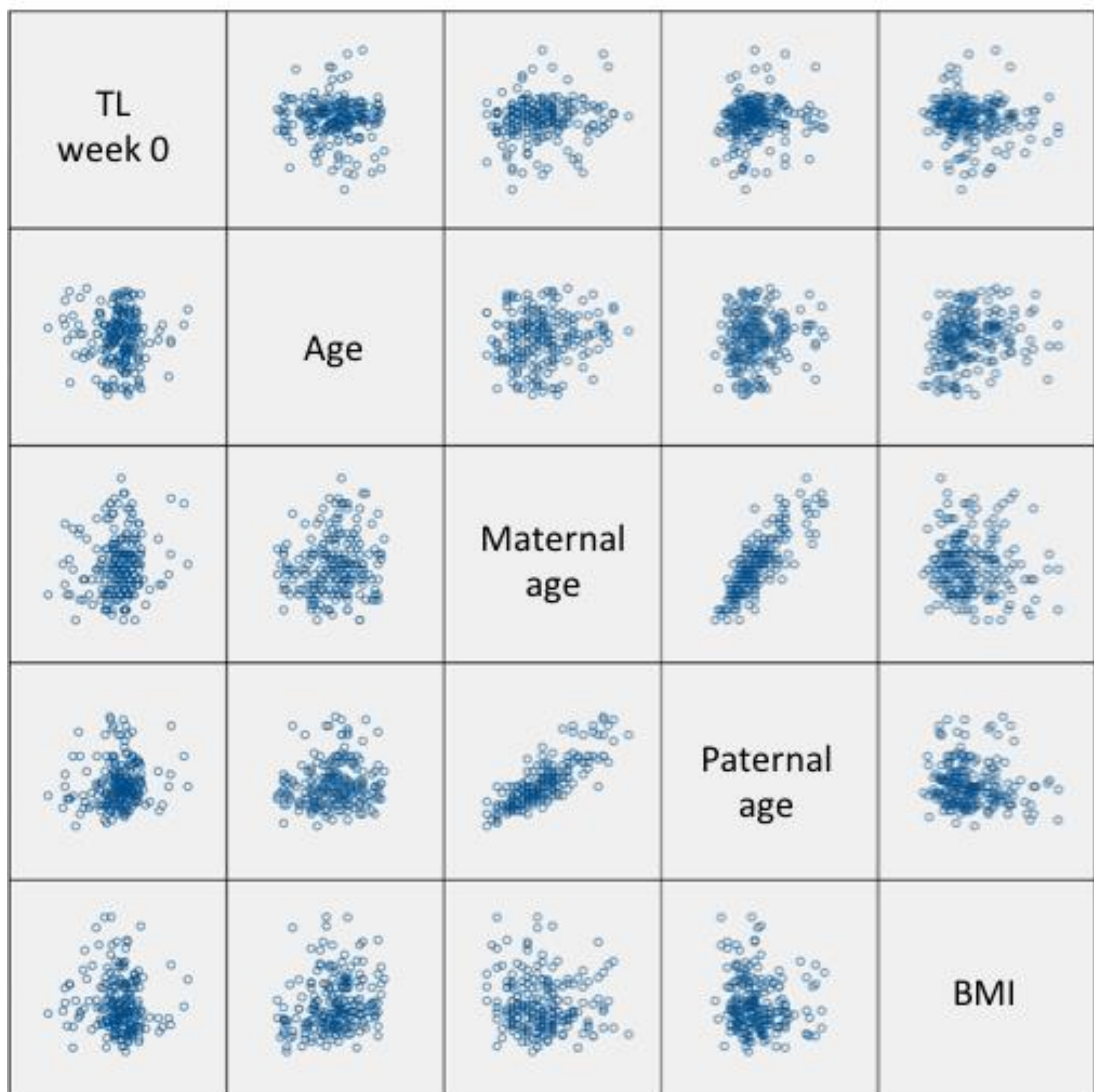

Figure 4.3 – Scatterplot matrix of bivariate correlation associations reported in Table 4.7

Abbreviations; BMI, body mass index; TL, telomere length

The graphical representation of age, BMI, maternal age and paternal age with telomere length (the top line of this scatterplot matrix) have been depicted also in Figure 4.4 with trendlines and a larger graph area.

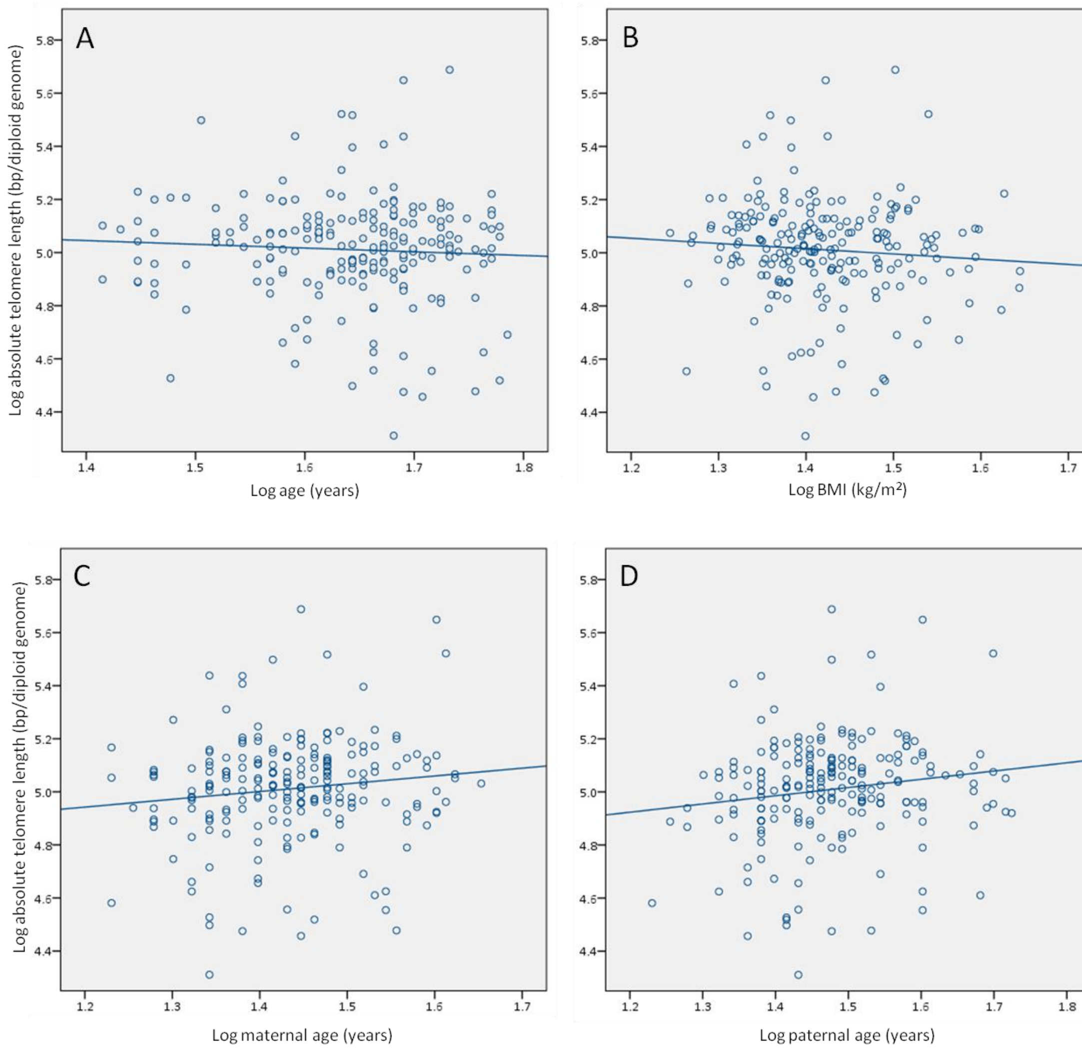

Figure 4.4 – Scatterplot of absolute telomere length against age, BMI, maternal age and paternal age at week 0

Absolute telomere length at baseline (log transformed) is graphed against A, Age (log transformed; R^2 = 0.004, P = 0.39, n = 212); B, BMI (log transformed; R^2 = 0.006, P = 0.25, n = 212) C, Maternal age (log transformed; R^2 = 0.016, P = 0.06, n = 211); and D, Paternal age (log transformed; R^2 = 0.023, P = 0.03, n = 209).

Next, bivariate correlation of telomere length with measured plasma micronutrients was performed (Table 4.8 and Figure 4.5). Bivariate correlation showed that homocysteine was negatively correlated with telomere length ($P = 0.004$; see also Figure 4.6), however this observation did not persist after the Bonferroni-correction for statistical significance in multiple correlation analyses was applied to the 0.05 alpha level ($P = 0.00076$). A number of micronutrients were positively and negatively associated with each other (Table 4.8).

Table 4.8 – Bivariate correlation matrix of telomere length with measured plasma micronutrient status at week 0

		TL week 0	FOL	HCY	B_{12}	αTOC	RET	NIA	Ca	Mg	Se	Zn	VITD
TL at week 0	*R*	1	0.061	-0.199	0.041	-0.016	-0.009	-0.018	0.030	0.073	-0.012	-0.033	0.118
	P	-	0.381	**0.004**	0.557	0.822	0.904	0.805	0.672	0.293	0.863	0.633	0.092
	n	207	206	206	206	198	198	198	207	207	207	207	206
Folate	*R*		1	-0.407	0.153	0.039	0.062	-0.087	0.123	0.173	0.123	0.197	0.039
	P	-	-	**<0.0001**	**0.028**	0.590	0.384	0.223	0.079	**0.013**	0.078	**0.005**	0.574
	n		206	206	206	198	198	198	206	206	206	206	206
Homocysteine	*R*			1	-0.258	0.096	0.264	-0.004	0.115	-0.035	0.134	0.002	0.013
	P	-	-	-	**0.0002**	0.178	**<0.0001**	0.961	0.101	0.616	0.056	0.978	0.857
	n			206	206	198	198	198	206	206	206	206	206
Vitamin B_{12}	*R*				1	0.143	0.016	-0.090	0.154	0.048	0.145	0.232	0.165
	P	-	-	-	-	**0.045**	0.819	0.207	**0.028**	0.495	**0.038**	**0.001**	**0.018**
	n				206	198	198	198	206	206	206	206	206
Alpha-tocopherol	*R*					1	0.426	-0.036	0.137	0.049	0.297	0.148	-0.052
	P	-	-	-	-	-	**<0.0001**	0.615	**0.054**	0.497	**<0.0001**	**0.038**	0.468
	n					198	198	198	198	198	198	198	198
Retinol	*R*						1	-0.057	0.254	0.035	0.347	0.182	0.136
	P	-	-	-	-	-	-	0.426	**0.0003**	0.621	**<0.0001**	**0.010**	0.057
	n						198	198	198	198	198	198	198

	R							1	-0.043	0.099	-0.172	0.024	-0.090
Niacin number	*P*	-	-	-	-	-	-	-	0.551	0.164	**0.015**	0.734	0.208
	n							198	198	198	198	198	198
	R								1	0.308	0.386	0.344	0.084
Calcium	*P*	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.232
	n								207	207	207	207	206
	R									1	0.162	0.264	0.072
Magnesium	*P*	-	-	-	-	-	-	-	-	-	0.020	**0.0001**	0.306
	n									207	207	207	206
	R										1	0.300	0.181
Selenium	*P*	-	-	-	-	-	-	-	-	-	-	**<0.0001**	**0.009**
	n										207	207	206
	R											1	0.017
Zinc	*P*	-	-	-	-	-	-	-	-	-	-	-	0.809
	n											207	206
	R												1
Vitamin D	*P*	-	-	-	-	-	-	-	-	-	-	-	-
	n												206

R, Pearson correlation coefficient

To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00076

n = 198 to 207 as five extreme cases excluded for these correlation analyses (original n = 212)

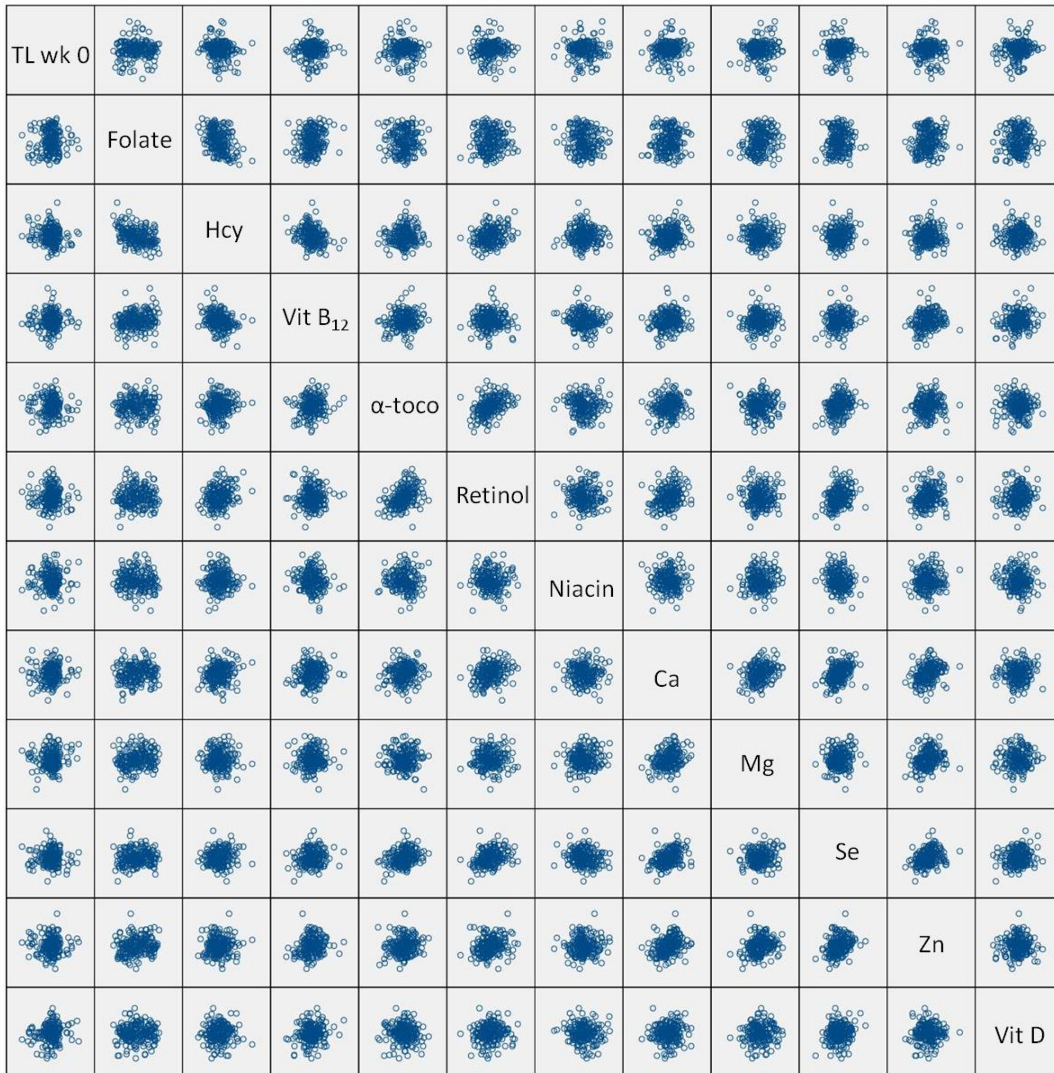

Figure 4.5 – Scatterplot matrix of bivariate correlation associations between telomere length and measured plasma micronutrients at week 0, as reported in Table 4.8

n ≥ 198 as five extreme cases excluded for these correlation analyses (original n = 212).
Abbreviations; α-toco; α-tocopherol; Ca, calcium; Hcy, homocysteine; Mg, magnesium; Se, selenium; TL, telomere length; vit, vitamin; Zn, zinc.

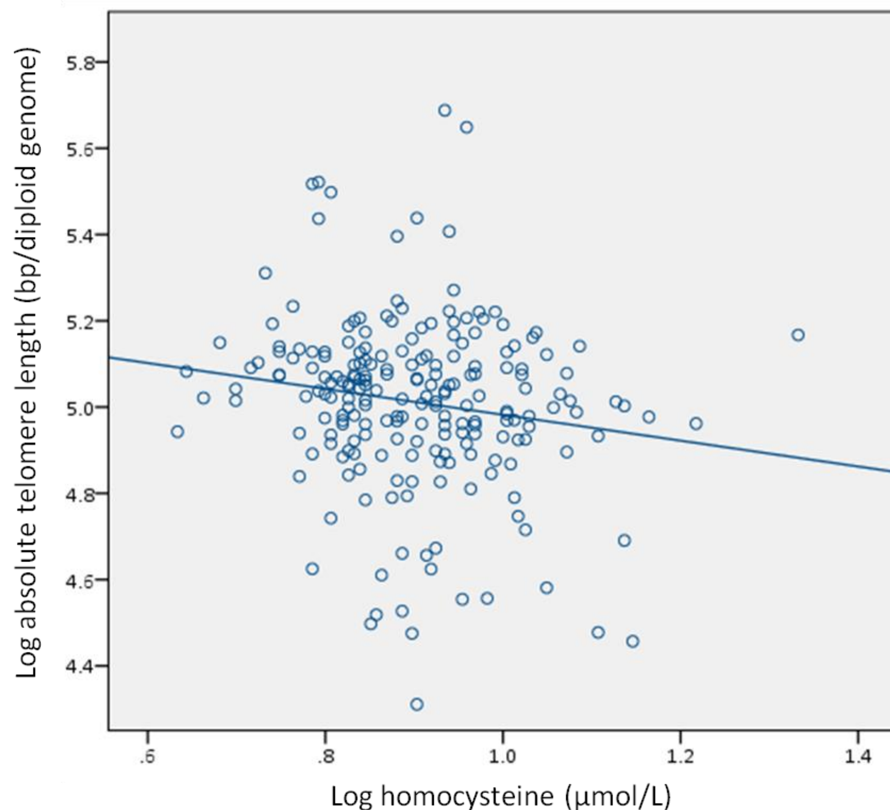

Figure 4.6 – Absolute telomere length against plasma homocysteine at week 0

Absolute telomere length at week 0 (log transformed) is graphed against plasma homocysteine at week 0 (log transformed; R^2 = 0.039, P = 0.004, n = 206)

Following from bivariate correlation analyses, partial correlation of each of the micronutrients with telomere length was performed. Table 4.9 shows partial correlation results where adjustment was made for participant age, gender, maternal age, paternal age and BMI. The negative correlation between plasma homocysteine and telomere length persisted (Pearson *R* = -0.154, *P* = 0.34), and a further significant positive association of plasma vitamin D with telomere length is returned (Pearson *R* = 0.174, *P* = 0.017), an observation which was not significant in bivariate correlation analysis. The bivariate correlation for the entire cohort was R^2 = 0.004 *(P* = 0.11, *n* = 211*)* and appeared to be modified by gender with correlation in females of R^2 = 0.0005, *(P* = 0.81*, n* = 124) and males R^2 = 0.145 (*P* <0.001, *n* = 87). These associations are graphed separately in Figure 4.7.

Table 4.9 – Partial correlation matrix of telomere length with measured plasma micronutrient status at week 0, with adjustment for participant age, gender, maternal age, paternal age and BMI

		TL week 0	FOL	HCY	B_{12}	αTOC	RET	NIA	Ca	Mg	Se	Zn	VITD
TL at week 0	*R*	1	0.041	-0.154	0.078	0.067	0.067	-0.057	0.063	0.091	0.072	-0.006	0.174
	P	-	0.575	**0.034**	0.286	0.362	0.357	0.437	0.385	0.214	0.326	0.937	**0.017**
	df	0	188	188	188	188	188	188	188	188	188	188	188
Folate	*R*		1	-0.448	0.179	0.099	0.045	-0.054	0.138	0.137	0.094	0.210	-0.087
	P	-	-	**<0.0001**	**0.014**	0.176	0.538	0.456	0.058	0.060	0.196	**0.004**	0.234
	df		0	188	188	188	188	188	188	188	188	188	188
Homocysteine	*R*			1	-0.374	-0.102	0.132	0.078	0.046	-0.093	0.007	-0.124	-0.046
	P	-	-	-	**<0.0001**	0.163	0.070	0.283	0.527	0.202	0.919	0.090	0.528
	df			0	188	188	188	188	188	188	188	188	188
Vitamin B_{12}	*R*				1	0.093	-0.053	-0.093	0.140	0.046	0.130	0.179	0.130
	P	-	-	-	-	0.201	0.465	0.204	0.055	0.532	0.074	**0.014**	0.074
	df				0	188	188	188	188	188	188	188	188
Alpha-tocopherol	*R*					1	0.407	0.029	0.149	-0.008	0.255	0.058	-0.083
	P	-	-	-	-	-	**<0.0001**	0.687	**0.040**	0.916	**0.0004**	0.426	0.257
	df					0	188	188	188	188	188	188	188
Retinol	*R*						1	0.015	0.182	-0.022	0.249	0.090	0.041
	P	-	-	-	-	-	-	0.835	**0.012**	0.759	**0.001**	0.218	0.577
	df						0	188	188	188	188	188	188

Niacin number	*R*							1	-0.025	0.163	-0.095	0.062	-0.033
	P	-	-	-	-	-	-	-	0.736	**0.025**	0.191	0.397	0.651
	df							0	188	188	188	188	188
Calcium	*R*								1	0.314	0.369	0.315	0.040
	P	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.588
	df								0	188	188	188	188
Magnesium	*R*									1	0.098	0.252	0.014
	P	-	-	-	-	-	-	-	-	-	0.180	**0.0005**	0.849
	df									0	188	188	188
Selenium	*R*										1	0.273	0.077
	P	-	-	-	-	-	-	-	-	-	-	**0.0001**	0.291
	df										0	188	188
Zinc	*R*											1	-0.058
	P	-	-	-	-	-	-	-	-	-	-	-	0.428
	df											0	188
Vitamin D	*R*												1
	P	-	-	-	-	-	-	-	-	-	-	-	-
	df												0

R, Pearson correlation coefficient

To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00076

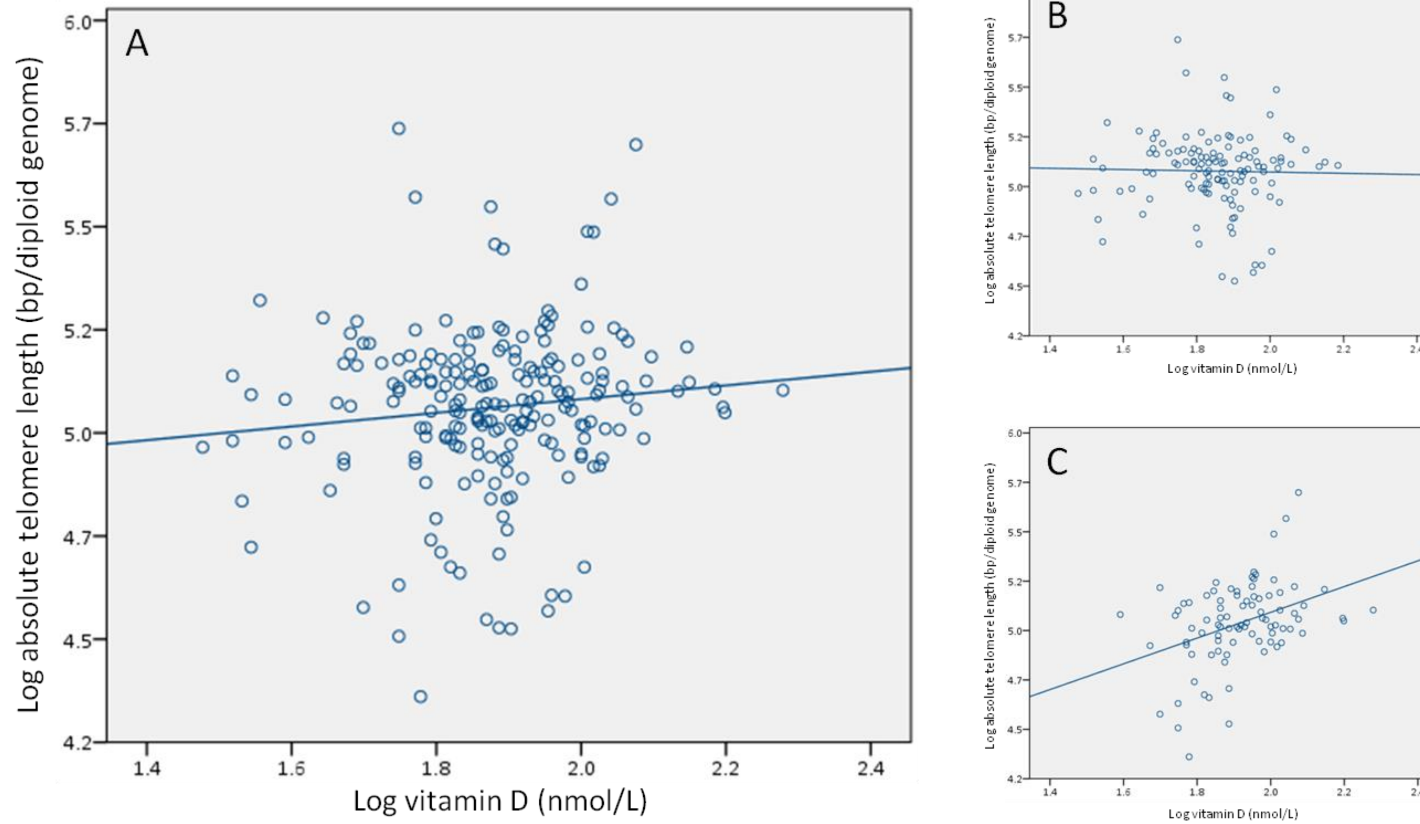

Figure 4.7 – Absolute telomere length against plasma vitamin D at week 0 for all participants, and individually for females and males

Absolute telomere length at baseline (log transformed) is graphed against A, plasma 25-OH-Vitamin D (log transformed; $R^2 = 0.004$, $P = 0.11$, $n = 211$)*; B, plasma 25-OH-Vitamin D in females (log transformed;* $R^2 = 0.0005$, $P = 0.81$, $n = 124$)*; C, plasma 25-OH-Vitamin D in males (log transformed;* $R^2 = 0.145$, $P < 0.001$, $n = 87$)

Next, linear regression was modelled with $n = 209$, the number of individuals with complete data for telomere length, all micronutrients and paternal age. Maternal age was excluded from this modelling in favour of paternal age (which was alone more strongly associated with telomere length than maternal age) as maternal and paternal age correlate too strongly with one another (Pearson $R = 0.83$, $P = 7.8 \times 10^{-55}$).

Initial modelling aimed to determine which demographic measures could explain the most variance in telomere length at baseline. The model incorporating age, gender, BMI, paternal age and previous supplement use explains 4% of the variance ($R^2 = 0.04$) in telomere length. The strongest predictor in this model was paternal age – individually accounting for 2% of the total 4% variance explained by the model ($P = 0.04$). There were three individual cases (1.4% of 209) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (98.6%).

Plasma micronutrients were modelled as independent variables to predict the dependent outcome variable, telomere length. This model contained all 11 measured micronutrients, which were found to collectively explain 6.2% of the variance observed in telomere length (Table 4.11). Plasma homocysteine significantly explained 3.0% of the variance in the model ($P = 0.014$) while vitamin D accounted for 1.7% of the explained variance in the model, although this did not reach statistical significance ($P = 0.067$). There were three individual cases (1.5% of 203) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (98.5%).

The incorporation of micronutrients to aforementioned model one (Table 4.10) did not significantly improve upon the fit of the model to the data overall; $R^2 = 0.042$ to 0.099 ($P = 0.12$ to $P = 0.23$), data not shown.

Table 4.10 – Multiple linear regression model one: demographic factors, BMI and previous supplement use as predictors of telomere length at week 0

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	4.91	0.39				
Age#	-0.13	0.17	-0.06	0.43	-0.05	0.29
Gender	0.04	0.03	0.10	0.14	0.10	1.06
BMI#	-0.11	0.12	-0.04	0.56	-0.04	0.17
Paternal age#	0.304	0.15	0.15	**0.037**	0.14	2.07
Previous supplement use	-0.01	0.03	-0.04	0.61	-0.04	0.12

 # log transformed variable

R^2 = 0.042, adjusted R^2 = 0.018, ANOVA P = 0.122

Model: $\text{log telomere length week } 0_i = b_0 + b_1\text{log age}_i + b_2\text{gender}_i + b_3\text{log BMI}_i + b_4\text{log paternal age}_i + b_5\text{previous supplement use}_i$

Model: $\text{log telomere length week } 16_i = 4.91 + (-0.13\text{log age}_i) + (0.04\text{gender}_i) + (-0.11\text{log BMI}_i) + (0.304\text{log paternal age}_i) + (-0.01\text{previous supplement use}_i)$

Table 4.11 – Multiple linear regression model two: measured plasma micronutrients as predictors of telomere length at week 0

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	4.71	0.66				
Folate	-0.001	0.00	-0.05	0.52	-0.05	0.20
Homocysteine	-0.40	0.16	-0.21	**0.014**	-0.17	3.03
Vitamin B_{12}	-0.04	0.09	-0.04	0.62	-0.04	0.12
α-tocopherol	-0.04	0.16	-0.02	0.82	-0.02	0.03
Retinol	0.12	0.16	0.06	0.47	0.05	0.26
Niacin number	-0.003	0.20	-0.001	0.99	-0.001	0.0001
Calcium	0.14	0.17	0.06	0.43	0.06	0.30
Magnesium	0.00	0.00	0.08	0.28	0.08	0.59
Selenium	-0.04	0.10	-0.03	0.68	-0.03	0.07
Zinc	-0.003	0.01	-0.03	0.73	-0.02	0.06
Vitamin D	0.20	0.11	0.14	0.067	0.13	1.66

R^2 = 0.062, adjusted R^2 = 0.008, ANOVA P = 0.320

Model: $log\ telomere\ length\ week\ 0_i = b_0 + b_1 Folate_i + b_2 Homocysteine_i + b_3 Vitamin\ B_{12i} + b_4 Alpha\text{-}tocopherol_i + b_5 Retinol_{i\ +}\ b_6 Niacin\ number_i + b_7 Calcium_i + b_8 Magnesium_i + b_9 Selenium_i + b_{10} Zinc_{i\ +}\ b_{11} Vitamin\ D_i$

Model: $log\ telomere\ length\ week\ 0_i = 4.71 + (-0.001 Folate_i) + (-0.40 Homocysteine_i) + (-0.04 Vitamin\ B_{12i}) + (-0.04 Alpha\text{-}tocopherol_i) + (0.12 Retinol_i) + (-0.003 Niacin\ number_i) + (0.14 Calcium_i) + (0.00 Magnesium_i) + (-0.04 Selenium_i) + (-0.003 Zinc_i) + (0.2 Vitamin\ D_i)$

4.4 Discussion

4.4.1 Recent supplementation use did not influence telomere length

As study recruitment was voluntary and participants responded positively to take part in the "*Polypill* to prevent genome damage study", it was of interest to assess previous use of dietary supplements. Self-reported supplement use data was generated from the personal questionnaire completed upon entry to the study. While 53% of the study cohort supplied no recent supplement use, 47% of the cohort responded to taking one or more supplements at frequencies ranging from sporadically to daily. This may not be a true representation of use as those currently taking dietary supplements may have been more likely to be engaged with the study, leading to a potential bias of previous use estimates in the population.

A study of 4862 individuals from the 1999 – 2000 United States National Health and Nutrition Examination Survey found that in the month leading up to participation, 52% of Americans took dietary supplements, while 35% took multivitamin/multimineral supplements (Radimer *et al.*, 2004). There was a higher prevalence of supplement use in women (56.7% took any dietary supplement; 38.0% took multivitamin/multimineral) than men (46.9% took any dietary supplement; 31.7% took multivitamin/multimineral) and an increasing prevalence of supplement and multivitamin/multimineral use with age (Radimer *et al.*, 2004). In this present sample, 46.7% of the cohort reported using any supplements while 21.2% of the cohort reported recent multivitamin/multimineral use (Table 4.3). These figures are below the respective 52% and 35% prevalence reported in US adults from the 1999 – 2000 NHANES cohort approximately 8 years prior to the current study period. There is a lack of recent data from Australia with which to compare this result, however reported studies from Adelaide dating back to 1984, 1993 and 2000 show the prevalence of using any supplement was 47%, 48.5% and 52%, respectively (Worsley and Crawford, 1984, MacLennan *et al.*, 2002).

Participants were asked to cease their dietary supplementation regimens in order to adequately assess baseline micronutrient levels from dietary sources only. There was a 16-week washout period prior to blood collection. Differences in demographic and anthropometric factors by use of dietary supplements were investigated and it was found that there were no differences in age, gender or BMI

between the two groups. However, there appeared to be a difference in the prevalence of obesity between the two groups in that there were less obese individuals among those who reported recent supplement use ($P = 0.078$). Although not significant, this difference likely reflects diet and health consciousness of current supplementers. There was no significant difference in any of the surrogate socioeconomic status measures between supplementers and non-supplementers.

A cross sectional study of American women reported that multivitamin use was significantly associated with 5% longer relative leukocyte telomere length, corresponding to approximately 9.8 years of age-related telomere loss in the sample (Xu *et al.*, 2009). In the current study there was no significant difference in mean telomere length between those who reported recent supplement use (117.6 ± 68.1 kb/diploid genome) and those who did not (109.9 ± 46.4 kb/diploid genome; $P = 0.51$).

At present, supplement use in Australia remains common, with an extensive range of supplements readily available in pharmacies, supermarkets, convenience, and health food stores. These dietary supplements and herbal medicines are classified as complementary medicines by the Australian Therapeutic Goods Administration (TGA) (Brownie, 2006). In 2000, the Australian national expenditure on alternative medicine, including dietary and herbal supplements was an estimated $1.7B and almost 4 times more than the patient contribution on pharmaceutical medications (MacLennan *et al.*, 2002). As of 15 March 2013, the TGA has started work on a series of reforms to complementary medicines which aim to improve community confidence in the safety and quality of these medicines (Australian Government Department of Health and Ageing Therapeutic Goods Administration, 2013). However, widespread use of supplements in Australia remains concerning as popular standard multivitamin/multiminerals provide ≥ 100% of the recommended daily allowance (RDA) of many micronutrients, and further there may be no requirement for supplemental micronutrients if this RDA is met through di*et al*one.

This present study was not designed to thoroughly investigate supplement use, type and frequency or duration of intake; indeed this study provides only an indication of previous recent supplement usage, which may have been open to interpretation by

responders. To best capture this information, participants could have been asked to bring their supplements to the clinic with them, in order to reduce discrepancy of misreporting, in particular of multivitamins which also often contain minerals and herbal extracts. This approach would further allow for the RDA of individual micronutrients to be calculated from supplemental sources, as the amount of individual micronutrients and other ingredients is provided on product labels as legislated. The indication of recent supplement use acquired in the current study may be too crude to identify the effect of dietary supplements, and indeed types of supplements or amounts of supplemental micronutrients, on telomere length.

4.4.2 Telomere length did not significantly differ with gender

On average, telomere length in females was measured to be 9 kb longer than telomere length in males, but this finding was not statistically significant ($P = 0.15$). The observation that females have longer telomeres than their male counterparts of the same age is not new (Benetos *et al.*, 2001, Cawthon *et al*., 2003, Nawrot *et al*., 2004) and whilst ageing is complex, it is thought that telomere length differences between the sexes may in part explain the increased longevity in women compared to men (Aviv *et al.*, 2005).

Telomere length among male and female newborns has been observed to be comparable and has led to the hypothesis that the gender difference in adult telomere length may be the result of slower attrition of sequence with ageing (Okuda *et al.*, 2002). Subsequently it was supported that males have shorter telomeres than females, but also that males had higher rates of telomere attrition than females (Mayer *et al.*, 2006). Although telomere length was heterogeneous across chromosome arms and there were distinct telomere erosion patterns, the arm-specific telomere lengths were similar between men and women, implying a shared mechanism may be responsible for interchromosomal variation of telomere length (Mayer *et al.*, 2006).

A similar relationship between female and male telomere length shown in the present study as a parallel decline with ageing, stratified by gender was recently reported (Ahola *et al.*, 2012). The decrease in telomere length with ageing in the present study was at 11.2 bp/diploid genome/y for males and 11.7 bp/diploid

genome/y for females; however the two studies could not be compared as Ahola *et al.* reported relative telomere length but did not report the respective regression equations. The Ahola *et al.* (2012) study of work related exhaustion and telomere length comprised 2911 individuals with 25% prevalence of smoking, 57% prevalence of somatic illness and 13% prevalence of mental illness and is therefore not directly comparable to the present study.

4.4.3 BMI and obesity did not significantly affect telomere length

The mean BMI of 26.8 kg/m^2 in this cohort is consistent with data from 2008 which showed the majority (> 60%) of males and females from Australasia were overweight with a BMI ≥ 25-30 kg/m^2 (Finucane *et al.*, 2011). Twenty seven percent of this current population was obese (BMI ≥ 30 kg/m^2), and this is also in agreement with the same 2011 study which observed an obesity prevalence in 2008 of 27% in Australasian men and women (Finucane *et al.*, 2011). This prevalence of obesity in Australia and this Australian cohort is lower than that reported for the United States in a more recent study of 2009 – 2010 data, which stated 36% of adults were obese (Flegal *et al.*, 2012). Although there were individuals included in the study with body weight at both extremes of the index (underweight and obese II and III) the great majority of cases (92%) were categorised as normal weight, overweight or obese I.

BMI was not significantly correlated with telomere length in this cohort however the association was inverse (R = -0.079, P = 0.25) as has been previously described (Al-Attas *et al.*, 2010, Lee *et al.*, 2011, Cui *et al.*, 2013). After dichotomising individuals as having a BMI above or below 30 kg/m^2, telomere length in obese and non-obese individuals was compared. However, there was no significant difference between the two groups with the mean telomere length in the obese population being 112.2 ± 68.3 kb, while the mean telomere length of non-obese individuals was 114.7 ± 55.3 kb (P = 0.51).

A recent meta-analysis of 97 published studies with reported BMI data sampled 2.88 million individuals with >270,000 cases of death. It was determined the lowest all-cause mortality compared to normal weight was observed for overweight, with a summary random effects hazard ratio (HR) of 0.94 (95% CI, 0.91 – 0.96) (Flegal *et*

al., 2013). Additional summary HRs were 0.95 (95% CI, 0.88 – 1.01) for obese I, and 1.29 (95% CI, 1.18 – 1.41) for obese II and III, with a combined HR of 1.18 (95% CI, 1.12 – 1.25) for all obesity. This study was limited in the geographical coverage, and in reporting all-cause mortality only, and not morbidity or cause-specific mortality (Flegal *et al.*, 2013). In addition, there are many recognised limitations of BMI. Categorisation of individuals in BMI categories is independent of gender, age and ethnic status which are known to influence body proportions. Additionally, BMI does not capture bone density, body fat mass, lean muscle mass, or the distribution of these body mass components. Notwithstanding, BMI has been reported consistently to be negatively associated with telomere length (Valdes *et al.*, 2005, Kim *et al.*, 2009, Lee *et al.*, 2011, Strandberg *et al.*, 2011), with suggestion that inflammation, oxidative stress could contribute to the accelerated telomere length attrition in these individuals (von Zglinicki, 2002, Aviv, 2004, Epel *et al.*, 2004, Valdes *et al.*, 2005).

4.4.4 Telomere length was not correlated with age

As a consequence of the DNA end-replication problem (Olovnikov, 1971, Watson, 1972, Olovnikov, 1973), telomere attrition occurs naturally during the ageing process (Lindsey *et al.*, 1991, Vaziri *et al.*, 1993). The relationship between age and telomere length in this study was determined by correlation analysis. Age was weakly, negatively correlated with telomere length, however this well-established association was non-significant in this population ($P = 0.39$). The negative relationship between age and telomere length has long been reported in the literature. A recent meta-analysis incorporating 124 cross-sectional studies estimated a decrease in telomere length with ageing at 24.7 bp/y by weighted linear regression (Muezzinler *et al.*, 2013). However, these studies included individuals from 0 – 104 years of age while the age in this study was narrower, comprising those aged 26 – 61 only. The overall estimated decrease in telomere length with ageing in this cross-sectional study was lower at 11.5 bp/diploid genome/y and was not balanced for gender (42% males).

4.4.5 Telomere length was associated with parental age

The effect of parental age on telomere length was probed individually for both maternal and paternal age. Maternal age was weakly correlated with telomere

length (Table 4.7), $R = 0.128$) however this association did not reach statistical significance. Paternal age was slightly more strongly correlated with telomere length ($R = 0.151$) and this observation, unlike for maternal age, was significant ($P = 0.029$). Maternal and paternal age were strongly correlated with each other ($R = 0.832$, $P = 7.8 \times 10^{-55}$) however their individual relationships with telomere length were not significant when controlling for the age of the other parent at birth. This suggests that the individual associations of telomere length with maternal or paternal age are surrogates for the combined effect of parental age on telomere length in the offspring. In this study, non-paternities could not be excluded and correlation between parental and offspring telomere length were not made as DNA was only sampled from offspring.

Many studies have reported this association for longer telomeres in offspring with increasing parental age at time of conception and later birth (Choi *et al.*, 2005a, Unryn *et al.*, 2005, De Meyer *et al.*, 2007, Kimura *et al.*, 2008). The mechanism behind this phenomenon is unknown; however it has been shown that telomere length in sperm is maintained (Allsopp *et al.*, 1992) or increases (Kimura *et al.*, 2008) with the age of the male. Additionally, the swim-up procedure applied in the selection of sperm for assisted reproductive technologies has been shown to select for sperm with longer than average telomere length (Santiso *et al.*, 2010). Taken together, these findings may suggest an evolutionary mechanism for selection of sperm with longer telomere length or some selection against sperm with shorter telomeres and/or older fathers who possess shorter telomere lengths. Evidence illustrating that infertile men have shorter telomeres than controls (Thilagavathi *et al.*, 2013) appears to lend early support to such a mechanism, however the involvement of telomere length and ageing on male fertility requires further investigation.

Telomere length in humans is known to be associated with methylation of the subtelomeric region of DNA immediately proximal to the TTAGGG repeats found at the chromosome ends. Separately, DNA methylation is known to decrease with ageing (Drinkwater *et al.*, 1989). As patterns of DNA methylation are recognised to have an element of heritability (Holliday, 1987), it might be that offspring of older parents inherit less methylated subtelomeric DNA and that this could be related to

longer telomere length in these individuals. There is insufficient information on whether subtelomeric methylation is associated with global DNA methylation. In one recent study, LINE-1, pericentromeric, and subtelomeric methylation were measured in patients with dyskeratosis congenita (DC; n = 40) and their mutation-negative relatives (n = 51). There were no detected differences in subtelomeric, LINE-1, or pericentromeric methylation between DC patients and relatives, although DC patients with a telomerase-complex mutation (*TERC*, *TERT*, *DKC1*, or *TCAB1*) were observed to have a greater extent of subtelomeric methylation (Gadalla *et al.*, 2012). Even though three types of methylation were measured, there was no reported association of these endpoints with each other in the publication. Following correspondence with the authors, there was no significant correlation between LINE-1 (a measure of methylation on a global genome scale) and subtelomere-specific methylation; the sample size was small and a possible positive correlation may exist in DC patients; $R = 0.22$, $P = 0.18$ for DC patients and $R = 0.05$, $P = 0.72$ for controls (Shahinaz Gadalla, personal correspondence, June 2013).

The effect of maternal and paternal age has been probed further, with individual assessment of mother-son, mother-daughter, father-son, and father-daughter intrafamilial pairings. Telomere length has been suggested to possibly be X-linked as there was no observed correlation between telomere length in father-son pairs, while significant positive relationships were observed in father-daughter, mother-daughter and mother-son pairs as well as for sister-sister, sister-brother and brother-brother pairings (Nawrot *et al.*, 2004). However another study exploring the same four intrafamilial pairings found no correlation of mother's telomere length with either son or daughter's, albeit in a small sample ($n \leq 23$ pairs for each observation) but significant correlations between father-son and father-daughter telomere length suggest paternal inheritance which is not solely linked to either the X or Y chromosome (Nordfjall *et al.*, 2005). Paternal inheritance of telomere length has been further identified and is postulated to be the result of genomic imprinting (Njajou *et al.*, 2007). A recent meta-analysis of telomere length heritability concluded heritability of included studies was on average 70%, with 95% confidence of heritability between 64 and 76% (Broer *et al.*, 2013). This study suggested stronger maternal effects on telomere length and emphasised the possible

mechanisms of imprinting and mitochondrial DNA (Broer *et al.*, 2013) which has sole maternal inheritance (Giles *et al.*, 1980).

While increased parental age is positively associated with telomere length in offspring and is associated with lifespan, there are no doubt other risks and considerations surrounding increased age at conception and birth. It has been suggested that older paternal age increases the risk of breast cancer in their female offspring, where women whose fathers were aged ≥ 40 y at birth had 1.6-fold increased risk of breast cancer compared to those with fathers aged < 30 y at their birth (Choi *et al.*, 2005a). A similar effect of increased risk of breast cancer in female offspring has been observed with increased maternal age at delivery (Xue *et al.*, 2007) however results from these studies appear to be inconsistent, and possibly affected or influenced by race (Hodgson *et al.*, 2004). In males, there has been an observed increased risk in prostate cancer with increasing paternal age, but not for maternal age (Zhang *et al.*, 1999).

Breast cancer risk has been shown to be increased with increasing telomere length in PBMC (Svenson *et al.*, 2008) but also increased independently with shorter telomere length (Shen *et al.*, 2009), or short chromosome-specific telomere length of 9p in lymphocytes (Zheng *et al.*, 2009b). Other studies have found no association of leukocyte telomere length with breast cancer risk in women (Zheng *et al.*, 2009a) or the risk of prostate cancer in men (Mirabello *et al.*, 2009). Thus the suggested possible increased risks of breast and prostate cancers with increased parental age may not be solely dependent on telomere length as the association of telomere length with these cancers is so far inconsistent.

4.4.5.1 Telomere length was associated with plasma biochemical measures

The mean levels of plasma micronutrients and homocysteine in this population was adequate and indicative of the South Australian population which has access broad and ready access to a range of foods. Although most cereals in Australia are fortified with some vitamins and minerals including folic acid – as well as other popular products such as Vegemite – blood samples were collected for micronutrient measurements at a time prior to mandatory folic acid fortification of flour in Australia. The 16-week washout phase post recruitment permitted for the assessment of plasma micronutrient levels in the population from diet sources

alone with the exception, perhaps, for some vitamins (e.g. folate, vitamin B_{12}) and homocysteine which may take several weeks to return to baseline following supplementation (Stites *et al.*, 1997, Brouwer *et al.*, 1999, Henning *et al.*, 2001). In order to investigate if dietary micronutrients are associated with telomere length in this population, pairwise bivariate correlation was conducted for telomere length and each measured vitamin and mineral.

4.4.5.2 Telomere length was negatively associated with homocysteine

Plasma homocysteine was observed to be weakly negatively associated with telomere length (Table 4.8; $R = -0.199$, $P = 0.004$). Partial correlation analysis was then performed to control for known confounders of telomere length which were captured in this study. As with bivariate correlation, partial correlation of homocysteine with telomere length was of a weak, negative nature ($R = -0.154$, $P = 0.034$). Although the Pearson correlation coefficient is devoid of indicating the direction of relationship or causality in two correlated variables, i.e. which variable is causing the change in the other variable, it is unlikely that telomere length could impact upon plasma levels of homocysteine and that the direction of the relationship instead sees homocysteine impacting upon telomere length.

This negative relationship between plasma homocysteine and telomere length has been observed previously in a number of cross-sectional cohort studies. Richards and colleagues observed the relationship in a cohort of 1200 who were predominantly female (> 90% females) (2008). The Pearson correlation coefficient between plasma homocysteine and leukocyte telomere length was -0.15 ($P < 0.0001$) in their study, a slightly weaker negative relationship than observed in the present study, but a statistically robust association. The mean and standard deviation of tertiles of homocysteine in that study were 6.0 ± 0.85 µmol/L, 8.1 ± 0.59 µmol/L and 11.8 ± 4.2 µmol/L. Homocysteine was analysed as a continuous variable in this study and so tertiles were not used, however were computed in order to compare the two studies. The mean and standard deviation of tertiles of homocysteine in this study were 6.3 ± 0.67 µmol/L, 8.0 ± 0.47 µmol/L and 10.6 ± 2.1 µmol/L. The third tertile of highest homocysteine is the largest difference between the two cohorts, with the Richards study having higher level in this tertile, perhaps due to a higher mean age and standard deviation of

participants (49 ± 12.5 y versus 45 ± 8.2 y) and as homocysteine is known to increase with ageing (El-Sammak *et al.*, 2004).

In the same year as Richards and colleagues, Salpea *et al*. correlated plasma homocysteine with telomere length in a case-control study of myocardial infarction, where cases were included if they experienced an event before the age of 55. The partial Pearson correlation coefficient associating homocysteine with telomere length was -0.15 ($P = 0.007$) for cases, -0.04 ($P = 0.40$) for controls and -0.10 ($P = 0.01$) for all study participants. The partial Pearson correlation included adjustment for age and region and further adjusted for case–control status when the two groups were pooled. Homocysteine levels between cases and controls were not significantly different, means of 10.15 µmol/L and 10.31 µmol/L, respectively (Salpea *et al.*, 2008a), however are higher than mean levels observed in the current study population (8.2 ± 2.2 µmol/L).

In a following study of 47 older males and females, there was a negative relationship between plasma homocysteine and telomere length in peripheral blood lymphocytes, however this did not reach statistical significance ($\beta = -0.37$, $0.05 \leq P \leq 0.10$) but in older males the relationship was significant ($\beta = -0.83$, $P < 0.005$, $n = 24$) (Bull *et al.*, 2009). The observations in this study are not consistent, varying in effect size and direction of effect for the different groups of all young participants, all old participants, older females only or older men only. The observation in the cohort ($n = 90$, but restricted to 86 in regression modelling) was weakly negative ($\beta = -0.03$) and not significant ($P > 0.1$). The Pearson correlation coefficient for PBL and homocysteine in older men was the only significant observation, and is notably stronger in effect than that observed in previous studies, and the present study ($R = -0.57$, $P = 0.004$, $n = 24$). The level of plasma homocysteine for the older men was 10.3 ± 2.36 µmol/L, which is much higher than that in the present study and closest to the level observed in the highest tertile of homocysteine in the Richards study population.

There were no age-adjusted associations between mean TRF values and total plasma homocysteine ($P > 0.25$) in a large cohort comprising 1218 men and 1291 women (Bekaert *et al.*, 2007). Although as levels of homocysteine are not reported, it is not possible to compare exposure of homocysteine in that population to the

current or other previous studies. A later study of 195 Italian men reported mean plasma homocysteine of 9.8 ± 5.4 µmol/L (Paul *et al.*, 2009). Men in the highest quartile of homocysteine (16.2 ± 7.6 µmol/L, *n* = 49) were found to have the longest telomeres, contrary to each of the studies which show decreased telomere length with increased homocysteine. This result was suggested to perhaps be explained by the inhibition of DNA methylation by a high concentration of S-adenosyl homocysteine (Paul *et al.*, 2009).

The most recent report in the literature, a cohort of 1715 women from the Nurses' Health Study showed that there was no significant differences in plasma homocysteine by quartiles of relative telomere length in peripheral blood lymphocytes measured by qPCR (Liu *et al.*, 2013a). Mean levels of homocysteine in the population were not supplied, and were reported instead by quartile of relative telomere length, where the lowest mean level was 11.0 ± 3.5 µmol/L and the highest was 12.0 ± 7.8 µmol/L, again observed to be greater than the highest tertile in the current and Richards *et al.* (2008) study.

That homocysteine levels in the populations differ and telomere length was measured in different cell types from blood and with various assays means that each of these studies cannot be directly compared. While these cross-sectional associations between homocysteine have been reported, there is no clear relationship between telomere length and plasma homocysteine, as present it appears there may be an effect of homocysteine on telomere length in blood cell subpopulations but that this effect may be subject to influence of other factors, including age, gender, genotype and other micronutrients, such as B_{12} and folate. As the relationship remains unclear, the mechanism by which homocysteine may impact telomere length is also undetermined. It has been shown *in vitro* that homocysteine accelerates cell senescence and this was postulated to be symptomatic of chronic oxidative stress which can lead to telomere attrition (Xu *et al.*, 2000).

Homocysteine is therefore thought to increase oxidative stress in the cell. As the telomere is high in guanine (25% of double stranded human telomere hexamer sequence is guanine), the telomere has been identified as a region which may be more prone to oxidative damage to guanine than other sequences (Henle *et al.*,

1999). It has also been shown that oxidative damage to the telomere is repaired with less efficiency that damage elsewhere in the genome (von Zglinicki, 2002), and this may explain the effect of increased levels of homocysteine correlating with a shorter telomere length. Additionally, cellular stress has been shown to cause telomere shortening in the absence of DNA replication by inducing a high frequency of double-strand breaks within the telomere (Bar-Or *et al.*, 2001, Oikawa *et al.*, 2001, von Zglinicki, 2002).

Richards *et al.* hypothesised two mechanisms through which homocysteine may negatively affect telomere length. They hypothesized elevated homocysteine accelerates telomere length attrition in leukocytes by increasing demand for proliferation of hematopoietic stem cells and that homocysteine mediates an increase in oxidative stress which results in greater telomere loss per cycle of DNA replication (Richards *et al.*, 2008). Homocysteine is known to promote proliferation of some cell types including vascular smooth muscle cells (Tsai *et al.*, 1994), mesangial cells (Yang and Zou, 2003), splenic B lymphocytes (Zhang *et al.*, 2001) and microglia (Zou *et al.*, 2010), however appears to inhibit proliferation of others such as fibrosarcoma cells (Chavarria *et al.,* 2003), hepatocytes (Yu *et al.*, 2013), and neural progenitor cells (Rabaneda *et al.*, 2008). The effect of homocysteine on proliferation of leukocytes and other peripheral blood mononuclear cells *in vivo* should hence be thoroughly explored.

Further discussion of the evidence and suggested mechanisms by which oxidative stress impacts telomere length has been extensively reviewed (von Zglinicki, 2000, von Zglinicki, 2002, Kawanishi and Oikawa, 2004). With suggestion that the mechanism of homocysteine mediating an increase in oxidative stress may explain observations of shorter telomere length in individuals with higher homocysteine levels there is need to control for factors known to affect oxidative stress, including smoking status or history and levels of plasma antioxidants. Subsequently, it is therefore difficult to compare results from the aforementioned cross-sectional associations in the literature as these data are not consistently captured or reported.

4.4.5.3 *Telomere length was positively associated with vitamin D*

In addition to a significant negative relationship with homocysteine, a weak, positive association with plasma 25-hydroxyvitamin D was observed ($R = 0.118$, $P = 0.092$). This relationship has been previously alluded to by Richards *et al.* (2007) where in 2160 women aged 18-79 y (mean age of 49.4 y), there was a positive correlation between serum 25-hydroxyvitamin D and leukocyte telomere length ($R = 0.07$, $P = 0.0010$). In this cohort with one tenth of the participants, this positive correlation was also observed. The bivariate correlation analysis was repeated individually for males and females to tease out any difference there may be between the sexes, and because the Richards *et al.* (2007) cohort consisted entirely of women. In this repeated correlation analysis with stratification based on sex, it appeared that the relationship was being driven by the effect observed in males ($R = 0.381$, $P = 0.003$) since there was no correlation observed in females alone ($R = -0.022$, $P = 0.81$).

Correlation of vitamin D with telomere length was weakly positive, and significant with adjustment for the aforementioned confounders, including gender ($R = 0.174$, $P = 0.017$). Significant positive association of serum 25-hydroxyvitamin D with leukocyte telomere length ($R = 0.09$, $P < 0.0001$) has been shown to persist after adjustment for age, season of vitamin D measurement, menopausal status, use of hormone replacement therapy, and physical activity in a much larger study of 2160 women (Richards *et al.*, 2007). To compare levels of vitamin D between these two studies, tertiles of plasma vitamin D were computed for the present study; 56.0 ± 10.5 nmol/L, 76.9 ± 4.3 nmol/L and 105.6 ± 19.6 nmol/L compared to 40.9 ± 11.0 nmol/L, 72.7 ± 9.0 nmol/L and 124 ± 37.3 nmol/L for the Richards cohort (2007).

Recently the intake of calcium was shown to significantly modify the association between plasma 25-hydroxyvitamin D and leukocyte telomere length in 1424 women for the Nurses' Health Study whereby the association between increased 25-hydroxyvitamin D and longer telomere length was stronger for persons with lower calcium intakes (Liu *et al.*, 2013b). In these women, plasma 25-hydroxyvitamin D was positively associated with leukocyte telomere length, supporting the previous cross-sectional studies and current results observed in

PBMCs. As total daily intakes of folate and retinol, in addition to calcium as measured by dietary and supplement questionnaire, were observed to increase by quartiles of plasma 25-hydroxyvitamin D concentration ($P < 0.0001$ for all trends; Liu *et al.*, 2013b) it is plausible these micronutrients modify or contribute to the positive association of vitamin D with telomere length. SNPs located in vitamin D-related genes including *GC*, *VDR*, *DHCR7* were not associated with telomere length ($n = 480$) (Liu *et al.*, 2013b).

Vitamin D can be produced in the skin or absorbed in the gut from dietary sources. In the liver, vitamin D is hydroxylated to 25-OH vitamin D, the major vitamin D metabolite in the serum (Heldenberg *et al.*, 1992)., and the metabolite which was measured in this study. In the kidney, this metabolite, 25-OH vitamin D, can be subsequently hydroxylated to either 1,25-dihydroxyvitamin D or 24,25-dihydroxyvitamin D3. *In vitro* 1,25(OH)2 vitamin D3 has been shown to protect cells from UV-B radiation (Tremezaygues *et al.*, 2009) and low dose ionising radiation (Tremezaygues *et al.*, 2010). A metabolite of vitamin D, 1alpha,25-dihydroxy-vitamin D3, has been demonstrated to be an inhibitor of polyadenosine diphosphate ADP-ribose (PARP) and this action is suggested as the mechanism by which vitamin D exerts anti-inflammatory effects (Mabley *et al.*, 2007). PARP-1 is the most abundant isoform of the PARP enzyme family (Pacher and Szabo, 2007) and its action is modulated by vitamin D (Szabo *et al.*, 2006, Mabley *et al.*, 2007). The modulation of PARP by vitamin D as well as further evidence of vitamin D otherwise preventing DNA damage has been recently reviewed (Nair-Shalliker *et al.*, 2012).

Inflammation can initiate proliferation of T-cells, which is one mechanism by which inflammation can cause telomere shortening (Aviv, 2004, Gardner *et al.*, 2005, Carrero *et al.*, 2008). Of course oxidative stress and inflammation are intricately linked; oxidative stress provokes inflammation and vice versa (Richards *et al.*, 2008). Oxidative stress can stimulate the synthesis of pro-inflammatory cytokines (Aviv, 2006, Lipcsey *et al.*, 2008, Kiecolt-Glaser *et al.*, 2013). Oxidative stress has been shown to correspond with a reduction in telomerase activity (Borras *et al.*, 2004, Kurz *et al.*, 2004), however the mechanism for this remains to be explained.

Double-blind supplementation of 60 000 IU vitamin D3 monthly (equivalent to ≈2000 IU per day) for four months was shown to significantly increase serum 25-OH vitamin D levels from 40.7 ± 15.7 nmol/L to 48.1 ± 17.5 nmol/L in the active group (Zhu *et al.*, 2012). There were 19 individuals in the vitamin D3 treatment group, aged 31.1 ± 10.0 y; telomerase activity in PBMC of these volunteers increased by 19.2% from baseline levels (Zhu *et al.*, 2012). Nevertheless, vitamin D is not the only dietary micronutrient which may enhance telomerase activity. In a small pilot study of intensive three-month lifestyle change including omega-3 polyunsaturated fatty acids supplementation, there was a significant increase in telomerase activity of men with prostate cancer (Ornish *et al.*, 2008). In addition, the omega-3 polyunsaturated fatty acids are known to reduce inflammation and decrease oxidative stress (Mori, 2004, Calder, 2006) and it is thought these mechanisms explain the positive association of omega 3 polyunsaturated fatty acids with telomere length (Ornish *et al.*, 2008, Farzaneh-Far *et al.*, 2010b, Kiecolt-Glaser *et al.*, 2013). Changes in telomere length were not reported to be measured in these publications (Ornish *et al.*, 2008, Zhu *et al.*, 2012), but as PBMC telomerase activity has been significantly and independently associated positively with leukocyte telomere length (Boccardi *et al.*, 2013), it is plausible that interventions which increase telomerase activity may consequently stimulate changes in telomere length.

4.4.5.4 Appropriateness of plasma micronutrients as biomarkers of exposure

Semi-quantitative FFQ have strengths over other methods of nutrient and food intake reporting in that there is a low burden placed upon respondents, unlike weighed food records and can be completed by the individual with no requirement for an interviewer, as required for 24 hour recall (Wrieden *et al.*, 2003). This self-completion among other qualities make the FFQ method for dietary assessment suitable for large studies however there is risk of both observation and reporting effects, where observation effect describes to the change in eating behaviour which occurs when individuals are required to report their food intake, and the report effect relates to misreporting of food intake (Wrieden *et al.*, 2003). Both the observation effect and reporting effect phenomena may have confounded the FFQ data in this study. As such, it was affirmed that plasma micronutrients would be

used solely in determining the associations of micronutrients on telomere length. That said, there may be benefit to investigating the associations of dietary patterns or foods on telomere length as previous studies have found processed meat consumption (Nettleton *et al.*, 2008), increased alcohol intake (Pavanello *et al.*, 2011), low fruit and vegetable consumption and high meat consumption (Diaz *et al.*, 2010) are negatively associated with telomere length.

Telomere length and plasma micronutrients were each measured once only from the same sample of blood, separated into PBMC and blood plasma components. As such the micronutrient levels at this time may hence not represent biological exposure to the micronutrient or dietary intake due to confounding of nutrient biomarkers (Giovannucci, 2013). Although these biomarkers are useful in piecing together relation to disease, nutrient biomarkers can also be subject to confounding, with variations in concentrations of biomarkers known to reflect other exposures such as alcohol, smoking, and/or metabolic, genetic, physiologic, or pathophysiologic processes (Giovannucci, 2013). Additionally, it should be noted that plasma micronutrient sufficiency might not exclude a functional deficiency in some individuals (Vugteveen *et al.*, 2011).

4.4.6 Additional covariates of telomere length identified in recent literature were unmeasured

These correlation analyses are restricted in that correlation does not necessarily indicate causation, and there may be one or more additional factors which may account for the correlation result. It may be that either vitamin D or homocysteine are impacted by an additional variable which is responsible for the significant association with telomere length. For example, in this study there were no measures of iron status in red blood cells (Hb) or in plasma (ferritin) however, it has been shown that elevated iron is associated with shortened telomeres (Mainous *et al.*, 2013). Excessive iron can lead to DNA strand breaks, DNA hypermethylation and reduction in telomere length (Pra *et al.*, 2012). Iron storage protein ferritin is suggested to catalyse folate turnover and be important in regulating the concentration of intracellular folate where it is observed that high intracellular ferritin corresponds with low folate availability (Suh *et al.*, 2000). As such, it appears ferritin-mediated folate catabolism can result in higher homocysteine by

minimizing the availability of one-carbon units for re-methylation (Suh *et al.*, 2000, Sullivan, 2006). In addition, iron deficiency has been shown to affect vitamin A absorption and it is also possible that iron deficiency may impair vitamin D absorption (Heldenberg *et al.*, 1992) though this finding complicates the relationship of micronutrients differentially associated with telomere length. Nutrient-nutrient interactions in this study were not explored past bivariate and partial correlation analysis of measured plasma micronutrients.

Indeed there are also nutrient–gene interactions which were also not examined in the present study due to time and other constraints. Relative telomere length was recently investigated for association with a number of one-carbon metabolism SNPs in 475 American Caucasian women from an endometrial cancer case-control set within the large Nurses' Health Study (Prescott *et al.*, 2010, Liu *et al.*, 2013a). Liu *et al.* (2013a) conducted genotyping of 29 non-synonymous SNPs across 16 genes – *ALDH1L1*, *ATIC*, *BHMT*, *CHDH*, *CTH*, *DNMT1*, *FOLH1*, *GART*, *GGH*, *MTHFD1*, *MTHFR*, *MTR*, *MTRR*, *PEMT*, *SHMT1*, and *SLC19A1* – involved in one-carbon metabolism protein functionality, or choline metabolism (Zeisel, 2008, Carr *et al.*, 2009). Logistic regression showed a significant association of relative telomere length with the rs2372536 SNP (*ATIC* gene involved in the *de novo* purine biosynthetic pathway, $P = 0.02$), however this association did not remain statistically significant when taking into account the false-discovery rate of SNP analyses (Liu *et al.*, 2013a). Although genotyping for these one-carbon and choline metabolism genes was not conducted in the present study, it is unlikely that any significant association would be observed in this smaller cohort of 212, as no robust associations were found between 29 SNPs and telomere length in a cohort more than twice the size of the present ($n = 475$). Other constituents of the one-carbon metabolism pathway explored in the Liu *et al*. (2013a) study, such as vitamin B_6, were not measured in plasma samples from the *Polypill* study due to the volume of the blood plasma collected and economical constraints. In future studies, it would be most valuable to measure B_6 in addition to other B-vitamins (folate, B_{12}) as B_6 intake estimated from FFQ has been shown to be positively associated with telomere length (Xu *et al.*, 2009).

Outside of nutrient metabolising enzymes, there are a number of genes which are known to be involved in telomere structure and function, including *TERC*, *TERT*, *NAF1*, *OBFC1* and *RTEL1* which were recently associated with mean telomere length (Codd *et al.*, 2013). These and other genes such as those encoding telosome complex proteins (*TRF1*, *TRF2*, *TIN2*, *Rap1*, *TPP1*, and *POT1*; de Lange, 2005), those which are otherwise involved in telomere homeostasis (*UP1*, *TNKS1BP1*, *EST1*, *EST2*, and *EST3*; Codd *et al.*, 2013) and others which encode non-telosome telomere binding proteins (*YKU70*, *SIR4*, and *RIF2*; Saldanha *et al.*, 2003) may impact upon telomere length, through their roles in telomere homeostasis and related biology.

Education and other direct personal-level measures which may indicate socio-economic status were not captured in this population. The SEIFA scores were used as surrogates for socio-economic status of the individuals in the cohort, however these scores represent the average of people and households within a particular postcode and are hence not individual level data. The four various SEIFA measurements allow us to make various inferences about the population, for example the index of economic resources (IER) could indicate financial security and the access to money for a diverse, healthy diet or use of dietary supplements while the index of education and occupation (IEO) may infer knowledge of healthy diet and lifestyle behaviours reflecting overall health consciousness. That said, personal-level data such as income, home ownership, profession and education attainment would be more appropriate, however this data was not captured and estimates on socio-economic status of individuals was derived purely from postcode of residence. Personal level SES and health behaviour data has been associated with telomere length in a large sample (n = 5360) (Needham *et al.*, 2013), however the overall association between various SES measures and biological ageing as measured by telomere length is generally weak and inconsistent (Robertson *et al.*, 2012).

Further indicators of health and wellbeing which were not measured, but have been associated with telomere length and hypothesised to affect cellular ageing include intimate partner violence or abuse (Humphreys *et al.*, 2012), trauma during childhood trauma (O'Donovan *et al.*, 2011), depression (Hoen *et al.*, 2011), life stress (Epel *et al.*, 2004) and psychological stress (Epel, 2009). Moreover, maternal psychosocial stress during pregnancy has been associated with telomere length in

newborns perhaps through exerting a programming effect on the developing telomere biology system *in utero* (Entringer *et al.*, 2013). Whilst not available data to consider at present, it is compelling that telomere biology may be impacted upon *in utero*, and as telomere length is certainly subject to inheritance, one may need to understand the influence of previous generations to determine health or stability of telomere length and other biology.

Longer telomeres have been noted in women who engage in moderate or vigorous activity for 2-4 h/wk (Du *et al.*, 2012), a finding which agrees with previous studies linking activity to longer telomeres (Cherkas *et al.*, 2008, Ludlow *et al.*, 2008, Zhu *et al.*, 2011). However, some studies have reported null associations between activity or exercise and telomere length (Woo *et al.*, 2008, Mirabello *et al.*, 2009, Cassidy *et al.*, 2010). Differences in these findings may be due to inconsistencies in assessment of physical activity, adjustment for covariates, study populations and sample sizes.

4.4.7 There is evidence of telomere length heterogeneity across different chromosomes, chromosome arms, and tissue types

Mean leukocyte telomere length is used in many studies as the cells are easily collected from a venous blood sample, and can be readily extracted from whole blood. Although telomere length is known to be heterogeneous across tissue types in the body (Friedrich *et al.*, 2000), leukocyte telomere length is regarded as an appropriate surrogate tissue as it is highly correlated with telomere length in other tissues and is easily accessible (Friedrich *et al.*, 2000, Wilson *et al.*, 2008). However, the large inter-individual variation of telomere length (Hastie *et al.*, 1990, Frenck *et al.*, 1998, Iwama *et al.*, 1998), including that of peripheral blood cells and leukocytes, requires that cross-sectional study cohorts consist of large study numbers to detect the modest effects on telomere length (Richards *et al.*, 2008). Human population variation in telomere length is not well understood, and is recognised to be simultaneously impacted by genetic, epigenetic and environmental factors. Considerable inter-individual variation in telomere length has been previously detected and the reasons explored (Aviv *et al.*, 2006). Moreover, evidence of geographical diversity of telomere length was demonstrated in

European populations (Salpea *et al.*, 2008b, Eisenberg *et al.*, 2011) and is yet to be explored for Australian populations.

Presently, there are few studies that correlate telomere length with biomarkers of DNA damage and chromosomal instability in humans. However, telomere length measured by TRF has been correlated with micronuclei in a study of patients with chronic B viral cirrhosis such that the number of micronuclei were significantly inversely correlated with telomere length; $R = -0.227$, $P = 0.016$ (Lee *et al.*, 2009b). In a separate study investigating polycyclic aromatic hydrocarbon exposure, study subjects with shorter telomere length – as measured by qPCR – had significantly higher levels of micronuclei (Pavanello *et al.*, 2010). The authors suggest this result reflects a role for telomere erosion in the formation of micronuclei (Pavanello *et al.*, 2010).

The human genome contains 23 pairs of chromosomes with 92 telomeres with heterogeneity of telomere length observed across chromosomes and chromosome arms. The longest telomere has been observed at 4q (Mayer *et al.*, 2006) and the shortest telomere has been reported at 17p (Martens *et al.*, 2000), however in at least one other study, the shortest telomere was observed at 19p (Mayer *et al.*, 2006). Cellular senescence may be the result of a critical telomere loss to just one or a few chromosomes (Harley, 1991). The chromosome 17p telomere is noted as the critical telomere in humans because of its comparatively short telomeric sequence (Martens *et al.*, 1998). This means that methods which can detect short telomeres in a cell (Vera and Blasco, 2012) may be more insightful than telomere length measured by qPCR methods which yield a global absolute or average telomere length and do not supply any information about arm specific telomere length. Other methods such as fluorescent *in situ* hybridisation permit the use of chromosome probes which would facilitate the determination of local, arm-specific telomere length.

4.5 Conclusions

4.5.1 Significance

This is the first study to report telomere length measured by qPCR in middle-aged South Australians and to associate telomere length in this population with demographic and biochemical nutritional data. The key significant findings in this current study support previous observations in the scientific literature that telomere length is influenced by parental age, and is associated positively with plasma vitamin D and negatively with plasma homocysteine. However, the observation that the relationship between plasma vitamin D and PBMC telomere length may be modified by gender has not previously been reported (males, $R = 0.381$, $P = 0.003$; females, $R = -0.022$, $P = 0.81$) and should be investigated further.

4.5.2 Strengths and weaknesses

This study is strengthened by the use of biological markers of micronutrient status, as opposed to FFQ data which is cheaper and easier to obtain but valuable depending on the subject reliably completing the FFQ. A large number of covariates investigated for their association with telomere length, and as such were adjusted for confounding in appropriate statistical analyses. Nonetheless, there are a number of covariates which have been associated with telomere length that were not measured in this cohort. These include physical activity (Cherkas *et al.*, 2008), ethnicity (Diez Roux *et al.*, 2009) and sleep quality (Prather *et al.*, 2011), as well as other dietary factors such as polyunsaturated and saturated fatty acids (Cassidy *et al.*, 2010, Farzaneh-Far *et al.*, 2010b, Kang, 2010, Kiecolt-Glaser *et al.*, 2013) and iron (Mainous *et al.*, 2013) to name a few.

Through the recruitment process, individuals receiving medical treatment for life-threatening diseases at the time of recruitment or in the immediate past six months were excluded from participating in the study. As such, those who remained eligible for the study were assumed to be healthy, however the absence of life-threatening disease does not necessarily indicate health, which cannot easily be quantified or measured. Therefore, it cannot be concluded that our cohort was free of chronic disease including diabetes. As measured by BMI, the prevalence of obesity in this cohort was 27% however abdominal obesity – one of five indicators

of metabolic syndrome (US National Institutes of Health, 2011) – was not specifically measured. Indeed there were no indicators of metabolic disorder or inflammation as there was no measure of blood pressure, blood sugar, triglycerides, HDL/LDL profile, haemoglobin A1c, CRP, interleukin-6 or TNFα. Biomarkers of obesity and insulin resistance have been associated with telomere length (Al-Attas *et al.*, 2010).

Finally, telomere length was the sole aspect of telomere biology measured, which does not give any indication of telomere function, sequence integrity or other factors known to affect telomere length such as subtelomeric methylation status, telomerase expression, abundance and activity, or the alternative lengthening of telomeres mechanism (Blackburn *et al.*, 1989, Bryan *et al.*, 1997, Lee *et al.*, 2009a, Osterhage and Friedman, 2009, Conomos *et al.*, 2013).

4.5.3 Future studies

While the scientific literature on telomere biology is rapidly expanding, there are still questions about telomere length as biomarker, specifically whether it is an appropriate biomarker of ageing and risk of age-related disease (Mather *et al.*, 2011, Der *et al.*, 2012, Boonekamp *et al.*, 2013, Sanders and Newman, 2013).

In future studies it would be beneficial to determine a suggested level or specified range of intake for dietary and supplemental micronutrients to provide optimum telomere length and function. It may be fruitful to approach this in the same way as combined nutrigenomics and nutrigenetics applications to minimise genome damage at an individual level (Fenech, 2008).

The accumulation of senescent cells and ensuing vascular ageing is postulated to be influenced by various factors including oxidative stress, activation of inflammatory pathways, impairment of the NO pathway as well as cellular telomere length and telomerase activity (Marin *et al.*, 2013) however these mechanisms are associated with one another and their order of action remains to be elucidated.

As a final point, it may be more insightful to investigate changes in telomere length and function over time in a longitudinal cohort instead of probing cross-sectional associations which are known to be impacted by a range of other confounders, effect modifiers and reverse causation.

5 The impact of a micronutrient *Polypill* on telomere length *in vivo* over time

5.1 Introduction

There exists strong evidence for heritability of telomere length (Broer *et al.*, 2013), however it also appears that telomere length can be affected by lifestyle factors including diet and as such is potentially modifiable. Cross-sectional associations have detailed the positive correlation of telomere length with diet components or behaviours including the Mediterranean diet (Boccardi *et al.*, 2013), fruit and vegetable intake (Marcon *et al.*, 2012), Chinese tea (Chan *et al.*, 2010), fruits, berries and juice (Tiainen *et al.*, 2012), fibre and cereal fibre (Cassidy *et al.*, 2010) and with dietary intake of vitamins A, C, E, B_9 (folate) and β-carotene as measured by FFQ (Marcon *et al.*, 2012). Conversely, processed meat consumption (Nettleton *et al.*, 2008), cooking fats and oils (Chan *et al.*, 2010) and intakes of total fat, saturated fatty acid and butter (Tiainen *et al.*, 2012) have been negatively associated with telomere length. That a generally healthy lifestyle –as defined by five factors including diet – was associated with longer leukocyte telomere length in 5863 women (Sun *et al.*, 2012) there is some evidence to suggest that lifestyle modifications may impact telomere length.

5.1.1 Intervention studies or RCTs and telomere length

Considering the limitations of cross-sectional studies, intervention studies and randomised controlled trials (RCTs) are more appropriate to study the effects of diet on telomere length. As yet, there are few longitudinal studies or RCTs which assess the impact of diet and/or micronutrient status on telomere length.

A short term RCT involving four monthly doses of 60000 IU vitamin D3 (equivalent to ≈2000 IU per day) resulted in a 19% increase in PBMC telomerase activity over the 16-week intervention period (Zhu *et al.*, 2012). An additional holistic 3-month lifestyle intervention which included a modified diet with restrictions on fat (10% energy), and emphasis on a wholefood, plant-based diet high in fruits, vegetables, unrefined grains, legumes, and low in refined carbohydrates, daily supplementation with soy (58 g tofu and powdered soy protein beverage), fish oil (3 g), vitamin E,

(100 IU), selenium (200 µg), and vitamin C (2 g), as well as exercise and stress management 6 days a week also elicited an increase in PBMC telomerase expression, in addition to improvements in LDL and psychological stress (Ornish *et al.*, 2008). As there were multiple components of this lifestyle intervention, it is unknown which has the strongest individual impact upon telomerase, or if the complete lifestyle overhaul as a routine was responsible and required for the increased telomerase expression.

Modifying diet to achieve weight loss in obese men through 30% calorie restricted (7000 kJ) high-protein or high-carbohydrate diets was shown to increase telomere length in rectal mucosa 4-fold after 12 weeks ($n = 54$), and 10-fold after 52 weeks ($n = 12$) on the diet, when compared to baseline telomere length before any weight loss (O'Callaghan *et al.*, 2009). Furthermore this increase in telomere length was correlated significantly with change in weight and change in both percentage body fat and abdominal fat (O'Callaghan *et al.*, 2009).

In the Finnish Diabetes Prevention Study, 150 individuals (cases) received tailored dietary advice aimed at reducing weight and intakes of total and saturated fats and increasing the intake of dietary fibre, as well as one-on-one guidance and encouragement to increase physical activity, while 161 controls received less advice and support (Hovatta *et al.*, 2012). Telomere length was measured at two time points about 4.5 y apart (Table 5.1). This intervention appeared to have no effect on telomere length, nor change in the prevalence of diabetes in the treated group.

In a recent RCT of omega-3 PUFA supplementation, active treatment was shown to lower the concentration of pro-inflammatory cytokines in serum (Kiecolt-Glaser et al., 2012). Later in this cohort, leukocyte telomere length, telomerase and oxidative stress were measured to assess whether the supplementation affected these measures in the healthy middle-aged and older adults (Kiecolt-Glaser *et al.*, 2013). Between groups, there were non-significant changes in both telomerase activity and telomere length, however there was an inverse relationship between PBL telomere length and plasma omega-6: omega-3 ratios whereby telomere length increased with decreasing *n*-6: *n*-3 PUFA ratios ($n = 106$) (Kiecolt-Glaser *et al.*, 2013). F2-isoprostanes were measured as a biomarker of oxidative stress in plasma

samples, and were lowest in those supplemented with 1.25 g/day *n*-3 fatty acids compared to 2.5 g/day or the placebo control.

5.1.2 Longitudinal studies of telomere length

Recently, in a meta-analysis of 124 cross-sectional studies, an estimated yearly telomere loss of 24.7 bp was determined by weighted linear regression ($P = 0.0071$) (Muezzinler *et al.*, 2013). This attrition rate is higher than that observed in the present cohort (11.5 bp/diploid genome/y, $P = 0.39$) and this difference could be attributed to the limited range of age in the present sample (26 – 61 y) or differences in the method used for telomere length determination (Chapter 4). Table 5.1 summarises identified longitudinal studies from the literature. Many of these investigations determined an annual rate of telomere length attrition, which ranged from 24.7 to 47.7 bp/y. Of these studies, just one includes data on nutrition in addition to longitudinal change in telomere length. In this report, there was a reported slower telomere length attrition rate over the 5-year follow-up period in individuals who had higher levels of marine omega-3 fatty acid levels (Farzaneh-Far *et al.*, 2010a, Farzaneh-Far *et al.*, 2010b).

Table 5.1 – Longitudinal telomere length investigations from the literature

COHORT	STUDY FOLLOW-UP PERIOD	MEASURED TELOMERE LENGTH	CHANGE OR ATTRITION RATE	NOTES	REFERENCES
Bogalusa Heart Study, USA *n* = 635; *n* = 185 AA *n* = 450 W Mean baseline age: 31.4 y	≈6 y	Baseline AA: 7847 ± 734 bp W: 7228 ± 735 bp All: 7451 ± 777 bp Follow up AA: 7603 ± 767 bp W: 7076 ± 721 bp All: 7230 ± 772 bp	AA: -47.7 ± 55.3 bp/y W: -37.8 ± 41.3 bp/y All: -40.7 ± 46.0 bp/y	Values: mean ± SD Measured by TRF AA, African American; W, white	Aviv *et al.* (2009)

Bogalusa Heart Study, USA *n* = 271; *n* = 48 ♀AA *n* = 131 ♀W *n* = 19 ♂AA *n* = 73 ♂W Baseline age range: 19.9 – 41.5 y	≈12 y mean: 12.4 y	Sample 1 ♀AA: 7650 ± 0.732 bp ♀W: 7114 ± 0.699 bp ♂AA: 7713 ± 0.688 bp ♂W: 7005 ± 0.617 bp Sample 2 ♀AA: 7450 ± 769 bp ♀W: 6938 ± 711 bp ♂AA: 7525 ± 688 bp ♂W: 6844 ± 645 bp Sample 3 ♀AA: 7215 ± 792 bp ♀W: 6749 ± 711 bp ♂AA: 7196 ± 634 bp ♂W: 6612 ± 582 bp	-32.2 bp/y	Values: mean ± SD Measured by TRF ♀, female; ♂, male; AA, African American; W, white	Chen *et al.* (2011)
Bruneck *n* = 510 Baseline age range: 51 – 66 y	10 y	1995: 1.49 (1.07 – 2.15) 2005: 1.05 (0.8 – 1.47)	Δ -0.33 (-0.10 – -0.79) -455 bp/10 y = -45.5 bp/y	RTL Values: median (IQR) Measured by qPCR	Ehrlenbach *et al.* (2009)
Heart and Soul *n* = 608	Median: 6.0 y Range: 5.0 – 8.1 y	Baseline: 0.89 ± 0.19 – 0.96 ± 0.25 5-year follow up: 0.82 ± 0.15 – 0.86 ± 0.15	-42 bp/y	Data are T/S ratio min – max of marine omega 3 fatty acid level quartiles Values: mean ± SD Measured by qPCR	Farzaneh-Far *et al.* (2010a) and Farzaneh-Far *et al.* (2010b)

Zutphen *n* = 75 Baseline age range: 72 – 91 y	7 y	1993: 5.03 kb 2000: 4.76 kb	-40.2 bp/y	Values: mean Measured by qPCR	Houben *et al.* (2011), Rius-Ottenheim *et al.* (2012)
Finnish Diabetes Prevention Study (*n* = 311) Mean baseline age: Intervention: 57.1 ± 7.1 y *n* = 150 Controls: 56.7 ± 7.0 y *n* = 161	Mean follow up time Intervention: 4.56 ± 0.57 y Controls: 4.53 ± 0.68 y	Baseline: Intervention: 0.84 ± 0.18 Controls: 0.86 ± 0.18 Follow up Intervention: 0.96 ± 0.23 Controls: 0.97 ± 0.26	Mean change Intervention: 0.027 ± 0.052 Controls: 0.022 ± 0.048	Values: mean ± SD Measured by qPCR All participants had impaired glucose tolerance; the intervention group received advice on weight reduction, physical activity and healthy diet.	Hovatta *et al.* (2012)
Normative Ageing Study, USA *n* = 165 men Baseline age Mean: 73.6 ± 7.1 y Range: 56 – 94 y	≈7 y	visit 1: 1.27 ± 1.34 (*n* = 165) visit 2: 1.25 ± 1.54 (*n* = 90) visit 3: 1.02 ± 1.41 (*n* = 19)	-	T/S ratio Values: mean ± SD Measured by qPCR	McCracken *et al.* (2010)

Flemish Study on Environment, Genes & Health Outcomes, Belgium *n* = 344 *n* = 136 hypertensive *n* = 198 normotensive Mean baseline age: 51.9 y	Median: 7.4 y IQR: 6.2 – 8/5 y	Baseline Normotensive: 1.84 ± 0.27 Hypertensive: 1.78 ± 0.26 All: 1.82 ± 0.26 Follow-up Normotensive:1.84 ± 0.27 Hypertensive:1.71 ± 0.25 All: 1.76 ± 0.26	Normotensive: -0.0063 ± 0.019 Hypertensive: -0.0097 ± 0.016 All: -0.0077 ± 0.018	Data are T/S ratio or T/S annual attrition rate Values: mean ± SD Measured by qPCR	Kuznetsova *et al.* (2010)
North Sweden Health and Disease Study (NSHDS) *n* = 959 Baseline age range: 30 – 61 y	≈10 y Range: 9 – 11 y	-	Age-related decline in TL over time (R = –0.164, P <0.001)	qPCR	Nordfjall *et al.* (2009)
Longitudinal Study of Ageing Danish Twins (LSADT) *n* = 80 56 females (73 – 81 y at baseline) 24 males (73 – 79 y at baseline)	Mean: 10.9 y	Baseline (1997) Females: 5.9 kb Males: 5.6 kb Follow-up (2007) Females: 5.6 kb Males: 5.4 kb	Females: Δ -0.37 (-0.42, -0.30) = -29 bp/y (-12, -47) Males: Δ -0.26 (-0.37, -0.16) = -18 bp/y (-12, -49) Overall: 24.7 bp/y	Measured by TRFL Values: mean (95% CI LB, UB)	Steenstrup *et al.* (2013b)

5.1.3 *Polypill* intervention study rationale

Previous studies have shown that increased dietary intake of folate, vitamin B_{12}, nicotinic acid, retinol, vitamin E and calcium was associated with reduced DNA damage measured using the cytokinesis-block micronucleus assay in lymphocytes from South Australian adults (Fenech *et al.*, 2005). Furthermore, high homocysteine was associated with increased micronuclei, a chromosome damage biomarker, which was later reduced along with plasma homocysteine following folic acid and vitamin B_{12} supplementation (Fenech *et al.*, 1998). In the previous chapter, it was demonstrated that telomere length was negatively associated with plasma homocysteine, and positively associated with plasma vitamin D in a cross-sectional study. Whilst vitamin D was not a component within the *Polypill* micronutrient supplement, folate and vitamin B_{12} were included and these B-group vitamins are known to reduce homocysteine *in vivo*. The other micronutrients included in the *Polypill* micronutrient supplement were alpha-tocopherol, retinol, nicotinic acid and calcium. These micronutrients and their concentrations in the *Polypill* supplement were selected for their observation with the lowest levels of DNA damage as measured *ex vivo* by cytokinesis-block micronucleus assay (CBMN; Table 3.6). The *Polypill* study was designed and powered to detect significant a reduction of micronuclei, a marker of DNA damage measured in the CBMN assay, in those treated with the level of micronutrients associated with the lowest DNA damage. Because a low micronucleus frequency was associated with longer telomeres in previous studies (Lee *et al.*, 2009b), it was hypothesised that the *Polypill* may also influence telomere length.

A study of multivitamin type, frequency of use and telomere length showed a generally longer telomere length in women who reported use of any multivitamins, once-a-day type multivitamins and antioxidant combinations (Xu *et al.*, 2009). In these multivitamin users, telomere length was significantly associated positively with the intake of micronutrients vitamins A, C, E, B_9 (folate), β-carotene, and minerals calcium and magnesium (Xu *et al.*, 2009). In non-users, telomere length was significantly associated positively with intakes of vitamin C and E only (Xu *et al.*, 2009).

5.1.4 Aims and hypotheses

This study aimed to investigate the impact that a *Polypill* micronutrient supplement (containing six micronutrients previously associated with reduced chromosomal DNA damage) has on mean longitudinal changes in telomere length and telomere length trajectories, compared with placebo control treatment administered in a randomised-controlled trial of healthy, middle-aged South Australians.

It was hypothesised that change in telomere length may vary with *Polypill* supplementation of micronutrients which were selected for their effectiveness in minimising chromosomal DNA damage. Specifically, it is hypothesised that telomere length is longer in those supplemented with micronutrients important for genome damage.

5.2 Methods

Details of the study including ethics, supplement composition and blood sample processing is as described in Section 3.2. The human trial was completed in its entirety in the 2008 calendar year with week 0 samples collected from 4th February to 13th May and week 16 samples collected from 20th May to 8th September.

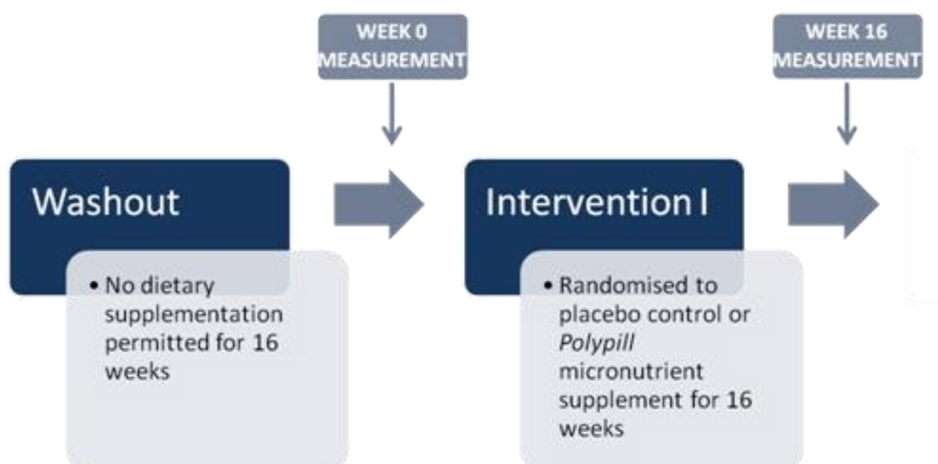

Figure 5.1 – Timeline of study defining week 0 and week 16

5.2.1 Randomisation to treatment groups

Individuals were randomised to their treatment groups on the basis of age and gender. Randomisation was performed by a person who was not involved with the intervention. As the study was double-blinded, this allocation was unknown to participants and other personnel until all trial data was collected.

5.2.2 Missing samples

Telomere length was measured in isolated PBMCs as previously detailed (Section 3.1). Due to insufficient sample, four individual week 16 PBMC samples were not available for DNA isolation. Although 203 volunteers finished phase two and supplied a sample, there are only 199 telomere length measures for this time point. The missing sample data for other parameters were not included in the telomere length analyses and models presented in this chapter.

5.2.3 Definition of seasons

In order to investigate the effect of a season on the change in micronutrients over time, the season of blood sample collection was determined from the calendar

date. Based on the international meteorological definition of typical calendar seasons in the Southern Hemisphere

- Summer was designated as season of collection if a blood sample was collected in December, January or February;
- Autumn was designated as season of collection if a blood sample was collected in March, April, or May;
- Winter was designated as season of collection if a blood sample was collected in June, July, or August; and
- Spring was designated as season of collection if a blood sample was collected in September, October, or November.

5.2.4 Longitudinal-specific calculations and analyses

Change in micronutrient concentrations were calculated after the intervention for all measured micronutrients. The formula was also used to calculate change in telomere length:

$$\Delta_{0-16} telomere\ length = week\ 16\ telomere\ length - week\ 0\ telomere\ length.$$

Additionally, percent change in telomere length was calculated using this formula:

$$\Delta_{0-16}\ telomere\ length\ [\%] = \frac{week\ 16\ telomere\ length - week\ 0\ telomere\ length}{week\ 0\ telomere\ length} \times 100$$

This percent change was used to determine telomere length trajectory as described by a previous study (Farzaneh-Far *et al.*, 2010a). Henceforth, change in telomere length was regarded as either telomere shortening (if there was a >10% loss of telomere length from week 0 to week 16), telomere maintenance (if there was a ± 10% change from week 0 to week 16) or telomere lengthening (if there was a >10% gain of telomere length from week 0 to week 16).

5.2.5 Statistical analyses

Results presented are typically reported as mean ± standard deviation, range (minimum observation – maximum observation), number [% cases] or median (IQR; $25^{th} - 75^{th}$ percentile). The threshold for statistical significance was set at $P < 0.05$ and these significant associations are highlighted in bold typeface throughout.

Relationships between measured variables were assessed by bivariate and partial Pearson correlation analyses. The Pearson product-moment correlation coefficient was squared in order to give R^2, the coefficient of determination. This value was then multiplied by 100 to yield a percentage which characterises the amount of shared variability between two correlated values. In order to correct for multiple correlation analyses, Bonferroni-adjusted P thresholds for statistical significance were computed by dividing the alpha value for type I error (0.05) by the total number of comparisons conducted in any correlation matrix. These adjusted P thresholds for statistical significance are provided as a footnote below correlation matrices, although reported P values < 0.05 are highlighted in bold typeface.

Standard multiple linear regression was performed with multiple predictor variables expected to influence telomere length. The accuracy of the model was assessed by the conformity of the measured dependent variables, where 99% of standardised residuals within ± 3.0 was acceptable. In regression modelling, dichotomous variables were coded as 0 or 1 with gender modelled as male (0) or female (1) and active *Polypill* treatment modelled as no (0) or yes (1).

Generalised linear modelling in the form of repeated measures analysis of variance was conducted with time as the repeated-measure factor and telomere length as the dependent quantitative variable. These statistical tests were performed in IBM SPSS Statistics 20.0 where the threshold for statistical significance was set at $P = 0.05$. Power analyses were conducted using Stata version 12 (StataCorp, College Station, Texas, USA).

Supplementary data is contained in the Appendix.

5.3 Results

5.3.1 Description of the study population

The characteristics of the study population are described in Table 5.2. As cross-sectional observations were made in this cohort at week 0, the population descriptives in the longitudinal and cross-sectional study are very similar (Table 4.1).

Table 5.2 – Demographic and anthropometric descriptives of the study population at week 16

VARIABLE	VALUE	RANGE (MIN – MAX)
Age (y)	45 ± 8.2	26 – 61
Gender *n* males [% cases]	86 [43.2]	-
BMI (kg/m^2)	27.1 ± 5.1	17.9 – 44.7
Obese *n* [% cases]	54 [27.1]	-
Maternal age (y)	27.8 ± 5.6	17 – 45
Paternal age (y)	30.7 ± 7.1	17 – 53
IRSED decile Median (IQR)	7 (5 – 9)	1 – 10
IRSD decile Median (IQR)	7 (4 – 9)	1 – 10
IER decile Median (IQR)	5 (2 – 8)	1 – 10
IEO decile Median (IQR)	8 (5 – 9)	1 – 10

n = 199; Values supplied are mean ± standard deviation, number of cases [% cohort] or median (IQR).

These factors were assessed with stratification by randomised treatment group to ensure the randomisation of individuals to each group resulted in comparable populations. The active *Polypill* group and the placebo control group were balanced for age, BMI and other data as seen in Table 5.3, below with the exclusion of

maternal age at birth where those in the *Polypill* group had mean maternal age at birth 1.8 y higher than the placebo group (P = 0.03).

Table 5.3 – Demographic and anthropometric descriptives of the study population at week 16, by treatment group

VARIABLE	*POLYPILL* (*n* = 98, 49.25%)	PLACEBO (*n* = 101, 50.75%)	*P* (ANOVA, χ^2 OR MANN-WHITNEY)
Age at week 16 (y)	44.1 ± 8.3 (26 – 60)	44.9 ± 8.0 (26 – 61)	0.49
Gender: *n* males [% cases]	42 [42.9]	44 [43.6]	1
BMI at week 16 (kg/m^2)	27.2 ± 5.4 (19.0 – 44.7)	27.0 ± 5.0 (17.9 – 43.6)	0.66
Obese at week 16 *n* [% cases]	27 [27.6]	27 [26.7]	0.85
Maternal age (y)	28.7 ± 5.9 (17 – 45)	26.9 ± 5.1 (17 – 40)	**0.03**
Paternal age (y)	31.2 ± 7.5 (19 – 52)	30.2 ± 6.7 (17 – 53)	0.35
IRSED decile Median (IQR)	7.5 (4 – 9)	7 (5 – 9)	0.18
IRSD decile Median (IQR)	7 (4 – 9)	7 (4 – 9)	0.21
IER decile Median (IQR)	5 (3 – 7.25)	5 (2 – 8)	0.34
IEO decile Median (IQR)	8 (5 – 9)	8 (5 – 9)	0.06

Values supplied are mean ± standard deviation with range of values as minimum – maximum in parentheses, number of cases [% cohort] or median (IQR).

In addition to balance of many demographic characteristics, balance in plasma micronutrients and homocysteine between the two groups at baseline was observed with no significant differences in mean micronutrient or homocysteine levels at week 0 as reported in Appendix Table 10.7.

5.3.2 Effect of *Polypill* supplementation on micronutrient levels

In order to assess whether the *Polypill* micronutrient supplement increased levels of supplemented micronutrients in plasma, the change in plasma micronutrient concentrations were calculated and compared across both groups (Table 5.4). There were increases in plasma folate, vitamin B_{12}, alpha-tocopherol, retinol and niacin in the *Polypill* treatment group, which were significantly different to reductions or smaller increases observed in the placebo group. When comparing mean levels of plasma calcium in the *Polypill* group, there was a significant decrease in mean calcium from week 0 to 16 (Appendix Table 10.6) however no significant difference in mean levels between groups at either week 0 or week 16 (Appendix Table 10.7). In comparing changes in plasma micronutrients from week 0 to week 16, there were greater reductions in plasma selenium and vitamin D in the *Polypill* group than there was in the placebo group.

Table 5.4 – Change in plasma micronutrient levels and homocysteine from week 0 to 16, by treatment group

VARIABLE	*POLYPILL* (*n* = 98, 49.25%)	PLACEBO (*n* = 101, 50.75%)	*P* (ANOVA)
$\Delta_{0\text{-}16}$Folate (nmol/L)	9.5 ± 7.3 (-6.6 – 30.7)	-0.3 ± 6.1 (-19.2 – 22.5)	**<0.0001**
$\Delta_{0\text{-}16}$Homocysteine (µmol/L)	-0.59 ± 1.25 (-3.7 – 3.7)	0.14 ± 1.26 (-4.5 – 3.9)	**<0.0001**
$\Delta_{0\text{-}16}$Vitamin B_{12} (pmol/L)	80.2 ± 160.2 (-931 – 295)	16.8 ± 50.8 (-106 – 173)	**<0.0001**
$\Delta_{0\text{-}16}$Alpha-tocopherol (µmol/L)	2.01 ± 4.45 (-10.7 – 24.0)	-0.32 ± 4.23 (-16.6 – 8.1)	**<0.0001**
$\Delta_{0\text{-}16}$Retinol (µmol/L)	0.08 ± 0.30 (-1.05 – 0.61)	-0.01 ± 0.32 (-1.45 – 0.67)	**0.04**
$\Delta_{0\text{-}16}$Niacin number $\left(\frac{\mathrm{NAD}}{\mathrm{NAD}+\mathrm{NADP}} \times 100\right)$	3.68 ± 9.74 (-17.5 – 42.1)	-0.25 ± 11.53 (-32.0 – 44.0)	**0.01**
$\Delta_{0\text{-}16}$Calcium (mmol/L)	-0.04 ± 0.14 (-0.57 – 0.21)	-0.01 ± 0.10 (-0.26 – 0.22)	**0.05**
$\Delta_{0\text{-}16}$Magnesium (µmol/L)	-11.7 ± 59.3 (-258 – 123)	-0.46 ± 42.8 (-97 – 147)	0.13
$\Delta_{0\text{-}16}$Selenium (µmol/L)	-0.05 ± 0.15 (-0.50 – 0.70)	-0.002 ± 0.138 (-0.34 – 0.56)	**0.03**
$\Delta_{0\text{-}16}$Zinc (µmol/L)	-0.48 ± 1.31 (-4.4 – 2.6)	-0.31 ± 1.71 (-5.0 – 6.2)	0.43
$\Delta_{0\text{-}16}$Vitamin D (µmol/L)	-25.0 ± 18.3 (-70 – 31)	-19.4 ± 17.3 (-81 – 33)	**0.03**

Values supplied are mean ± standard deviation with range of values as minimum – maximum in parentheses. Where negative, there is a reduction in the micronutrient level over 16 weeks from week 0 to week 16; where positive, there is an increase in the micronutrient level over 16 weeks from week 0 to week 16; P (ANOVA) reports statistic for differences in the treatment groups

5.3.3 Effect of *Polypill* supplementation on telomere length

After analysing the change in micronutrients over the 16-week study period, the change in telomere length was investigated. In all 199 participants, there was a mean loss of 1.25 kb/diploid genome over the 16-week period, with an average loss of 3.9 kb observed in the *Polypill* treatment group and a mean gain of 1.3 kb/diploid genome seen in the placebo control group, however these differences were not statistically significant (Table 5.5).

Table 5.5 – Change in telomere length from week 0 to week 16, by treatment group

VARIABLE	*POLYPILL* (*n* = 98, 49.25%)	PLACEBO (*n* = 101, 50.75%)	*P* (ANOVA)
TL at week 0 (kb/diploid genome)	113.9 ± 54.7 (30.0 – 332.2)	108.7 ± 50.8 (20.5 – 445.3)	0.56
TL at week 16 (kb/diploid genome)	110 ± 45.9 (33.2 – 305.3)	110 ± 39.7 (13.5 – 304.3)	0.79
P (paired samples t-test) week 0 – 16	0.25	0.74	-
$\Delta_{0\text{-}16}$ Telomere length (kb/diploid genome)	-3.9 ± 32.8 (-121.1 – 82)	1.33 ± 39.5 (-201.1 – 143.3)	0.31

Values supplied are mean ± standard deviation with range of values as minimum – maximum in parentheses.

The mean changes in telomere length reported above are graphically represented in Figure 5.2 for each treatment group, and for the mean change in the entire cohort.

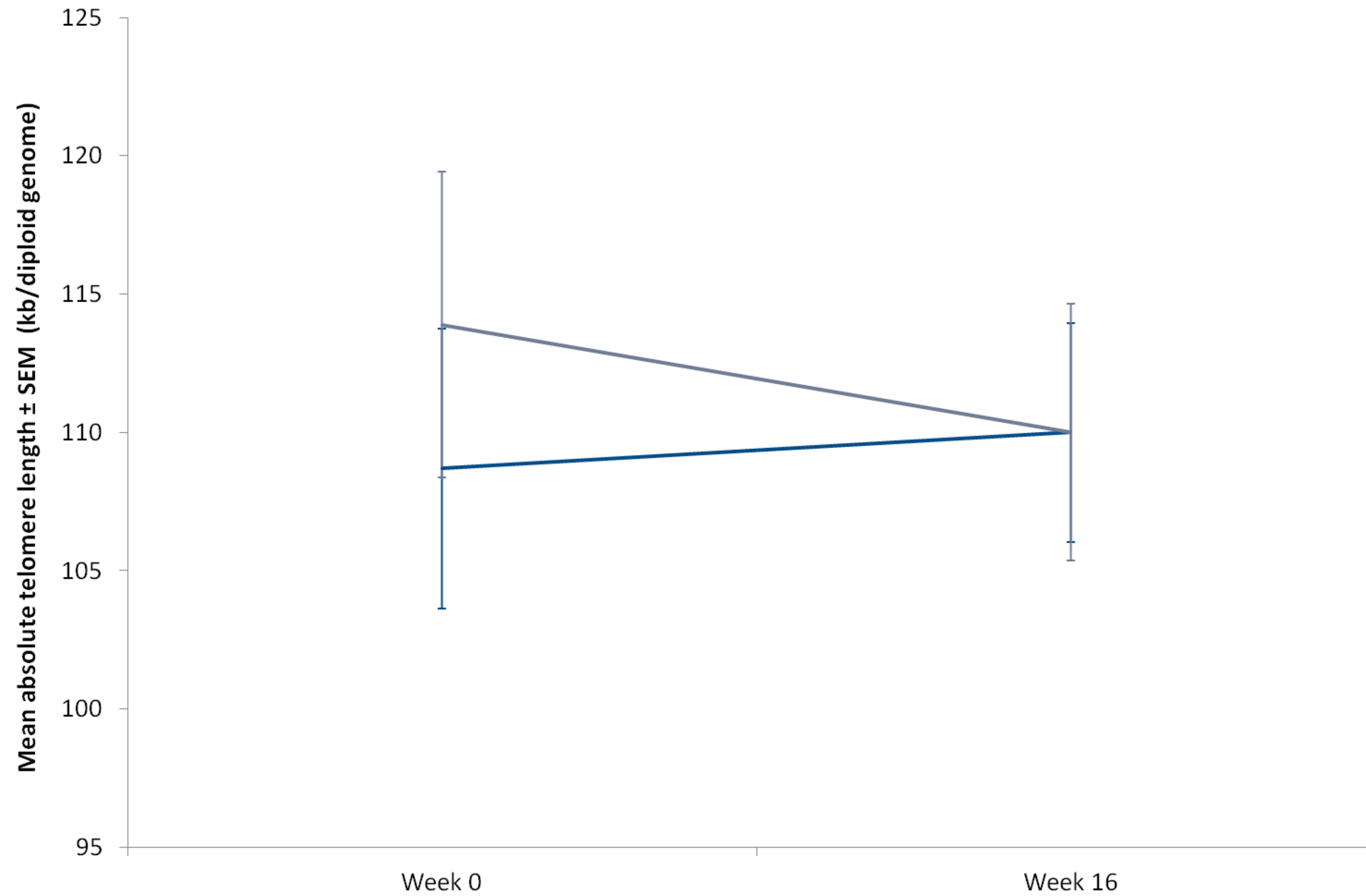

Figure 5.2 – Graphical representation of the mean change in absolute telomere length over time and by treatment group

Grey line indicates Polypill group mean ± SEM (n = 98), dark blue line indicates placebo control group mean ± SEM (n = 101).

5.3.4 Analyses by telomere length trajectory

In order to better investigate for differences in telomere length, telomere length trajectory analysis was opted for. The participants were stratified into three telomere length trajectory groups; those whose telomeres shortened (> 10% loss), were maintained (± 10% change) or lengthened (> 10% gain). These different telomere length trajectories are represented graphically in Figure 5.3. A separate figure illustrates these same telomere length trajectories in the two treatment groups (Figure 5.4).

In order to discern if demographic characteristics might be responsible for the changes in trajectory, they were compared by treatment group (Table 5.6). There were no significant group differences in the proportion of individuals in each of the telomere length trajectories. In addition there was no difference within groups of age, gender, BMI, obesity prevalence, maternal and paternal age across the three defined telomere length trajectories. There was a significant difference in telomere length at week 0 across the telomere length trajectories in both the *Polypill* and placebo groups. In both groups, telomere length was highest at week 0 in the telomere length shortened trajectory. While conversely, telomere length was shortest at week 0 for those whose telomere length increased over the 16 week period irrespective of randomised treatment group. For both groups, mean telomere length in the maintained telomere length trajectory was between the shortest telomere length observed in lengthened trajectory and the highest telomere length observed in shortened trajectory. *Post hoc* comparisons show that only telomere length in maintained and shortened trajectory groups were not significantly different from each other in either treatment group.

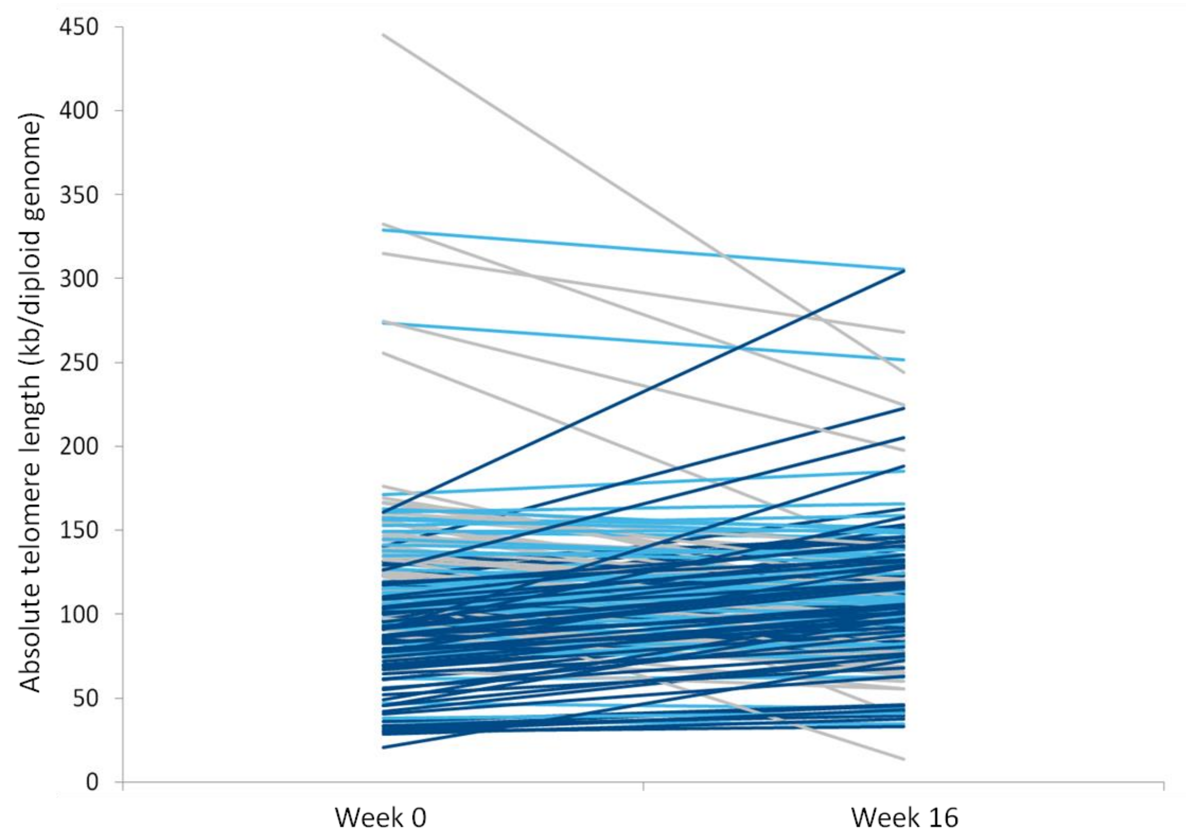

Figure 5.3 – Graphical representation of individual telomere length trajectory from week 0 to-, for all participants

Measured absolute telomere length in PBMC over the 16 week intervention period (week 0 – week 16) for all individuals (n = 199). Grey lines indicate telomere length shortening (>10% loss), sky blue lines indicate telomere length maintenance (± 10% change), and dark blue lines indicate telomere length lengthening (>10% gain).

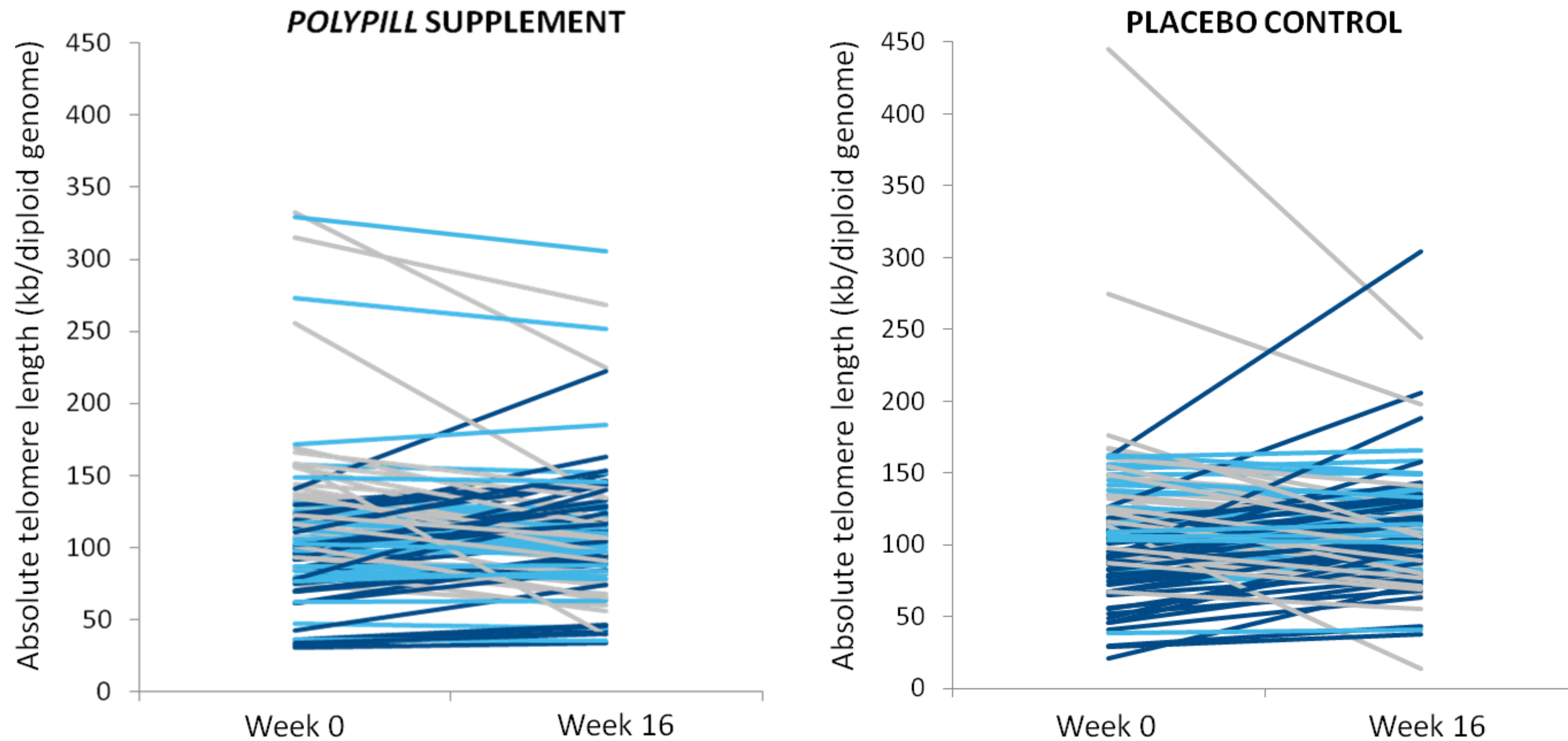

Figure 5.4 – Graphical representation of individual telomere length trajectory from week 0 to 16, for all participants, by treatment group

Measured absolute telomere length in PBMC over the 16-week intervention period (week 0 – week 16) for individuals randomised to Polypill micronutrient supplement (n = 98) or the placebo control group (n = 101). Grey lines indicate telomere length shortening (> 10% loss), sky blue lines indicate telomere length maintenance (± 10% change), and dark blue lines indicate telomere length lengthening (> 10% gain).

Table 5.6 – Demographic and anthropometric descriptives and telomere length of the study population, by treatment group and telomere length trajectory from week 0 to week 16

VARIABLE	GROUP	SHORTENED > 10% LOSS	MAINTAINED ± 10% CHANGE	LENGTHENED > 10% GAIN	*P* (ANOVA OR χ^2)
Number [% cases]	*Polypill* (*n* = 98)	34 [34.7]	31 [31.6]	33 [33.7]	0.93[a]
	Placebo (*n* = 101)	29 [28.7]	33 [32.3]	39 [38.6]	0.47[a]
Age (y)	*Polypill*	43.7 ± 8.9	44.1 ± 7.3	44.4 ± 8.8	0.95
	Placebo	47.2 ± 7.6	44.0 ± 7.5	43.9 ± 8.6	0.19
Gender: males [%]	*Polypill*	13 [38.2]	12 [38.7]	17 [51.5]	0.47
	Placebo	11 [37.9]	14 [42.4]	19 [48.7]	0.67
BMI (kg/m^2)	*Polypill*	27.7 ± 5.8	25.4 ± 4.4	27.9 ± 5.9	0.12
	Placebo	27.7 ± 6.4	26.4 ± 4.2	26.2 ± 4.4	0.44
Obese *n* [% cases]	*Polypill*	10 [29.4]	5 [16.1]	11 [33.3]	0.27
	Placebo	9 [31.0]	10 [30.3]	10 [25.6]	0.86
Maternal age (y)	*Polypill*	27.4 ± 5.8	30.2 ± 6.3	28.6 ± 5.5	0.15
	Placebo	27.0 ± 5.8	26.5 ± 4.0	27.2 ± 5.5	0.91
Paternal age (y)	*Polypill*	30.0 ± 6.8	32.9 ± 8.8	30.8 ± 6.7	0.34
	Placebo	30.3 ± 5.8	29.4 ± 5.8	30.9 ± 7.9	0.74

TL at week 0 (kb)	*Polypill*	139.8 ± 57.2	117.5 ± 57.4	83.7 ± 30.7	**<0.0001**[b]
	Placebo	141.3 ± 69.9	114.1 ± 27.8	79.8 ± 29.2	**<0.0001**[c]

[a] *P = 0.63 for χ^2 interaction between telomere length trajectory and treatment group*

[b] *Bonferroni post hoc multiple comparisons show telomere length at week 0 was significantly different among telomere loss and gain groups (P <0.0001) and significantly different between telomere maintenance and gain groups (P = 0.004), however there was no significant difference between telomere loss and maintenance groups (P = 0.13).*

[c] *Bonferroni post hoc multiple comparisons show telomere length at week 0 was significantly different among telomere loss and gain groups (P <0.0001) and significantly different between telomere maintenance and gain groups (P <0.0001), however there was no significant difference between telomere loss and maintenance groups (P = 0.20).*

Subsequent comparison of telomere length trajectory groups involved comparing the change in micronutrient levels over the 16-week intervention period for significant differences in plasma micronutrients which may have influenced the change in telomere length (Table 5.7). There were no significant differences in the change of micronutrients across telomere length trajectories in the *Polypill* group. In the placebo group only, there was a significant difference in the change in plasma zinc from week 0 to week 16 across the three telomere length trajectories, however there were no significant changes across trajectory groups for any other micronutrient. In the placebo group, it appeared that a greater reduction in plasma zinc over the 16-week period was observed in the telomere length shortened trajectory group (-0.61 µmol/L; Table 5.7). There was a small decrease in plasma zinc (-0.09 µmol/L) in the maintained trajectory group and a small increase (+0.17 µmol/L) in zinc in the telomere lengthened trajectory. The trend across the three trajectories was statistically significant, and *post hoc* analyses revealed significant differences for telomere shortened and maintained, and telomere shortened and lengthened trajectories, but no significant difference between telomere length in maintained and lengthened trajectory groups.

Table 5.7 – Change in plasma micronutrient levels of the study population, by treatment group and telomere length trajectory from week 0 to week 16

VARIABLE	GROUP	SHORTENED >10% LOSS	MAINTAINED ± 10% CHANGE	LENGTHENED >10% GAIN	*P* (ANOVA)
$\Delta_{0\text{-}16}$Folate (nmol/L)	*Polypill*	9.16 ± 6.39	10.16 ± 6.48	9.12 ± 8.90	0.82
	Placebo	-0.93 ± 4.73	0.64 ± 6.34	-0.61 ± 6.91	0.56
$\Delta_{0\text{-}16}$Homocysteine (µmol/L)	*Polypill*	-0.39 ± 1.31	-0.93 ± 1.21	-0.48 ± 1.20	0.19
	Placebo	-0.09 ± 1.33	0.26 ± 1.20	0.22 ± 1.27	0.49
$\Delta_{0\text{-}16}$Vitamin B_{12} (pmol/L)	*Polypill*	91.26 ± 74.74	71.74 ± 191.95	76.88 ± 193.01	0.88
	Placebo	15.52 ± 46.69	8.00 ± 49.55	25.26 ± 54.67	0.36
$\Delta_{0\text{-}16}$Alpha-tocopherol (µmol/L)	*Polypill*	2.02 ± 5.26	2.76 ± 3.79	1.30 ± 4.12	0.43
	Placebo	-0.74 ± 4.56	-0.84 ± 3.82	0.44 ± 4.30	0.36
$\Delta_{0\text{-}16}$Retinol (µmol/L)	*Polypill*	0.004 ± 0.36	0.13 ± 0.25	0.11 ± 0.27	0.19
	Placebo	-0.04 ± 0.33	-0.02 ± 0.21	0.02 ± 0.40	0.72
$\Delta_{0\text{-}16}$Niacin number $\left(\frac{\text{NAD}}{\text{NAD} + \text{NADP}} \times 100\right)$	*Polypill*	4.01 ± 11.62	3.98 ± 9.60	3.05 ± 7.81	0.90
	Placebo	-1.47 ± 11.96	0.61 ± 10.93	-0.06 ± 11.92	0.78
$\Delta_{0\text{-}16}$Calcium (mmol/L)	*Polypill*	-0.02 ± 0.13	-0.05 ± 0.11	-0.05 ± 0.17	0.66
	Placebo	-0.04 ± 0.11	0.008 ± 0.11	0.002 ± 0.09	0.11

Δ_{0-16}Magnesium (µmol/L)	*Polypill*	-1.69 ± 60.32	-13.55 ± 44.29	-20.06 ± 67.00	0.45
	Placebo	-6.79 ± 41.46	1.48 ± 47.11	2.60 ± 40.49	0.64
Δ_{0-16}Selenium (µmol/L)	*Polypill*	-0.03 ± 0.19	-0.07 ± 0.11	-0.04 ± 0.14	0.55
	Placebo	0.007 ± 0.17	-0.02 ± 0.11	0.005 ± 0.14	0.74
Δ_{0-16}Zinc (µmol/L)	*Polypill*	-0.61 ± 1.48	-0.42 ± 1.15	-0.40 ± 1.31	0.78
	Placebo	-1.21 ± 1.69	-0.09 ± 1.57	0.17 ± 1.61	**0.002**[a]
Δ_{0-16} Vitamin D (µmol/L)	*Polypill*	-22.68 ± 17.84	-26.61 ± 21.37	-25.82 ± 15.90	0.69
	Placebo	-21.76 ± 17.25	-20.88 ± 18.18	-16.34 ± 16.69	0.38

Where negative, there is a reduction in the micronutrient level; where positive, there is an increase in the micronutrient level;
for Polypill group, shortened n = 34, maintained n = 31, lengthened n = 33
for Placebo group, shortened n = 29, maintained n = 33, lengthened n = 39
[a] Bonferroni post hoc multiple comparisons show change in zinc was significantly different among telomere loss and maintenance groups (P = 0.024) and significantly different between telomere loss and gain groups (P = 0.002), however there was no significant difference in the change in zinc between telomere gain and maintenance groups (P = 1.0).

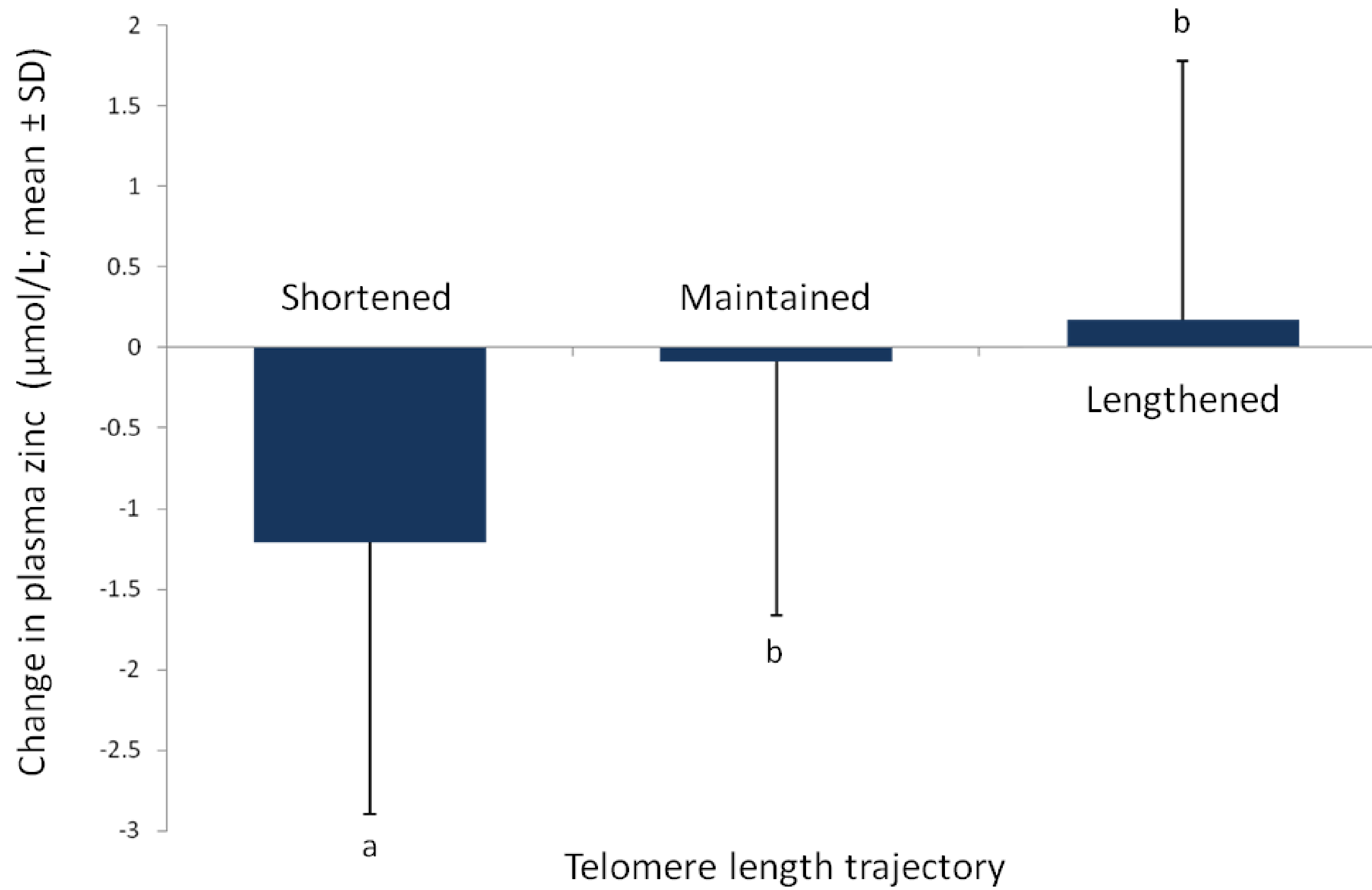

Figure 5.5– Telomere length trajectory is associated with change in plasma zinc

Placebo group only; P-trend = 0.002, n = 101
Bars not sharing the same letter are statistically significantly different from each other (Bonferroni post hoc multiple comparisons; P < 0.05).

5.3.5 Partial and bivariate correlation

Among the *Polypill* group, there was no significant correlation of change in any plasma micronutrients and telomere length at week 16 or change in telomere length from week 0 to week 16 (Table 5.8 and Appendix Figure 10.1). In the placebo group, the individual changes in plasma calcium and magnesium from week 0 to week 16 was significantly negatively associated with telomere length at week 16 (Table 5.9 and Appendix Figure 10.2). Both of these relationships were of a similar, weak magnitude; $R = -0.241$ for calcium ($P = 0.021$) and -0.262 for magnesium ($P = 0.012$). Magnesium and calcium were moderately correlated with each other, $R = 0.5$ ($P < 0.0001$).

Bivariate correlation of telomere length at week 16 was performed with age, maternal age, paternal age and BMI (as similarly performed with telomere length at week 0 in chapter 6 Table 4.7) however the change in telomere length from week 0 to 16 was also incorporated as a continuous variable. In the *Polypill* group, there was no significant association of telomere length at week 0 and week 16 (Table 5.10 and Appendix Figure 10.3). In the placebo group, telomere length was significantly negatively associated with age, and significantly positively associated with both maternal and paternal age at birth (Table 5.11).

In an attempt to control for these known confounders of telomere length and the apparent different associations of these with telomere length between the two treatment groups, partial correlation was performed. In the *Polypill* group there was no significant association of any change in plasma micronutrients from week 0 to week 16 with either telomere length at week 16 or change in telomere length from week 0 to week 16 (Table 5.12). Corresponding analysis in the placebo control group revealed a significant association of change in plasma calcium and magnesium from week 0 to week 16 with telomere length at week 16. These two relationships were negative whereby an increase in calcium or magnesium was associated with a decrease in telomere length (Table 5.13).

Table 5.8 – Bivariate correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, for *Polypill* group

		TL week 16	ΔTL	ΔFOL	ΔHCY	ΔB_{12}	ΔαTOC	ΔRET	ΔNIA	ΔCa	ΔMg	ΔSe	ΔZn	ΔVITD
TL at week 16	*R*	1	0.155	-0.021	0.043	0.022	-0.042	0.118	0.117	-0.179	-0.191	-0.118	-0.162	-0.055
	P	-	0.148	0.844	0.688	0.838	0.696	0.273	0.277	0.098	0.076	0.277	0.133	0.608
	n	88	88	88	88	88	88	88	88	87	87	87	87	88
$\Delta_{0\text{-}16}$Telomere length	*R*		1	-0.080	-0.012	0.030	-0.087	0.057	-0.107	-0.002	-0.048	0.110	0.078	-0.073
	P	-	-	0.457	0.911	0.779	0.422	0.598	0.322	0.988	0.658	0.310	0.472	0.502
	n		88	88	88	88	88	88	88	87	87	87	87	88
$\Delta_{0\text{-}16}$Folate	*R*			1	-0.457	0.111	0.167	0.095	-0.001	-0.047	-0.020	0.053	0.029	-0.072
	P	-	-	-	**<0.0001**	0.302	0.120	0.377	0.994	0.666	0.856	0.623	0.791	0.505
	n			88	88	88	88	88	88	87	87	87	87	88
$\Delta_{0\text{-}16}$Homocysteine	*R*				1	-0.034	0.174	0.170	0.020	0.143	0.163	0.133	-0.033	0.023
	P	-	-	-	-	0.755	0.104	0.114	0.856	0.187	0.131	0.220	0.761	0.830
	n				88	88	88	88	88	87	87	87	87	88
$\Delta_{0\text{-}16}$Vitamin B_{12}	*R*					1	0.094	0.101	0.115	0.058	0.118	0.065	0.228	0.030
	P	-	-	-	-	-	0.386	0.350	0.288	0.593	0.276	0.547	**0.034**	0.784
	n					88	88	88	88	87	87	87	87	88
$\Delta_{0\text{-}16}$Alpha-tocopherol	*R*						1	0.530	-0.024	0.062	0.172	0.238	0.039	-0.200
	P	-	-	-	-	-	-	**<0.0001**	0.823	0.568	0.111	**0.027**	0.719	0.062
	n						88	88	88	87	87	87	87	88

	R							1	0.006	0.143	0.087	0.213	0.195	-0.079
Δ0-16Retinol	*P*	-	-	-	-	-	-	-	0.958	0.185	0.424	**0.047**	0.070	0.466
	n							88	88	87	87	87	87	88
	R								1	-0.110	-0.210	-0.069	0.009	0.101
Δ0-16Niacin number	*P*	-	-	-	-	-	-	-	-	0.309	0.051	0.525	0.933	0.351
	n								88	87	87	87	87	88
	R									1	0.641	0.599	0.528	0.113
Δ0-16Calcium	*P*	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.299
	n									87	87	87	87	87
	R										1	0.599	0.392	-0.028
Δ0-16Magnesium	*P*	-	-	-	-	-	-	-	-	-	-	**<0.0001**	**0.0002**	0.794
	n										87	87	87	87
	R											1	0.454	-0.152
Δ0-16Selenium	*P*	-	-	-	-	-	-	-	-	-	-	-	**<0.0001**	0.161
	n											87	87	87
	R												1	-0.019
Δ0-16Zinc	*P*	-	-	-	-	-	-	-	-	-	-	-	-	0.859
	n												87	198
	R													1
Δ0-16Vitamin D	*P*	-	-	-	-	-	-	-	-	-	-	-	-	-
	n													88

R, Pearson correlation coefficient;
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00064
n ≥87 as 10 visible extreme outliers removed from n = 98

Table 5.9 – Bivariate correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, for Placebo group

		TL week 16	**ΔTL**	**ΔFOL**	**ΔHCY**	**ΔB_{12}**	**ΔαTOC**	**ΔRET**	**ΔNIA**	**ΔCa**	**ΔMg**	**ΔSe**	**ΔZn**	**ΔVITD**
TL at week 16	*R*	1	0.180	0.001	-0.123	-0.043	0.119	-0.108	-0.127	-0.241	-0.262	-0.172	-0.072	-0.145
	P	-	0.086	0.989	0.244	0.687	0.260	0.308	0.226	**0.021**	**0.012**	0.100	0.495	0.169
	n	92	92	92	92	92	92	92	92	92	92	92	92	92
$\Delta_{0\text{-}16}$Telomere length	*R*		1	-0.027	0.125	0.033	0.110	0.121	0.066	-0.029	-0.017	-0.060	0.164	0.127
	P	-	-	0.796	0.237	0.756	0.297	0.251	0.535	0.786	0.873	0.573	0.118	0.227
	n		92	92	92	92	92	92	92	92	92	92	92	92
$\Delta_{0\text{-}16}$Folate	*R*			1	-0.189	0.199	-0.009	-0.004	-0.105	0.163	0.105	0.089	-0.016	-0.051
	P	-	-	-	0.071	0.058	0.931	0.973	0.318	0.120	0.317	0.397	0.879	0.630
	n			92	92	92	92	92	92	92	92	92	92	92
$\Delta_{0\text{-}16}$Homocysteine	*R*				1	0.065	-0.015	0.112	-0.105	0.130	0.116	0.151	0.294	0.070
	P	-	-	-	-	0.541	0.884	0.288	0.321	0.218	0.271	0.150	**0.004**	0.506
	n				92	92	92	92	92	92	92	92	92	92
$\Delta_{0\text{-}16}$Vitamin B_{12}	*R*					1	0.281	0.070	-0.103	0.276	0.317	0.176	0.270	0.064
	P	-	-	-	-	-	**0.007**	0.510	0.326	**0.008**	**0.002**	0.093	**0.009**	0.547
	n					92	92	92	92	92	92	92	92	92
$\Delta_{0\text{-}16}$Alpha-tocopherol	*R*						1	0.455	-0.047	0.049	-0.043	0.084	0.164	-0.003
	P	-	-	-	-	-	-	**<0.0001**	0.659	0.643	0.681	0.428	0.119	0.979
	n						92	92	92	92	92	92	92	92

Δ_{0-16}Retinol	*R*							1	0.069	0.031	0.155	0.169	0.242	0.006
	P	-	-	-	-	-	-	-	0.513	0.768	0.141	0.107	**0.020**	0.955
	n							92	92	92	92	92	92	92
Δ_{0-16}Niacin number	*R*								1	-0.100	-0.073	-0.211	0.017	0.032
	P	-	-	-	-	-	-	-	-	0.344	0.490	**0.043**	0.875	0.759
	n								92	92	92	92	92	92
Δ_{0-16}Calcium	*R*									1	0.497	0.433	0.385	0.059
	P	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**0.0002**	0.576
	n									92	92	92	92	92
Δ_{0-16}Magnesium	*R*										1	0.398	0.459	0.112
	P	-	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	0.288
	n										92	92	92	92
Δ_{0-16}Selenium	*R*											1	0.426	0.192
	P	-	-	-	-	-	-	-	-	-	-	-	**<0.0001**	0.066
	n											92	92	92
Δ_{0-16}Zinc	*R*												1	0.180
	P	-	-	-	-	-	-	-	-	-	-	-	-	0.086
	n												92	92
Δ_{0-16}Vitamin D	*R*													1
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
	n													92

R, Pearson correlation coefficient;
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00064
n = 92 as 9 extreme outliers removed from n = 101.

Table 5.10 – Bivariate correlation matrix of telomere length at week 16, change in telomere length from week 0 to week 16, participant age, maternal age, paternal age and BMI, for *Polypill* group

GROUP		TL week 16	$\Delta_{0\text{-}16}$TL	AGE	MAT AGE	PAT AGE	BMI
	R	1	0.167	-0.096	0.070	0.075	-0.150
TL at week 16	*P*	-	0.105	0.354	0.499	0.470	0.146
	n	95	95	95	95	95	95
	R		1	0.022	0.050	-0.016	0.084
$\Delta_{0\text{-}16}$Telomere length	*P*	-	-	0.831	0.629	0.880	0.419
	n		95	95	95	94	95
	R			1	0.180	0.173	0.228
Age	*P*	-	-	-	0.081	0.096	**0.026**
	n			95	95	94	95
	R				1	0.867	-0.199
Maternal age	*P*	-	-	-	-	**<0.0001**	0.054
	n				95	94	95
	R					1	-0.207
Paternal age	*P*	-	-	-	-	-	**0.045**
	n					94	94
	R						1
BMI	*P*	-	-	-	-	-	-
	n						95

R, Pearson correlation coefficient
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.003

Table 5.11 – Bivariate correlation matrix of telomere length at week 16, change in telomere length from week 0 to week 16, participant age, maternal age, paternal age and BMI, for placebo group

GROUP		TL week 16	Δ_{0-16}TL	AGE	MAT AGE	PAT AGE	BMI
TL at week 16	*R*	1	0.170	-0.204	0.240	0.285	-0.150
	P	-	0.094	**0.044**	**0.018**	**0.005**	0.141
	n	98	98	98	97	96	98
Δ_{0-16}Telomere length	*R*		1	-0.173	0.054	0.020	-0.069
	P	-	-	0.088	0.602	0.845	0.501
	n		98	98	97	96	98
Age	*R*			1	0.131	0.213	0.235
	P	-	-	-	0.200	**0.037**	**0.020**
	n			98	97	96	98
Maternal age	*R*				1	0.773	-0.041
	P	-	-	-	-	**<0.0001**	0.693
	n				97	96	97
Paternal age	*R*					1	-0.125
	P	-	-	-	-	-	0.226
	n					96	96
BMI	*R*						1
	P	-	-	-	-	-	-
	n						98

R, Pearson correlation coefficient

To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.003

Table 5.12 – Partial correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, with adjustment for participant age, gender, maternal age, paternal age and BMI, for *Polypill* group

		TL week 16	ΔTL	ΔFOL	ΔHCY	ΔB_{12}	ΔαTOC	ΔRET	ΔNIA	ΔCa	ΔMg	ΔSe	ΔZn	ΔVITD
TL at week 16	*R*	1	0.149	-0.021	0.032	0.001	-0.082	0.082	0.086	-0.158	-0.170	-0.096	-0.181	-0.043
	P	-	0.183	0.854	0.778	0.991	0.466	0.464	0.446	0.159	0.129	0.393	0.106	0.703
	df	0	79	79	79	79	79	79	79	79	79	79	79	79
$\Delta_{0\text{-}16}$Telomere length	*R*		1	-0.058	-0.030	0.062	-0.065	0.072	-0.099	0.004	-0.030	0.126	0.067	-0.068
	P	-	-	0.610	0.791	0.584	0.562	0.526	0.378	0.972	0.789	0.261	0.554	0.546
	df		0	79	79	79	79	79	79	79	79	79	79	79
$\Delta_{0\text{-}16}$Folate	*R*			1	-0.467	0.142	0.136	0.044	-0.033	-0.070	-0.014	0.039	0.060	-0.082
	P	-	-	-	**<0.0001**	0.206	0.228	0.698	0.767	0.534	0.904	0.731	0.597	0.467
	df			0	79	79	79	79	79	79	79	79	79	79
$\Delta_{0\text{-}16}$Homocysteine	*R*				1	-0.035	0.191	0.227	0.006	0.150	0.177	0.140	-0.033	0.091
	P	-	-	-	-	0.757	0.087	**0.042**	0.960	0.182	0.114	0.212	0.772	0.420
	df				0	79	79	79	79	79	79	79	79	79
$\Delta_{0\text{-}16}$Vitamin B_{12}	*R*					1	0.031	0.073	0.117	0.074	0.097	0.065	0.220	-0.021
	P	-	-	-	-	-	0.782	0.517	0.297	0.512	0.389	0.564	**0.049**	0.856
	df					0	79	79	79	79	79	79	79	79
$\Delta_{0\text{-}16}$Alpha-tocopherol	*R*						1	0.502	-0.069	0.070	0.187	0.247	0.011	-0.206
	P	-	-	-	-	-	-	**<0.0001**	0.538	0.533	0.094	**0.026**	0.920	0.064
	df						0	79	79	79	79	79	79	79

	R							1	-0.035	0.137	0.120	0.201	0.103	-0.060
Δ_{0-16}Retinol	*P*	-	-	-	-	-	-	-	0.758	0.223	0.284	0.072	0.362	0.595
	df							0	79	79	79	79	79	79
	R								1	-0.091	-0.192	-0.049	0.040	0.140
Δ_{0-16}Niacin number	*P*	-	-	-	-	-	-	-	-	0.417	0.086	0.662	0.724	0.213
	df								0	79	79	79	79	79
	R									1	0.640	0.570	0.541	0.149
Δ_{0-16}Calcium	*P*	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.185
	df									0	79	79	79	79
	R										1	0.593	0.407	-0.037
Δ_{0-16}Magnesium	*P*	-	-	-	-	-	-	-	-	-	-	**<0.0001**	**0.0002**	0.746
	df										0	79	79	79
	R											1	0.449	-0.139
Δ_{0-16}Selenium	*P*	-	-	-	-	-	-	-	-	-	-	-	**<0.0001**	0.216
	df											0	79	79
	R												1	-0.001
Δ_{0-16}Zinc	*P*	-	-	-	-	-	-	-	-	-	-	-	-	0.995
	df												0	79
	R													1
Δ_{0-16}Vitamin D	*P*	-	-	-	-	-	-	-	-	-	-	-	-	-
	df													0

Partial correlation with adjustment for individual age, gender, BMI, maternal age and paternal age
R, Pearson correlation coefficient
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00064

Table 5.13 – Partial correlation matrix of telomere length, change in telomere length and plasma micronutrient status from week 0 to week 16, with adjustment for participant age, gender, maternal age, paternal age and BMI, for placebo group

		TL week 16	ΔTL	ΔFOL	ΔHCY	ΔB_{12}	ΔαTOC	ΔRET	ΔNIA	ΔCa	ΔMg	ΔSe	ΔZn	ΔVITD
TL at week 16	*R*	1	0.222	0.050	-0.114	-0.073	0.063	-0.157	-0.046	-0.245	-0.227	-0.188	-0.110	-0.103
	P	-	**0.042**	0.649	0.297	0.505	0.567	0.150	0.674	**0.024**	**0.037**	0.086	0.317	0.349
	df	0	83	83	83	83	83	83	83	83	83	83	83	83
Δ_{0-16}Telomere length	*R*		1	-0.074	0.135	0.019	0.112	0.098	0.021	-0.050	-0.017	-0.083	0.180	0.138
	P	-	-	0.501	0.217	0.862	0.307	0.372	0.852	0.647	0.876	0.452	0.099	0.209
	df		0	83	83	83	83	83	83	83	83	83	83	83
Δ_{0-16}Folate	*R*			1	-0.203	0.213	0.021	-0.019	-0.157	0.179	0.083	0.086	0.010	-0.070
	P	-	-	-	0.063	**0.050**	0.852	0.862	0.152	0.100	0.450	0.433	0.924	0.526
	df			0	83	83	83	83	83	83	83	83	83	83
Δ_{0-16}Homocysteine	*R*				1	0.061	-0.018	0.098	-0.129	0.144	0.113	0.141	0.251	0.025
	P	-	-	-	-	0.577	0.872	0.375	0.238	0.188	0.302	0.198	**0.020**	0.823
	df				0	83	83	83	83	83	83	83	83	83
Δ_{0-16}Vitamin B_{12}	*R*					1	0.270	0.064	-0.111	0.260	0.331	0.159	0.270	0.066
	P	-	-	-	-	-	**0.012**	0.559	0.313	**0.016**	**0.002**	0.146	**0.012**	0.551
	df					0	83	83	83	83	83	83	83	83
Δ_{0-16}Alpha-tocopherol	*R*						1	0.463	-0.038	0.031	-0.040	0.076	0.146	0.008
	P	-	-	-	-	-	-	**<0.0001**	0.732	0.779	0.714	0.489	0.183	0.944
	df						0	83	83	83	83	83	83	83

Δ_{0-16}Retinol	*R*							1	0.068	0.049	0.162	0.168	0.233	-0.002
	P	-	-	-	-	-	-	-	0.539	0.653	0.139	0.125	**0.032**	0.989
	df							0	83	83	83	83	83	83
Δ_{0-16}Niacin number	*R*								1	-0.147	-0.161	-0.243	0.019	0.003
	P	-	-	-	-	-	-	-	-	0.180	0.142	**0.025**	0.862	0.978
	df								0	83	83	83	83	83
Δ_{0-16}Calcium	*R*									1	0.512	0.413	0.431	0.052
	P	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.636
	df									0	83	83	83	83
Δ_{0-16}Magnesium	*R*										1	0.403	0.497	0.085
	P	-	-	-	-	-	-	-	-	-	-	**0.0001**	**<0.0001**	0.438
	df										0	83	83	83
Δ_{0-16}Selenium	*R*											1	0.438	0.184
	P	-	-	-	-	-	-	-	-	-	-	-	**<0.0001**	0.091
	df											0	83	83
Δ_{0-16}Zinc	*R*												1	0.161
	P	-	-	-	-	-	-	-	-	-	-	-	-	0.142
	df												0	83
Δ_{0-16}Vitamin D	*R*													1
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
	df													0

Partial correlation with adjustment for individual age, gender, BMI, maternal age and paternal age
R, Pearson correlation coefficient
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00064

5.3.6 Linear regression

Maternal age was excluded from modelling in favour of paternal age only as maternal and paternal age correlate strongly with each other, indicating multicollinearity and violating this assumption of linear regression (Pearson $R = 0.83$, $P = 7.8 \times 10^{-55}$; Table 5.10).

Initial modelling aimed to determine which demographic measures could explain the most variance in telomere length at week 16. The model incorporating age, gender, BMI, paternal age, phase 2 treatment allocation and telomere length at week 0 explains 51% of the variance ($R^2 = 0.51$) in telomere length at week 16 (Table 5.14) and is statistically significant (ANOVA $P = 3.2 \times 10^{-27}$). The strongest predictors in this model are telomere length at week 0, individually accounting for 44% of the total 51% variance explained by the model in addition to gender and BMI, which both individually account for 1% of the total variance. Each of these three dependent variables – telomere length at week 0, gender and BMI – were statistically significant in the variance of the outcome variable – telomere length at week 16 – they explained. In assessing model fit, there were two individual cases (1% of 209) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (99%).

Table 5.14 – Multivariate linear regression to explain telomere length at week 16, for all participants

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	2.53	0.332				
Age[#]	-0.14	0.11	-0.07	0.20	-0.07	0.44
Gender	-0.04	0.02	-0.11	**0.03**	-0.11	1.28
BMI[#]	-0.23	0.12	-0.02	**0.05**	-0.10	1.02
Paternal age[#]	0.14	0.09	0.08	0.13	0.08	0.58
Phase 2 treatment group	-0.03	0.02	-0.08	0.14	-0.08	0.56
TL at week 0[#]	0.59	0.05	0.68	**<0.001**[a]	0.66	44.1

[#] *log transformed data*
[a] *$P = 2.6 \times 10^{-28}$*
$R^2 = 0.51$
Adjusted $R^2 = 0.50$
Model: $\text{log telomere length week } 16_i = b_0 + b_1\text{log age}_i + b_2\text{gender}_i + b_3\text{log BMI}_i + b_4\text{log paternal age}_i + b_5\text{phase two treatment}_i + b_6\text{log telomere length at week } 0_i$
Model: $\text{log telomere length week } 16_i = 2.53 + (-0.14\text{log age}_i) + (-0.04\text{gender}_i) + (-0.23\text{log BMI}_i) + (0.14\text{log paternal age}_i) + (-0.03\text{phase two treatment}_i) + (0.59\text{log telomere length at week } 0_i)$

Subsequent linear regression modelling was performed individually on the treatment groups, whether active *Polypill* supplementation or placebo control.

In *Polypill* supplementers, the model incorporating age, gender, BMI, paternal age and telomere length at week 0 explains 62% of the variance (R^2 = 0.62) in telomere length at week 16 (Table 5.15) and is statistically significant (ANOVA $P = 8.0 \times 10^{-18}$). The strongest predictor in this model was telomere length at week 0, significantly individually accounting for 59% of the total 62% variance explained by the model ($P = 2.6 \times 10^{-20}$). No other dependent variables significantly explained any variance in the independent variable, telomere length at week 16. In assessing model fit, there was one individual case (1% of 97) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (99%).

Among the participants randomised to the placebo control group, the same model incorporating age, gender, BMI, paternal age and telomere length at week 0 explains 41% of the variance (R^2 = 0.41) in telomere length at week 16 (Table 5.16) and is statistically significant (ANOVA $P = 1.5 \times 10^{-9}$). The strongest predictor in this model is telomere length at week 0, significantly individually accounting for 25% of the total 41% variance explained by the model ($P = 9.4 \times 10^{-9}$). Unlike in the *Polypill* supplementers, paternal age significantly accounted for approximately 3% of the variance in telomere length at week 16 (P = 0.04). In assessing model fit, there was one individual case (1% of 99) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (99%).

Table 5.15 – Multivariate linear regression to explain telomere length at week 16, for *Polypill* group

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	1.67	0.44				
Age[#]	-0.02	0.14	-0.01	0.88	-0.02	0.01
Gender	-0.03	0.02	-0.09	0.20	-0.135	0.71
BMI[#]	-0.13	0.14	-0.06	0.36	-0.10	0.35
Paternal age[#]	0.04	0.12	0.02	0.77	0.03	0.04
TL at week 0[#]	0.70	0.06	0.78	**<0.001[a]**	0.78	59.3

[#] *log transformed data*
[a] *2.6×10^{-20}*
$R^2 = 0.62$
Adjusted $R^2 = 0.60$
Model: $\log \text{telomere length week } 16_i = b_0 + b_1 \log \text{age}_i + b_2 \text{gender}_i + b_3 \log \text{BMI}_i + b_4 \log \text{paternal age}_i + b_5 \log \text{telomere length at week } 0_i$
Model: $\log \text{telomere length week } 16_i = 1.67 + (-0.02 \log \text{age}_i) + (-0.03 \text{gender}_i) + (-0.13 \log \text{BMI}_i) + (0.04 \log \text{paternal age}_i) + (0.70 \log \text{telomere length at week } 0_i)$

Table 5.16 – Multivariate linear regression to explain telomere length at week 16, for placebo group

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	3.07	0.51				
Age[#]	-0.31	0.18	-0.15	0.08	-0.18	2.04
Gender	-0.04	0.03	-0.12	0.16	-0.15	1.30
BMI[#]	-0.24	0.18	-0.12	0.17	-0.14	1.21
Paternal age[#]	0.33	0.16	0.18	**0.04**	0.22	2.86
TL at week 0[#]	0.47	0.07	0.52	**<0.001[a]**	0.56	25.3

[#] log transformed data
[a] 9.4×10^{-9}
$R^2 = 0.41$
Adjusted $R^2 = 0.38$
Model: $\log \text{telomere length week } 16_i = b_0 + b_1 \log \text{age}_i + b_2 \text{gender}_i + b_3 \log \text{BMI}_i + b_4 \log \text{paternal age}_i + b_5 \log \text{telomere length at week } 0_i$
Model: $\log \text{telomere length week } 16_i = 3.07 + (-0.31 \log \text{age}_i) + (-0.04 \text{gender}_i) + (-0.24 \log \text{BMI}_i) + (0.33 \log \text{paternal age}_i) + (0.47 \log \text{telomere length at week } 0_i)$

The subsequent model aimed to investigate whether the change in the 11 measured plasma micronutrients from week 0 to 16 ($\Delta_{0\text{-}16}$) could explain telomere length in PBMCs at week 16.

In *Polypill* supplementers, the $\Delta_{0\text{-}16}$ measured micronutrients were found to explain 5% of the variance in telomere length at week 16 (Table 5.17). However, this model did not fit the data significantly (ANOVA $P = 0.96$). The largest contributor in the model was $\Delta_{0\text{-}16}$ retinol which accounted for under 3% of the variance, though this was not statistically significant ($P = 0.13$). In this model there was one individual case (1% of 97) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed acceptably (99%).

In the placebo control group, the equivalent $\Delta_{0\text{-}16}$ measured micronutrients model was found to explain 15% of the variance in telomere length at week 16 (Table 5.18). However, this model did not fit the data significantly (ANOVA $P = 0.19$). The largest contributors in the model included $\Delta_{0\text{-}16}$ zinc which accounted for 6% of the variance ($P = 0.01$), $\Delta_{0\text{-}16}$ niacin which accounted for 5% of the variance ($P = 0.02$), and $\Delta_{0\text{-}16}$ homocysteine which accounted for 3% of the variance ($P = 0.07$). In this model there was one individual case (1% of 101) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed acceptably (99%).

Table 5.17 – Multivariate linear regression to explain telomere length at week 16 with change in micronutrients from week 0 to week 16, for *Polypill* group

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	5.01	0.04				
$\Delta_{0\text{-}16}$Folate	-0.001	0.003	-0.05	0.70	-0.04	0.16
$\Delta_{0\text{-}16}$Homocysteine	0.005	0.02	0.03	0.81	0.03	0.07
$\Delta_{0\text{-}16}$Vitamin B_{12}	0.00003	0.000	0.02	0.84	0.02	0.02
$\Delta_{0\text{-}16}$Alpha-tocopherol	-0.006	0.005	-0.16	0.22	-0.13	1.69
$\Delta_{0\text{-}16}$Retinol	0.11	0.07	0.19	0.13	0.17	2.66
$\Delta_{0\text{-}16}$Niacin number	0.001	0.002	0.03	0.78	0.03	0.09
$\Delta_{0\text{-}16}$Calcium	-0.22	0.24	-0.17	0.37	-0.10	0.90
$\Delta_{0\text{-}16}$Magnesium	0.000	0.001	0.12	0.52	0.07	0.46
$\Delta_{0\text{-}16}$Selenium	0.003	0.19	0.002	0.99	0.002	0.0004
$\Delta_{0\text{-}16}$Zinc	-0.007	0.02	-0.05	0.72	-0.04	0.15
$\Delta_{0\text{-}16}$Vitamin D	-0.00006	0.001	-0.007	0.95	-0.006	0.004

$R^2 = 0.05$

Adjusted $R^2 = -0.08$

Model: $\log \text{telomere length week } 16_i = b_0 + b_1\Delta_{0\text{-}16}\text{Folate}_i + b_2\Delta_{0\text{-}16}\text{Homocysteine}_i + b_3\Delta_{0\text{-}16}\text{Vitamin } B_{12i} + b_4\Delta_{0\text{-}16}\text{Alpha-tocopherol}_i + b_5\Delta_{0\text{-}16}\text{Retinol}_i + b_6\Delta_{0\text{-}16}\text{Niacin number}_i + b_7\Delta_{0\text{-}16}\text{Calcium}_i + b_8\Delta_{0\text{-}16}\text{Magnesium}_i + b_9\Delta_{0\text{-}16}\text{Selenium}_i + b_{10}\Delta_{0\text{-}16}\text{Zinc}_i + b_{11}\Delta_{0\text{-}16}\text{Vitamin } D_i$

Model: $\log \text{telomere length week } 16_i = 5.01 + (-0.001\Delta_{0\text{-}16}\text{Folate}_i) + (0.005\Delta_{0\text{-}16}\text{Homocysteine}_i) + (0.00003\Delta_{0\text{-}16}\text{Vitamin } B_{12i}) + (-0.006\Delta_{0\text{-}16}\text{Alpha-tocopherol}_i) + (0.11\Delta_{0\text{-}16}\text{Retinol}_i) + (0.001\Delta_{0\text{-}16}\text{Niacin number}_i) + (-0.22\Delta_{0\text{-}16}\text{Calcium}_i) + (0.000\Delta_{0\text{-}16}\text{Magnesium}_i) + (0.003\Delta_{0\text{-}16}\text{Selenium}_i) + (-0.007\Delta_{0\text{-}16}\text{Zinc}_i) + (-0.00006\Delta_{0\text{-}16}\text{Vitamin } D_i)$

Table 5.18 – Multivariate linear regression to explain telomere length at week 16 with change in micronutrients from week 0 to week 16, for placebo group

VARIABLE	*B*	*SE B*	β	*P*	SEMI PARTIAL CORRELATION COEFFICIENTS	% VARANCE EXPLAINED
Constant	5.02	0.03				
$\Delta_{0\text{-}16}$Folate	0.00008	0.003	0.003	0.98	0.003	0.0009
$\Delta_{0\text{-}16}$Homocysteine	-0.03	0.01	-0.20	0.07	-0.19	3.17
$\Delta_{0\text{-}16}$Vitamin B_{12}	0.000	0.000	-0.05	0.67	-0.05	0.18
$\Delta_{0\text{-}16}$Alpha-tocopherol	0.004	0.005	0.10	0.40	0.09	0.71
$\Delta_{0\text{-}16}$Retinol	-0.02	0.06	-0.03	0.77	-0.03	0.08
$\Delta_{0\text{-}16}$Niacin number	-0.004	0.002	-0.25	**0.02**	-0.24	5.34
$\Delta_{0\text{-}16}$Calcium	-0.11	0.21	-0.07	0.61	-0.05	0.25
$\Delta_{0\text{-}16}$Magnesium	-0.001	0.000	-0.16	0.21	-0.13	1.54
$\Delta_{0\text{-}16}$Selenium	-0.14	0.15	-0.11	0.36	-0.10	0.79
$\Delta_{0\text{-}16}$Zinc	0.03	0.01	0.32	**0.01**	0.26	6.15
$\Delta_{0\text{-}16}$Vitamin D	0.000	0.001	-0.05	0.65	-0.05	0.20

$R^2 = 0.15$
Adjusted $R^2 = 0.04$

Model: $log\ telomere\ length\ week\ 16_i = b_0 + b_1\Delta_{0\text{-}16}Folate_i + b_2\Delta_{0\text{-}16}Homocysteine_i + b_3\Delta_{0\text{-}16}Vitamin\ B_{12i} + b_4\Delta_{0\text{-}16}Alpha\text{-}tocopherol_i + b_5\Delta_{0\text{-}16}Retinol_i + b_6\Delta_{0\text{-}16}Niacin\ number_i + b_7\Delta_{0\text{-}16}Calcium_i + b_8\Delta_{0\text{-}16}Magnesium_i + b_9\Delta_{0\text{-}16}Selenium_i + b_{10}\Delta_{0\text{-}16}Zinc_i + b_{11}\Delta_{0\text{-}16}Vitamin\ D_i$

Model: $log\ telomere\ length\ week\ 16_i = 5.02 + (0.00008\Delta_{0\text{-}16}Folate_i) + (-0.03\Delta_{0\text{-}16}Homocysteine_i) + (0.000\Delta_{0\text{-}16}Vitamin\ B_{12i}) + (0.004\Delta_{0\text{-}16}Alpha\text{-}tocopherol_i) + (-0.02\Delta_{0\text{-}16}Retinol_i) + (-0.004\Delta_{0\text{-}16}Niacin\ number_i) + (-0.11\Delta_{0\text{-}16}Calcium_i) + (-0.001\Delta_{0\text{-}16}Magnesium_i) + (-0.14\Delta_{0\text{-}16}Selenium_i) + (0.03\Delta_{0\text{-}16}Zinc_i) + (0.000\Delta_{0\text{-}16}Vitamin\ D_i)$

The independent variables from these two models were combined to assess whether the incorporating the change in plasma micronutrients could improve upon the 60% and 40% explained variance for week 16 telomere length, for *Polypill* and placebo groups, respectively, which was obtained for in modelling with age, gender, BMI, paternal age and telomere length at week 0.

When the independent variables from these two regression models (Table 5.15 and Table 5.17) were combined in the same regression model, 66% of the variance in telomere length at week 16 for *Polypill* supplementers was explained ($R^2 = 0.66$, adjusted $R^2 = 0.59$, ANOVA combined model $P = 1.1 \times 10^{-12}$, data not shown). Significant predictors in this combined model were telomere length at week 0, explaining 59.4% of the 66% total variance ($P = 5.0 \times 10^{-19}$) and $\Delta_{0\text{-}16}$ niacin number, which accounted for 1.8% of the variance in the model ($P = 0.04$). An approximately 6% improvement upon the explained variance in model one was achieved by incorporating the change in micronutrient levels with age, gender, BMI, paternal age and telomere length at week 0. There was one individual case (1% of 96 cases) with standardised residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (99%).

Model: $\text{log telomere length week } 16_i = b_0 + b_1\text{log age}_i + b_2\text{gender}_i + b_3\text{log BMI}_i + b_4\text{log paternal age}_i + b_5\text{log telomere length at week } 0_i + b_6\Delta_{0\text{-}16}\text{Folate}_i + b_7\Delta_{0\text{-}16}\text{Homocysteine}_i + b_8\Delta_{0\text{-}16}\text{Vitamin B}_{12i} + b_9\Delta_{0\text{-}16}\text{Alpha-tocopherol}_i + b_{10}\Delta_{0\text{-}16}\text{Retinol}_i + b_{11}\Delta_{0\text{-}16}\text{Niacin number}_i + b_{12}\Delta_{0\text{-}16}\text{Calcium}_i + b_{13}\Delta_{0\text{-}16}\text{Magnesium}_i + b_{14}\Delta_{0\text{-}16}\text{Selenium}_i + b_{10}\Delta_{0\text{-}16}\text{Zinc}_i + b_{15}\Delta_{0\text{-}16}\text{Vitamin D}_i$

Model: $\text{log telomere length week } 16_i = 1.60 + (0.006\text{log age}_i) + (-0.02\text{gender}_i) + (-0.16\text{log BMI}_i) + (0.28\text{log paternal age}_i) + (0.72\text{log telomere length at week } 0_i) + (-0.001\Delta_{0\text{-}16}\text{Folate}_i) + (0.002\Delta_{0\text{-}16}\text{Homocysteine}_i) + (0.000003\Delta_{0\text{-}16}\text{Vitamin B}_{12i}) + (-0.005\Delta_{0\text{-}16}\text{Alpha-tocopherol}_i) + (0.06\Delta_{0\text{-}16}\text{Retinol}_i) + (-0.003\Delta_{0\text{-}16}\text{Niacin number}_i) + (-0.17\Delta_{0\text{-}16}\text{Calcium}_i) + (-0.00008\Delta_{0\text{-}16}\text{Magnesium}_i) + (0.04\Delta_{0\text{-}16}\text{Selenium}_i) + (0.006\Delta_{0\text{-}16}\text{Zinc}_i) + (0.000\Delta_{0\text{-}16}\text{Vitamin D}_i)$

When the independent variables from these two regression models (Table 5.16 and Table 5.18) were combined in the same regression model, 50% of the variance in telomere length at week 16 in the placebo control group was explained ($R^2 = 0.50$, adjusted $R^2 = 0.40$, ANOVA combined model $P = 4.0 \times 10^{-7}$, data not shown). Significant predictors in this combined model were telomere length at baseline was the strongest predictor individually explaining 25% of the 50% total variance ($P = 1.0 \times 10^{-8}$) and change in zinc (5.2%, $P = 0.005$). An approximately 9% improvement upon the explained variance in model one was achieved by incorporating the change in micronutrient levels with age, gender, BMI, paternal age and telomere length at baseline. There was one individual case (1% of 99 cases) with residuals beyond ± 3.0 however the remainder of the cases conformed to the model acceptably (99%).

Model: $\text{log telomere length week } 16_i = b_0 + b_1\text{log age}_i + b_2\text{gender}_i + b_3\text{log BMI}_i + b_4\text{log paternal age}_i + b_5\text{log telomere length at week } 0_i + b_6\Delta_{0\text{-}16}\text{Folate}_i + b_7\Delta_{0\text{-}16}\text{Homocysteine}_i + b_8\Delta_{0\text{-}16}\text{Vitamin B}_{12i} + b_9\Delta_{0\text{-}16}\text{Alpha-tocopherol}_i + b_{10}\Delta_{0\text{-}16}\text{Retinol}_i + b_{11}\Delta_{0\text{-}16}\text{Niacin number}_i + b_{12}\Delta_{0\text{-}16}\text{Calcium}_i + b_{13}\Delta_{0\text{-}16}\text{Magnesium}_i + b_{14}\Delta_{0\text{-}16}\text{Selenium}_i + b_{10}\Delta_{0\text{-}16}\text{Zinc}_i + b_{15}\Delta_{0\text{-}16}\text{Vitamin D}_i$

Model: $\text{log telomere length week } 16_i = 2.76 + (-0.20\text{log age}_i) + (-0.05\text{gender}_i) + (-0.19\text{log BMI}_i) + (0.27\text{log paternal age}_i) + (0.51\text{log telomere length at week } 0_i) + (0.000\Delta_{0\text{-}16}\text{Folate}_i) + (-0.01\Delta_{0\text{-}16}\text{Homocysteine}_i) + (0.000\Delta_{0\text{-}16}\text{Vitamin B}_{12i}) + (0.002\Delta_{0\text{-}16}\text{Alpha-tocopherol}_i) + (0.004\Delta_{0\text{-}16}\text{Retinol}_i) + (-0.002\Delta_{0\text{-}16}\text{Niacin number}_i) + (0.31\Delta_{0\text{-}16}\text{Calcium}_i) + (0.000\Delta_{0\text{-}16}\text{Magnesium}_i) + (-0.17\Delta_{0\text{-}16}\text{Selenium}_i) + (0.03\Delta_{0\text{-}16}\text{Zinc}_i) + (0.001\Delta_{0\text{-}16}\text{Vitamin D}_i)$

5.4 Discussion

5.4.1 The *Polypill* study was a successful double-blinded RCT

This study followed a double-blinded, placebo-controlled, randomised trial design. A great strength of randomised controlled trials (RCTs) is that they balance participants between treatment groups in an effort to reduce the effects of both identified and unidentified confounders (Kestenbaum *et al.*, 2009). Potentially confounding demographic and anthropometric factors were assessed by randomised treatment group to ensure that the randomisation of individuals to groups on the basis of age and gender was comparable for other measures. The *Polypill* treatment group and the placebo control group were balanced for age, anthropometric and other data as seen in Table 5.3 with the exclusion of maternal age at birth in which a significant difference between the two groups was detected with a younger age at birth for those in the placebo control group. At week 0, there was no significant difference in plasma micronutrients measured (Appendix Table 10.7). This is perhaps the effect of randomisation of diet and lifestyle factors which could affect levels of these micronutrient levels in the two groups. This may also reflect randomisation of other exposures such as alcohol, smoking, and/or metabolic, genetic, physiologic, or pathophysiologic processes which are also known to influence plasma biomarkers of nutrient intake (Giovannucci, 2013). This indicates that the randomisation worked well as even though diet or other demographic/anthropometric data were not included in the randomisation protocol, there were no differences between the two groups, reducing the influence of these factors on the main outcome measures.

5.4.2 *Polypill* supplementation increased plasma micronutrients and decreased homocysteine

Plasma levels of micronutrients in the *Polypill* were compared from week 0 to week 16 in the treatment group to assess the efficacy of the supplement in increasing plasma levels of these micronutrients. There were significant increases in folate, vitamin B_{12}, α-tocopherol, retinol and niacin number from week 0 to week 16 in the *Polypill* supplementers (Table 5.4). A significant decrease in plasma homocysteine from week 0 to 16 was also observed in *Polypill* supplementers. A statistically significant decrease in calcium from 2.3 mmol/L in week 0 to 2.2 mmol/L in week 16

was observed in the *Polypill* treatment group. This decrease in plasma calcium was unexpected as these individuals were supplemented with 500 mg of calcium daily, as per the *Polypill* formulation. Increases in other *Polypill* plasma micronutrients, daily supplement checklists and the number of supplements returned at the end of the study indicate that the active *Polypill* treatment group were compliant in taking their three *Polypill* supplements daily as instructed and hence that this result was not caused through non-compliance. As calcium is tightly regulated within the body, it is likely that this change, while statistically significant, may not be biologically significant. However a basis for the decrease in calcium over the 16-week supplementation period was sought, considering factors such as season and plasma vitamin D concentration. The season of collection was largely matched for participants in each group (Appendix Table 10.13) with the bulk of blood sample collections at week 16 taking place in winter, compared to summer/autumn measurements from week 0. As such, there was a reduction in plasma vitamin D in all participants from week 0 to week 16 (Appendix Table 10.7) however it appeared that there was a slightly greater decrease in plasma vitamin D in the *Polypill* supplementers (Appendix Table 10.6). As vitamin D is known to influence calcium uptake, it is thought the greater decrease in vitamin D may have been responsible for the statistically significant, but not biologically significant decrease in plasma calcium of 100 µmol/L in the *Polypill* group (Appendix Table 10.7). As no instruction was given to participants regarding whether they should consume their supplements with or without food, it is possible that absorption and uptake of the *Polypill* micronutrients may not have been uniform in the active treatment group and it is unknown what effect this may have had on plasma micronutrient status in the *Polypill* group.

In addition to *Polypill* micronutrients, homocysteine and vitamin D, there was a significant change in selenium between the two groups, which may be a reflection of changes in dietary intake of selenium-rich foods, however this finding was not further explored. It appeared there was a greater reduction in plasma selenium amongst those in the *Polypill* supplement group compared to the placebo control group. Plasma selenium did not appear to vary with season in this cohort (Appendix Table 10.13 and Table 10.14) but it is possible that seasonal variation in diet could affect plasma levels of selenium, as well as other micronutrients.

Meta-analyses of studies involving antioxidant supplements (e.g. β-carotene, vitamin A, vitamin C, vitamin E, and selenium) have shown that β-carotene and vitamins E and A may increase mortality and that there is no significant detrimental effect of vitamin C or selenium nor evidence to support the use of antioxidant supplements for primary or secondary prevention (Bjelakovic *et al.*, 2007, Bjelakovic *et al.*, 2008, Bjelakovic *et al.*, 2012). However, in a large double-blind placebo-controlled randomised trial in men with a median follow up time of 11.2 y, multivitamins were shown to significantly reduce the incidence of total cancer when compared to placebo treatment (Gaziano *et al.*, 2012). In these same men, there appeared to be no evidence of an effect on cardiovascular events, myocardial infarction, non-fatal stroke, and CVD mortality (Sesso *et al.*, 2012). This suggests that the effect of multivitamin use on some disease and mortality may be differential and that supplementation of some micronutrients in those who are not deficient does not provide benefit, and moreover can instead be harmful. The *Polypill* supplementation dosage is based on previous studies where these levels of micronutrients were associated with chromosomal DNA damage reduction (Table 3.6) (Fenech *et al.*, 1998, Fenech *et al.*, 2005). These micronutrient doses do not exceed the RDI, with the exception of B_{12} which has no suggested upper limit of intake and no evidence of toxic effects at the dose used in the *Polypill* (Table 3.7).

5.4.3 Telomere length change over time was used to determine telomere length trajectory

There was no difference in mean telomere length at week 0 between the two randomised treatment groups, and after 16 weeks of *Polypill* or placebo treatment, there was no significant difference in telomere length between the groups (Table 5.5). Within each group, the mean change in telomere length over the 16-week period was small; in *Polypill* supplementers there was a mean loss of 3.9 kb and in the placebo control group there was a mean gain of 1.3 kb however these changes were not significantly different within or between groups (Figure 5.2).

Due to the inherent inter-individual variation in telomere length observed in human studies, and as these data were longitudinal in nature, telomere length changes were determined to be one of three trajectories. Either there was an increase in telomere length in the individual, a decrease in telomere length or maintenance of

telomere length over the 16 weeks. Such a trajectory analysis is based on that reported by Farzaneh-Far *et al.* (2010a) where >10% decrease was characterised as a telomere length loss trajectory, >10% increase was considered telomere length gain trajectory and where ± 10% is considered telomere length maintenance. The distribution and scale of telomere length trajectories in the entire cohort (n = 199) is seen in Figure 5.3.

The distribution of these telomere length trajectory groups were compared across the two randomised treatment groups and was not significantly different (P = 0.63; Table 5.6 and Figure 5.4). A number of covariates were assessed for differences across the telomere length trajectory groups in an attempt to characterise factors that may have affected change in telomere length. It was determined that age, gender, BMI, obesity, maternal age and paternal age was not significantly different across the trajectory groups, in either treatment group. Interestingly, there was a significant difference in telomere length at week 0 where those in the telomere shortened trajectory group had the longest telomeres at week 0 and conversely those in the telomere lengthened trajectory group had the shortest telomeres at week 0.

Farzaneh-Far *et al.* (2010a), whose study provided the source of the telomere length trajectory analysis employed here, showed after a five year follow-up period that there was telomere length shortening in 45%, maintenance in 32% and telomere lengthening in 23%. In the present study, telomere length shortening was detected in 32% of the cohort, with maintenance in 32% and telomere lengthening in 36% (Appendix Table 10.5). When these trajectories were compared between treatment groups the observed prevalence of telomere shortening was 25% in the *Polypill* group, with 32% maintenance and 34% lengthening. This is compared to 29% telomere shortening in the placebo group, with 32% maintenance and 39% lengthening (Table 5.6). The Farzaneh-Far *et al.* study of 608 individuals measured telomere length after a 5-year follow-up, and there are some similarities in the trajectory groups between this study and the present study. The prevalence of telomere maintenance was 32% for both studies, and the higher prevalence of telomere lengthening in the current study may have been influenced by the short follow-up time – 16 weeks versus 5 years – which perhaps reduced the influence of

age-related telomere shortening in the short-term. In another longitudinal study of telomere length with a ten year follow up period, the prevalence of telomere length stability (maintenance) or increase was 34% (Nordfjall *et al.*, 2009). Telomere length maintenance and elongation over time has been detected in subgroups of people in the absence of tumourigenesis (Aviv *et al.*, 2009, Nordfjall *et al.*, 2009), however the plausibility of telomere length elongation over years of follow-up has been criticized and such results have been suggested to be measurement artefacts (Chen *et al.*, 2011).

At least one study has identified phases of telomere shortening characterised by various rates of telomere loss (Frenck *et al.*, 1998). Here, telomere loss was shown to be rapid in early life, with stabilisation of telomere length between 5 years of age and young adulthood with a slower rate of telomere loss during later adulthood than in early life. As such, these defined phases of variable telomere loss rate may have impacted upon the telomere length trajectory analysis employed as the study included both young adults possibly placed in telomere length stabilisation phase and older adults experiencing sustained telomere loss. Additionally, while the PBMC samples used in the present study contain PBLs, they also contain neutrophils and monocytes and it is unknown whether the Frenck *et al.* (1998) -identified phases of telomere shortening in PBLs would be comparable to that in a more diverse sample of PBMC.

Previous findings that telomere attrition rate is inversely correlated with baseline telomere length ($R = -0.752$, $P < 0.001$) (Nordfjall *et al.*, 2009) is in agreement with these trajectory results which show that those who telomeres shorten, have the longest baseline telomere length. In addition, this study showed that the rate of age-associated telomere attrition was highly correlated with telomere length at a young age ($R = -0.691$, $P = 0.009$), further suggesting that baseline or inherited telomere length strongly influences telomere length trajectory throughout adulthood (Nordfjall *et al.*, 2009).

An increase in telomere length over the 16 weeks may have been brought about by an increase in telomerase expression or the ALT mechanism. Telomerase is known to preferentially elongate short telomeres in the cell and as such there may have

been a greater propensity for telomerase to increase the short baseline telomere length observed in the telomere lengthening subgroup.

Chronic infection with *Salmonella enterica* has been shown to cause telomere attrition in mice (Ilmonen *et al.*, 2008), but the effects of bacterial infection on human telomere length remains unknown bearing in mind the differences in telomere biology between the two species. Ageing induces a shift from naïve to memory T-cells, which have a shorter telomere length (Svenson *et al.*, 2011, Kiecolt-Glaser *et al.*, 2013). Peripheral T-lymphocytes possess varied levels of telomerase activity depending on whether they are resting or activated (Weng *et al.*, 1998). When activated, the telomerase activity rises in these T-cells in an effort to maintain telomere length. While correlated with cellular proliferation, telomerase activity is not restricted by cell cycle phase (Weng *et al.*, 1996). T-lymphocyte stimulation and subsequent proliferation may have caused decreases in telomere length measured from PBMC samples while the activation of telomerase in these cells to increase or maintain T-lymphocyte telomere length may have affected the telomere length results in PBMCs. Further, there is some evidence that micronutrients could modulate T-lymphocyte-mediated immune responses and cellular proliferation (for example selenium; Roy *et al.*, 1994) and this phenomenon may have confounded the present results. In order to capture cell population information in the future, one could measure percentages of cell types such as the strategy employed by McCracken *et al.* (2010) who measured percentages of neutrophils and lymphocytes in total leukocyte samples. Cell populations could be separated in order to compare relative changes in each type or sub-type over time.

5.4.3.1 There is potential for regression toward the mean in telomere length trajectory analyses

Regression toward the mean (RTM) is a common statistical phenomenon that can make natural variation in repeated data appear to be real change. RTM is most prevalent with increased measurement error and when study participants are selected for intervention and follow-up measures on the basis of an initial baseline measurement. In this study, there was no pre- or post-selection of individuals on the basis of their baseline telomere length or other biological measure. Telomere length was not measured in isolated PBMC samples until after the study had

concluded, while independent randomisation on the basis of gender and age was used to define and balance study intervention groups.

As RTM can occur in any measurement that is observed with error, where possible measurement error was minimised to reduce the effect of RTM. Briefly, DNA from longitudinal PBMC samples was extracted using one established and optimised extraction method in order to reduce variation in DNA quality. In addition, DNA extraction and qPCR for telomere length of all samples was performed by the same person with consistency across used equipment, reagents and consumables. Telomere length of PBMC samples from week 0 and week 16 were measured on the same PCR plate and each was so measured in triplicate in order to further minimise measurement error.

However, only one blood sample was taken at each visit and so the detected mean absolute telomere length from PBMC may differ from the actual mean absolute telomere length depending on blood volume and cellular populations therein. In future studies it would serve well to collect multiple samples over time, and to measure blood cell subpopulations in assessing whether individual telomere length may fluctuate over short and extended periods of follow-up.

In published longitudinal studies of telomere length, there is a strong inverse relationship between baseline telomere length and the telomere length change (Epel, 2012). Some have suggested that this association may be measurement error or RTM and have questioned the observation that telomeres lengthen and maintain more so than shorten over time (Chen *et al.*, 2011). A range of longitudinal studies report various percentages of individuals who were detected to have leukocyte telomere length increases in follow-up time ranging from 6 months to >10 years (Steenstrup *et al.*, 2013a). The change in telomere length over a shorter period of time, such as that in the present study of 16 weeks or <4 months follow-up, has not been described in the literature. In a study of 6 months duration, authors suggested an oscillation pattern of telomere length may be evident in white blood cells (Svenson *et al.*, 2011). This oscillation pattern, similar to that of a sinus curve, suggests dynamic changes in telomere length in the short term but a certain decline in telomere length over extended periods of time. It may be that telomere length maintenance and lengthening trajectories identified in the present study may

represent the short term dynamic changes or fluctuations described in this oscillation model.

Telomere lengthening has appeared to be dependent on telomere length (Nordfjall *et al.*, 2009, Farzaneh-Far *et al.*, 2010a), whereby shorter telomeres are preferentially increased, fitting with a previous mathematical model confirming the length-dependency of telomere shortening (op den Buijs *et al.*, 2004). This is in consonance with the preferential action of human telomerase to increase telomere length of the shortest telomeres (Ouellette *et al.*, 2000, Steinert *et al.*, 2000, Teixeira *et al.*, 2004). Telomere shortening trajectories may be defined by the absence of telomerase or ALT to increase telomere length, as well as the decline of telomere length over time with cellular division and ageing. A telomere length maintenance trajectory may result from the balance of telomere lengthening mechanisms, such as telomerase and ALT, with age-related telomere shortening and other factors known to influence telomere attrition, such as stress. Even so, it is of fundamental importance to determine the mechanisms of telomere length homeostasis and other trajectories *in vivo* to better understand and interpret the results from longitudinal studies of telomere length.

Due to the fact that there was low measurement error with the telomere length assay used, that similar results have been identified in independent longitudinal study populations and that the mechanism of telomerase can explain short-term telomere length increases and lengthening trajectory, RTM is unlikely to exclusively account for telomere length trajectories determined in this study.

5.4.4 Telomere length trajectory is associated with change in plasma zinc

There were no significant differences in the changes of many micronutrients across telomere length trajectory groups in both treatment groups (Table 5.7). Indeed the only significant difference was observed in the change in plasma levels of zinc in the placebo group only, where there was a greater reduction in plasma zinc from week 0 to week 16 in the telomere length shortened trajectory. This change in plasma zinc was significantly different to the change in zinc observed in the maintenance ($P = 0.024$) and gain ($P = 0.002$) trajectories. The change in plasma zinc in maintenance and gain trajectories were not significantly different from each other

($P = 1.0$), however there was a trend in the data which showed greater reduction in change of plasma zinc from shortened to maintained trajectories and an increase in plasma zinc among the gain trajectory group.

In vitro experimentation with various trace elements including zinc has revealed differential effects of these elements on telomere length in human hepatocyte and hepatoma cell lines (Liu *et al.*, 2004). A concentration of 40 µmol/L zinc sulphate over 4 weeks of culture caused an increase in telomere fluorescence in human hepatocyte cells, but a significant decrease in fluorescence in hepatoma cells when compared to 0 µmol/L zinc sulphate (Liu *et al.*, 2004). The decrease in telomere signal in hepatoma cells may have reflected the prevention of growth by zinc in these transformed cells (Liu *et al.*, 2004) while the association of increased zinc with increased telomeres in human hepatocytes, though not significant, mirrors the result observed in telomere length trajectory groups.

In another *in vitro* study, WIL2-NS cells acquired a 4-fold increase in FPG-sensitive sites within the telomeric DNA under zinc deficiency challenge (0 µM) compared to 4 µM zinc (O'Callaghan *et al.*, 2011, Sharif, 2012). The FPG enzyme (formamidopyridine DNA glycosylase) possesses both N-glycosylase and AP-lyase activities and cleaves oxidised purines such as 8-oxoguanine (Tchou *et al.*, 1994). It is suggested that this increase in FPG-sensitive sites within the telomere under conditions of zinc deficiency is a result of increased oxidative stress damaging the guanine residues within the telomere (O'Callaghan *et al.*, 2011, Sharif *et al.*, 2011, Sharif, 2012), which is 25% guanine. The hypothesis that such guanine base damage could lead to telomere sequence deletion if DNA strand breaks are induced may explain why a reduction in zinc over time was coupled with telomere length shortening in the present study.

Zinc is required in the cell for the catalytic function of more than 100 enzymes (Sharif *et al.*, 2011), while the metallothioneins group of proteins modulate intracellular zinc homeostasis (Mocchegiani *et al.*, 2007). Although plasma levels of zinc were measured, there was no measure of intracellular zinc in PBMCs which has been shown to be positively associated with mean telomere length ($R = 0.502$, $P < 0.05$) as well as inversely associated with the percentage of short telomeres ($R = -0.456$, $P < 0.05$) less than 6 kb as measured by qFISH (Cipriano *et al.*, 2009).

DNA damage observed under *in vitro* zinc deficiency which causes low intracellular zinc, has been hypothesised to be the result of the combined effects of increased oxidative stress and impairment of DNA-repair pathways (Ho and Ames, 2002). However, as there exists an inverse correlation between plasma zinc and age (Mariani *et al.*, 2006), it is possible this detected association with zinc is representing the effect of ageing on telomere length, or may be due to zinc acting as a measure for other age-related change in biology or behaviour.

5.4.5 Correlation of week 16 telomere length with covariates differed with treatment group

Bivariate correlation of telomere length at week 16 was performed with age, maternal age, paternal age and BMI (as similarly performed with telomere length at week 0 in Table 4.7) however the change in telomere length from week 0 to 16 was also incorporated as a continuous variable. In the *Polypill* group, there were no significant associations of these measures with telomere length at week 16 (Table 5.10 and Appendix Figure 10.3), unlike that observed in Chapter 4 and in the placebo control group (Table 5.11 and Appendix Figure 10.4), where telomere length at week 16 was significantly associated with age ($R = -0.2$, $P = 0.044$), maternal age ($R = 0.2$, $P = 0.018$) and paternal age ($R = 0.3$, $P = 0.005$). This correlation result indicates that the 16-week *Polypill* treatment may have affected telomere length such that it was no longer significantly associated with parental age.

5.4.6 Telomere length at week 16 was correlated with change in plasma micronutrients

In the placebo group, the change in plasma calcium and magnesium from week 0 to week 16 was significantly associated with telomere length at week 16 (Table 5.9 and Appendix Figure 10.2). Both of these relationships were of a similar, weak magnitude, $R = -0.241$ for calcium ($P = 0.021$) and -0.262 for magnesium ($P = 0.012$). Magnesium and calcium were moderately correlated with each other, $R = 0.5$ ($P < 0.0001$). Following partial correlation analysis which controlled for measured telomere length covariates, there was no significant association of any change in plasma micronutrients with either telomere length at week 16 or change in telomere length within the *Polypill* group (Table 5.12). Corresponding analysis in the placebo control group revealed a significant association of change in plasma

calcium and magnesium from week 0 to week 16 with telomere length at week 16. These two relationships were negative whereby an increase in calcium or magnesium were negatively associated with telomere length at the end of the intervention (Table 5.13).

5.4.6.1 Increase in plasma calcium over time was negatively associated with telomere length at week 16

Although there is evidence of an influence on telomere length by diet and dietary nutrients (Paul, 2011), there is insufficient evidence for calcium intake. Briefly, calcium intake from self-reported FFQ has been significantly positively associated with telomere length in 586 women, with multivariate adjustment (Xu *et al.*, 2009). However the use of plasma calcium biochemistry as a marker of calcium nutrition status has previously shown unsatisfactory specificity and sensitivity (Seibel, 2005, Seibel, 2006, Wang *et al.*, 2013b).

Intake of dairy foods as determined by FFQ may have had a negative influence on telomere length in 56 men and women, however this finding from multivariate logistic analysis did not reach statistical significance ($P = 0.092$) (Marcon *et al.*, 2012). In mice, calcium intake has been shown to reduce inflammatory and oxidative stress (Bruckbauer and Zemel, 2009) while in humans, high intakes of dietary calcium have been associated with the lower chromosomal DNA damage (Fenech *et al.*, 2005). The negative association of a greater increase in calcium over 16 weeks with decreased telomere length at the end of the intervention was only detected in the placebo group. There were no significant observations of change in micronutrient level with telomere length in the *Polypill* group. Although the direction of the relationship is consistent in correlation analyses of each treatment group, the observation was not significant in the *Polypill* group, who were supplemented daily with 500 mg calcium. Therefore the association of changes in plasma calcium over time with telomere length should be further investigated in a larger cohort as the association may be significant under conditions with and without micronutrient supplementation or alternatively, the association may be modified by micronutrient supplement use.

5.4.6.2 Increase in plasma magnesium over time was negatively associated with telomere length at week 16

The binding of the telomerase reverse transcriptase enzyme to DNA is mediated by magnesium such that binding of telomerase to short DNAs is magnesium-dependent while binding of telomerase to long DNAs is magnesium-independent (Lue, 1999). In a situation where there is inadequate base-pairing of telomerase to DNA, magnesium may serve to mediate interaction required for successful binding (Lue, 1999). As magnesium may mediate binding of telomerase, the inverse association of increased magnesium with decreased telomere length in the present study is curious.

In addition to a possible involvement with telomerase, magnesium is known to stabilise DNA, through involvement in DNA replication and transcription and as cofactor or component of DNA repair enzymes together conferring magnesium requirement for genome stability (Hartwig, 2001). For this reason, it has been suggested that correction of magnesium deficiency through supplementation may prolong life (Rowe, 2012).

Just 1% of magnesium is found in serum, so where serum or plasma measures of magnesium may be in the normal range, or may change over time as observed here, the status of intra-erythrocyte magnesium, intracellular free magnesium or magnesium stored in bone and muscle is not known (Rowe, 2012). There is further evidence magnesium regulates cell-cycle and apoptosis and as intracellular magnesium is tightly regulated (Hartwig, 2001) perhaps intracellular magnesium would be a more appropriate measure of status than that in plasma.

That magnesium deficiency can induce cellular senescence (Ferre *et al.*, 2007, Killilea and Ames, 2008) could mean that maintenance of a low level of magnesium may have induced cell-cycle arrest, contrasting with those who had an increase in magnesium levels over time to give the appearance of a negative association of increased plasma magnesium with telomere length. This finding requires further investigation, beginning with cross-sectional studies and *in vitro* experimentation.

5.4.7 Week 0 telomere length significantly explained week 16 telomere length in regression models

Multivariate regression modelling of demographic factors as independent variables to explain the dependent variable telomere length at week 16 revealed the strongest factor associated with telomere length at week 16 was telomere length 16 weeks prior, at week 0 (Table 5.14). This independent variable solely explained 44% of the 50% variance explained by the model and was strongly significant. Other significant contributors in the model of the entire cohort were gender and BMI, which each individually explained 1% of the variance. A dichotomous indicator variable for treatment allocation was included in the model and was found to explain only 0.6% of the variance in a non-significant manner ($P = 0.14$). Instead of including treatment group allocation in the regression model, individual regression was performed with these same independent variables, but individually for the treatment groups. In the *Polypill* treatment group, telomere length at week 0 accounted for 59% of the total 62% variance explained in the model for telomere length at week 16 ($P = 2.6 \times 10^{-20}$, Table 5.15) compared to telomere length at week 0 which significantly accounted for 25% of the total 41% variance explained in the model for telomere length at week 16 for those in the placebo group ($P = 9.4 \times 10^{-9}$, Table 5.16). The potential for regression toward the mean effects in these longitudinal telomere length measures is previously discussed in Section 5.4.3.1.

5.4.8 Changes in plasma zinc and niacin explained change in telomere length in regression models

In modelling change in plasma micronutrients, there were no significant predictors of change in telomere length in the *Polypill* group (Table 5.17). However, when the same modelling was performed for the placebo group, there were two significant micronutrients whose change in plasma over time were predictive of change in telomere length over time (Table 5.18). As previously determined in trajectory analysis (Section 5.4.4), change in plasma zinc in the placebo group was positively associated with change in telomere length ($\beta = 0.32$, $P = 0.01$, explaining 6.15% variance). In addition, change in niacin number was associated negatively with change in telomere length ($\beta = -0.25$, $P = 0.02$, explaining 5.34% variance).

Niacin is a precursor for nicotinamide adenine dinucleotide (NAD) which is required for synthesis of DNA and for activity of poly(ADP-ribose) polymerase (PARP) family

of enzymes (Hageman and Stierum, 2001). PARP-1 is activated by DNA strand breaks, is a component of the base excision repair pathway (Hageman and Stierum, 2001). High intakes of nicotinic acid have been associated with lower frequencies of DNA damage as measured my micronuclei (Fenech *et al.*, 2005) and by reducing DNA damage and amongst other actions, niacin is essential for genome stability (Kirkland, 2012). Telomere length can be associated with niacin through PARPs tankyrase 1 and 2 which are telomere binding proteins that function to regulate telomere length and separation of sister chromatids (Hsiao and Smith, 2008). In diabetic mice, niacin has been shown to decrease reactive oxidative stress, apoptosis and senescence in endothelial progenitor cells (Huang *et al.*, 2012). In the present study, it appeared that positive change in niacin number was significantly negatively associated with telomere length. Evidence of a direct relationship between plasma niacin and telomere length in humans has not been demonstrated previously and so the possible influence of niacin on genomic stability and telomere length maintenance remains to be determined. Given the lack of a plausible explanation for this relationship, it is possible that this observation may have occurred by chance and requires further investigation.

5.4.9 Study limitations and power

In the present study, *a priori* power and sample size calculations for the telomere length outcome were not performed as the effect size of micronutrient supplementation on telomere length was unknown, and as telomere length was not the primary outcome measure in the *Polypill* study. Instead, *a priori* power calculations were conducted for the primary outcome measure – micronuclei – as measured by the cytokinesis block micronucleus assay, and as previously described in the *Polypill* study methods (Section 3.2.4).

As *post hoc* power calculations are not recommended in this situation, the confidence interval for the observed treatment effect is instead explored (Walters, 2009). The mean (± SD) change in telomere length for the *Polypill* group was -3.88 ± 32.8 kb/diploid genome (n = 98) while the corresponding change in the place group was +1.33 ± 39.5 kb/diploid genome (n = 101). The mean difference in telomere length between the two groups was -1.23 kb/diploid genome (95% CI; -6.32 kb to +3.85 kb, n = 199, P = 0.31). Although the minimum clinically important

difference in the change in telomere length of this intervention is unknown, the mean difference in the change in telomere length between the two groups (-1234 bp/diploid genome) corresponds to >100 y of ageing using the regression result from Section 4.4.4 (mean annual attrition of telomere length for all participants is -11.46 bp/diploid genome/y; 95% CI; -16.16 to +11.94 bp/diploid genome/y, $n = 212$, $P = 0.39$). Although the effect size appears large, the large width of the confidence intervals in both the regression estimate of annual telomere length attrition and in the mean difference of the change in telomere length between the two treatment groups in this sample indicate that the clinical relevance of this intervention is unknown. This study provides further knowledge and insight in to telomere length changes in a micronutrient intervention RCT, but there is a need to cautiously use the results from this study to inform future clinical interventions (Kraemer *et al.*, 2006).

5.5 Conclusion

The most significant factor explaining telomere length at week 16 or telomere length trajectory from week 0 to week 16 was week 0 baseline telomere length. In the *Polypill* group, regression modelling showed week 0 telomere length accounted for 59% of variation in week 16 telomere length, while in the placebo group week 0 telomere length accounted for 25% of the variation of telomere length at week 16.

5.5.1 Significance

This investigation may be the first such study investigating the effect of a mixed micronutrient supplement on telomere length in an RCT. In addition, this study has the shortest follow-up time between longitudinal telomere length measures (16 weeks) of any other study reported in the current literature. Longitudinal changes in calcium, magnesium, niacin and zinc over the 16 week intervention period were associated with telomere length, but only in the placebo group. This novel study provides the first evidence to suggest that changes in plasma vitamins and minerals may be associated with telomere length, or telomere length trajectory *in vivo,* but these effects were mainly seen in the placebo group. The null effect in the *Polypill* group suggests that any effects in the placebo group are likely due to dietary changes and that *Polypill* supplementation provides no benefit to telomere length in well-nourished individuals.

5.5.2 Strengths and weaknesses

As this intervention study was a RCT, there was effective randomisation for measured and unmeasured confounders of telomere length which may have been biological, nutritional, environmental, genetic, social or otherwise. There was an acceptable rate of both volunteer compliance (> 90% in both groups; Appendix Table 10.3) to the study protocol and retention throughout the trial (≈ 87% of those who commenced the washout period also completed this RCT phase; Appendix Table 10.2). The double-blind placebo-control design minimised bias in measurement of telomere length.

Considering that the size of the cohort was relatively small, the age range large, in addition to the inherently large inter-individual variation of telomere length collectively meant the small changes in telomere length which may have been

induced through micronutrient supplementation were not detected. The short length of time of both the intervention and collection of longitudinal samples for telomere length measurement perhaps restricted the detection of telomere length change, and indeed identification of longer-term telomere length trajectory. Furthermore, telomere length was a secondary outcome measure of the study and therefore the intervention was not optimally powered to detect small changes in telomere length.

5.5.3 Future directions

In future, a longer micronutrient intervention or a study of a greater number of participants may build upon these results. Certainly a study with a more refined range of participant age may remove the impact of variable telomere maintenance and subsequent age-related acceleration in telomere attrition during adulthood. Alternatively, targeted intervention of individuals with micronutrient deficiencies or those with short telomeres may be more informative of the potential of micronutrients to influence telomere length *in vivo*.

6 A pilot study to investigate the impact of a modified micronutrient *Polypill* on telomere length *in vivo* over time

6.1 Introduction

There are few longitudinal studies of telomere length in the current literature (Table 5.1) however they are increasing in recent years. Of these limited longitudinal studies, there are just two reports which have described more than one follow up or repeated measure over time (McCracken *et al.*, 2010, Chen *et al.*, 2011). Moreover, to date there are no reported nutritional interventions or RCTs to investigate the influence of diet on telomere length. In Chapter 5, the effect of a micronutrient *Polypill* on telomere length was assessed in a double-blinded randomised placebo-controlled trial. The *Polypill* study design included a following pilot phase to further interrogate whether removal of a single micronutrient from the original FBERNC *Polypill* formulation had a greater effect on telomere length and DNA damage.

6.1.1 Aims and hypotheses

The aim of this pilot phase of the trial was to determine the effects of the individual micronutrients on telomere length through excluding one component of the FBERNC *Polypill*. It was hypothesised that the *Polypill* formulations may have differential effects on telomere length change and trajectory over time, depending on which micronutrient was excluded.

6.2 Methods

Details of the study including ethics, supplement composition and blood sample processing is as described in Section 3.2. The human trial was completed in its entirety in the 2008 calendar year with week 16 samples collected from 20th May to 8th September; and week 32 samples collected from 11th August to 16th December.

There were three distinct phases of this human study (Figure 6.1). For 16 weeks to week 0, there was a washout period prohibiting any use of micronutrient supplements (Chapter 4). For 16 weeks from week 0 to week 16, participants were randomised to either the placebo control group or a *Polypill* micronutrient (Chapter 5). Finally for 16 weeks from week 16 to week 32, the participants were allocated to one of seven groups, either a placebo, or a modified *Polypill* supplement which contained only five of the original six (FBERNC) micronutrients with each of the six groups having a different micronutrient removed from the *Polypill*. This phase was included as a pilot investigation to assess whether the omission of one of the micronutrients may have impacted upon telomere length change in the group.

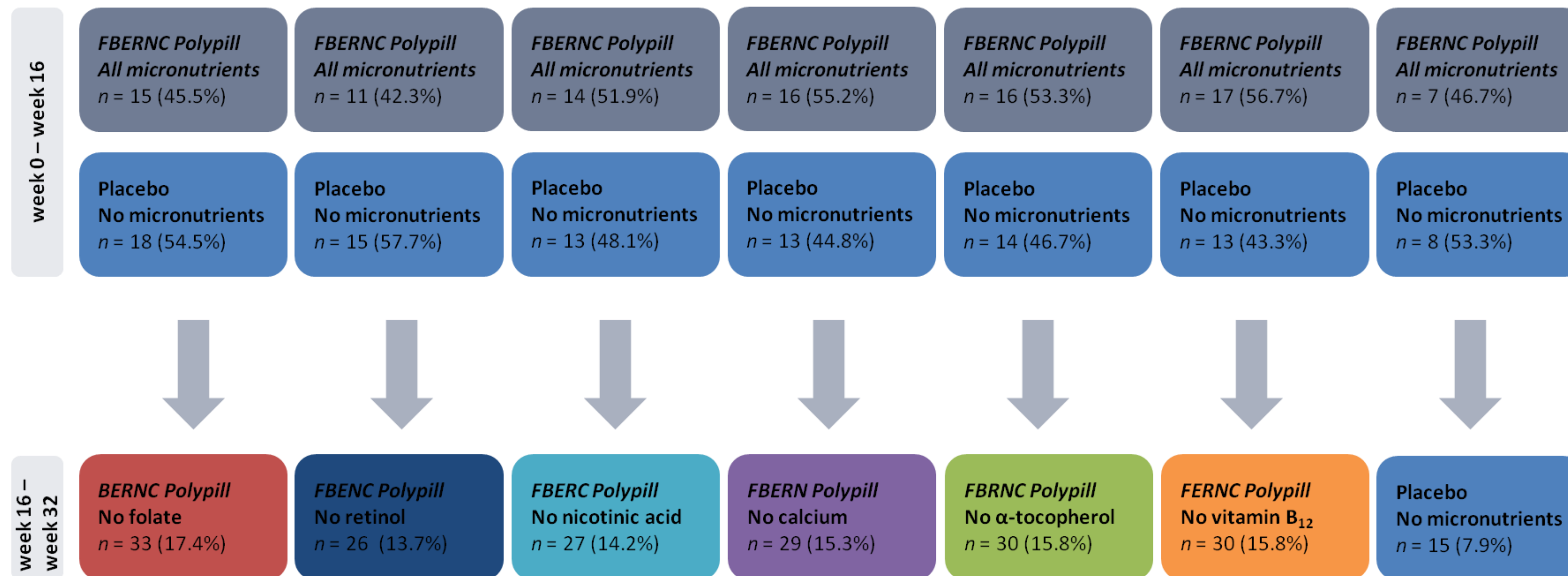

Figure 6.1 – Schematic of study detailing the six modified *Polypill* active treatments used in the second intervention

Abbreviations: F, folic acid; B, vitamin B_{12}; E, vitamin E; R, retinol; N, nicotinic acid; C, calcium.
Week 0 – week 16 indicates treatment group during initial FBERNC Polypill RCT intervention; numbers in parentheses indicate the distribution of individuals within the groups during this phase, by treatment group from week 16 – week 32;
Week 16 – week 32 indicates treatment group during this pilot investigation of a modified Polypill; numbers in parentheses indicate the proportion of individuals within treatment group by total number of participants who completed this phase (n = 190).

6.2.1 Missing samples

Telomere length was measured in isolated PBMCs as previously detailed (Section 3.1). Four individual week 16 PBMC samples for four subjects were not available for DNA isolation. Although 194 volunteers completed the study to supply a sample at week 32, there are only 190 week 16 – 32 telomere length dyads due to these missing samples from week 16.

6.2.2 Randomisation to treatment groups

Individuals were allocated to their treatment group in a listwise manner and as the study was double-blinded, this allocation was unknown to participants and other personnel until all trial data was collected.

6.2.3 Longitudinal-specific calculations and analyses

Change in telomere length was calculated using the below formula:

$$\Delta_{16-32}\, telomere\ length\ [\%] = \frac{week\ 32\ telomere\ length - week\ 16\ telomere\ length}{week\ 16\ telomere\ length} \times 100$$

Change in telomere length was regarded as telomere shortening (>10% loss), telomere maintenance (within ± 10% change) or telomere lengthening (>10% gain) as described in a previous study (Farzaneh-Far *et al.*, 2010a).

6.2.4 Statistical analyses

Results presented are typically reported as mean ± standard deviation, range (minimum observation – maximum observation) or number [% cases]. The threshold for statistical significance was set at $P < 0.05$ and these significant associations are highlighted in bold typeface throughout. Statistical tests were performed in IBM SPSS Statistics 20.0. Additional data is contained in the Appendix.

6.3 Results

6.3.1 Descriptives of the treatment population

In Chapters 4 and 5, it was shown that demographic and anthropometric indicators were associated with telomere length and telomere length trajectories in this population. As such, the variation in these measures for the seven treatment groups in the second intervention was determined. There appeared to be differences in some telomere length determinants compared to baseline means observed in the initial cohort (Table 6.1). For example, week 0 and week 16 telomere length in the FBENC group were significantly different from that observed in the whole cohort at week 0 or in the placebo group at week 16, respectively. Male participants were significantly underrepresented in the FBRNC group ($P = 0.017$) with just 6 of 86 males in this group. In addition, there was an imbalance in the number of participants in each treatment group and fewest volunteers were allocated to the placebo treatment group ($n = 15$).

6.3.2 Telomere length changes and trajectories over time

The change in telomere length over time was compared for each of the *Polypill* formulations and the placebo in order to detect whether micronutrient supplementation may have been associated with telomere length (Table 6.2). Analysing the mean telomere length from week 16 to week 32 revealed no significant change in any group, however it appeared mean reductions in telomere length observed in both the FERNC ($P = 0.06$) and the placebo group ($P = 0.051$) were approaching statistical significance. These telomere length changes by treatment group are represented graphically in Figure 6.2.

The distribution of telomere length trajectory phenotypes for each group was explored in Table 6.3. There was no statistically significant deviation from even distributions of telomere length trajectory proportions in each treatment group. Overall the ratio of shortened: maintained: lengthened telomere length trajectories was 33:33:34 and not statistically significant ($P = 0.11$).

Table 6.1 – Demographic and anthropometric descriptives and telomere length, by treatment group from week 16 to week 32

VARIABLE	BERNC *n* = 33 [17.4%]	FBENC *n* = 26 [13.7%]	FBERC *n* = 27 [14.2%]	FBERN *n* = 29 [15.3%]	FBRNC *n* = 30 [15.8%]	FERNC *n* = 30 [15.8%]	PLACEBO *n* = 15 [7.9%]
Age (y)	43.1 ± 7.0 (*P* = 0.35)	43.4 ± 7.0 (*P* = 0.51)	41.3 ± 7.3 (*P* = 0.055)	47.5 ± 7.4 (*P* = 0.06)	47.2 ± 9.5 (*P* = 0.10)	43.6 ± 9.1 (*P* = 0.58)	46.9 ± 8.7 (*P* = 0.28)
Gender: males [% cases]	16 [48.5] (*P* = 0.41)	12 [46.2] (*P* = 0.63)	14 [51.9] (*P* = 0.27)	15 [51.7] (*P* = 0.26)	6 [20] (*P* = **0.017**)	14 [46.7] (*P* = 0.56)	9 [60.0] (*P* = 0.15)
BMI at week 32 (kg/m^2)	27.0 ± 5.1 (*P* = 0.84)	27.7 ± 6.0 (*P* = 0.41)	26.8 ± 5.0 (*P* = 1.0)	26.8 ± 4.9 (*P* = 1.0)	25.4 ± 4.8 (*P* = 0.17)	27.7 ± 4.8 (*P* = 0.37)	27.0 ± 5.6 (*P* = 0.89)
Obese at week 32 *n* [% cases]	8 [24.2] (*P* = 0.68)	9 [34.6] (*P* = 0.41)	9 [33.3] (*P* = 0.49)	7 [24.1] (*P* = 0.69)	6 [20.0] (*P* = 0.36)	8 [26.7] (*P* = 0.93)	3 [20.0] (*P* = 0.52)
Maternal age (y)	27.9 ± 6.3 (*P* = 0.85)	28.0 ± 5.3 (*P* = 0.80)	26.7 ± 5.5 (*P* = 0.39)	27.9 ± 5.0 (*P* = 0.86)	27.6 ± 6.0 (*P* = 0.93)	28.9 ± 5.1 (*P* = 0.28)	26.5 ± 6.3 (*P* = 0.43)
Paternal age (y)	31.6 ± 8.9 (*P* = 0.52)	31.0 ± 6.9 (*P* = 0.84)	29.7 ± 6.3 (*P* = 0.49)	31.4 ± 6.6 (*P* = 0.62)	29.6 ± 5.2 (*P* = 0.42)	32.0 ± 7.4 (*P* = 0.36)	28.9 ± 8.1 (*P* = 0.35)
Polypill week 0 – 16 *n* [% cases]	15 [45.5]	11 [42.3]	14 [51.9]	16 [55.2]	16 [53.3]	17 [56.7]	7 [46.7]
Placebo week 0 – 16 *n* [% cases]	18 [54.5] (*P* = 0.61)	15 [57.7] (*P* = 0.43)	13 [48.1] (*P* = 0.84)	13 [44.8] (*P* = 0.58)	14 [46.7] (*P* = 0.72)	13 [43.3] (*P* = 0.46)	8 [53.3] (*P* = 0.80)
Week 0 telomere length (kb/diploid genome)	94.6 ± 41.9 (*P* = 0.070)	148.8 ± 109.8 (*P* = **0.012**)	104.3 ± 22.6 (*P* = 0.40)	114.2 ± 26.7 (*P* = 0.99)	121.0 ± 20.4 (*P* = 0.52)	111.9 ± 36.3 (*P* = 0.85)	67.6 ± 39.8 (*P* = **0.003**)

Week 16 telomere length (kb/diploid genome)	89.4 ± 27.0 (*P* = **0.006**)	137.4 ± 71.3 (*P* = **0.010**)	111.3 ± 23.4 (*P* = 0.87)	110.0 ± 29.7 (*P* = 1.0)	116.2 ± 44.7 (*P* = 0.47)	111.8 ± 26.0 (*P* = 0.82)	89.6 ± 55.8 (*P* = 0.082)
Week 32 telomere length (kb/diploid genome)	93.9 ± 34.9	132.7 ± 69.3	109.3 ± 25.6	122.3 ± 37.7	128.1 ± 39.7	103.2 ± 26.0	70.0 ± 41.2

For treatment group columns: F, folic acid; B, vitamin B_{12}; E, vitamin E; R, retinol; N, nicotinic acid; C, calcium

Overall P for distribution in groups is not significantly different when tested against even proportion n = 27 (14.29%) per group (P = 0.28)

P in parentheses reports significance for differences in group compared to cohort means observed in Table 4.1, Table 4.5, and week 16 telomere length in the placebo group as per Table 5.5

P for placebo reports significance for difference in proportions between Polypill and placebo treatment groups using test proportion of 0.50.

Table 6.2 – Change in telomere length from week 16 to week 32, by treatment group

GROUP*	WEEK 16 TELOMERE LENGTH	WEEK 32 TELOMERE LENGTH	*P* (PAIRED SAMPLES T-TEST)	Δ_{16-32} TELOMERE LENGTH	PERCENT Δ_{16-32} TELOMERE LENGTH
BERNC (ex. folic acid)	89.4 ± 27.0 (55.5 – 197.7)	93.9 ± 34.9 (44.6 – 247.5)	0.36	4.5 ± 0.0 (-29.1 – 49.8)	5.7 ± 22.4 (-39.5 – 60.5)
FBENC (ex. retinol)	137.4 ± 71.3 (43.2 – 305.3)	132.7 ± 69.3 (51.2 – 299.4)	0.56	-4.7 ± 0.0 (-61.7 – 58.8)	0.001 ± 24.6 (-31.4 – 70.5)
FBERC (ex. nicotinic acid	111.3 ± 23.4 (65.3 – 162.8)	109.3 ± 25.6 (59.2 – 172.2)	0.60	-2.0 ± 0.0 (-51.9 – 44.7)	0.007 ± 21.8 (-35.6 – 52.7)
FBERN (ex. calcium)	110.0 ± 29.7 (13.5 – 149.8)	122.3 ± 37.7 (38.9 – 247.8)	0.17	12.3 ± 0.0 (-44.6 – 167.6)	37.0 ± 146.6 (-31.6 – 764.6)
FBRNC (ex. vitamin E)	116.2 ± 44.7 (71.3 – 304.3)	128.1 ± 39.7 (63.0 – 207.1)	0.18	11.9 ± 0.0 (-133.4 – 130.3)	19.1 ± 48.0 (-51.8 – 169.6)
FERNC (ex. vitamin B_{12})	111.8 ± 26.0 (45.9 – 205.4)	103.2 ± 26.0 (32.5 – 160.1)	0.06	-8.6 ± 0.0 (-93.4 – 44.2)	-5.9 ± 23.0 (-45.5 – 46.0)
PLACEBO (ex. all micronutrients)	89.6 ± 55.8 (33.2 – 222.6)	70.0 ± 41.2 (21.2 – 137.4)	0.051	0.0002 ± 0.0 (-107.2 – 24.5)	-16.0 ± 27.4 (-79.6 – 30.5)

* *F, folic acid; B, vitamin B_{12}; E, vitamin E; R, retinol; N, nicotinic acid; C, calcium; Ex., excludes;*
Telomere length units are kb/diploid genome;
Values reported as mean ± SD (minimum – maximum).

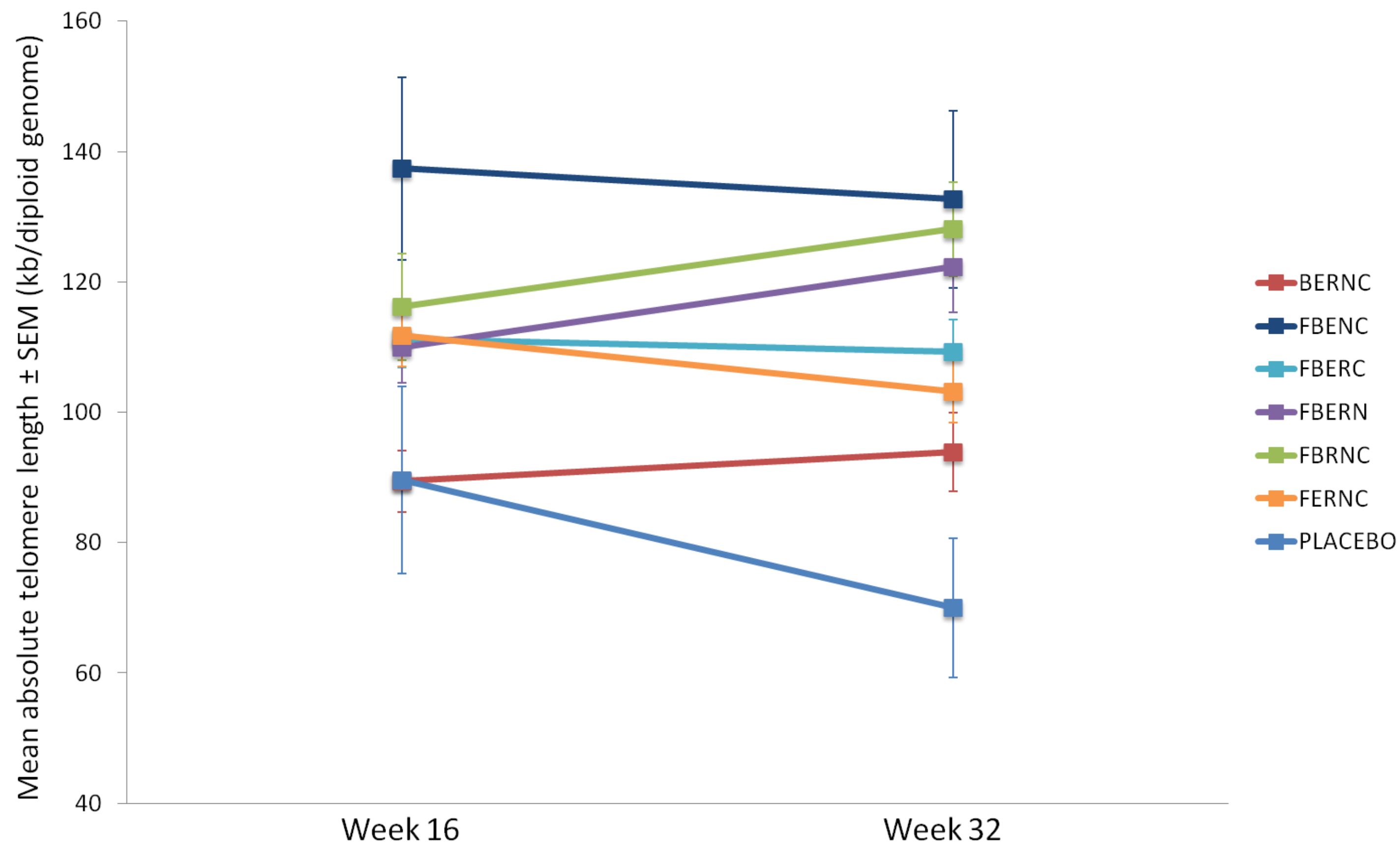

Figure 6.2 – Graphical representation of change in telomere length from week 16 to week 32, by treatment group

***BERNC**, n = 33 (17.4%); **FBENC**, n = 26 (13.7%); **FBERC**, n = 27 (14.2%); **FBERN**, n = 29 (15.3%); **FBRNC**, n = 30 (15.8%); **FERNC**, n = 30 (15.8%); **Placebo**, n = 15 (7.9%);*

F, folic acid; B, vitamin B_{12}; E, vitamin E; R, retinol; N, nicotinic acid; C, calcium.

Table 6.3 – Telomere length trajectory from week 16 to week 32, by treatment group

GROUP[*]	*n* [% CASES] *n* = 190	SHORTENED (*n* = 63, 33.16%)	MAINTAINED (*n* = 62, 32.63%)	LENGTHENED (*n* = 65, 34.21%)	*P*[**] (χ^2)
BERNC (ex. folic acid)	33 [17.4]	7 [21.2] (11.1)	15 [45.5] (24.2)	11 [33.3] (16.9)	0.23
FBENC (ex. retinol)	26 [13.7]	11 [42.3] (17.5)	7 [26.9] (11.3)	8 [30.8] (12.3)	0.61
FBERC (ex. nicotinic acid)	27 [14.2]	10 [37.0] (15.9)	11 [40.7] (17.7)	6 [22.2] (9.2)	0.46
FBERN (ex. calcium)	29 [15.3]	7 [24.1] (11.1)	9 [31.0] (14.5)	13 [44.8] (20.0)	0.38
FBRNC (ex. vitamin E)	30 [15.8]	8 [26.7] (12.7)	6 [20.0] (9.7)	16 [53.3] (24.6)	0.06
FERNC (ex. vitamin B_{12})	30 [15.8]	11 [36.7] (17.5)	11 [36.7] (17.7)	8 [26.7] (12.3)	0.74
PLACEBO (ex. all micronutrients)	15 [7.9]	9 [60.0] (14.3)	3 [20.0] (4.8)	3 [20.0] (4.6)	0.09

[*] *F, folic acid; B, vitamin B_{12}; E, vitamin E; R, retinol; N, nicotinic acid; C, calcium; Ex., excludes;*
[**] *P for trajectory ratios in entire cohort = 0.11*
Within each treatment, percentage of individuals in each trajectory are reported within square brackets;
Within each trajectory, percentage of individuals in each treatment are reported within parentheses.

The *Polypill* or placebo allocation from week 0 to week 16 was considered for telomere length trajectories within each treatment group (Table 6.4). There appeared to be a shift in the most prominent telomere length trajectory for some formulations, for example the BERNC *Polypill* which excluded folate (P = 0.0004). Within this group, of those who took the placebo from week 0 to week 16 (n = 18), there were significantly more individuals displaying a telomere length maintained trajectory (± 10%) from week 16 to 32 (P = 0.002). For those who took the *Polypill* from week 0 to week 16 (n = 15), there were significantly more people displaying a telomere length gain trajectory (>10%) from week 16 to 32 (P = 0.02).

Table 6.5 summarises demographic and anthropometric descriptives of the volunteers who completed the third 16-week phase of the *Polypill* intervention study, by telomere length trajectory over week 16 to week 32. There were significant differences across the trajectory groups for gender, BMI and prevalence of obesity. More males than females (57: 43%) displayed a telomere length shortening phenotype over the 16 weeks (P = 0.05). Mean BMI was greater in telomere length maintained and lengthened trajectory groups (P = 0.04), and prevalence of obesity also followed this trend (P = 0.006). The difference in the proportion of individuals who were randomised to any *Polypill* formulation from week 16 to 32 in the telomere length trajectory appeared to be approaching statistical significance (P = 0.071).

Table 6.4 – Week 16 to week 32 treatment allocation for week 16 and week 32 completers

GROUP*	*n* [% CASES]	FORMULATION EXCLUDES	PHASE 2 INDICATION**	*n* [% CASES]	TRAJECTORY LOSS: MAIN: GAIN	*P* (χ^2)	*P* (χ^2)
BERNC	33 [17.4]	Folic acid	BERNC 1	18 [9.5]	4: 13: 1	**0.002**	**0.0004**
			BERNC 2	15 [7.9]	3: 2: 10	**0.02**	
FBENC	26 [13.7]	Retinol	FBENC 1	15 [7.9]	5: 3: 7	0.45	0.12
			FBENC 2	11 [5.8]	6: 4: 1	0.18	
FBERC	27 [14.2]	Nicotinic acid	FBERC 1	13 [6.8]	5: 5: 3	0.74	0.97
			FBERC 2	14 [7.4]	5: 6: 3	0.61	
FBERN	29 [15.3]	Calcium	FBERN 1	13 [6.8]	2: 5: 6	0.37	0.67
			FBERN 2	16 [8.4]	5: 4: 7	0.65	
FBRNC	30 [15.8]	Vitamin E	FBRNC 1	14 [7.4]	3: 4: 7	0.40	0.52
			FBRNC 2	16 [8.4]	5: 2: 9	0.10	
FERNC	30 [15.8]	Vitamin B_{12}	FERNC 1	13 [6.8]	4: 6: 3	0.58	0.64
			FERNC 2	17 [8.9]	7: 5: 5	0.80	
Placebo	15 [7.9]	All micronutrients	PLA 1	8 [4.2]	5: 2: 1	0.20	0.70
			PLA 2	7 [3.7]	4: 1: 2	0.37	

* *F, folic acid; B, vitamin B_{12}; E, vitamin E; R, retinol; N, nicotinic acid; C, calcium;*
** *Phase 2 indication: 1 = placebo, 2 = FBERNC Polypill*

Table 6.5 – Demographic and anthropometric descriptives and telomere length of the study population, by telomere length trajectory from week 16 to week 32

VARIABLE	SHORTENED (*n* = 63, 33.16%)	MAINTAINED (*n* = 62, 32.63%)	LENGTHENED (*n* = 65, 34.21%)	*P* (ANOVA OR χ^2)
Age (y)	44.6 ± 8.5	43.1 ± 6.7	46.0 ± 9.0	0.13
Gender: males [% cases]	36 [57.1]	27 [43.5]	23 [35.4]	**0.05**
BMI at week 16 (kg/m^2)	25.6 ± 3.8	27.3 ± 5.1	28.1 ± 6.0	**0.04**[a]
Obese at week 16 *n* [% cases]	8 [12.7]	19 [30.6]	24 [36.9]	**0.006**
Maternal age[b] (y)	28.0 ± 5.4	27.9 ± 5.8	27.4 ± 5.6	0.84
Paternal age[b] (y)	30.5 ± 6.4	31.0 ± 7.7	30.7 ± 7.2	0.99
Week 16 telomere length (kb/diploid genome)	121.3 ± 50.4	108.3 ± 41.8	100.6 ± 33.8	**0.02**[c]
Week 0 telomere length (kb/diploid genome)	105.7 ± 60.0	114.2 ± 50.0	113.4 ± 50.5	0.62
Active *Polypill* treatment during phase *n* [% cases]	54 [85.7]	59 [95.2]	62 [95.4]	0.071[d]

[a] *Robust tests of equality of means used; Welch P = 0.024, Brown-Forsythe P = 0.035; Bonferroni post hoc multiple comparisons reveal BMI at week 16 in the lengthened trajectory group is 1.08* kg/m^2 *higher than in the shortened trajectory group (P = 0.037);*
[b] *Maternal age in shortened group n = 62; Paternal age n = 62 in shortened group; and n= 63 in lengthened group;*

[c] *Bonferroni post hoc multiple comparisons reveal telomere length in shortened trajectory group is 13 kb longer than that in lengthened trajectory group (P = 0.019);*
[d] *2 cells (33.3%) have expected count less than 5. The minimum expected count is 4.89.*

6.4 Discussion

6.4.1 There were no significant changes in telomere length within modified *Polypill* or placebo groups

There were no significant changes in telomere length from week 16 to week 32 for any of the randomised treatment groups, however the decrease in telomere length observed in the FERNC formulation (excluding vitamin B_{12}) and the placebo appeared to approach statistical significance (P = 0.06 and 0.052, respectively; Table 6.2). While it is possible that this result was obtained by chance, it is feasible that supplementation with B_{12} prevented telomere length decrease when the telomere length trajectory of the FERNC supplement and the change in the placebo group is compared to other *Polypill* formulations. Additionally, as participants were allocated to treatment groups in a list-wise fashion, there are differences in demographic and anthropometric measures which have been shown to be associated with telomere length in this cohort (Table 6.1).

6.4.2 Modified *Polypill* supplements differentially influenced telomere trajectories

There seemed to be a difference in the effect of the various *Polypill* formulations on the telomere length trajectories (Table 6.3 and Figure 6.2). For the formulations excluding retinol, niacin, B_{12} and all micronutrients (placebo), the majority of individuals were placed in telomere length shortened or shortened/maintained trajectories. For the formulations which excluded folate, vitamin E and calcium, the majority of participants had telomere length lengthened or maintained/lengthened trajectories. These results indicate that the different micronutrients may differential effects on telomere biology. As this was a short study, with few participants in each group these differential effects certainly warrant further investigation. Yet as there are differences in known confounders in telomere length across the groups (Table 6.1), as well as telomere length at week 16 which could direct trajectory for the proceeding 16 weeks, it is unlikely that changes in telomere length over the 16 weeks from week 16 to week 32 could be attributed to treatment group or *Polypill* formulation alone. In addition, there are a great number of other covariates which have been associated with telomere length in the literature which were not measured and as such could not be considered or controlled for in these analyses. These covariates including genetic SNPs and

lifestyle and environment factors including stress could be measured in future studies.

6.4.3 Previous treatment allocation influenced telomere length trajectory with modified *Polypill* formulations

For some *Polypill* formulations, there appeared to be an effect of treatment during the first intervention from week 0 to week 16 on telomere length trajectory during the second intervention from week 16 to week 32 (Table 6.4). For example, for the BERNC formulation which excluded folic acid, the loss: maintain: gain trajectory ratio was 4: 13: 1 for those who were randomised to the placebo group during the first intervention, placing the majority in the telomere length maintained trajectory. For those who were randomised to the FBERNC *Polypill* for 16 weeks from week 0 to week 16, the ratio of 3: 2: 10 placed the majority in the trajectory of telomere length gain from week 16 to 32. As such, it appears that the effect of the treatment from week 0 to week 16 may have influenced the trajectory observed from week 16 to week 32 for some *Polypill* formulations. That there was no washout period between initial intervention and this second intervention phase means that it cannot be certain that there was no residual effect of the FBERNC *Polypill* during the second intervention phase for half of the volunteers.

It is known that following mixed vitamin B supplementation, serum homocysteine remained significantly lower than pre-supplementation levels 248 days after the cease of supplementation (Henning *et al.*, 2001). This implies that in the 16 week period immediately following the FBERNC *Polypill* intervention, there was a likely persistent influence of this previous supplementation on plasma homocysteine in those who were randomised to the *Polypill* group. That plasma homocysteine does not rapidly return to pre-supplementation or baseline levels is not unanticipated, as the turnover of folate within the body is less than 1% per day (Stites *et al.*, 1997). Therefore, it is expected that plasma homocysteine would remain lower and folate concentrations higher than baseline levels for at least 3 months (Brouwer *et al.*, 1999).

6.4.4 Those in the telomere shortened trajectory were most likely to be male, have lower BMI and longer telomeres at week 16

Comparing telomere length trajectory groups based on telomere change phenotype from week 16 to week 32 revealed significant differences in gender, BMI, obesity and week 16 telomere length (Table 6.5). The significant difference in the ratio of males to females in the telomere trajectory groups, was such that there were more males (57% prevalence) in the telomere length shortened group compared to maintained (44%) and lengthened groups (35%) (*P* = 0.05). This result is in consonance with findings that males have a greater rate of telomere attrition or telomere sequence loss than females (Okuda *et al.*, 2002, Mayer *et al.*, 2006), a fact which may mean that over the 16 weeks, more men displayed a telomere loss phenotype than women. The differences in obesity prevalence and BMI trended the same way with a higher BMI, and increased prevalence of obesity observed in maintained group compared to telomere length shortened group, with the highest BMI and obesity prevalence observations in the telomere length gain trajectory (BMI *P trend* = 0.04, obesity *P* = 0.006). This result was not observed in either randomised treatment group of the first intervention from week 0 to week 16 (Table 5.6) nor when these treatment groups were combined for increased statistical power (Appendix Table 10.8). Active *Polypill* treatment during the 16-week phase appeared to place fewer supplementers in the telomere length shortened trajectory group (*P* = 0.07), however the low number of placebo controls in this phase is problematic.

The significant difference in telomere length at week 16 (*P* = 0.02) is of a similar fashion to that observed in trajectory analysis from week 0 to 16 (Table 5.6) where telomere length was shortest at week 16 in those whose telomeres increased in length from week 16 to week 32, with the opposite true for those in the telomere length shortened trajectory who has the longest telomeres at week 16. However, there was no difference in telomere length at week 0 across the trajectory groups. This result indicates that there is potential for regression toward the mean to occur in these repeated analyses (Section 5.4.3.1). Additionally, this result potentially reflects *in vivo* homeostasis of telomere length which is not yet well characterised.

6.4.5 Limitations of this study design

This second *Polypill* intervention was purely exploratory and was not intended to be a definitive study. The rationale was that when studying a *Polypill* it is not possible to know which factor contributed to any biological effects that are observed. The strategy used to probe this was to remove one of each of the micronutrients and test whether the biological effect persisted or was lost. Admittedly, the design of the study could have been improved by a washout (or depletion) phase followed by repletion with a reconstituted *Polypill* deficient in one of the six original *Polypill* components. Such a washout stage would have minimised any carryover effects from the first intervention phase, particularly for those micronutrients that are retained longer in the body, such as retinol and vitamin B_{12}. Additionally, a cross-over trial design would have had its merits in this study, as the differences in telomere length change during supplementation and placebo treatments could be investigated for each participant in the study. This exploratory phase is further limited by an unbalanced number of participants in each of the modified *Polypill* groups.

6.5 Conclusions

Due to the small number of individuals in each treatment group, there was insufficient power to detect statistically significant changes in telomere length over time with treatment of the varied *Polypill* formulations. Indeed, if there was an observed difference between FBERNC *Polypill* supplementation compared to placebo treatment in the preceding phase, there may have been additional value in this pilot investigation. However, this preliminary analysis indicated that there may be micronutrient-dependent differences influencing telomere length change and trajectory with micronutrient supplementation.

6.5.1 Significance

Of the limited longitudinal studies of telomere length in the literature, this is the third which has measured individual telomere length on more than two occasions and appears to be the longitudinal study with the shortest follow-up time.

6.5.2 Strengths and weaknesses

As the number of participants in the study was limited, by allocating individuals to one of seven treatments – six *Polypill* formulations and a placebo control group – there was great reduction in statistical power to detect any difference in the effect of the *Polypill* micronutrient supplements. In addition, this allocation of volunteers to several groups resulted in heterogeneity of known covariates of telomere length, such as gender, age and parental age as well as differences in telomere length which is known to influence proceeding telomere length trajectory.

6.5.3 Future directions

Among suggested future investigations could be an RCT of micronutrient supplements with a longer period of intervention and follow-up. There would also be great benefit in a similar study containing an increased number of participants or a study which adheres to a cross-over design where each individual receives both the placebo and treatment such that biological responses to each could be compared within individuals.

7 A quantitative PCR method for the detection of uracil bases within the telomere

7.1 Introduction

7.1.1 Uracil in DNA

The RNA base uracil is not an archetypal DNA base. Instead, DNA typically consists of the four bases adenine, cytosine, guanine and thymine. Yet, there are two primary pathways within the cell which can lead to the presence of non-canonical uracil bases in DNA; spontaneous deamination of cytosine or misincorporation of uracil in place of thymine under conditions of limited dTTP relative to dUTP supply (Figure 7.1).

In the first instance, the DNA base cytosine may undergo spontaneous deamination to uracil. As cytosine complementarily pairs with the purine guanine, the deamination of cytosine to uracil causes a U: G mispairing in the cell. This U: G mispairing can develop a C → T transition mutation which results in an altered DNA sequence. An estimated 100 – 500 uracil residues per cell, per day may arise in DNA through this process (Lindahl, 1993).

Secondly, in the situation of a skewed dUTP: dTTP ratio, uracil can be misincorporated into the DNA instead of thymine during DNA replication (Ahmad *et al.*, 1998). This misincorporated uracil base can be excised from the DNA *via* the action of uracil DNA glycosylase (UDG) enzyme and later, the base excision repair (BER) pathway which leads to an apyrimidinic or abasic (AP) site, and hence may generate both single and double-strand DNA breaks (Ahmad *et al.*, 1998). The typical dUTP: dTTP ratio within the cell is below 1%, with physiological concentration of ≈37 µM dTTP and 0.2 µM dUTP (Traut, 1994). It has been estimated that $\approx 10^4$ uracil residues may be misincorporated into human DNA following S-phase DNA synthesis (Mosbaugh and Bennett, 1994).

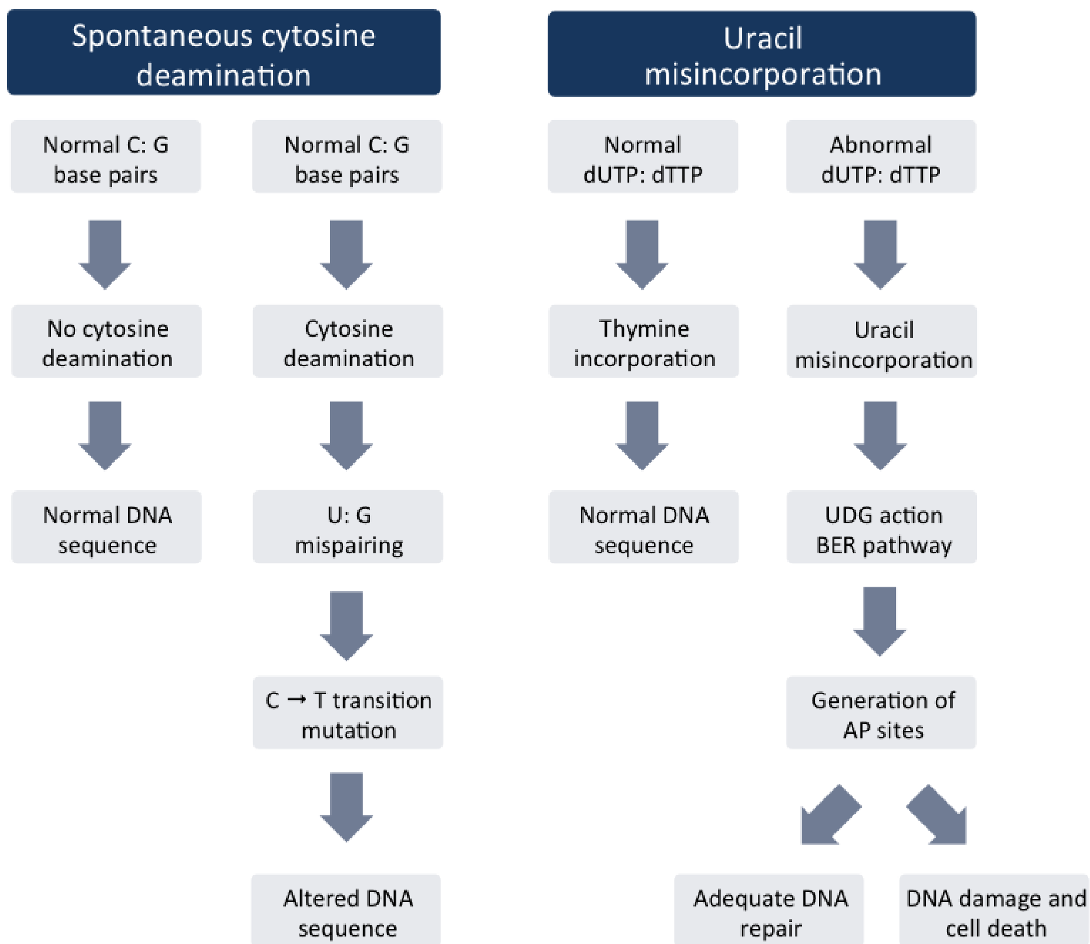

Figure 7.1 – Incorporation of uracil in DNA

Uracil may arise in DNA through spontaneous cytosine deamination or misincorporation of dUTP when cellular dUTP: dTTP pools are altered from their usual <1% ratio (Traut, 1994). Image modified from Olinski et al. (2010).

Since mammalian replicative DNA polymerases do not differentiate between dUTP and dTTP (Mosbaugh and Bennett, 1994), the background level of uracil in DNA is likely dependent on the activities of dUTP pyrophosphatase (dUTPase) and uracil DNA glycosylase (UDG) enzymes (Olinski *et al.*, 2010) as well as the dUTP: dTTP ratio. dUTPase hydrolyses dUTP to dUMP (as seen in Figure 7.2) and pyrophosphate (Ladner *et al.*, 1996), thereby eliminating the utilisation of dUTP by DNA polymerase during DNA replication and repair (Ladner, 2001). The enzyme UDG catalyses the excision of uracil from DNA. UDG cleaves uracil and forms an apyrimidinic site whilst leaving the phosphodiester backbone intact (Lindahl *et al.*, 1977).

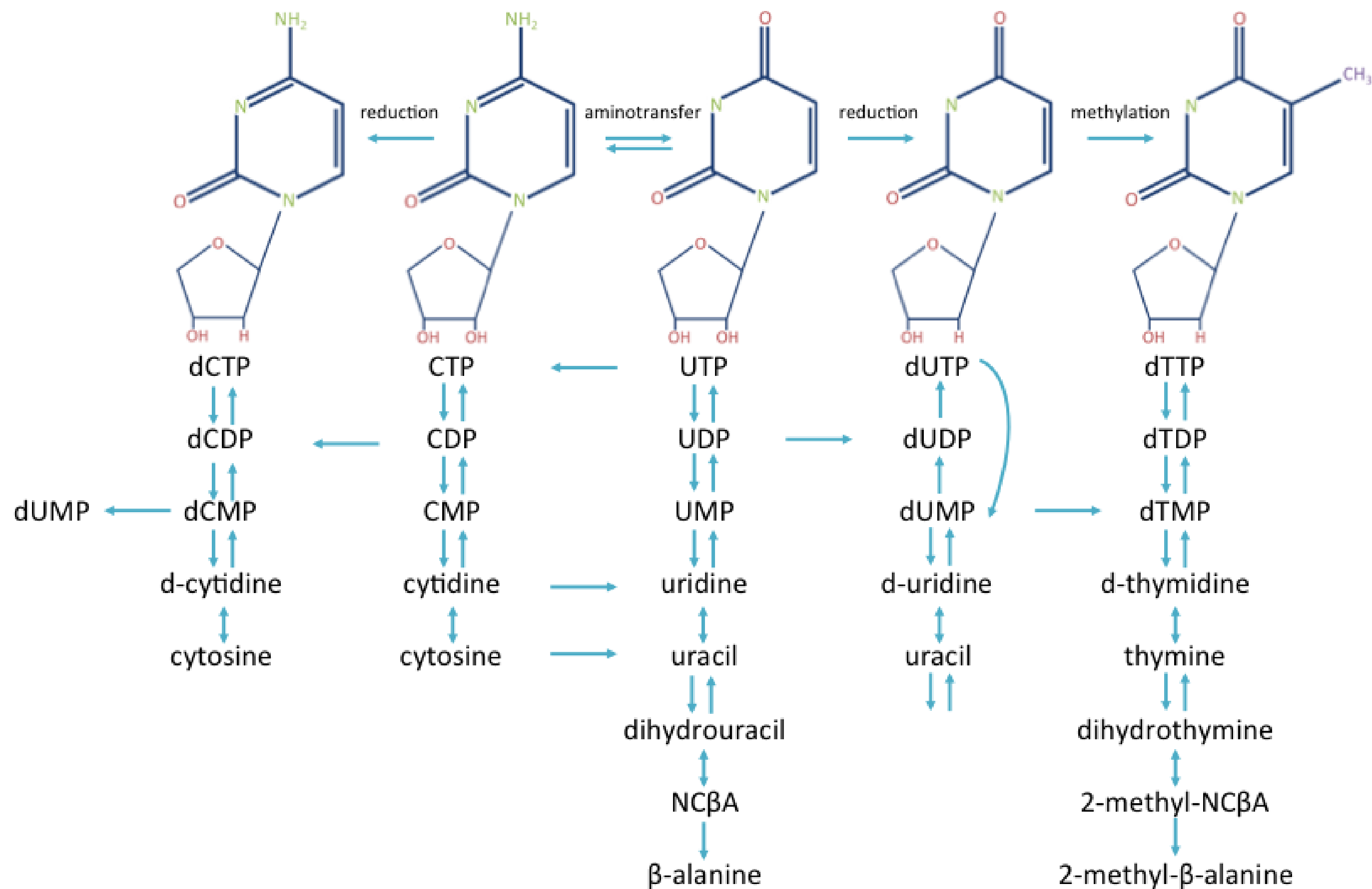

Figure 7.2 – Pyrimidine metabolism

Pyrimidine base or ribose modifications are represented horizontally, while phosphorylation, nucleoside cleavage or base catabolism is represented vertically. Individual arrows correspond to distinct enzymes. Image modified from Traut (1994)
Abbreviations; NCβA, N-carbamoyl-β-alanine.

7.1.2 Uracil in DNA and folate

Within the cell, folate is involved in DNA synthesis, repair and methylation pathways, and furthermore is an essential methyl donor in the conversion of dUMP to dTTP and hence the maintenance of dUMP: dTTP ratios within the cell (Benesh and Carl, 1978, Appling, 1991) (Figure 1.4). Low levels of the folate enzyme 5,10-MTHFR, a cofactor of thymidylate synthase, cause a decrease in the synthesis of thymidylate (dTMP) (Das and Herbert, 1989) and an increase in the ratio of dUMP: dTMP in the cell (Blount et al., 1997). This increase in the dUMP: dTMP ratio may predispose to uracil misincorporation and DNA repair-related DNA strand breaks (James *et al.*, 1997).

DNA from folate-deficient individuals (erythrocyte folate < 140 ng/mL) has been shown to have a high uracil content, with an average of 4×10^6 uracils/diploid cell (Blount *et al.*, 1997). This level of uracil was significantly reduced to 1.9×10^5 uracils/diploid cell following an 8 week period of folate supplementation ($P = 0.0003$) (Blount *et al.*, 1997). In a separate study, the *ex vivo* culture of normal human lymphocytes under low folate conditions again demonstrated that misincorporation of uracil is increased with folate deficiency (Duthie and Hawdon, 1998). In one *in vivo* model of folic acid deficiency, lymphocytes from rats fed a folate-deficient diet for 4 weeks showed significantly higher uracil misincorporation compared to a control diet of 0.005 g/kg folic acid mix (Duthie *et al.*, 2000).

7.1.3 Uracil in the telomere

Few studies have probed the impact that limited TTP supply, or uracil misincorporation may have on telomere length and function. One group has shown that a low TTP supply can cause shortening of telomeric repeat tracts, concluding that lowered TTP synthesis can accelerate telomere shortening and cellular growth arrest (Toussaint *et al.*, 2005). That telomere homeostasis may be sensitive to dNTP supplies, in particular TTP supply (Toussaint *et al.*, 2005) is plausible as the telomere elongating enzyme telomerase requires TTP for 25% of bases in the double-stranded mammalian repeat. Additionally, it is postulated misincorporation of uracil into the telomere may cause accelerated telomere attrition as UDG action and BER may lead to single and double-stranded DNA breaks within the telomeric

DNA, hence causing loss of telomere sequence. Still, in modelling of the yeast *Saccharomyces cerevisiae*, these conceivable mechanisms appeared not to be responsible for the observed shortened telomere phenotype induced with lowered TTP supply (Toussaint *et al.*, 2005). At present it remains unknown whether uracil content within the telomere is correlated with folate deficiency or with telomere length as there is no existing methodology that permits the measurement of uracil residues specifically within the telomeric repeat sequence.

7.1.4 Measurement of base damage within the telomere

It has previously been demonstrated that oxidative and alkylative DNA damage preferentially accumulates within single-stranded telomeric sequence (Petersen *et al.*, 1998). This vulnerability of telomeres to damage was displayed by the faster accumulation of single-stranded nicks and/or gaps in regions of telomere sequence relative to other interstitial repetitive variable number tandem repeats (Petersen *et al.*, 1998). Repeated observations that telomere shortening under mild stressors requires DNA replication has led to the hypothesis that presence of base damage or AP sites within the telomere interferes with the replication fork, leading to replication stress by stalling DNA replication and increasing the proportion of unreplicated DNA ends (von Zglinicki, 2002). This is thought to be the main cause of damage-induced telomere shortening, more so than the induction of double-strand DNA breaks within telomeric sequence (von Zglinicki, 2002).

That base damage within the telomere appears to be repaired with much less efficiency than that DNA in the remainder of the genome, highlights a need to quantify damage specifically within the telomere as this may confer more information about cellular and telomere welfare and homeostasis. Until recently, there was a lack of accessible and practical methods that allowed for the quantification of base damage within the telomeric sequence. A qPCR assay for absolute telomere length which first employs an incubation with the enzyme formamidopyridine DNA glycosylase (FPG), allowed for the quantification of FPG-sensitive lesions such as 7,8-dihydro-8-oxyguanine when the qPCR cycle threshold for the digested and sham-digested products (ΔC_T) are analysed (O'Callaghan *et al.*, 2011). In addition to quantifying oxidative damage to guanine bases within the telomere (O'Callaghan *et al.*, 2011), other oxidised lesions have been detected

within the telomeric sequence using a similar approach entailing qPCR for telomere length following pre-amplification digestion with DNA glycosylase Nth1 to compare the ΔC_T of digested and undigested DNA (Vallabhaneni *et al.*, 2013). These approaches provided the rationale for the development of this assay to detect uracil.

7.1.5 Aims and hypotheses

The aim of this study was to develop a method that can reliably detect uracil within telomere sequence. Additionally, *in vitro* experimentation aimed to establish the effect of varying folic acid concentration and dUTP challenge on telomere length and telomeric uracil in WIL2-NS cells.

It is hypothesised that telomeric uracil content may be highest in lower folate concentrations due to reduced conversion of dUTP to dTTP. Moreover, it is hypothesised that cultures supplemented with dUTP may also have greater uracil content due to the utilisation of these supplied bases preferentially over synthesis of or conversion to dTTP, which is energetically more expensive. Telomeric uracil content is hypothesised to be negatively associated with telomere length, as UDG action and BER of the aberrant base has the potential to influence telomere attrition by causing replication stress.

7.2 Methods

7.2.1 USER™ enzyme

Uracil-Specific Excision Reagent (USER™) enzyme is a commercially prepared mix of UDG and the DNA glycosylase-lyase enzyme Endonuclease VIII (New England Biolabs, Ipswich, MA, USA). Endonuclease VIII contains both N-glycosylase and AP lyase activities, and subsequently cleaves the phosphodiester backbone 3' and 5' to the abasic site (Melamede *et al.*, 1994). The result of these enzymes working in combination is the generation of a single nucleotide gap and DNA break at the location of UDG-sensitive uracil residues.

In mammalian cells, thymine may itself be oxidised to 5-hydroxymethyluracil or hydroxyuracil however as these aberrant bases are not recognised by UDG, rather they are excised by hydroxymethyluracil DNA glycosylase (Hollstein *et al.*, 1984) and Endonuclease III-like protein 1 (Nth1) (Hazra *et al.*, 2003), respectively, these such base aberrations would not be detected with this assay incorporating USER.

7.2.2 Deoxyuridine oligonucleotide standards

A series of oligonucleotide standards containing deoxyuridine (dU) were designed and used in the optimisation of the USER™ qPCR assay (Table 3.12).

7.2.3 A standard curve of telomere standards containing uracil

The telomere standards containing 0, 1, 2 and 4 dU per 84 oligomers were either overnight digested with 1 U of USER™ mixture or sham-treated with an equivalent volume of TE buffer. The products of USER™ digestion of the 4 dU oligomer is shown in Figure 7.3. The digests were diluted to 15 pg/µL and 60 pg of digested or undigested oligonucleotide was amplified by qPCR as described in Section 3.1. The ΔC_T was calculated as C_T digested – C_T undigested.

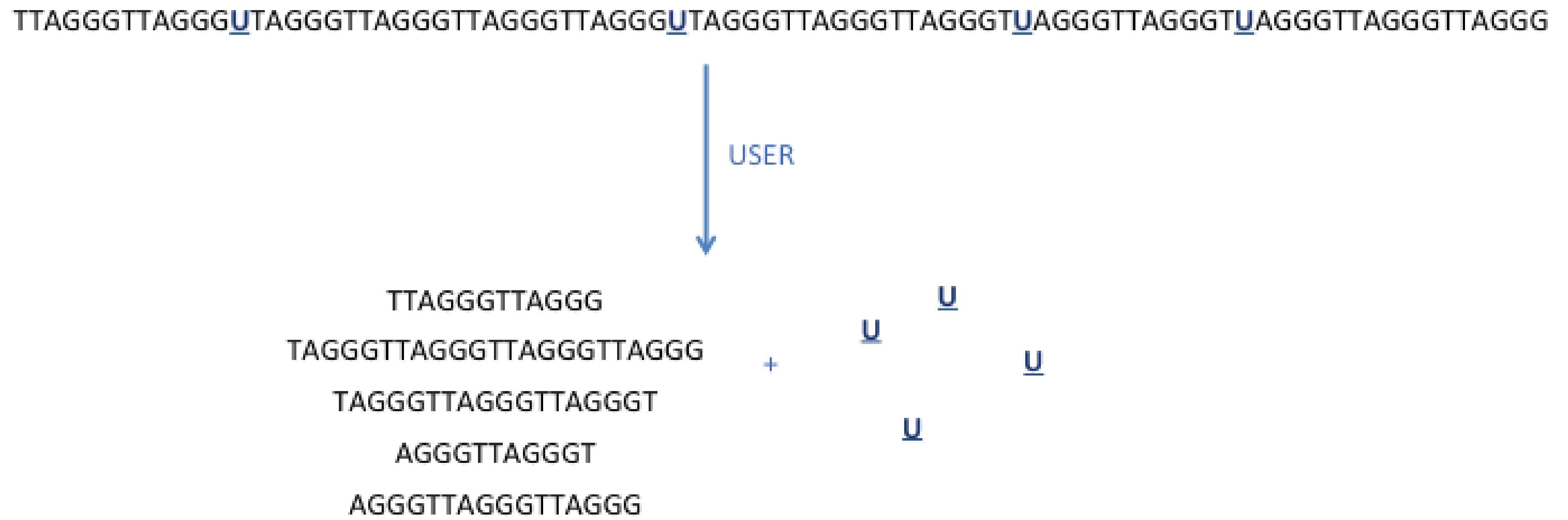

Figure 7.3 – Products from complete USER digestion of the 4U-containing telomere oligomer

7.2.4 Sensitivity of the USER-qPCR method

The telomere standards containing 0 and 4 dU bases per 84 oligonucleotides were mixed in the following ratios: 100: 0, 90: 10, 80: 20, 70: 30, 60: 40, 50: 50, 40: 60, 30: 70, 20: 80, 10: 90, and 0: 100. Then, 200 ng of oligonucleotide mixes were either digested with 1 U of USER™ mixture or sham-treated with a corresponding volume of TE buffer. The digests were diluted to 15 pg/µL and 60 pg of digested or undigested oligonucleotide was amplified by qPCR as described in Section 3.1. The ΔC_T was calculated as C_T digested – C_T undigested.

7.2.5 WIL2-NS *in vitro* experimentation

An *in vitro* model for 2´-Deoxyuridine, 5´-Triphosphate (dUTP) incorporation was developed, based on the commonly used 5-bromo-2-deoxyuridine (BrdU) DNA replication assay to quantify cellular proliferation. BrdU incorporates into dividing cells as an analogue of the pyrimidine deoxynucleoside thymidine during the S-phase of the cell cycle which involves DNA synthesis and replication (Cavanagh *et al.*, 2011). WIL2-NS cells were cultured under two concentrations of the thymidine analogue dUTP – 15 µM and 150 µM – or no dUTP control. With cell growth and during the S-phase of the cell cycle, supplied dUTP should incorporate in to the DNA in place of thymidine. However these cultured WIL2-NS cells were further challenged with folic acid deficiency which aimed to alter cellular dUTP: dTTP pools and contribute to DNA uracil misincorporation. The specific experimental treatments are represented in Figure 7.4. WIL2-NS cell culture conditions, harvest and USER-qPCR protocols are as described in Section 3.3. Absolute telomere length was performed as per Section 3.1.

7.2.6 Statistical analysis

ANOVA and correlation analyses were performed in IBM SPSS Statistics version 20.0 where the threshold for statistical significance was set at $P < 0.05$. Graphical representations of data were prepared in Microsoft Excel. Additional data is contained in the Appendix.

Figure 7.4 – Schematic representation of the experimental cultures

Three 24 well plates were used with separate cultures of WIL2-NS cells in differing folic acid and dUTP concentrations. There were six replicates of each treatment. Abbreviations: dUTP, 2´-Deoxyuridine, 5´-Triphosphate.

7.3 Results

7.3.1 The USER assay with synthetic telomere oligonucleotides containing deoxyuridine displayed proof of concept

Oligonucleotides of telomere sequence ($TTAGGG_{14}$) containing 0, 1, 2 and 4 dU residues were treated with USER enzyme mix and amplified by qPCR as described. The number of dU bases per kilobase (U/kb) of telomere length was calculated and a standard curve of ΔC_T was computed (Figure 7.5). An exponential trendline was modelled to fit the standard curve data, $R^2 = 0.96$.

Next, the sensitivity of the USER enzyme and qPCR assay for ΔC_T was investigated by conducting the USER assay with 0 – 100% mixes of the 4 dU telomere oligonucleotide with the unmodified oligonucleotide ($TTAGGG_{14}$) (Figure 7.6). The oligonucleotides were mixed prior to digestion and mock-digestion to simulate the differences in ΔC_T which would occur with USER treatment of samples with varying uracil content. The number of dU bases per kilobase (U/kb) of telomere length was calculated and the standard curve was shown to best fit an exponential trendline, $R^2 = 0.91$.

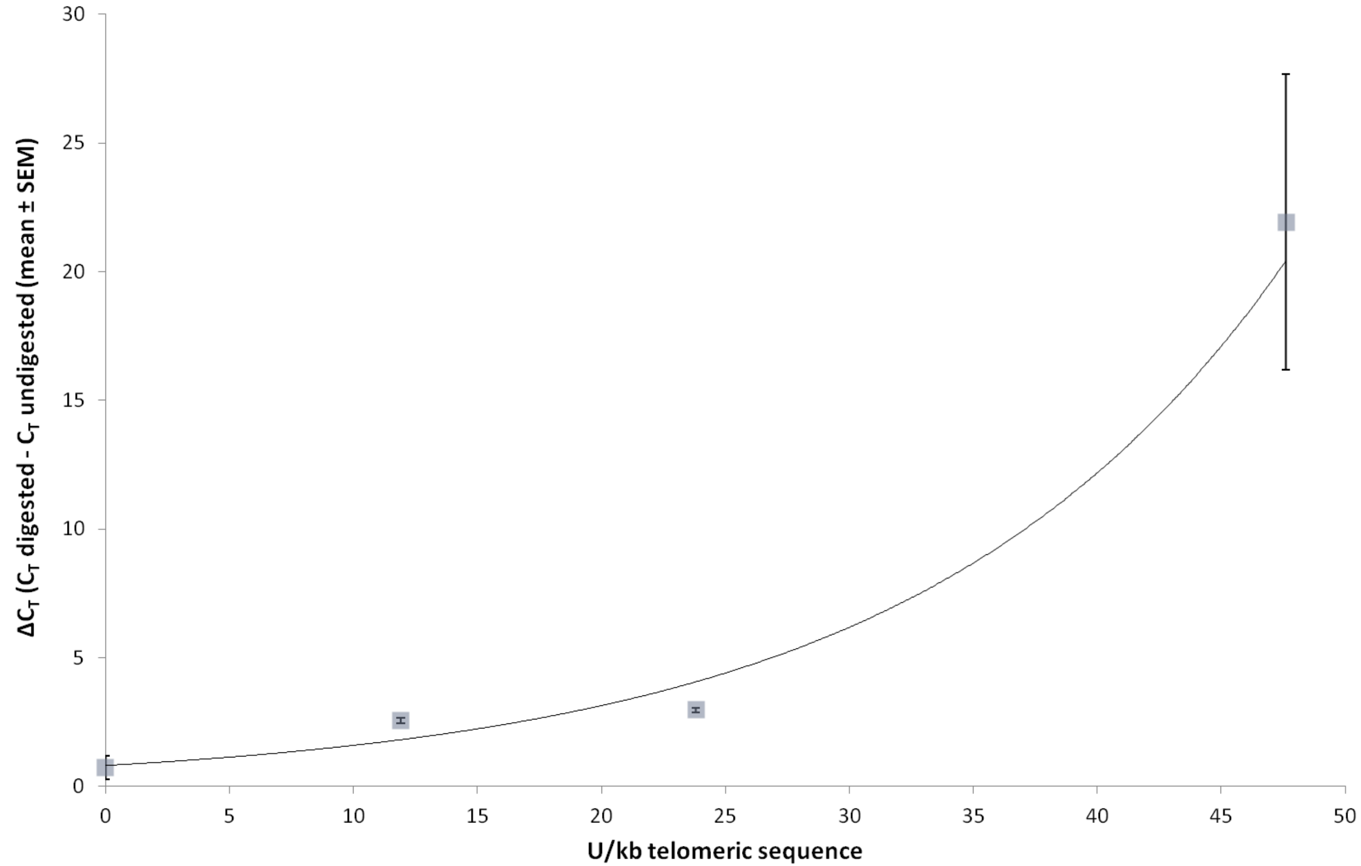

Figure 7.5 – Standard curve of telomere oligonucleotide containing 0, 1, 2 and 4U

200 ng of oligonucleotide mixes containing 0, 1, 2 and 4U per 84 bases were USER-digested or undigested overnight. 60 pg of each digested or undigested oligonucleotide mixture was amplified by qPCR with ΔC_T calculated as C_T digested - C_T undigested.
R^2 for exponential trendline = 0.96.

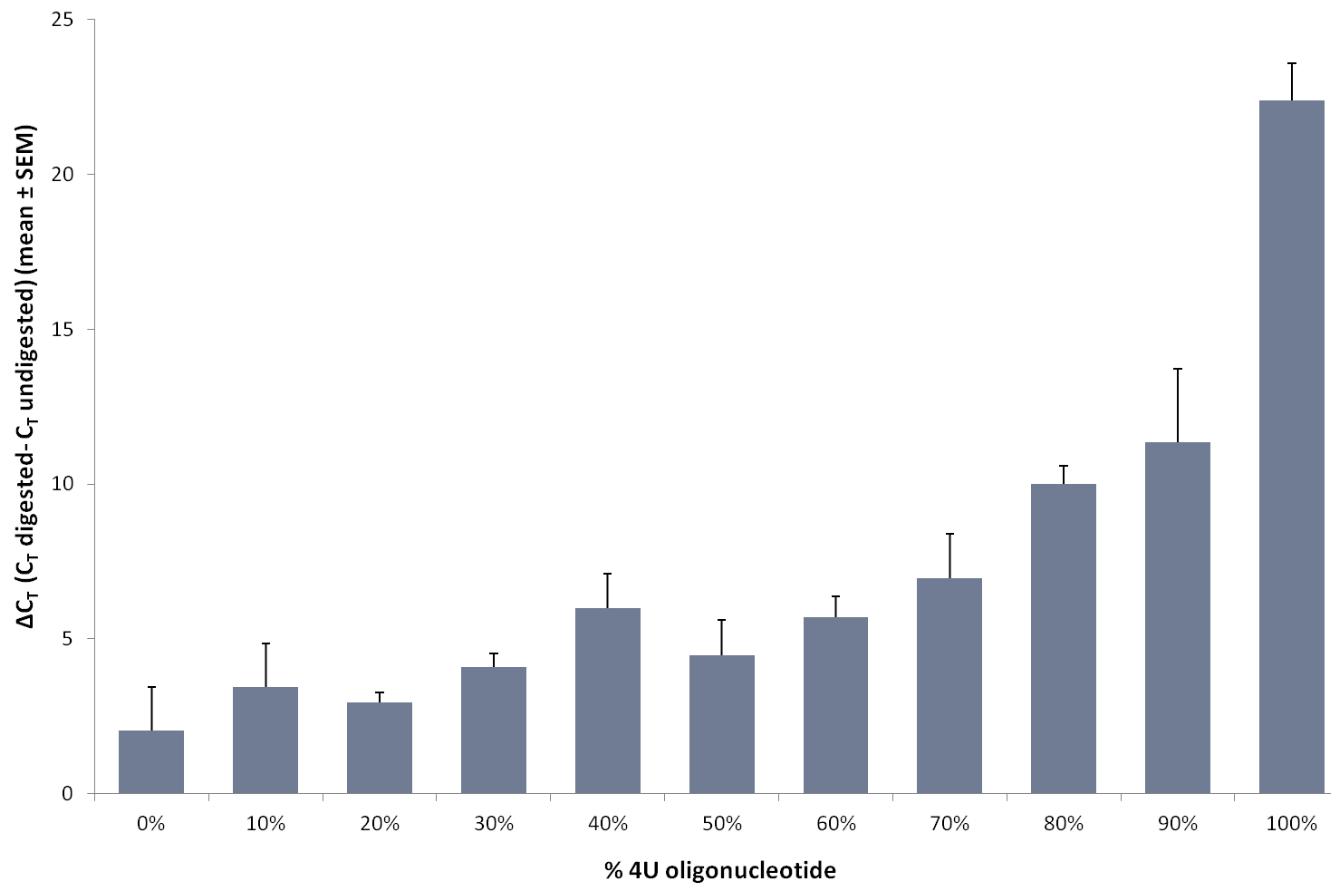

Figure 7.6 – Standard curve of 0 – 100% 4U telomere oligonucleotide

200 ng of oligonucleotide mixes containing 0% 4U to 100% 4U were USER-digested or mock digested overnight. 60 pg of each digested or undigested oligonucleotide mixture was amplified by qPCR with ΔC_T calculated as C_T digested - C_T undigested.
R^2 for exponential trendline = 0.91.

7.3.2 Verification with *in vitro* challenge

Together, these previous results indicated the feasibility of the USER assay to reliably detect uracil within the telomeric DNA sequence. *In vitro* modelling was then conducted to test the method on natural DNA as opposed to synthetically derived oligonucleotides. A 3 × 3 factorial model of folic acid and dUTP challenge was performed *in vitro* with WIL2-NS cells.

7.3.2.1 Live cell concentration was decreased with reduced folic acid concentration and increased dUTP concentration

Live cell concentration following 7-day culture was significantly higher with increasing concentrations of folic acid (ANOVA $P = 2.02 \times 10^{-14}$) (Figure 7.7). Additionally, there was a significant negative effect of increasing dUTP on live cell concentration ($P = 3.44 \times 10^{-4}$). However overall, and individually in each group of folic acid treatment, there was no significant difference between the live cell concentration between 15 µM and 150 µM dUTP-challenged cells. Finally, there appeared to be no interactive effect of folic acid and dUTP on total cell concentration ($P = 0.277$).

7.3.2.2 There was a significant effect of dUTP on telomere length

There were no significant differences in telomere length brought about by dUTP challenge in very low folic acid ($P = 0.074$), low folic acid ($P = 0.669$) or high folic acid ($P = 0.305$) treatment groups (Figure 7.8). There was no significant difference in telomere length with folic acid concentration ($P = 0.144$) however there was a significant effect of dUTP concentration ($P = 0.038$) with significantly longer telomere length observed with 15 µM dUTP treatment compared to the 0 µM dUTP control (3.6 kb mean difference, $P = 0.040$). There appeared to be no significant interaction of folic acid and dUTP concentration and telomere length ($P = 0.649$).

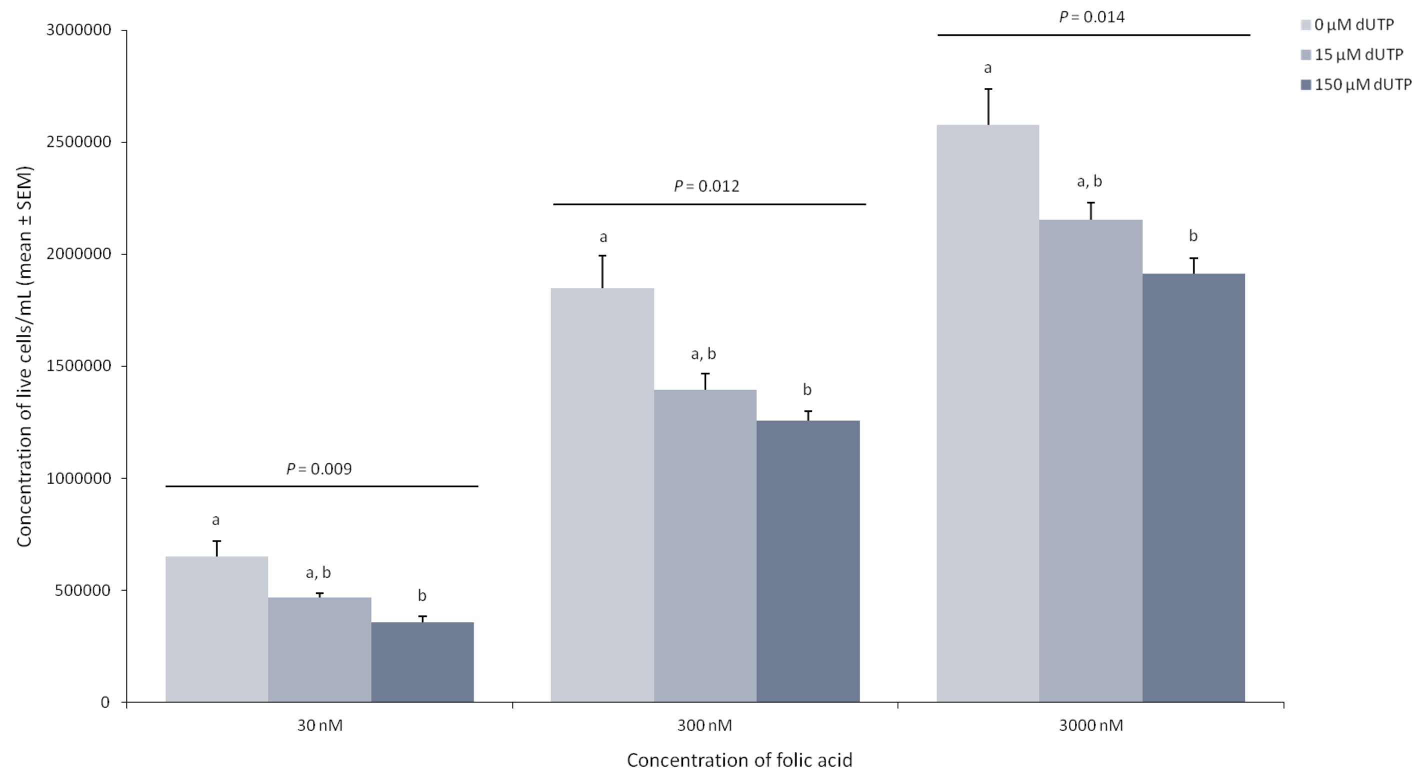

Figure 7.7 – Live cell concentration following 7 day *in vitro* culture

ANOVA P for differences within groups of FA treatment are shown; bars not sharing the same letter are statistically significantly different from each other ($P < 0.05$);
$P_{FA} = 2.02 \times 10^{-14}$; (Bonferroni post hoc multiple comparisons 30 – 300 nM $P = 1.17 \times 10^{-14}$; 300 – 3000 nM $P = 2.94 \times 10^{-8}$; 30 – 3000 nM $P = 1.24 \times 10^{-14}$)
$P_{dUTP} = 3.95 \times 10^{-6}$ (Bonferroni post hoc multiple comparisons 0 – 15 µM $P = 3.44 \times 10^{-4}$; 15 – 150 µM $P = 0.108$; 0 – 150 µM $P = 3.38 \times 10^{-6}$
$P_{interaction} = 0.277$.

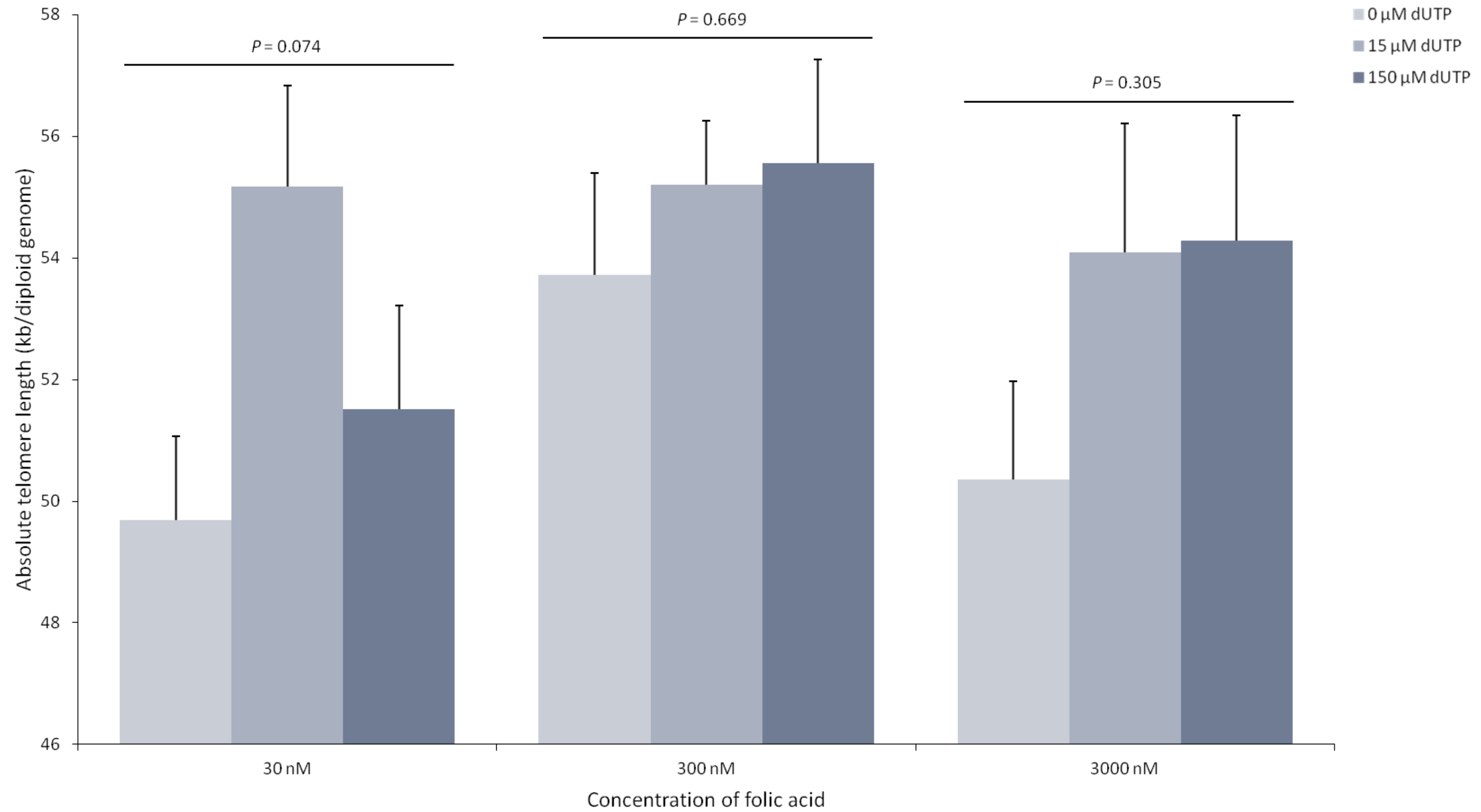

Figure 7.8 – Telomere length

Bars represent mean ± SEM; ANOVA P_{FA} = 0.144; P_{dUTP} = 0.038 (Bonferroni post hoc multiple comparisons 0 – 15 µM P = 0.040); $P_{interaction}$ = 0.649.

7.3.2.3 There were no significant effects of folic acid or dUTP on uracil/kb telomere sequence

As previously detailed (Section 3.3.8), the telomere length qPCR C_T for the mock-digested control was subtracted from the C_T for the USER-digested DNA sample. Then, the number of uracils/kb telomere sequence (Figure 7.9) was calculated using the formula for the exponential equation from the standard curve (Figure 7.5). In these experiments, the 4U containing telomere standard was used as a positive control. The mean ± SD of ΔC_T for the 4U oligomer was 24.6 ± 1.6 (n = 5, CV = 6.5%). There were no significant differences in uracil bases/kb telomere sequence in very low folic acid ($P = 0.386$), low folic acid ($P = 0.491$) or high folic acid ($P = 0.822$) treatment groups. There was no significant difference in uracil bases/kb telomere sequence with folic acid concentration ($P = 0.894$), dUTP concentration ($P = 0.407$) and there was no significant interaction of folic acid and dUTP concentration ($P = 0.773$).

7.3.2.4 Telomere length was non-significantly correlated with uracil/kb telomere

There was a negative association between telomere length and uracil content within the telomere, where telomere length was shorter with increased uracil/kb telomere, however the correlation was weak and not statistically significant $R^2 = 0.004$, $P = 0.654$ (Figure 7.10 and Table 7.1). Treatment group averages of endpoints were partially correlated with control for folic acid and dUTP concentration, but there was no significant association of telomere length with USER ΔC_T or uracil bases/kb telomere sequence (Table 7.2).

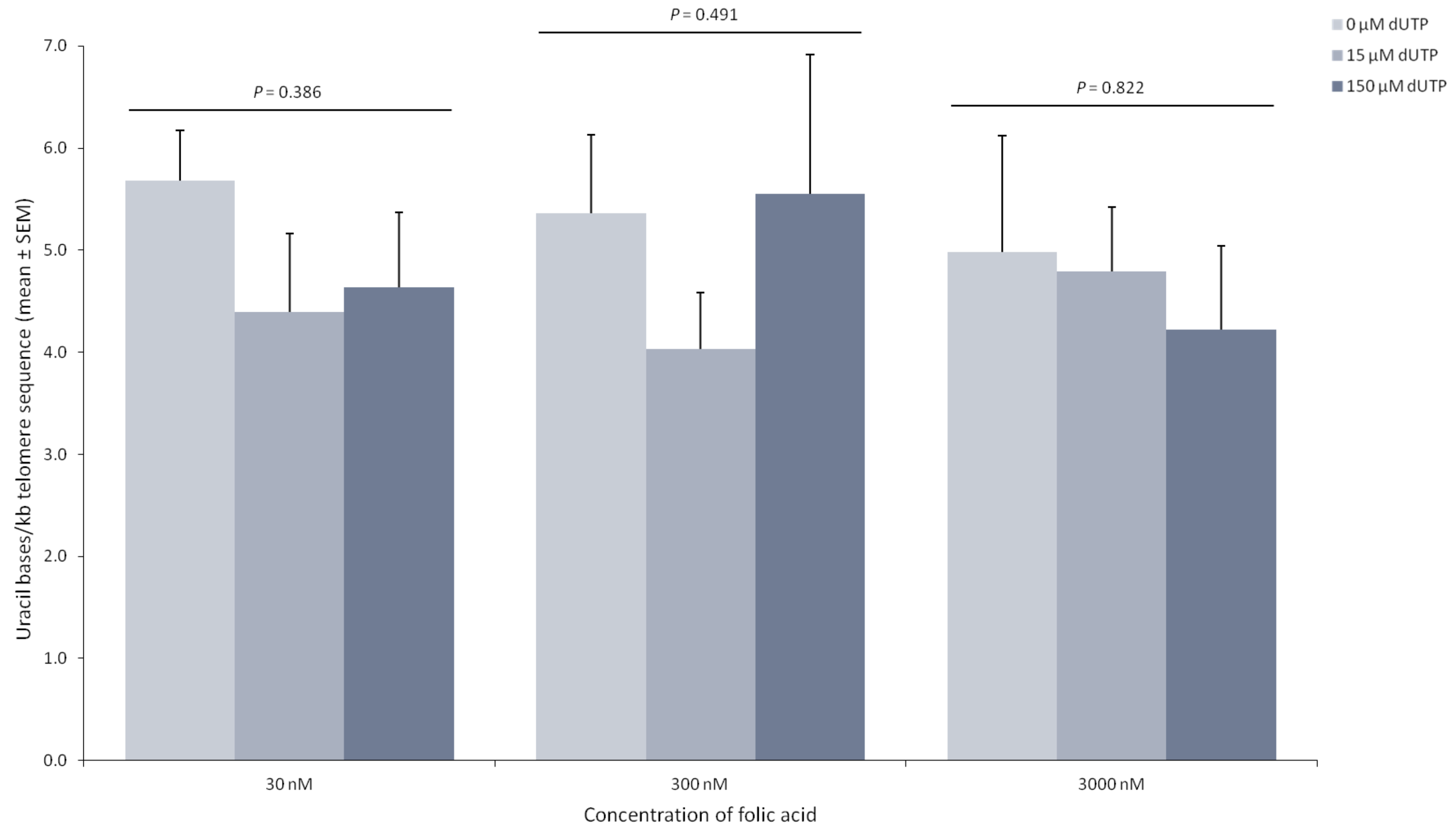

Figure 7.9 – Number of uracil bases/kb telomere sequence

ANOVA $P_{FA} = 0.894$; $P_{dUTP} = 0.407$; $P_{interaction} = 0.773$.

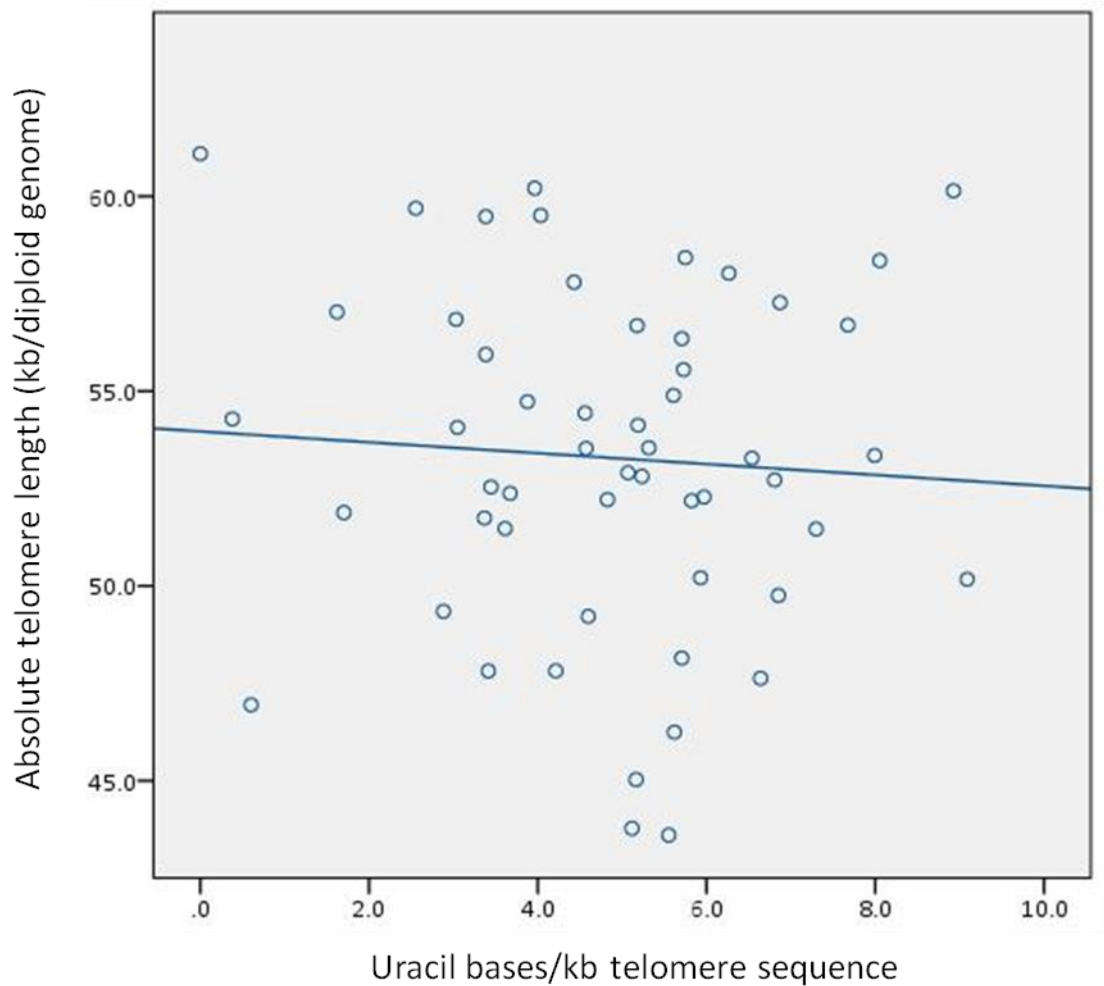

Figure 7.10 – Correlation of telomere length with uracil bases/kb telomere sequence

$P = 0.654$, $R^2 = 0.004$, $n = 54$.

Table 7.1 – Partial correlation of individual WIL2-NS endpoint measures

ENDPOINT	VALUE	TELOMERE LENGTH	USER ΔC_T	U/KB TELOMERE
Telomere length	*R* *P* *df*	1 - 0	-0.034 0.813 50	-0.064 0.654 50
USER assay ΔC_T	*R* *P* *df*	-	1 - 0	0.981 **<0.0001**[a] 50
U/kb telomere	*R* *P* *df*	-	-	1 - 0

[a] $P = 3.3 \times 10^{-37}$

Partial correlation with control for FA and dUTP

R, Pearson correlation coefficient

To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.017

Table 7.2 – Partial correlation of group WIL2-NS endpoint measures

ENDPOINT	VALUE	TOTAL CELLS DAY 7	TELOMERE LENGTH	USER ΔC_T	U/KB TELOMERE	HOMOCYSTEINE/10^6 CELLS DAY 7
Total cell concentration (day 7)	*R* *P* *df*	1 - 0	0.315 0.491 5	0.274 0.552 5	0.271 0.557 5	-0.920 **0.003** 5
Telomere length	*R* *P* *df*	-	1 - 0	-0.360 0.428 5	-0.407 0.365 5	-0.240 0.604 5
USER assay ΔC_T	*R* *P* *df*	-	-	1 - 0	0.962 **0.001** 5	-0.352 0.439 5
U/kb telomere	*R* *P* *df*	-	-	-	1 - 0	-0.384 0.394 5
Cellular generated homocysteine	*R* *P* *df*	-	-	-	-	1 - 0

Partial correlation with control for FA and dUTP
R, Pearson correlation coefficient
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.005

7.4 Discussion

7.4.1 Pre-qPCR digestion with USER enzyme mix enables the detection of uracil in telomeric sequence

USER-digestion of synthetic telomere oligonucleotides containing 1, 2 and 4 dU was shown to cause a higher telomere qPCR cycle threshold (C_T) than corresponding mock-digested controls comparatively incubated with additional buffer in place of the USER enzyme mix. Moreover, the ΔC_T between USER-digested and mock-digested controls was observed to increase exponentially with an increased number of dU per 84 base telomere sequence oligonucleotides which is expressed as uracils per kilobase of telomeric sequence (U/kb) ($R^2 = 0.96$; Figure 7.5). The high R^2 coefficient of determination indicates an acceptable goodness of fit of the exponential trendline to these data and as such, the standard curve generated can be used to calculate the number of USER-sensitive lesions (uracils) per kilobase of telomeric sequence. This original method can therefore be used to determine telomeric uracil content; either on a relative scale (comparing ΔC_T differences between samples) or on a quantitative scale (computing ΔC_T values into the exponential equation of the standard curve).

Alternative methods which capably detect oxidatively-damaged bases excised by FPG, such as 8-oxoguanine (O'Callaghan *et al.*, 2011), or Nth1, including thymine glycol (Vallabhaneni *et al.*, 2013) have been developed. The biological circumstances where this present method can be wholly utilised may be limited when compared to the applications previously described (O'Callaghan *et al.*, 2011, Vallabhaneni *et al.*, 2013) which have been utilised under conditions that induce oxidative damage or its accumulation through preventing cellular repair of certain DNA oxidation products, respectively. Uracil may arise from spontaneous cytosine deamination, or misincorporation in place of thymine, with the former route occurring much less frequent than the latter. As dUTP misincorporation is limited to occur during DNA replication processes within the cell, it is essential for the cells to be actively replicating DNA prior to cell division in order to accumulate misincorporated uracil. In addition, the detection of telomeric uracil may be limited by the *in situ* activities of native uracil DNA glycosylases which need to be

considered. However, whether uracil in the telomere sequence is repaired remains unknown.

The sensitivity of this method was demonstrated by using 11 pairs of USER-digested and mock-digested telomere oligonucleotide mixes containing from 0% 4U: 100% 0U to 100% 4U: 0% 0U oligonucleotides (Figure 7.6). An increasing proportion of 4U containing telomere oligomer was observed to cause a significant exponential increase in ΔC_T (R^2 = 0.91). Following this verification of the USER assay reliability, the method was then applied to an *in vitro* model of folate deficiency and dUTP supplementation designed to induce uracil misincorporation above sporadic levels.

7.4.2 Both folate deficiency and dUTP supplementation *in vitro* impacted live cell concentration at day 7

The concentration of live cells at day 7 was significantly reduced with decreasing concentrations of folic acid in the cell culture medium ($P = 2.02 \times 10^{-14}$; Figure 7.7). Folate deficiency (≤12 nM folic acid) compared to high folate (3000 nM folic acid) has been shown to significantly reduce cellular proliferation in primary human T lymphocytes following 10 days of culture, compared to 3000 nM folic acid (Courtemanche *et al.*, 2004). That folate deficiency can reduce proliferation and induce cell cycle arrest at S-phase as well as apoptosis (Courtemanche *et al.*, 2004), explains the dose-dependent reduction in live cell counts observed with reducing concentrations of folic acid in this study.

There was a significant decrease in the live cell concentration with dUTP supplementation ($P = 3.95 \times 10^{-6}$). Because there is evidence to suggest that cytotoxicity from aberrant DNA uracil incorporation occurs with inhibition of dTMP synthesis (Olinski *et al.*, 2010), increased DNA uracil incorporation under reduced folic acid and altered dUTP: dTTP pools will likely also affect cellular growth, as has been observed here. Within each group of folic acid treatment and between groups of dUTP supplementation, there was no significant difference between 15 µM and 150 µM doses of dUTP ($P > 0.05$ for each folic acid concentration). This finding illustrates that the effect of dUTP on the concentration of live cells was not significantly increased with this (10×) higher dose of dUTP. While there may certainly be significant effects of higher doses of dUTP (i.e. those > 150 µM) such

greater levels of dUTP were not administered in this study because higher dUTP concentrations may not be physiologically relevant. There was no noted significant interactive effect of folic acid and dUTP on cell concentration at day 7 ($P = 0.28$).

7.4.3 Folic acid deficiency did not significantly alter telomere length

There was no significant effect of folic acid on telomere length in WIL2-NS cells ($P = 0.14$; Figure 7.8). The concentration of folic acid was presumed to exert influence upon telomere length due to its crucial roles in genome stability and maintenance (Table 1.2), which may extend to telomeric DNA integrity and protective telomere function. *In vivo*, folate has been observed to be non-linearly associated with telomere length in male peripheral blood mononuclear cells (Paul *et al.*, 2009). As homocysteine, which is elevated under low folic acid states, has been associated with shorter telomeres (Bekaert *et al.*, 2007, Richards *et al.*, 2008), it is interesting that there was no significant association of folic acid concentrations across two orders of magnitude on telomere length. In a cross-sectional study of women, neither folate nor homocysteine were significantly associated with quartiles of peripheral blood leukocyte telomere length (Liu *et al.*, 2013a), however this study in humans assessed concentrations of folic acid within the physiological range and at present, these *in vitro* doses span the physiological to supraphysiological concentration of folic acid. In addition to the homocysteine-mediated increase in oxidative stress (Richards *et al.*, 2008) induced under low folic acid, there are other factors which might have influenced telomere length in these cells, including DNA methylation within CpG islands of promoters of telomere maintenance genes and at subtelomeres, as well as cell turnover and replicative history. Replicative history of the cells was uniform as each culture was initiated from the same cell stock maintained under optimal cryogenic storage conditions. However, the number of cell cycles at harvest time was likely to be lower under folic acid deficient conditions.

7.4.4 dUTP supplementation of 15 μM was associated with longer telomeres

There was a significant association of dUTP supplementation with telomere length (P trend = 0.038; Figure 7.8). *Post hoc* multiple comparisons showed the WIL2-NS cells treated with 15 μM dUTP had significantly longer telomeres than those cells

grown in 0 µM dUTP ($P = 0.040$). This finding is perplexing as supplementing cultures with dUTP was performed to alter the usual dUTP: dTTP ratio and persuade the cells to incorporate dUTP. This subsequent dUTP incorporation would require increased excision by UDG and BER, and could be converted to single-strand or double stranded DNA breaks if the AP sites are closely opposed (Harrison *et al.*, 2006), as could be potentially misincorporated at telomeric repeat DNA known to contain thymine on both strands. As such, dUTP supplementation was proposed to be associated with shorter telomeres, rather than longer telomeres. Yet, it is possible that these longer telomeres observed with dUTP supplementation may have been dysfunctional and that incorporation of telomeric uracil may have perturbed the protective function of the telosome thereby increasing vulnerability to ALT-recombination (see Conomos *et al.*, 2013 for a comprehensive review on the ALT phenotype). However, as such potentially dysfunctional longer telomeres were not also observed in the 150 µM dUTP cultures, there does not seem to be a clear dose-response influence of dUTP on telomere length. There was additionally no noted significant interactive effect of folic acid and dUTP on telomere length ($P = 0.65$).

7.4.5 Uracil/kb telomere length was not significantly altered with folic acid deficiency or dUTP supplementation

Using the previously developed validated assay (Figure 7.5 and Figure 7.6), uracil was detected within telomeric DNA sequences of WIL2-NS cells (Figure 7.9). This shows that uracil is present in the telomeres of cultured human cells, and that uracil content can be measured. There appeared to be no significant effects of folic acid ($P = 0.14$) and dUTP ($P = 0.41$) on telomeric uracil content in these WIL2-NS cells. There was also no interactive effect of folic acid and dUTP on uracil bases/kb telomere sequence ($P = 0.77$).

The RNA base uracil can arise in DNA through either of two primary pathways (Figure 7.1). As such, this assay for the detection of telomeric uracil may have captured uracil formed by spontaneous deamination of cytosine in addition to misincorporated uracil. The extent of uracil in telomeric DNA from *in situ* deamination of cytosine may not be insignificant as 25% of double stranded eukaryote telomere DNA is cytosine. Furthermore, as a measure of global uracil

content was not captured, the level of uracil within telomeric sequence cannot be compared to the extent of uracil in the genome. Oxidation-induced base lesions have been shown to be more prevalent within telomeric sequences relative to the total genome (O'Callaghan *et al.*, 2011) and less efficiently repaired (Kruk *et al.*, 1995, Rhee *et al.*, 2011). It remains to be seen whether telomeres are more vulnerable to misincorporated uracil – or cytosine deamination – and whether uracil is subsequently less proficiently repaired, as is the case with oxidative telomere damage. Overall, the results suggest that measurement of uracil in the telomere sequence is not a robust indicator of folate deficiency.

7.4.6 Telomere length may be influenced by the content of uracil in telomeric sequence

To explore whether telomere length may be associated with uracil content within the telomere, the two endpoints for each individual culture (n = 45) were tested for their correlation (Figure 7.10). There may be an influence of uracil within the telomere upon telomere length, however this was not shown in the correlation of these two endpoints in the present study (R^2 = 0.004, P = 0.65). Although cells supplemented with 15 µM dUTP had significantly longer telomeres than 0 µM dUTP controls, there was no significant effect of dUTP supplementation on telomeric uracil content. As such, this model of low folate and dUTP supplementation did not appear to significantly increase uracil misincorporation within the telomere.

Previously, thymine starvation in *Escherichia coli* was observed to cause the accumulation of Okazaki fragments, interpreted as an inefficiency in the assembly of these fragments essential for lagging strand DNA replication (Freifelder and Katz, 1971). At the time this finding was mistakenly interpreted to be that of DNA ligase inhibition in a thymine-starved cell until further work illustrated no inhibition of DNA ligase (Freifelder and Levine, 1972) which is required to join the Okazaki fragments. More recently than this, it has been suggested that the incorporation of uracil in thymine-starved cells, and the subsequent removal by uracil glycosylase which generates increased gaps in the DNA sequence, may impact upon Okazaki fragment assembly in the manner previously observed (Ahmad *et al.*, 1998). The G-rich strand of the telomere is replicated by the lagging-strand DNA replication machinery (Gilson and Geli, 2007). For this reason, it remains conceivable that

uracil incorporation in place of thymine on this strand $(TTAGGG)_n$ may cause Okazaki fragment accumulation and potentially, a loss of telomere sequence if there is incomplete replication and assembly. Independently, base damage and AP sites within the telomere are proposed to interfere with the DNA replication fork and increase the number of unreplicated DNA ends (von Zglinicki, 2002) concomitant with the suggestions of Ahmad *et al.* (1998) in molecular situations of excised aberrant bases other than uracil.

In addition to base damage, there are other factors which influence telomere length within the cell, for example recombination, telomerase expression and functionality and cell turnover. Therefore it is certain telomeric uracil content alone will not explain short or long telomeres.

7.5 Conclusion

A method for the quantification of uracil in telomeric DNA sequence was developed and applied in an *in vitro* model of short-term folate deficiency. Telomere length in WIL2-NS cells was not significantly altered with folic acid deficiency, although cells supplemented with 15 µM dUTP had significantly longer telomeres than 0 µM dUTP controls. The method for quantification of uracil in the telomere was performed on DNA cells grown in an *in vitro* model of folic acid deficiency. It was hypothesised that short-term deficiency of folate and supplementation of culture medium with dUTP would cause an increase in telomeric uracil content, however these conditions did not significantly modify the number of uracil bases/kb of telomeric sequence in WIL2-NS cells. Telomeric uracil content may be negatively associated with telomere length as was hypothesised, yet this was not convincingly demonstrated in the present study. For all biological endpoints investigated, there were no significant interactive effects of folic acid and dUTP concentration.

7.5.1 Significance

This is the first demonstration and application of a modified telomere qPCR which can detect and quantify the uracil base content specifically within telomeric DNA sequence.

7.5.2 Future directions

This novel assay allows for the investigation of telomeric uracil content and the impact this may have on telomere length homeostasis. *In vitro* conditions that perturb cytosolic dUTP: dTTP ratios can now be further investigated for changes in uracil content within the telomere which may be shown to impact telomere length and function. Since folic acid, after its conversion to 5-methylenetetrahydrafolate, acts also methyl-donor in generating SAM for DNA methylation, it is reasonable that DNA methylation capacity and extent may be reduced under limited folic acid conditions. As subtelomeric methylation and DNA methyltransferases have been associated with telomere length, it is possible that this *in vitro* model induced differences in DNA methylation which could have affected telomere length independently of DNA or telomere-specific uracil misincorporation. In future, DNA methylation should be measured and maintained to assess the sole impact of modified dUTP: dTTP ratios on telomere length *in vitro*.

8 The effects of folic acid, SAM and dUTP on telomere length, telomeric uracil and DNA methylation in WIL2-NS cells

8.1 Introduction

8.1.1 DNA methylation and folate

Folate is essential for minimising the ratio of dUMP: dTTP within the cell, and thus controlling the amount of uracil available for misincorporation into the DNA of dividing cells (Section 7.1.2). Yet additionally, folate is recognised as a crucial methyl donor for establishing and maintaining patterns of DNA methylation. In vertebrates, DNA methylation involves the addition of a methyl group (CH_3) to cytosine residues in CpG dinucleotides and consequently forms the stable modified base 5-methylcytosine (m^5C) (Figure 8.1). Specifically, DNA methyltransferase (DNMT) covalently binds to the 6-carbon position of cytosine and donates CH_3 to the 5-carbon position in the cytosine ring by cofactor S-adenosyl methionine (SAM) before DNMT is released (Santi *et al.*, 1984).

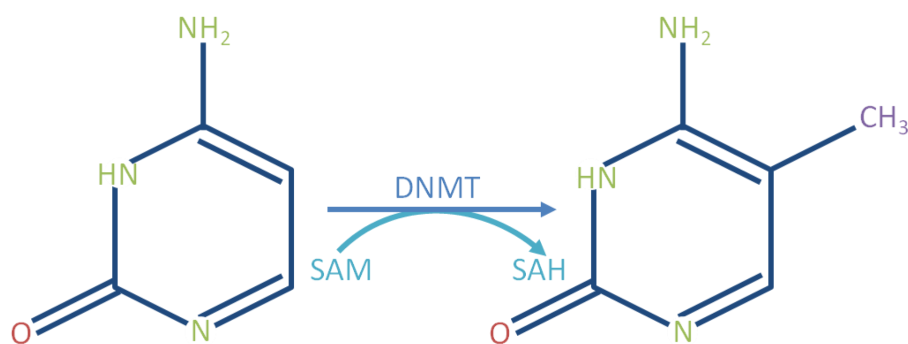

Figure 8.1 – DNA methylation: chemical conversion of cytosine to 5-methylcytosine

With the transfer of the methyl moiety from SAM to the 5-carbon position in the cytosine ring, SAH is produced. The cellular SAM: SAH ratio indicates the methylation capacity in the cell; when the ratio is low, there is a reduced cellular methylation potential (Chiang and Cantoni, 1979, Hoffman *et al.*, 1980, Cantoni, 1985). Folate deficiency may deplete cellular SAM levels, reducing the SAM: SAH ratio and methylation potential, and in turn cause DNA hypomethylation within the cell (Duthie and Hawdon, 1998).

The depletion of folate *in vivo* has been shown to correspond with increased homocysteine and DNA hypomethylation in leukocytes (Rampersaud *et al.*, 2000) and lymphocytes (Jacob *et al.*, 1998). The extent of DNA methylation in lymphocytes was restored with 3 weeks folate repletion (Jacob *et al.*, 1998) however in leukocytes, DNA methylation was not reversed after 7-week folate repletion (Rampersaud *et al.*, 2000). Due to the complex relationship between folate exposure and DNA methylation (Reviewed in Crider *et al.*, 2012, Ly *et al.*, 2012), which may differ with MTHFR 677 status (Crider *et al.*, 2011) there is a need to further investigate the molecular functions of folate.

8.1.2 DNA methylation and nutrients

In addition to folate, other dietary micronutrients can affect DNA methylation *via* a range of targets (Table 8.1), including polyphenols such as (-)-epigallocatechin 3-gallate from green tea and genistein from soybean (Fang *et al.*, 2007).

There may also be active gene-nutrient interactions which impact telomere length. An example of which is the *MTHFR* 677C→T polymorphism which has been shown to influence DNA methylation by impacting homocysteine and folate status through the reduced activity and thermolability of the mutant enzyme (Friso *et al.*, 2002). In another study, the polymorphism was weakly associated ($P = 0.065$) with increased telomere length when folate status is low (Paul *et al.*, 2009). Paul *et al.* (2009) hypothesise that the increase in telomere length observed in the lowest quartiles of folate is caused by DNA hypomethylation and the induction of a DNA damage response to critically short telomeres (d'Adda di Fagagna *et al.*, 2003) and the succeeding global decondensation of chromatin (Ziv *et al.*, 2006, Murga *et al.*, 2007).

Table 8.1 – Micronutrients which can influence DNA methylation

MICRONUTRIENT	BIOLOGICAL INVOLVEMENT WITH DNA METHYLATION
Folate	Methyl acceptors and donors in one carbon metabolism
Vitamin B_{12}	Coenzyme for MTR
Vitamin B_6	Coenzyme for SHMT, CBS and cystathionase
Vitamin B_2	Coenzyme for MTHFR
Methionine	Precursor of SAM
Choline	Homocysteine re-methylation after conversion to betaine
Betaine	Homocysteine re-methylation by BHMT
Serine	Methyl donor to THF by SHMT
Retinoic Acid	Increases activity of GNMT
Zinc	Coenzyme for MAT
Selenium	Increases the transsulfuration pathway
Genistein	Inhibition of DNMTs
Tea polyphenols	Inhibition of DNMTs

Table modified from Choi and Friso (2009)
Abbreviations; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathione-β-synthase; DNMT, DNA-methyltransferase; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SAM, S-adenosyl methionine; SHMT, Serine hydroxymethyltransferase; THF, tetrahydrofolate.

8.1.3 Subtelomeric DNA methylation and telomere length

Localised DNA methylation at the subtelomere has also been linked with telomere length. Unlike the mammalian telomere sequence $(TTAGGG)_n$ which is devoid of CpG dinucleotide DNA methylation substrates, sequences proximal to the telomere – aptly, the subtelomeric region – are reported to be CpG dense (Brock *et al.*, 1999, Steinert *et al.*, 2004). Mammalian telomeric and subtelomeric regions contain DNA and histone modifications which are exhibited by constitutive heterochromatin; namely these regions possess DNA hypermethylation, histone hypoacetylation and hypermethylation of histone H3 at various lysines, most notably at lysine 9 (H3K9) (Blasco, 2007). That the reduction of constitutive heterochromatin-characteristic histone hypermethylation in Suv39h1 and Suv39h2 histone methyltransferase-null mice is associated with changes in telomere length demonstrates the epigenetic regulation of mammalian telomere length (Garcia-Cao *et al.*, 2004).

Both maintenance (*DNMT1*) and *de novo* (*DNMT3A* and *DNMT3B*) DNA methyltransferases have been shown to negatively regulate telomere length (Gonzalo *et al.*, 2006). DNMT-deficient cells were observed to possess higher levels of telomeric recombination, as identified by the presence of ALT-associated PML bodies (APBs) (Gonzalo *et al.*, 2006), which can cause longer, and heterogeneous telomere lengths.

Immunodeficiency, centromere instability and facial anomalies syndrome (ICF) is a rare autosomal recessive disease of heterogeneous aetiology, however common mutations in *de novo* DNA methyltransferase *DNMT3B* and aberrant methylation have been noted (Jiang *et al.*, 2005). Specifically, individuals with ICF have been shown to possess hypomethylated subtelomeres, have abnormally short telomere length, and exhibit high expression of TERRA (Yehezkel *et al.*, 2008). Conversely, in women with Alzheimer's disease, relatively high subtelomeric methylation status was noted for peripheral blood leukocytes with the shortest telomere length (Guan *et al.*, 2013). However, these brief examples are observed under a state of disease, or have been noted in those with ICF syndrome.

In normal individuals, with ageing comes a decrease of long telomeres and an increase in the number of short telomeres and it has been proposed that telomeres with less methylated subtelomeric sequences may tend to shorten faster (Maeda *et*

al., 2009). Furthermore, when telomeres shorten to a critical length, there are epigenetic changes at mammalian telomeric and subtelomeric regions including decreased methylation at histones and DNA, and increased histone acetylation (Blasco, 2007).

The influence that folate may exert on telomere length and/or *via* DNA – and subtelomeric – methylation has been recently critically reviewed yet remains unclear (Moores *et al.*, 2011). In this review, it was postulated that insufficient folate may (1) cause accelerated telomere shortening, (2) intrinsically affect telomere function, and/or (3) cause increased telomere-end fusions and subsequent breakage-fusion-bridge cycles in the cell.

8.1.4 Aims and hypotheses

In vitro supplementation with SAM is aimed to allow WIL2-NS cells to maintain DNA methylation throughout culture in media containing reduced concentrations of the methyl donor folic acid which may typically cause hypomethylation. Through maintaining DNA methylation, the consequential or interactive impacts of reduced folic acid and dUTP on telomere length and uracil content within the telomere can be probed without the confounding effect of reduced SAM availability.

It is hypothesised that telomeric uracil content may be highest in lower folic acid concentrations, and in cultures supplemented with high dUTP and low SAM. Further, as telomeric uracil content is hypothesised to be negatively associated with telomere length, telomere length may be longer in cells grown in high folic acid concentrations without supplementation of dUTP and with SAM.

8.2 Methods

8.2.1 WIL2-NS *in vitro* experimentation

A 3 × 3 × 4 factorial model of various concentrations of folic acid (FA), dUTP and SAM was established with a total of 36 alternate treatment conditions, and $n = 6$ replicates per group (total $n = 216$). Each individual culture is represented schematically in Figure 8.2. The experiment was repeated once and results from both experiments are shown. WIL2-NS cell culture conditions, harvest and DNA extraction protocols are as described in Section 3.3. For some culture conditions, there were insufficient quantities of DNA harvested from cells at day 7 required to perform some of the following molecular analyses. The number of live cells following 7 d *in vitro* culture is reported in this chapter, while total cell counts, cell viability, population doublings and generated homocysteine are in Appendix Table 10.17 and Appendix Table 10.18. As there was no separation of cultures at day 7 to yield distinct populations of live and dead cells, subsequent molecular analyses depict observations from all cells within each culture condition.

8.2.2 Telomere length and telomere uracil content

Absolute telomere length was performed as per Section 3.1. Uracil content within the telomere measured as described in Section 3.3.8. The equation of the standard curve in Figure 7.5 was used to calculate the number of uracil residues per kb of telomere sequence from the qPCR ΔC_T values. For experiment one, the mean (± SD) ΔC_T for the 4U oligonucleotide control was 24.37 ± 0.89 ($n = 17$; inter-assay CV = 3.67%), and for the repeat experiment two the mean (± SD) ΔC_T for the 4U oligonucleotide control was 24.65 ± 0.97 ($n = 18$; inter-assay CV = 3.95%).

8.2.3 Global cytosine DNA methylation

Global cytosine DNA methylation (m^5C) of pooled DNA samples was determined by a commercially available kit as detailed in Section 3.3.9. In an effort to reduce the economic cost of DNA methylation determination, each 2 of 6 replicates were pooled for each treatment giving $n = 3$ pooled samples per treatment group, per experiment.

8.2.4 Statistical graphs and analyses

Data presented are mean ± SEM. ANOVA models were performed in IBM SPSS Statistics 20.0. Graphs were prepared in Microsoft Excel and annotated in Microsoft PowerPoint.

30 nM folic acid					
0 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM
15 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM
150 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM

300 nM folic acid					
0 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM
15 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM
150 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM

3000 nM folic acid					
0 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM
15 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM
150 µM dUTP					
0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM	0 µM SAM
5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM	5 µM SAM
10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM	10 µM SAM
50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM	50 µM SAM

Figure 8.2 – Schematic representation of the experimental cultures

Nine 24 well plates were used with separate cultures of WIL2-NS in media with differing folic acid, dUTP and SAM concentrations. There were six replicates of each treatment and the experiment was repeated once (n = 216 each).

Abbreviations: dUTP, 2´-Deoxyuridine, 5´-Triphosphate; SAM, S-(5'-Adenosyl)-L-methionine chloride dihydrochloride

8.3 Results

8.3.1 Experiment one: Folic acid, dUTP and SAM influenced WIL2-NS cell viability

Culture conditions of various folic acid (FA), dUTP and SAM were compared for their effect on WIL2-NS cell viability following 7 days of *in vitro* challenge. There was a significant dose dependent-effect of FA on cell viability (Figure 8.3), whereby there were 1.12×10^5 fewer viable cells in 300 nM FA than in 3000 nM FA ($P = 0.031$), and fewest viable cells in 30 nM FA (1.26×10^5 cells fewer than 300 nM FA; $P = 0.014$ and 2.38×10^5 cells fewer than 3000 nM FA; $P = 1.27 \times 10^{-6}$).

There was no significant influence of dUTP on cell viability overall or in 300 nM and 3000 nM FA, however there were significantly fewer cells in 30 nM FA cultures supplemented with 15 µM dUTP than 150 µM dUTP (difference of 1.46×10^5 cells; $P = 0.042$) and 0 µM dUTP (difference of 1.45×10^5 cells; $P = 0.040$; Figure 8.3A). Supplementation of 150 µM dUTP was associated with significantly increased viability in 30 nM FA with no SAM, however both 15 µM and 150 µM dUTP supplementation in 3000 nM FA with 50 µM SAM were significantly associated with a drastically reduced number of viable cells (Figure 8.3C).

Following 7 day culture, there were significantly fewer live cells with 50 µM SAM compared to each of 0 µM (difference of 3.91×10^5 cells; $P = 1.11 \times 10^{-10}$), 5 µM (difference of 2.23×10^5 cells; $P = 1.42 \times 10^{-4}$) and 10 µM SAM (difference of 3.281×10^5 cells; $P = 2.11 \times 10^{-8}$). There were also 1.68×10^5 fewer live cells in 5 µM SAM culture conditions compared to the 0 µM SAM control culture ($P = 0.007$).

Additionally, there were significant interactive effects observed with FA × dUTP, FA × SAM, SAM × dUTP and FA × dUTP × SAM. An ANOVA model incorporating FA, dUTP and SAM treatment with these FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 80% of the variance in live WIL2-NS cells at day 7.

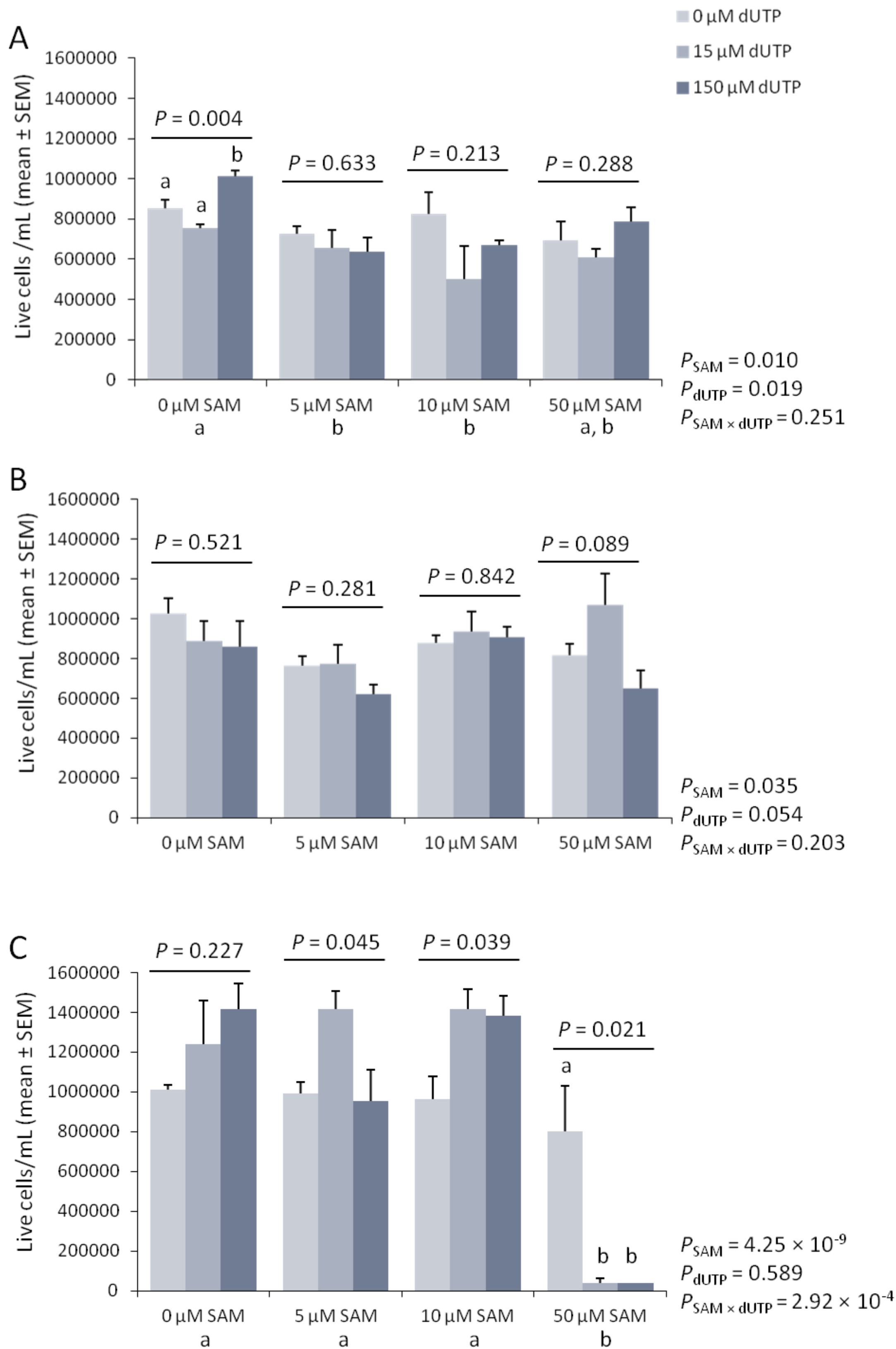

Figure 8.3 – Experiment one: viable cells at day 7

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 1.64 \times 10^{-6}$; $P_{SAM} = 2.74 \times 10^{-11}$; $P_{dUTP} = 0.710$; $P_{FA \times SAM} = 1.76 \times 10^{-13}$; $P_{FA \times dUTP} = 0.036$; $P_{SAM \times dUTP} = 0.004$; $P_{FA \times SAM \times dUTP} = 1.49 \times 10^{-5}$; $R^2 = 0.803$.

8.3.2 Experiment one: Folic acid, SAM and dUTP influenced WIL2-NS telomere length

Significantly shorter telomere length was observed in WIL2-NS cells cultured in 30 nM FA when compared to 300 nM FA (10.1 kb difference, $P = 4.63 \times 10^{-10}$) and 3000 nM FA (6.5 kb difference, $P = 1.51 \times 10^{-4}$) cultures (Figure 8.4). The longest telomere length was noted in those cells cultured in 300 nM FA, where telomere length was an average of 3.6 kb longer than those cultured in 3000 nM FA, although this was not a statistically significant difference ($P = 0.069$).

Supplementation of 150 µM dUTP was associated with significantly longer telomeres than 15 µM dUTP (5.4 kb difference, $P = 2.38 \times 10^{-6}$) and 0 µM dUTP control (4.1 kb difference, $P = 3.82 \times 10^{-4}$), however there was no significant difference in telomere length between the control culture and 15 µM dUTP treatment (1.4 kb difference; $P = 0.57$).

Telomere length of cells cultured in 50 µM SAM were significantly shorter than those cultured in 0 µM SAM (6.4 kb difference; $P = 5.78 \times 10^{-6}$) and 10 µM SAM (6.5 kb difference; $P = 4.24 \times 10^{-6}$). Cells cultured in 5 µM SAM had 5.1 kb shorter telomere length than cells cultured in both 0 µM ($P = 1.63 \times 10^{-4}$) and 10 µM SAM ($P = 1.21 \times 10^{-4}$).

Additionally, there were significant interactive effects observed with FA × dUTP, FA × SAM, SAM × dUTP and FA × dUTP × SAM. An ANOVA model incorporating FA, dUTP and SAM concentrations with these FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 68% of the variance in WIL2-NS cell telomere length at day 7.

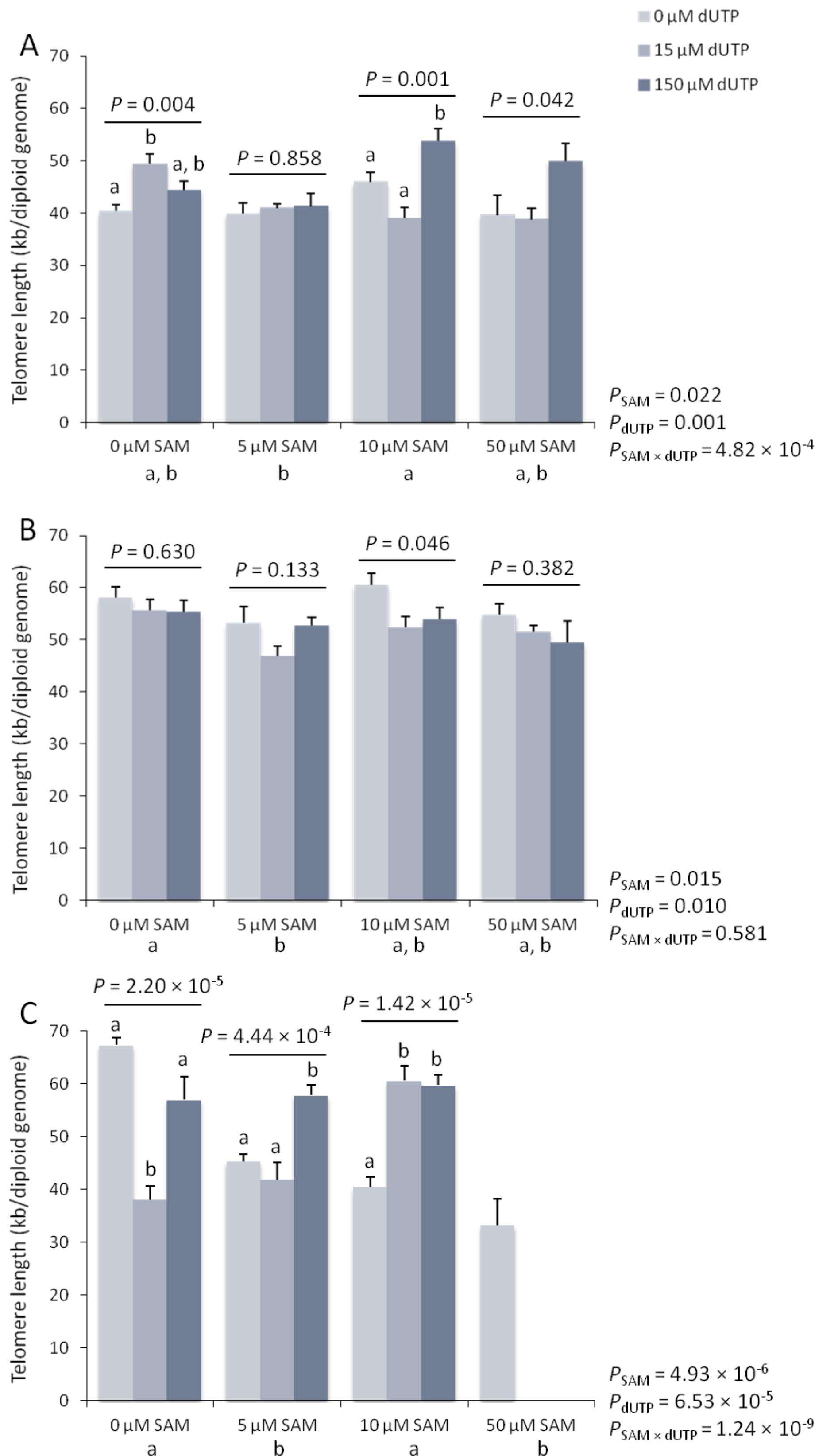

Figure 8.4 – Experiment one: telomere length

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 1.23 \times 10^{-17}$; $P_{SAM} = 5.22 \times 10^{-9}$; $P_{dUTP} = 1.12 \times 10^{-6}$; $P_{FA \times SAM} = 0.003$;
$P_{FA \times dUTP} = 2.93 \times 10^{-5}$; $P_{SAM \times dUTP} = 0.014$; $P_{FA \times SAM \times dUTP} = 6.56 \times 10^{-15}$; $R^2 = 0.683$.

8.3.3 Experiment one: Folic acid and SAM influenced telomeric uracil content in WIL2-NS cells

There was a significant effect of FA on uracil within the telomere whereby fewer uracil residues were detected within the telomeres of WIL2-NS cells cultured in 3000 nM FA compared to 30 nM (1.51 U/kb fewer, $P = 3.47 \times 10^{-5}$) and 300 nM FA (1.21 U/kb fewer, $P = 0.001$; Figure 8.5). However, there was no significant difference in telomeric uracil between cells from 30 nM and 300 nM FA cultures ($P = 0.16$).

There was no significant effect of dUTP on telomeric uracil overall, or within 30 nM, 300 nM and 3000 nM FA cultures. Yet, there were significant effects of dUTP observed only in 3000 nM FA cultures with 0 µM and 50 µM SAM (Figure 8.5C).

In 3000 nM FA, there was also a significant effect of SAM concentration on telomeric uracil such that there was significantly greater uracil content within the telomere for 10 µM SAM compared to 0 µM (1.78 U/kb greater, $P = 0.009$) and 50 µM SAM (1.518 U/kb greater, $P = 0.036$).

There were no significant interactive effects observed for either FA × dUTP, FA × SAM, SAM × dUTP or FA × dUTP × SAM. An ANOVA model incorporating FA, dUTP and SAM concentrations with FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 27% of the variance in telomeric uracil of WIL2-NS cells.

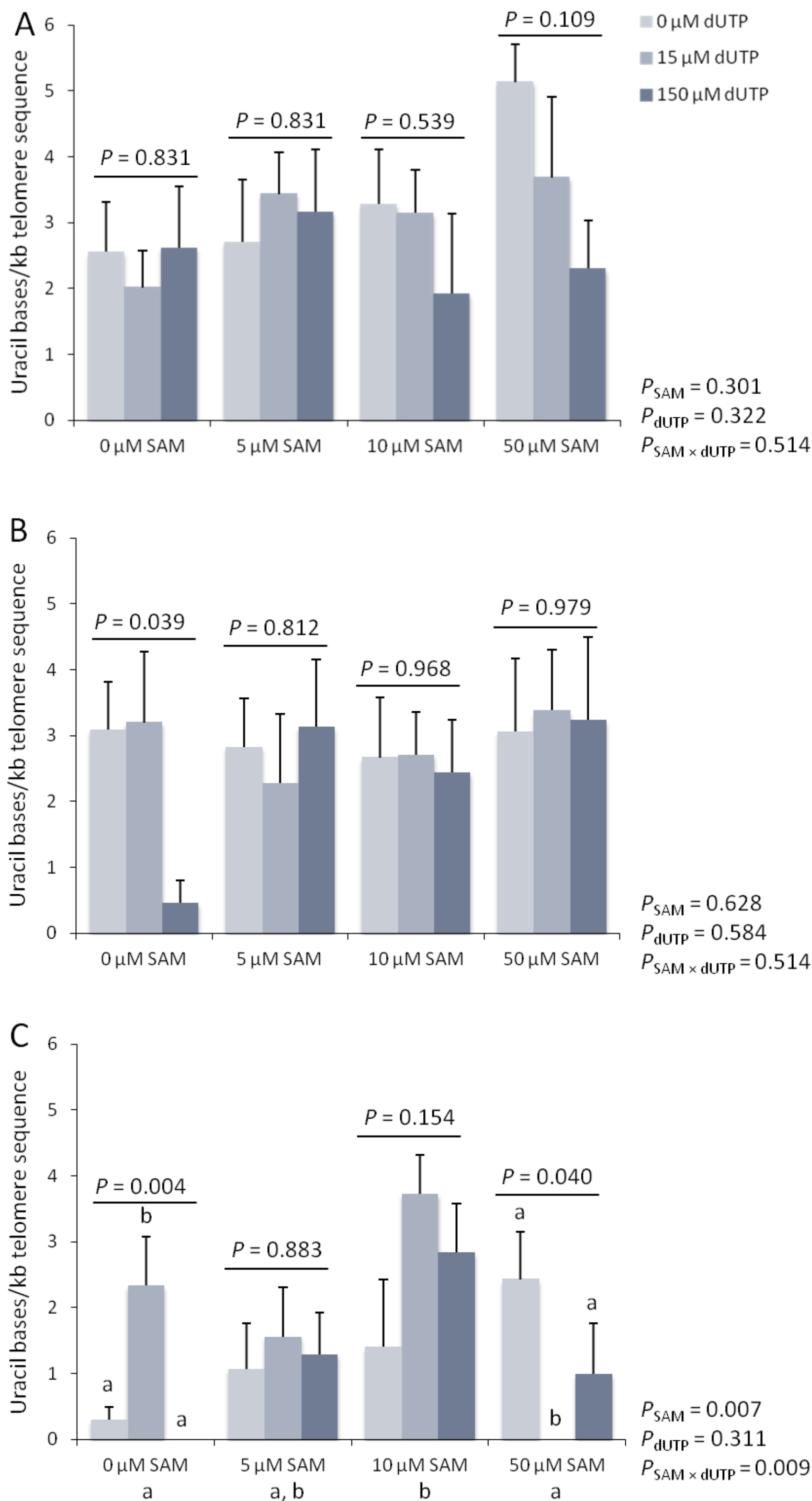

Figure 8.5 – Experiment one: uracil/kb telomeric sequence

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 2.09 \times 10^{-5}$; $P_{SAM} = 0.097$; $P_{dUTP} = 0.162$; $P_{FA \times SAM} = 0.215$; $P_{FA \times dUTP} = 0.779$; $P_{SAM \times dUTP} = 0.214$; $P_{FA \times SAM \times dUTP} = 0.200$; $R^2 = 0.273$.

8.3.4 Experiment one: Folic acid, SAM and dUTP influenced global 5-methylcytosine content in WIL2-NS cells

There was a significant effect of FA on global content of 5-methylcytosine (m^5C; expressed as % m^5C) whereby the highest level of m^5C was detected in 300 nM FA cells, which had greater m^5C than 30 nM FA (0.022 difference in % m^5C; $P = 1.95 \times 10^{-4}$) and 3000 nM FA (0.018 difference in % m^5C; $P = 0.003$) cultured cells (Figure 8.6).

There were no noted significant effects of dUTP supplementation overall, or in 30 nM FA, however there were significant differences in % m^5C for cells cultured in 300 nM and 3000 nM FA and supplemented with dUTP. In 300 nM FA, there was significantly greater m^5C for 15 µM (0.039 difference in % m^5C, $P = 4.57 \times 10^{-5}$) and 150 µM dUTP (0.026 difference in % m^5C, $P = 0.004$) and in 3000 nM FA there was significantly less m^5C in 15 µM dUTP, compared to 150 µM dUTP (0.015 difference in % m^5C; $P = 0.034$) and 0 µM dUTP control (0.020 difference in % m^5C; $P = 0.003$; Figure 8.6B and C).

Amongst all concentrations of FA, there was a significant difference in m^5C content between cells cultured in 5 µM SAM compared to those cultured in 50 µM SAM (0.018 difference in % m^5C; $P = 0.032$). In 300 nM FA, there was a significant increase in m^5C with the addition of 5 µM SAM compared to 0 µM SAM control (Figure 8.6B), however in both 300 nM and 3000 nM FA cultures, there were no observed dose-dependent increases in m^5C content with increased SAM concentration (Figure 8.6B and C).

There were significant interactive effects observed for FA × dUTP, SAM × dUTP and FA × dUTP × SAM interactive terms, however there was no significant interactive effect of FA and SAM concentration. An ANOVA model incorporating FA, dUTP and SAM concentrations with FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 65% of the variance in % m^5C of WIL2-NS cells.

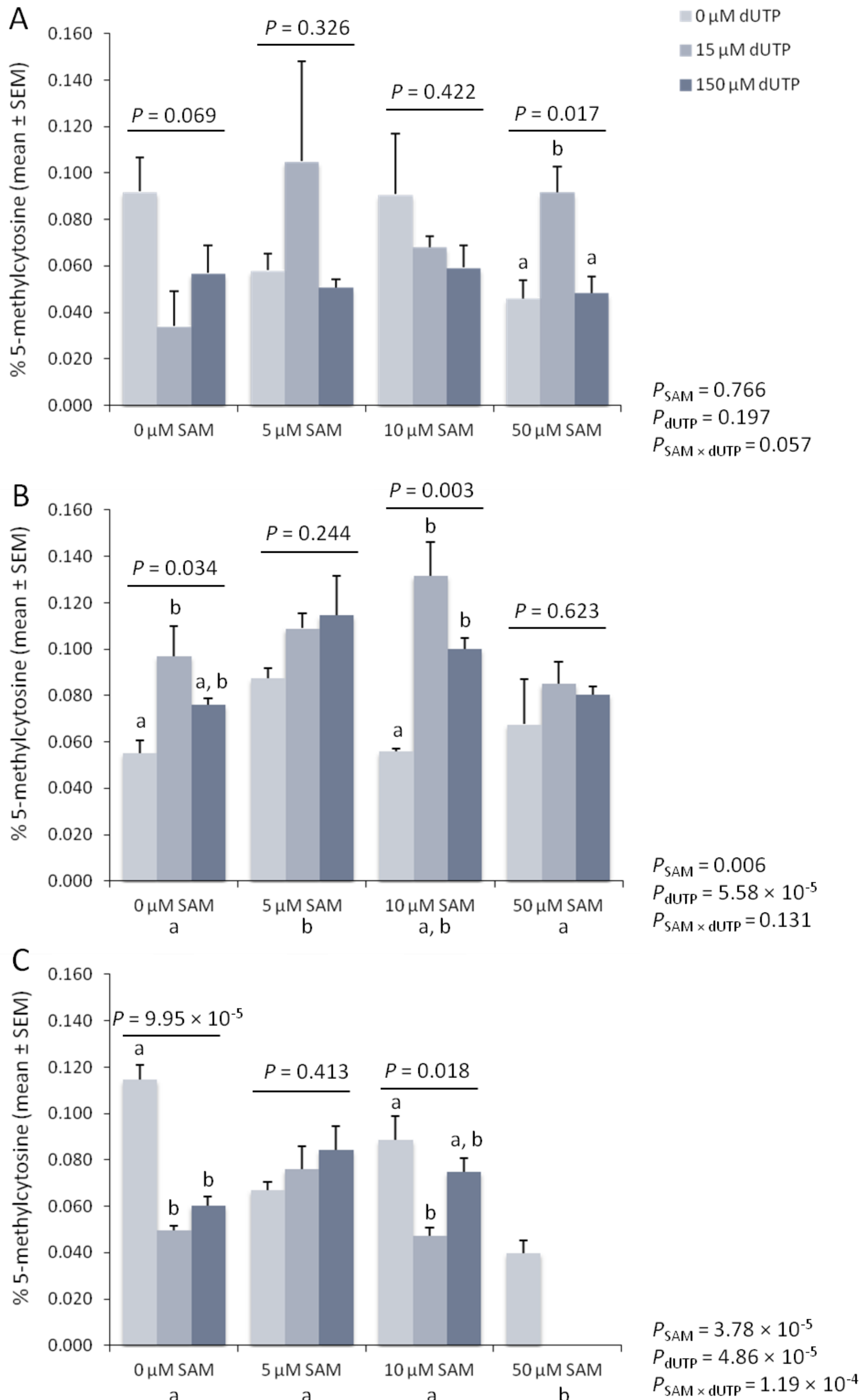

Figure 8.6 – Experiment one: percent global 5-methylcytosine

A, 30 nM folic acid; B, 300 nM folic acid; C, 3000 nM folic acid.
$P_{FA} = 4.77 \times 10^{-5}$; $P_{SAM} = 0.003$; $P_{dUTP} = 0.398$; $P_{FA \times SAM} = 0.199$; $P_{FA \times dUTP} = 3.59 \times 10^{-5}$; $P_{SAM \times dUTP} = 0.028$; $P_{FA \times SAM \times dUTP} = 0.002$; $R^2 = 0.648$.

8.3.5 Experiment two: Folic acid, and SAM influenced WIL2-NS cell viability

There were significantly fewer live cells in 30 nM FA at day 7 compared to 300 nM FA (4.17×10^5 fewer cells; $P = 2.36 \times 10^{-8}$) and 3000 nM FA (4.25×10^5 fewer cells; $P = 2.57 \times 10^{-9}$), however there was no significant difference in cell viability at day 7 between cells cultured in 300 nM and 3000 nM FA ($P = 1.0$; Figure 8.7).

There was no significant effect of dUTP concentration on WIL2-NS cell viability overall in all concentrations of FA, or within 30 nM, 300 nM or 3000 nM FA. There were significant influences of dUTP on cell viability only in those cells supplemented with 50 µM SAM; in 30 nM FA, 150 µM dUTP had 2.32×10^5 greater cells at day 7 compared to 15 µM dUTP ($P = 0.026$; Figure 8.7A). However, in 300 nM FA, there were 2.17×10^5 more live cells at day 7 in the 0 µM dUTP control compared to 15 µM dUTP (difference, $P = 0.046$; (Figure 8.7B).

There were significantly greater live cells after 7 days of culture in all cultures containing 0 µM SAM compared to those with 50 µM SAM (2.23×10^5 greater cells; $P = 0.023$). Additionally, there was a statistically significant interactive effect of SAM and dUTP concentrations on live cells in 300 nM FA (Figure 8.7B).

There was a significant interactive effect observed only for FA × SAM. An ANOVA model incorporating FA, dUTP and SAM concentrations with FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 59% of the variance in the viability of WIL2-NS cells at day 7.

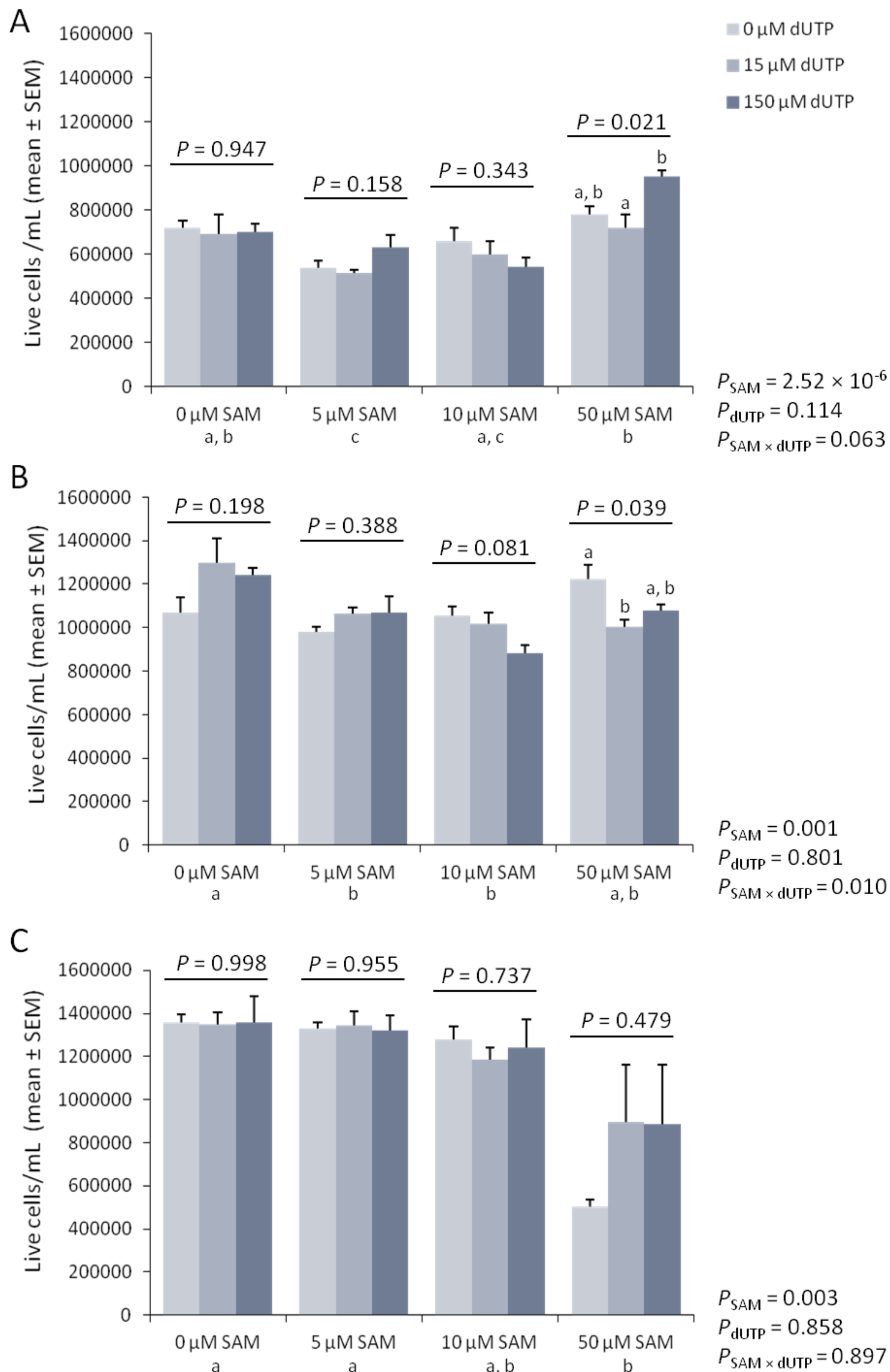

Figure 8.7 – Experiment two: viable cells at day 7

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 7.74 \times 10^{-12}$; $P_{SAM} = 0.068$; $P_{dUTP} = 0.860$; $P_{FA \times SAM} = 3.74 \times 10^{-12}$; $P_{FA \times dUTP} = 0.922$; $P_{SAM \times dUTP} = 0.902$; $P_{FA \times SAM \times dUTP} = 0.885$; $R^2 = 0.586$.

8.3.6 Experiment two: Folic acid, dUTP and SAM influenced WIL2-NS telomere length

There were significantly shorter telomeres observed in those cells cultured in 30 nM FA compared to 300 nM FA (22.0 kb shorter; $P = 9.68 \times 10^{-25}$) and 3000 nM FA (25.9 kb shorter; $P = 1.38 \times 10^{-30}$). There was no significant difference in telomere length between cells cultured in 300 nM and 3000 nM FA ($P = 0.1$; Figure 8.8).

Significantly shorter telomere length was noted in cells grown in 0 µM dUTP compared to 15 µM dUTP (10.1 kb shorter, $P = 3.13 \times 10^{-7}$) and 150 µM dUTP (7.8 kb shorter, $P = 8.97 \times 10^{-5}$). However, there was no significant difference in telomere length between cells cultured in 15 µM and 150 µM dUTP ($P = 0.66$).

There was no significant effect of SAM on telomere length in WIL2-NS cells cultured in all concentrations of FA, however there were significant effects observed within FA treatment of 30 nM and 3000 nM FA (Figure 8.8A and C, respectively). In 30 nM FA, cells treated with 50 µM SAM had significantly shorter telomeres compared to 0 µM SAM controls (7.9 kb shorter, $P = 0.042$) and 5 µM SAM treatment (10.2 kb shorter, $P = 0.004$). In 3000 nM FA, cells treated with 50 µM SAM had significantly longer telomeres than those cells cultured in 0 µM SAM (15.0 kb shorter, $P = 0.007$)

There were significant interactive effects observed for all interactive terms; FA × dUTP, FA × SAM, SAM × dUTP and FA × dUTP × SAM. An ANOVA model incorporating FA, dUTP and SAM concentrations with FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 75% of the variance in telomere length of WIL2-NS cells.

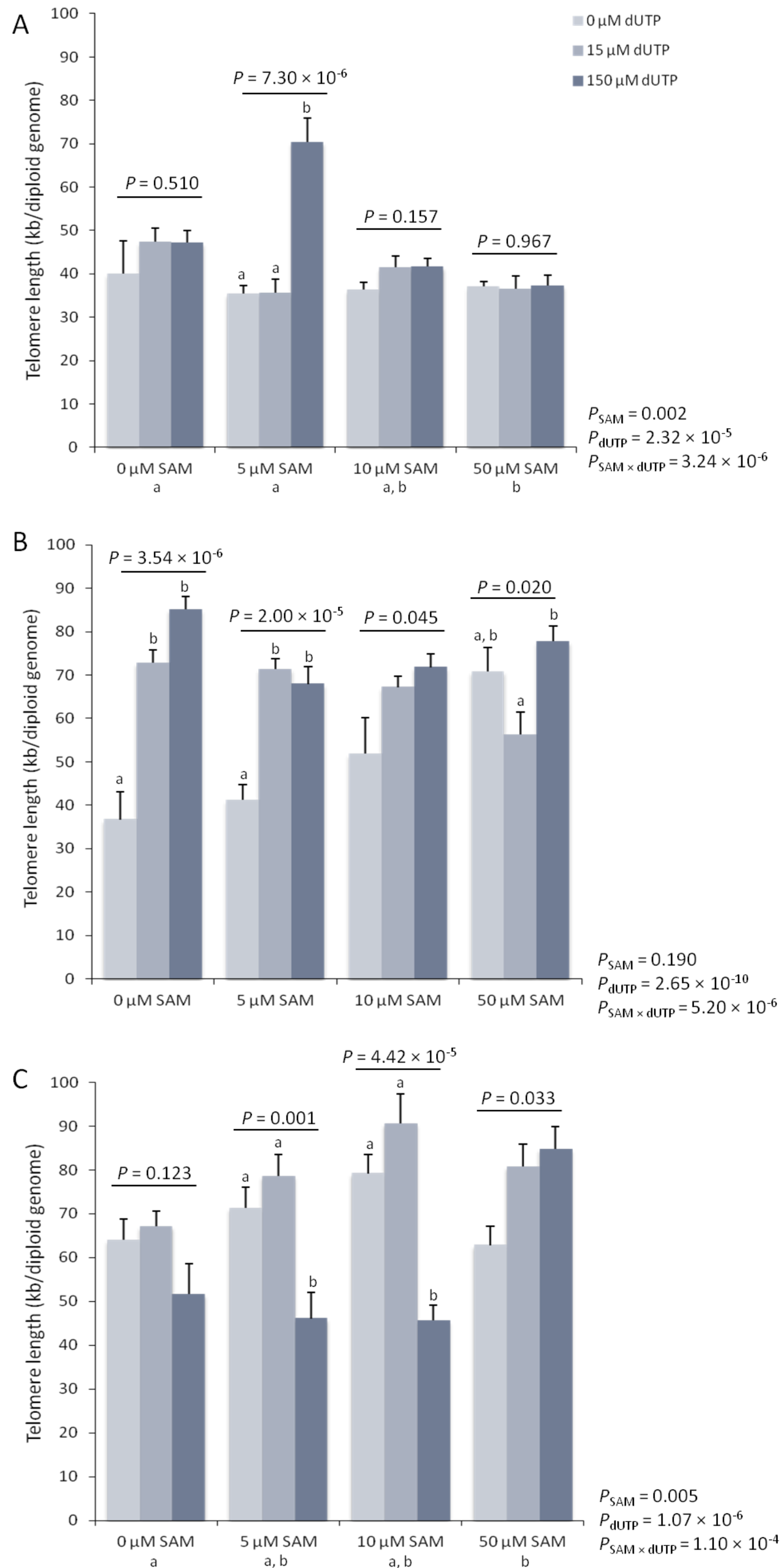

Figure 8.8 – Experiment two: telomere length

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 9.44 \times 10^{-34}$; $P_{SAM} = 0.365$; $P_{dUTP} = 1.54 \times 10^{-7}$; $P_{FA \times SAM} = 4.78 \times 10^{-5}$; $P_{FA \times dUTP} = 8.54 \times 10^{-16}$; $P_{SAM \times dUTP} = 2.47 \times 10^{-4}$; $P_{FA \times SAM \times dUTP} = 7.49 \times 10^{-12}$; $R^2 = 0.752$.

8.3.7 Experiment two: SAM, but not folic acid or dUTP influenced telomeric uracil content in WIL2-NS cells

There was no significant effect of FA ($P = 0.41$) or dUTP ($P = 0.28$) on uracil content within the telomere (Figure 8.9). Overall, there was significant influence of SAM on uracil content within the telomere, however there was a significant SAM effect in the 300 nM FA group only (Figure 8.9B). When compared to the 0 µM SAM control, there was 1.60 U/kb greater in the telomeres of WIL2-NS cells cultured in 50 µM SAM ($P = 0.036$). There were no significant interactions of SAM and dUTP concentration on telomeric uracil content in either 30 nM or 3000 nM FA subgroups yet there was in the 300 nM FA group ($P = 8.02 \times 10^{-5}$).

However overall, there was a significant interactive effect observed for SAM and dUTP concentration only. An ANOVA model incorporating FA, dUTP and SAM concentrations with FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 23% of the variance in uracil content within the telomeres of WIL2-NS cells.

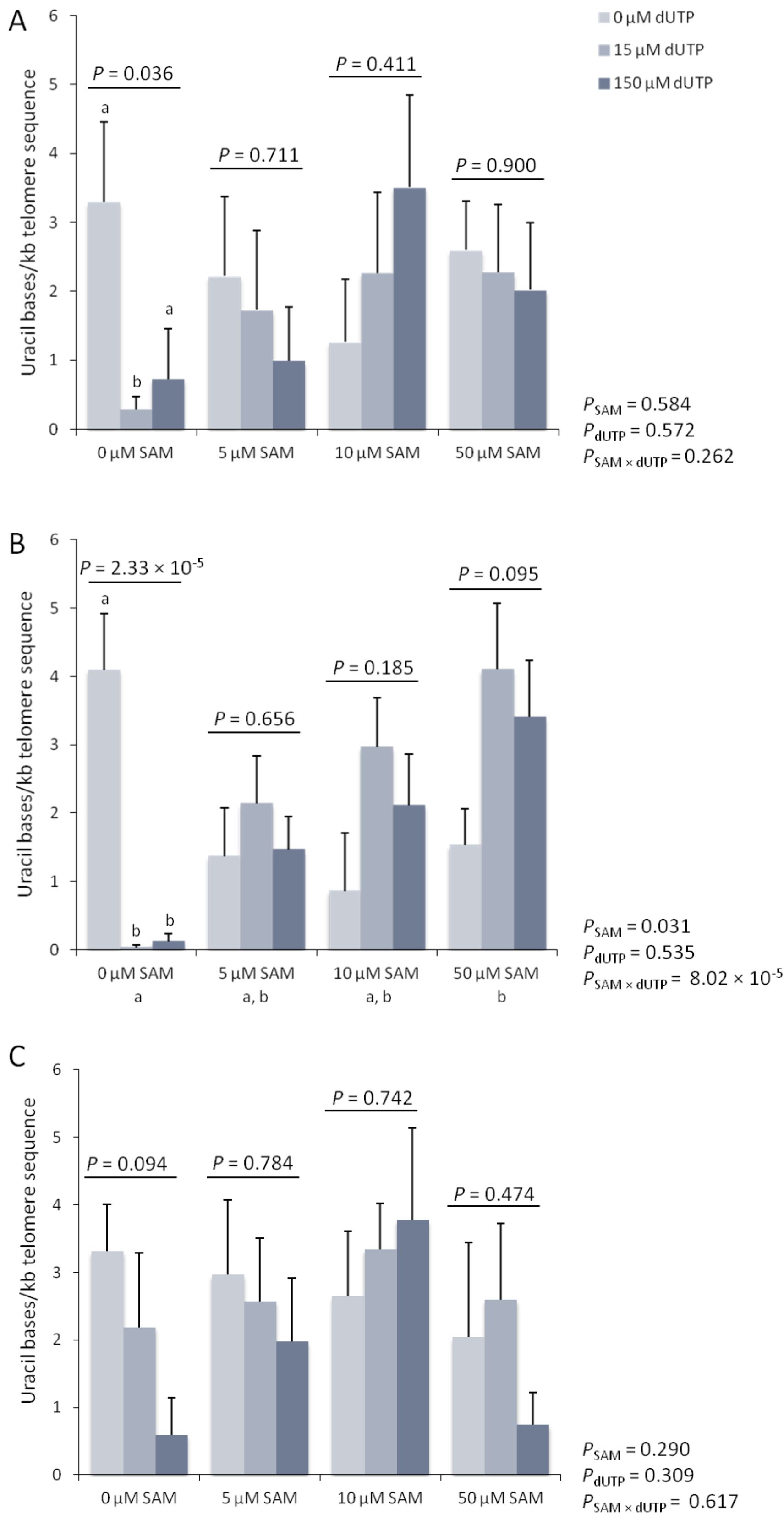

Figure 8.9 – Experiment two: uracil bases/kb telomere sequence

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 0.409$; $P_{SAM} = 0.135$; $P_{dUTP} = 0.280$; $P_{FA \times SAM} = 0.334$; $P_{FA \times dUTP} = 0.656$; $P_{SAM \times dUTP} = 2.64 \times 10^{-4}$; $P_{FA \times SAM \times dUTP} = 0.774$; $R^2 = 0.233$.

8.3.8 Experiment two: Folic acid, dUTP and SAM concentration influenced global 5-methylcytosine in WIL2-NS cells

There was significantly less global m^5C in 3000 nM FA treated cells, compared to 30 nM (0.044 difference in % m^5C, $P = 1.27 \times 10^{-5}$) and 300 nM (0.037 difference in % m^5C, $P = 2.86 \times 10^{-4}$) FA cultured WIL2-NS cells (Figure 8.10). However, there was no significant difference in global m^5C content for 30 nM and 300 nM FA treatments ($P = 1.0$).

There was significantly less methylation for cells grown in 150 µM dUTP compared to 15 µM dUTP (0.044 difference in % m^5C, $P = 1.51 \times 10^{-5}$) and 0 µM dUTP (0.049 difference in % m^5C, $P = 1.91 \times 10^{-6}$). However, there was no significant difference in global m^5C content for 0 µM and 15 µM dUTP treatments ($P = 1.0$).

There was no significant effect of SAM on global m^5C content overall ($P = 0.06$), however there were significant differences of SAM on methylation in 300 nM and 3000 nM FA. In 300 nM FA-cultured WIL2-NS cells, there was significantly higher m^5C content in 5 µM SAM cells compared to 0 µM (0.053 difference in % m^5C, $P = 0.003$) and less m^5C in 50 µM SAM compared to 5 µM (0.056 difference in % m^5C, $P = 0.002$) and 10 µM (0.038 difference in % m^5C, $P = 0.042$) SAM cultures (Figure 8.10B). In 3000 nM FA, there was a significantly higher level of m^5C in 10 µM SAM compared to 50 µM SAM (0.047 difference in % m^5C, $P = 0.020$; Figure 8.10C).

There were significant interactive effects observed for FA × SAM, SAM × dUTP and FA × dUTP × SAM interactive terms, however there was no significant interactive effect of FA and dUTP concentration. An ANOVA model incorporating FA, dUTP and SAM concentrations with FA × SAM, FA × dUTP, SAM × dUTP and FA × SAM × dUTP interactive terms explained 68% of the variance in the global m^5C content in these WIL2-NS cells.

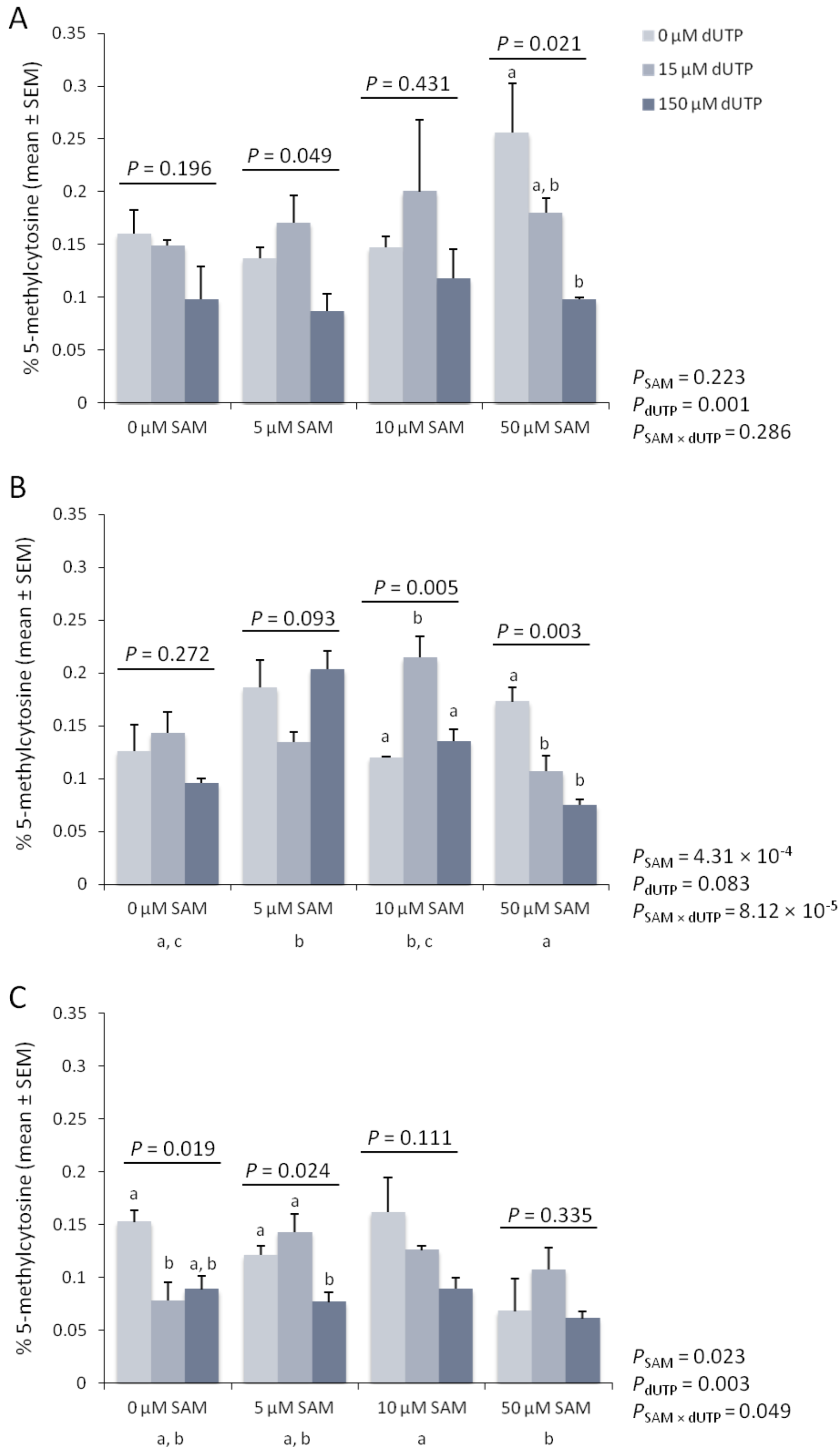

Figure 8.10 – Experiment two: percent global 5-methylcytosine

A 30 nM folic acid; B 300 nM folic acid; C 3000 nM folic acid.
$P_{FA} = 6.36 \times 10^{-6}$; $P_{SAM} = 0.059$; $P_{dUTP} = 4.14 \times 10^{-7}$; $P_{FA \times SAM} = 0.003$; $P_{FA \times dUTP} = 0.101$;
$P_{SAM \times dUTP} = 0.044$; $P_{FA \times SAM \times dUTP} = 0.002$; $R^2 = 0.680$.

8.4 Discussion

8.4.1 FA, dUTP and SAM may affect cell viability alone and interactively

There were significantly fewer cells in 30 nM FA which reflected the reduced viability of WIL2-NS cultured cells in a FA deficient state (Figure 8.3 and Figure 8.7). This present finding is in agreement with that shown in Figure 7.7. Folate deficiency is recognised to impair both DNA excision repair (Choi *et al.*, 1998) and dNTP pool balance (James *et al.*, 1997), induce uracil misincorporation (Blount *et al.*, 1997) and cause chromosomal and DNA damage (Melnyk *et al.*, 1999, Fenech and Crott, 2002, Wang and Fenech, 2003). *In vitro* folate depletion has been shown to affect proliferation and induce apoptosis in various cell types including erythroblasts from p53-null mice (Koury *et al.*, 2000), hepatoma HepG2 cells (Huang *et al.*, 1999) and human CD8-positive T-lymphocytes (Courtemanche *et al.*, 2004). Additionally, homocysteine is known to accelerate senescence in cultured endothelial cells (Xu *et al.*, 2000).

Increased uracil insertion in DNA is cytotoxic (Castillo-Acosta *et al.*, 2012) and for this reason, FA deficiency and dUTP supplementation was assumed to negatively influence cell viability. However, dUTP supplementation did not universally decrease the number of viable cells at day 7. For example, there appeared to be differences in the response to 15 µM and 150 µM dUTP supplementation in cells cultured in 3000 nM folic acid with 50 µM SAM. WIL2-NS cells did not grow under these conditions in experiment one (Figure 8.3) however thrived in this treatment in the repeat experiment two, where cell viability numbers were increased with 15 µM and 150 µM dUTP supplementation (Figure 8.7). It is possible that this *in vitro* supplementation with dUTP did not increase the amount of uracil in the DNA to the point of impacting cell viability, or if increased dUTP was misincorporated in the DNA of dividing cells, it may have been adequately identified, excised and replaced with thymine.

The concentration of dUTP in dividing cells has been reported to be approximately 0.2 µM (Traut, 1994). By supplementing 5×10^5 cells with 150 µM and 15 µM dUTP, there was a potential increase in dUTP per cell of ≈0.003 µM and 0.0003 µM, respectively. The consequence of these doses of dUTP on dUTP: dTTP ratios and cellular physiology in the WIL2-NS lymphoblastoid line is unknown. However, as

these doses of dUTP were previously observed to dose-dependently impact cell viability over 7 d of culture in WIL2-NS cells (Figure 7.7), augmented doses of dUTP were not included in these experiments.

The concentration of SAM administered to WIL2-NS cells did not appear to impact cell viability in a dose-dependent fashion. In a pilot study, a 100 µM dose of SAM in the form of S-(5'-Adenosyl)-L-methionine chloride dihydrochloride (Sigma Aldrich) was shown to greatly decrease WIL2-NS cell viability following 7 days of culture in 30 nM FA (data not shown). As such, the highest dose of SAM used in this present study was 50 µM as this dose did not appear to affect cell viability and as physiologically tolerated doses of SAM were sought. Previously, increasing SAM concentration from 0.5 mM to 1.0 mM to 5.0 mM has been shown to significantly increase apoptosis in both HT-29 and RKO cells (Li *et al.*, 2009). However, SAM has been demonstrated to be selectively pro-apoptotic on liver cancer cells and conversely anti-apoptotic on normal hepatocytes (Ansorena *et al.*, 2002). The WIL2-NS cell line used in this study encodes a non-functional mutant p53 protein which suppresses p53-induced apoptosis and hence permits the relative accumulation of DNA damage compared to p53-wild-type cell lines. However the pro-apoptotic influence of SAM was noted to be p53-independent as HT-29 cells express inactive p53 and RKO cells express wild-type p53 (Li *et al.*, 2009). Under FA deficiency and with the supplementation of methionine *in vitro*, it is possible to induce cell death by apoptosis (Kruman *et al.*, 2002). As SAM treatment in the aforementioned study resulted in the release of cytochrome *c*, there is likelihood for mitochondrial response and involvement (Li *et al.*, 2009).

In these experiments there were statistically significant interactions of FA and SAM on WIL2-NS viable cells at day 7. In Figure 8.3 there were additionally significant interactions of FA with dUTP, SAM with dUTP and FA, SAM with dUTP. This indicates, for example, that the impact of SAM and dUTP may be influenced by the status of FA. Such interactions are not unanticipated as both FA deficiency and dUTP supplementation can together impact cellular dUTP: dTTP ratios, uracil misincorporation and subsequent cytotoxic effects.

8.4.2 Telomere length

Cells cultured in FA-deficient medium (30 nM) were shown to have shorter telomeres than those cultured in 300 nM and 3000 nM FA (Figure 8.4 and Figure 8.8). However, in these experimental replicates, there were both observed longer telomeres in 300 nM FA-cultured cells compared to 3000 nM (Figure 8.4) and no significant difference between telomere lengths in greater FA concentrations (Figure 8.8). In previous human cross-sectional studies (Bekaert *et al.*, 2007, Richards *et al.*, 2008) and in this PhD thesis (Table 4.8 and Table 4.9), high levels of plasma homocysteine have been associated with short telomere length. As homocysteine is elevated in FA deficiency, the observed effect of FA on telomere length may arise from homocysteine-mediated elevations of oxidative stress. A U-shaped response curve has been previously shown for the association of plasma folate with telomere length in men (Paul *et al.*, 2009), however such a relationship was not observed for FA in these *in vitro* samples.

SAM and dUTP were associated with significant differences in telomere length in both experiments, however these effects do not appear to be increased with increasing dose, and in all cases, there was an influence of FA concentration. It is possible that dUTP may provide additional substrate for the synthesis of TTP and that this supplementation may be especially valuable to those cells cultured in low FA conditions (Figure 7.2). Variations in SAM and FA concentration may have induced changes in DNA methylation status at the subtelomere. Epigenetic modifications at the subtelomere are known to induce changes in chromatin structure and impact telomere length (Blasco, 2004), and it would be of great value to assess subtelomeric methylation in these cells cultured with varying levels of methyl donors.

8.4.3 Telomeric uracil

WIL2-NS cells cultured in replete FA (3000 nM) had significantly fewer number of uracil bases per kb of telomeric sequence compared to those cultured in deficient (30 nM) and sufficient FA (300 nM; Section 8.3.3, Figure 8.5) however this finding was not demonstrated in an experimental replication (Figure 8.9) and instead a significant SAM × dUTP interaction was observed. There was no apparent consequence of dUTP supplementation to increase telomeric uracil content (Figure

8.5 and Figure 8.9). It is possible that these WIL2-NS cells were able to repair misincorporated uracil in the genome and within the telomere brought about by dUTP treatment, but the activity of UDG and efficiency of BER in the WIL2-NS cell line was not measured in these experiments. It is possible that under high dUTP: dTTP ratios, uracil may not be successfully eliminated from the DNA by UDG and BER as it can be reincorporated during the repair process (Olinski *et al.*, 2010), however such an impact on telomeric uracil was not detected with increased dUTP availability through supplementation. Any repaired misincorporated uracil from within telomeric sequence was not detected with this assay.

Telomeric regions of DNA have been shown to be more susceptible to oxidative DNA damage (Petersen *et al.*, 1998, O'Callaghan *et al.*, 2011, Vallabhaneni *et al.*, 2013) and such damage may be less efficiently repaired compared to other genomic sequences (Kruk *et al.*, 1995, von Zglinicki, 2002). As there was no global measure of dUTP incorporation, it is not possible to specify whether the telomere may be more vulnerable to uracil misincorporation compared to other regions of the genome. It remains possible that telomeres could contain a higher proportion of uracil compared to other genomic sequences, and that this content could significantly influence telomere shortening as demonstrated with other telomeric base damage (Petersen *et al.*, 1998, von Zglinicki, 2000, von Zglinicki, 2002).

In addition, there are other factors which would influence UDG activity, BER and perhaps telomeric uracil content, for example cellular proliferation. DNA damage is known to delay the progression of the cell cycle (Alberts, 2002) and additionally, the p53 protein can regulate both the BER and NER DNA excision repair pathways (Smith and Seo, 2002, Lu *et al.*, 2004) as well as apoptosis (reviewed in Amaral *et al.*, 2010). Misincorporated uracil in DNA is typically repaired by the BER pathway during S-phase of the cell cycle (Sancar *et al.*, 2004, Branzei and Foiani, 2008). The WIL2-NS cell line contains a mutation in the *p53* gene such that it encodes a non-functional p53 protein and repression of p53-induced apoptosis (Xia *et al.*, 1995). In a damaged cell, p53 levels are increased and the cell cycle is stalled to permit time for the adequate repair of DNA damage. As WIL2-NS cells express non-functional and mutated p53, it is likely that DNA damage accumulates due to impaired signalling for DNA repair and continued cell cycle division.

The misincorporation of uracil in DNA *in vivo* has been shown to be influenced by the concentration of B_{12} and MTHFR 677 genotype (Kapiszewska *et al.*, 2005). In these RPMI1640 culture media of varying FA concentrations, there was abundant B_{12} (3.7 nM). However, reducing the concentration of B_{12} would exacerbate the deficiency of FA to cause increased accumulation of homocysteine (Ziegler *et al.*, 1996), and consequently increase also the misincorporation of uracil (Wickramasinghe and Fida, 1994) (Blount *et al.*, 1997) and hence this could be applied in future modelling. Aside from the described perturbations in FA, dUTP and SAM, the culture conditions of the cell cultures were matched for the concentration of vitamin B_{12} and other RPMI micronutrient levels and additionally WIL2-NS subcultures were established from the same cryogenically stored early passage cells. The ANOVA models applied to experiments one and two accounted for 27% and 23% of the variance in uracil bases per kb of telomeric sequence, respectively, suggesting greater impact of uncontrolled factors, including assay variation. However, the inter-assay coefficient of variation for the uracil assay in both experiment one and experiment two was reasonable (< 4%). Moreover, the wide variation of FA concentration over two orders of magnitude and additional supplementation with SAM may have induced epigenetic changes in the form of gene-specific methylation to reduce the expression of DNA repair genes.

8.4.4 Global 5-methylcytosine

The greatest level of global methylation, as measured by % m^5C content was detected in 300 nM FA (Figure 8.6) and in 300 nM and 30 nM FA-cultured cells (Figure 8.10). As FA is a methyl donor, it is reasonable to anticipate greater DNA methylation with abundant FA (3000 nM) than in lower concentrations of FA (300 nM and 30 nM). It is well recognised that homocysteine levels are elevated when FA is deficient, such as in 30 nM FA. Persistent elevation of homocysteine, such as in these FA-deficient conditions – causes an increase in intracellular SAH and inhibition of DNMTs (James *et al.*, 2002). Under FA deficiency, there are fewer methyl groups available for cytosine methylation, and in addition there is elevated homocysteine which through the inhibition of DNMT, would also impact global methylation status.

There was no dose-dependent increase in methylation with increased FA or SAM concentration. As SAM is generally unstable when prepared – as much as 10% loss of purity per day has been observed (Sigma Aldrich) – it is likely that SAM activity declined over time during this *in vitro* culture. SAM was supplied to WIL2-NS cells in an effort to maintain global DNA methylation, and hence stable the methylation status also at the subtelomere. SAM is known to inhibit MTHFR, consequently providing a feedback regulation that prevents the “folate methyl trap” and prioritises folate one-carbon units for DNA precursor synthesis when methionine is abundant (Herbig *et al.*, 2002).

Some DNA methylation methods detect other methylated cytosine bases such as 5-hydroxymethylcytosine (hm^5C), however this chosen method was selected for high specificity to m^5C with no cross-reactivity to unmethylated cytosine and negligible cross-reactivity to hm^5C. However, it is important to note that some m^5C captured with this method may have arisen in the DNA from the deamination of thymine and not solely from the covalent addition of CH_3 to cytosine.

8.5 Conclusion

One option was to combine the data obtained in experiment one and two, but due to the observed disparity between the experiments, this strategy was deemed inappropriate. The discrepancy of the results in this *in vitro* study highlight the variability of the system, and the need for altered culture conditions, such as time in culture and chronic dUTP and SAM exposure, in future experimentation. Additionally, the sampling of cells at one time point was restrictive and it may have been more fruitful to sample the cultures at multiple time points, especially as the current results may not be reflective of a robust model nor an achieved *in vitro* steady-state.

It is anticipated that this work may provide insight for planning studies designed at assessing the roles of methyl donors – such as FA and SAM – on telomere length and telomeric uracil content. Global cytosine DNA methylation may not be a sensitive marker of folate status in studies of telomere biology, instead genome-wide and telomere-specific measures of uracil or uracil-induced DNA breaks may be more insightful. In order to induce greater uracil misincorporation in the genome and at the telomere, UDG and BER inhibition or null models which negate repair should be utilised.

8.5.1 Future directions

It is reasonable to hypothesise global uracil may be correlated with telomeric uracil content, and that the telomere may be a more vulnerable region for uracil misincorporation and BER, however future study is required to address these knowledge gaps. It also remains to be seen whether there is a relationship between global and subtelomeric methylation. Consequently, methylation changes at the subtelomere may be more insightful in telomere studies as there are known associations between subtelomere methylation and telomere length and function. Immediate efforts should also focus on the optimisation and verification of this method for *in vivo* samples, such as human DNA or DNA from animal models of UDG knockout or deficiency to determine the impacts of telomeric uracil on telomere length at the organism level.

9 Discussion, conclusions and knowledge gaps

9.1 Discussion and conclusions

9.1.1 *In vivo*: cross-sectional associations from the *Polypill* study

In this cohort of middle-aged South Australians, plasma micronutrient levels were associated with PBMC telomere length; there was a significant negative correlation of plasma homocysteine with telomere length, in that telomere length was shortest when plasma homocysteine was elevated and conversely there was a significant positive association of plasma vitamin D with telomere length whereby telomere length was longest in those with higher plasma levels of vitamin D (Chapter 4).

Additionally, there were positive correlations of telomere length with parental age at birth of the volunteers which was strongest and significant for paternal age. The apparent inheritability of telomere length and influence of parental ages has been previously identified, however the mechanisms are unclear.

Although cross-sectional study designs such as this are convenient, they make it possible to study associations only, and do not allow for the inference of direction or causality. That said it is unlikely that telomere length of peripheral blood cells may have an influence on the level of micronutrients in blood plasma, and even more so unlikely that one's telomere length in middle age could influence the age of their parents at birth. The over-interpretation of such correlation results can be problematic (Maurage *et al.*, 2013), however the associations noted in this cohort have been detected in other populations and are likely *bona fide*.

This new information extends the previously limited body of knowledge surrounding the association of plasma micronutrients with telomere length in Australians.

9.1.2 *In vivo*: longitudinal observations from the *Polypill* study

The *Polypill* study was a double-blinded placebo controlled randomised intervention trial designed to assess changes in DNA damage over time with micronutrient supplementation (folic acid, vitamin B_{12}, vitamin E, retinol, nicotinic acid and calcium; Chapter 5). Sixteen-week supplementation with the micronutrient *Polypill* supplement was associated with significant increases in plasma folic acid, B_{12}, α-tocopherol, retinol and niacin number. However, there were no significant differences in telomere length changes over time between the placebo and *Polypill* micronutrient intervention group. The proportion of individuals who displayed distinct telomere length trajectories – whether shortened, maintained or lengthened – were also not notably different between the two groups. Thus, in this study, the *Polypill* did not affect telomere length or trajectory in this sample over the 16-week period. Amongst the subgroup of individuals randomised to the placebo, the change in plasma zinc was significantly associated with telomere length trajectory, whereby the greatest reduction in plasma zinc over time was observed in those whose telomere lengths decreased >10% in the same period.

Recent longitudinal observations of telomere length, including this one, report a proportion of individuals who exhibit telomere lengthening. In a recent review of these studies, the authors concluded that such leukocyte telomere length increases observed in longitudinal study designs was predominantly, if not entirely, an artefact of measurement error which is exacerbated by short follow-up periods (Steenstrup *et al.*, 2013a). Reported leukocyte telomere lengthening over time was said to be far less frequent in studies with long follow-up times (Steenstrup *et al.*, 2013a). However, this is to be expected, as age-related telomere attrition is more likely to be detected over increased periods of ageing. Shorter duration longitudinal studies which report a higher proportion of telomere lengthening – up to a third of participants as also observed in this present study – may reflect the short-term effect of lifestyle change or intervention where such effects may be greater than the age-associated decline in telomere length during the brief period. For example, short-term intervention studies of 12 – 16 weeks follow-up have reported detectable and significant changes in telomerase with vitamin D supplementation (Zhu *et al.*, 2012) and comprehensive lifestyle changes (Ornish *et al.*, 2008).

Telomere length is known to vary across chromosomes in the human genome (Martens *et al.*, 1998) and moreover, telomere length can differ between homologous chromosomes (Goldman *et al.*, 2005) with faster telomeric attrition in the homologous chromosome with greater telomere length (Aviv *et al.*, 2009). There are indeed biological observations and explanations which support telomere lengthening over time, especially for shorter duration follow-up measurements as well as a described mechanism by which telomere length attrition is impacted upon by telomere length itself. While RTM effects are of rational concern and can be addressed in longitudinal analyses, when they are controlled for, a significant association of longer baseline telomere length with higher telomere length attrition in LTLs persists (Verhulst *et al.*, 2013).

Although there was no effect of *Polypill* micronutrient supplementation on telomere length of the individuals in this cohort, there may be greater benefits of supplementation in other populations. As such there may be a greater effect of micronutrient supplementation in those malnourished, deficient, at risk of disease or those with short telomere length, and hence such targeted interventions should be explored.

9.1.3 *In vivo*: longitudinal observations from the pilot study of modified *Polypill* composition

It was hypothesised that a modified Polypill micronutrient supplement would impact changes in telomere length due to the known differential roles of micronutrients on genome maintenance (Chapter 6). Yet this hypothesis was not supported, as there were no significant changes in telomere length over the 16-week treatment phase observed in any group. However, it appeared that mean reductions in telomere length observed in both the FERNC *Polypill* group (excluding vitamin B_{12}) ($P = 0.06$) and the placebo group ($P = 0.051$) were approaching statistical significance. As this result may have arisen by chance, future investigations should determine whether vitamin B_{12} in particular has an effect on telomere length, which sees the prevention of telomere length shortening, and moreover whether supplementation with vitamin B_{12} is beneficial.

Additionally, it appeared that the effect of the treatment from week 0 to week 16 may have influenced the trajectory observed from week 16 to week 32 for some *Polypill* formulations, for example that excluding folic acid (BERNC). When groups were further split by their treatment from the previous phase of the RCT (*Polypill* versus placebo), there was a significant difference in the proportion of individuals in the trajectories for the *Polypill* formulation which did not contain folic acid (BERNC); those who initially received the *Polypill* were more likely to have increases in their telomere sequence while those who were initially randomised to placebo treatment were more likely to maintain telomere length with 16 weeks of BERNC supplementation.

Due to the small number of individuals in each treatment group, there was insufficient power to detect statistically significant changes in telomere length over time with treatment of the varied *Polypill* formulations. This phase of the RCT may have been more informative if there was a visible effect of FBERNC *Polypill* micronutrient supplementation on telomere length.

9.1.4 *In vitro*: uracil within the telomere can be detected by a novel qPCR method

As telomere integrity and function may be more insightful than telomere length alone, a method to feasibly detect uracil specifically within the telomeric sequence was conceived (Chapter 0). The modified telomere length qPCR assay to detect uracil within the telomere was developed and following validation with artificial oligonucleotide sequence, it was established to be quantitative and reproducible.

The novel assay to detect telomeric uracil content was then successfully applied to DNA from WIL2-NS cells, and it was found that uracil is present within the telomeric sequences of cultured human cells and that it can be detected in this way. The extent of uracil residues within the telomere did not appear to differ with short-term supplementation of 15 µM and 150 µM dUTP, nor with *in vitro* folic acid depletion to 30 nM.

9.1.5 *In vitro*: folic acid may influence telomere integrity, functionality and length

In addition to varying folic acid and dUTP concentration *in vitro* as per the previously employed model (Chapter 0), WIL2-NS cells were supplemented with SAM in an effort to maintain the methylation status, for example at subtelomeric regions, as folic acid deficiency can reduce the pool of methyl donors available for the conversion of cytosine to 5-methylcytosine (Chapter 8). Through maintaining DNA methylation, the consequential or interactive impacts of reduced folic acid and dUTP on telomere length and uracil content within the telomere were investigated in the absence of the confounding by reduced SAM availability.

It was hypothesised that telomeric uracil content may be highest in lower folic acid concentrations, and in cultures supplemented with dUTP. However, it was evident that uracil content within the telomere was not increased using this model. As such, uracil within the telomere may be tightly regulated in the cell and so uracil within the telomere is not a powerful biomarker of short-term folate deficiency in WIL2-NS cells cultured *in vitro*. Additionally, the short-term culture of seven days may not have been sufficient for cells to achieve steady state. Complex interactive effects of the factors; folic acid, dUTP and SAM were observed.

In order to study the influence of greater telomeric uracil content on telomere length, alternative models should be explored. For example, uracil DNA glycosylase-deficient cells or cells which inefficiently convert dUTP to dTTP could be utilised; or perhaps the combination of these defects could be explored. Alternatively, a similar *in vitro* model to that employed could be modified to include multiple time points of measurement, extended culture in folic acid deficient media and chronic exposure to dUTP.

As the modified qPCR assay to detect telomeric uracil was not previously established, it required *in vitro* verification before it could be utilised on *in vivo* samples. The method may be applied to human samples – such as those from the *Polypill* study – to investigate the typical level of telomeric uracil content *in vivo*, and whether the extent could be minimised with supplementation of micronutrients including folic acid and vitamin B_{12}.

9.2 Remaining knowledge gaps

9.2.1 What are the optimal micronutrient conditions for telomere length?

Plasma levels of micronutrients at a single given time, and changes in the levels over time have been associated with telomere length in this study. There are likely to be differential influences of micronutrients on telomere length and these mechanisms remain to be elucidated. The optimal concentration of micronutrients for peak telomere length maintenance and stability may be determined once these mechanisms are well understood. It is probable that individual differences in genetics, environment and lifestyle factors may influence telomere health and as such there may be inter-individual variations in the optimum concentration of micronutrients. It is conceivable that deficient or malnourished individuals may have greater benefits of micronutrient supplementation on genome maintenance and by extension to telomere integrity, function and length maintenance. Determining the range of dietary micronutrient intakes and plasma levels for optimal telomere length health remains the ultimate goal. Thenceforth, this knowledge can be applied to achieve successful ageing and longevity, and ultimately to delay the onset of ageing-related diseases.

In the growing body of literature on telomeres, there are few RCTs which explore the association of diet or micronutrients on telomere biology. As such, this study represents a substantial contribution to the existing body of knowledge. However, additional studies with larger numbers of individuals are needed to identify and characterise the effects of micronutrients or dietary factors on telomere length, and to determine the biological effects of these differences.

9.2.2 Are PBMCs the optimal tissue for measuring telomere length and how long should an intervention study examining telomere length be?

PBMC samples contain blood cells with a round nucleus; they contain granulocytes including neutrophils, eosinophils, basophils as well as agranulocytes lymphocytes and monocytes. Collectively granulocytes and agranulocytes are referred to as leukocytes. Blood cell formulation is regulated by haematopoietic cytokines including human growth factors and erythropoietin. Additionally, the proportions of these cells *in vivo* can be influenced by infection and immune function. In contrast to cancer cells and embryonic stem cells, human peripheral blood cells express less telomerase activity (Maeda *et al.*, 2009). The cellular subtype ratios and differential rates of cell turnover may complicate using PBMCs as a biomarker.

Of the cellular populations within a sample of PBMCs, the most likely fluctuations in cell subpopulations would perhaps occur in the lymphocytes. Within the population of lymphocytes in a PBMC sample, there are number of lymphocyte subsets with varying telomere length which can be attributed to differences in proliferative history and future replication potential. PBMC samples have been shown to comprise of the following lymphocyte proportions; 13% B lymphocytes, 48% $CD4^+$ T lymphocytes, 10% $CD8^+CD28^+$ T lymphocytes and 5.5% $CD8^+CD28^-$ T lymphocytes (Lin *et al.*, 2010). Of the lymphocyte subpopulations, B lymphocytes have the both longest telomere length and the highest telomerase activity (Lin *et al.*, 2010). While telomere length in $CD4^+$ naïve T cells is longer than that in memory T cells due to differences in *in vivo* replicative history (Weng *et al.*, 1995), the difference is telomere length between the cell types is small (2.5 kb) (Rufer *et al.*, 1998). The percentages of these aforementioned lymphocyte subtypes (B, $CD4^+$, $CD8^+CD28^+$, $CD8^+CD28^-$) is a weak indicator of PBMC telomere length, as only the $CD8^+CD28^-$ (senescent) T lymphocyte frequency was associated with PBMC telomere length (Lin *et al.*, 2010). Thus, the PBMC telomere length measured in the present study may most reflect $CD8^+$ T lymphocytes, which are indicative of an immunosenescent state (Lin *et al.*, 2010). Additionally, while shifts in lymphocyte subtypes may occur with ageing (Yan *et al.*, 2010) and in the event of a typical immune response, it is unlikely

that changes in telomere length observed in this study are solely attributable to such shifts as the changes in telomere length would be minimal.

Alternatively, there are other cell types, from which telomere length may instead be measured in substitution of whole PBMC samples, including buccal (cheek) cells or a specified subpopulation of PBMCs, e.g. lymphocytes. However, there are strengths and weaknesses in sampling each of these tissues. For example, buccal epithelial cells can be easily accessed and sampled non-invasively by a simple cheek swap, but sampling can result in a heterogenous sample of both proliferating and basal cells, and it is unknown whether there are differences in telomere length between these cell types. Due to the added sample processing time, separating PBMCs into cell specific populations may be logistically and economically challenging in large human studies. As such, the weight of evidence that associates telomere length with disease currently exists for telomere length measured in either whole blood or PBMC samples.

There are a number of critical decisions that must be considered in addressing the issue of optimal intervention timing and/or the effect size of an intervention which employs telomere length as the primary outcome measure. These include 1) what is the normal rate of telomere shortening in PBMCs *in vivo*; 2) what is the inter-individual variance in telomere shortening in PBMCs *in vivo*; and 3) what factors influence telomere shortening – whether amelioration or exacerbation in PBMCs *in vivo*. Indeed, there could be differences also across the tissue types, thus if PBMCs are not utilised, then these questions need to be addressed for the tissue to be studied.

9.2.3 Is telomere quality and functionality more important that telomere length?

It has been shown that DNA damage repair within the telomere is less efficacious than repair to other regions of the genome (Kruk *et al.*, 1995, Petersen *et al.*, 1998, von Zglinicki *et al.*, 2000). Moreover, the rate and degree of repair of telomere base damage declines with increasing age in cultured cells, which indicates the functional significance of telomeric repair to the recognised age-associated decline in genomic stability (Kruk *et al.*, 1995). Further, telomerase activity has been negatively modulated by inflammation and oxidative stress (Boccardi *et al.*, 2013) possibly due to the presence of associated oxidatively-damaged bases in the telomere.

Recently – and in Chapter 0 of this thesis – methods have been described which allow the measurement of base aberrations specifically within human telomeric hexanucleotide repeats (O'Callaghan *et al.*, 2011, Vallabhaneni *et al.*, 2013). Considering the potential for telomeric base damage to be less efficiently repaired, and interfere with telomere length maintenance and elongation by telomerase, there is a need for detailed understanding of the relationship between telomere base damage and telomere length. Indeed, it is plausible that telomere base damage may be more valuable than telomere length in some applications – for example in dietary intervention studies – as base damage has potential to affect telomere length maintenance and stability due to inefficient sequence repair, and putative telomerase interference.

9.3 Overview of main study findings and future directions

9.3.1 Diet

This study showed that plasma levels of the toxic metabolite homocysteine were inversely associated with telomere length. Alternatively, plasma vitamin D concentration was positively associated with telomere length, but this positive relationship was much stronger in males than in females. Future investigation should aim at investigating the association of vitamin D with longer telomere length, and the empirical gender difference. In the subsequent intervention study, there was limited evidence of an effect of micronutrient supplementation on telomere length. As such, there remains no solid evidence of supplementation to increase telomere length *in vivo*.

Generally, the causative mechanisms by which telomere length is associated with diet and nutrition – which may be easily targeted through aimed intervention – are not well understood at present. Correspondingly, it remains unknown which diets may be best, and moreover it is possible that optimal diet may be influenced by age, gender and other factors.

9.3.2 Base damage

A novel method to measure uracil within the telomere was developed, and this method was used to demonstrate that uracil is present in the telomere sequence of human lymphoblastoid (WIL2-NS) cells. This new method can be utilised in future studies of telomere length as the integrity of the telomere sequence may be additionally informative of telomere health and function. It is possible that an excessively long telomere sequence becomes an *in situ* target for base damage, and so the relationship between telomere length and telomere base damage should be investigated. Additionally, any association between genomic (global) base damage and telomere base damage should be explored, as base damage in the telomere may provide a protective effect to shield base damage in the coding DNA sequence.

While *in vitro* modelling of the WIL2-NS cell line in varied concentrations of folic acid, dUTP and SAM did not produce well-defined effects of these factors on telomere length, or possible influencers of telomere length – such as uracil base

damage in the telomere – the results may inform future experimentation, and contribute to existing mathematical models of folate metabolism.

9.3.3 Heritability

Parental age was shown to be positively associated with telomere length in offspring who have reached middle age (26 – 61 y), however the basis of the effect is unknown. It is postulated that the association or influence of parental age on telomere length could have a genetic, epigenetic or natural selection basis. For example, men who produce viable sperm at older age (hence an older paternal age) may have longer telomere lengths than men of the same age that are no longer reproductively successful. Indeed, sperm telomere length has been shown to increase with ageing in males. Alternatively, DNA methylation status may be inherited in the offspring and it is possible that children with older fathers could inherit a lower extent of methylation globally or perhaps at the subtelomere, as it is known that methylation in this region corresponds with telomere length. However, telomere length and methylation heritability is not well understood, as embryonic development involves the establishment of both new methylation patterns and telomere length in the foetus. Further understanding the inheritance of telomere length and methylation may help to characterise the described parental associations with longer telomere length.

9.3.4 Future directions

These described main findings of this doctoral study are outlined in Figure 9.1, under the headings of diet, base damage and heritability. However, the influences of these elements on telomere length are likely to be interconnected, rather than discrete. For example, lifestyle – specifically diet – may influence the extent of base damage in the genome and the telomere, or alternatively genetic inheritance may influence the capacity for base damage repair, or the activity of telomere length maintenance genes. Hence, methodological approaches which are utilised in the fields of nutrigenetics and nutrigenomics – which aim to characterise the complex relationship between the genome, diet and health – should be employed to study telomere health.

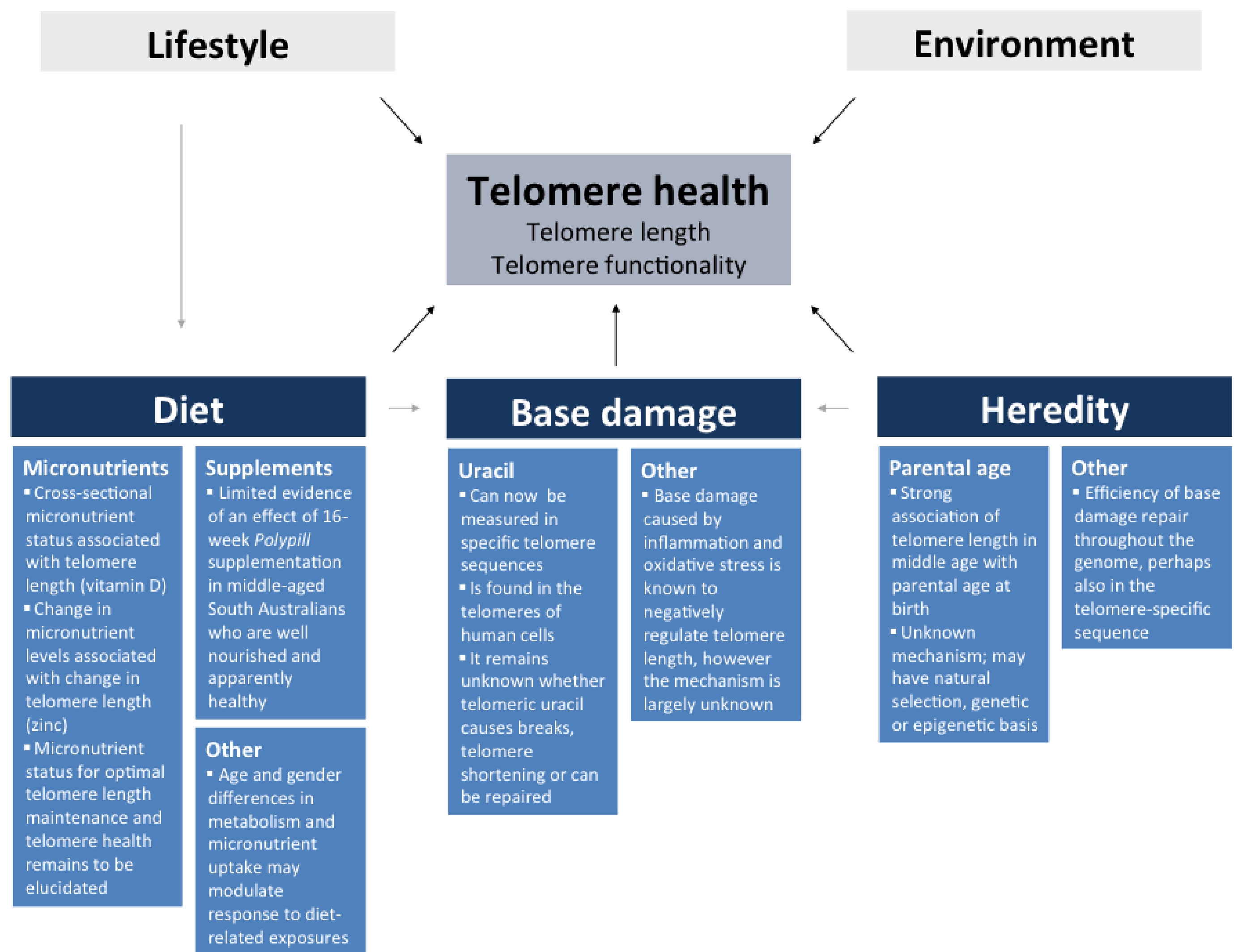

Figure 9.1 – Piecing together the main study findings to identify the remaining knowledge gaps

10 Appendices

10.1 Supplementary data

This section contains supplementary data that is referred to in the text but not presented in any chapter.

Table 10.1 – Volunteer withdrawals during the *Polypill* study: stage and reason for withdrawal, adverse events

STUDY STAGE	NUMBER OF VOLUNTEERS AT STAGE	NUMBER OF WITHDRAWALS DURING STAGE	PERCENT OF WITHDRAWAL AT STAGE	CUMULATIVE NUMBER OF DROPOUTS [%]	REASONS FOR WITHDRAWAL	NUMBER OF ADVERSE EVENTS
Recruited to participate	266	32	12.0	32 [12.0]	Did not meet study criteria: 1 Health/injury/medication/SAE: 1 Lost to contact: 11 Personal: 19	-
Commenced part one	234	22	9.4	54 [20.3]	Did not meet study criteria: 2 Health/injury/medication/SAE: 5 Lost to contact: 1 Personal: 14	6
Completed part one	212	1	0.5	55 [20.7]	Health/injury/medication/SAE: 1	1
Commenced part two	211	8	4.2	63 [23.7]	Health/injury/medication/SAE: 2 Lost to contact: 1 Personal: 5	2
Completed part two	203	1	0.5	64 [24.1]	Health/injury/medication/SAE: 1	-
Commenced part three	202	8	4.0	72 [27.1]	Health/injury/medication/SAE: 3 Lost to contact: 3 Personal: 2	1
Completed part three	194	-	-	-	-	-

Abbreviations; SAE, serious adverse event.

Table 10.2 – Volunteer withdrawals during the *Polypill* study, by study phase and treatment group

STUDY STAGE	NUMBER OF VOLUNTEERS COMMENCING STAGE	NUMBER OF VOLUNTEERS FINISHING STAGE	NUMBER OF WITHDRAWALS DURING STAGE [%]	NUMBER OF WITHDRAWALS FROM *POLYPILL* GROUP [%]	NUMBER OF WITHDRAWALS FROM CONTROL GROUP [%]	CUMULATIVE NUMBER OF DROPOUTS [%]
Recruitment	-	266	32 [12.0]	-	-	32 [12.0]
Phase 1	234	212	23 [9.8]	10 [43.5]	13 [56.5]	55 [20.7]
Phase 2	211	203	9 [4.3]	7 [77.8]	2 [22.2]	64 [24.1]
Phase 3[a]	202	194	8 [4.0]	8 [100]	0 [0]	72 [27.1]

[a] *of the eight participants who withdrew during phase three, 50% were from the phase two active Polypill group (data not shown).*

Table 10.3 – Compliance data from week 0 to week 16 *Polypill* intervention, by treatment group

	POLYPILL **(*n* = 98; 49.25%)**		**PLACEBO (*n* = 101; 50.75%)**	
VARIABLE	**MEAN ± SD**	**MIN - MAX**	**MEAN ± SD**	**MIN - MAX**
Number of supplements issued	360 ± 0	(360 – 360)	360 ± 0	(360 – 360)
Number of supplements returned[a]	38.5 ± 23.5	(5 – 185)	35.2 ± 17.9	(0 – 103)
Number of supplements consumed[a]	324.2 ± 23.5	(175 – 355)	324.8 ± 17.9	(257 – 360)
Percentage of supplements consumed[a] [%]	90.1 ± 6.5	(48.6 – 98.6)	90.2 ± 5.0	(71.4 – 100.0)

[a] the number of cases in the Polypill group is 96 for these variables

Table 10.4 – Compliance data from week 16 to week 32 *Polypill* intervention, by treatment group

VARIABLE	BERNC *n* = 33 [17.4%]	FBENC *n* = 26 [13.7%]	FBERC *n* = 27 [14.2%]	FBERN *n* = 29 [15.3%]	FBRNC *n* = 30 [15.8%]	FERNC *n* = 30 [15.8%]	PLACEBO *n* = 15 [7.9%]
Number of supplements issued	360 ± 0 (360 – 360)	360 ± 0 (360 – 360)	360 ± 0 (360 – 360)	360 ± 0 (360 – 360)	360 ± 0 (360 – 360)	360 ± 0 (360 – 360)	360 ± 0 (360 – 360)
Number of supplements returned	40.2 ± 19.1 (2 – 93)	40.4 ± 24.3 (18 – 127)	53.9 ± 57.5 (18 – 273)	36.8 ± 22.6 (3 – 135)	45.0 ± 29.1 (6 – 132)	46.8 ± 42.5 (0 – 203)	49.3 ± 66.8 (0 – 279)
Number of supplements consumed	319.9 ± 19.1 (267 – 358)	319.7 ± 24.3 (233 – 342)	306.1 ± 57.5 (87 – 342)	323.2 ± 22.6 (225 – 357)	315.0 ± 29.1 (228 – 354)	313.2 ± 42.5 (157 – 360)	310.7 ± 66.8 (81 – 360)
Percentage of supplements consumed [%]	88.8 ± 5.3 (74.2 – 99.4)	88.8 ± 6.8 (64.7 – 95.0)	85.0 ± 16.0 (24.2 – 95.0)	89.8 ± 6.3 (62.5 – 99.2)	87.5 ± 8.1 (63.3 – 98.3)	87.0 ± 11.8 (43.6 – 100.0)	86.3 ± 18.5 (22.5 – 100.0)

Data provided are mean ± SD (minimum – maximum)

Table 10.5 – Correlation of longitudinal telomere length measures for all participants, and separately for week 0 – week 16 groups

GROUP		WEEK 0 AND WEEK 16	WEEK 0 AND WEEK 32	WEEK 16 AND WEEK 32
All	*R*	0.772	0.761	0.741
	P	**<0.0001**	**<0.0001**	**<0.0001**
	n	196	192	188
Placebo week 0-16	*R*	0.710	0.638	0.576
	P	**<0.0001**	**<0.0001**	**<0.0001**
	n	100	96	95
Polypill week 0-16	*R*	0.832	0.859	0.846
	P	**<0.0001**	**<0.0001**	**<0.0001**
	n	96	96	93

R, Pearson correlation coefficient;
To penalise for multiple comparisons in each table row, Bonferroni-adjusted P threshold for statistical significance is 0.017

Table 10.6 – Differences in plasma micronutrient levels and homocysteine at week 0 and 16 in *Polypill* supplement group

VARIABLE	WEEK 0	WEEK 16	*P* (PAIRED SAMPLES T-TEST)
Folate (nmol/L)	24.2 ± 8.3 (8.2 – 41.7)	33.7 ± 6.4 (14.4 – 45.3)	**<0.0001**
Homocysteine (µmol/L)	8.1 ± 2.1 (4.6 – 16.5)	7.5 ± 2.0 (4.0 – 18.0)	**<0.0001**
Vitamin B_{12} (pmol/L)	314.5 ± 192.0 (71 – 1450)	394.7 ± 145.5 (81 – 1104)	**<0.0001**
α-tocopherol (µmol/L)	30.2 ± 6.3 (17.4 – 48.0)	32.2 ± 7.0 (19.7 – 66.5)	**<0.0001**
Retinol (µmol/L)	2.3 ± 0.6 (1.0 – 4.5)	2.4 ± 0.6 (1.2 – 4.8)	**0.003**
Niacin number (NAD/NADP ×100)	155.6 ± 26.7 (89.0 – 250.8)	159.3 ± 28.3 (97.6 – 271.7)	**<0.0001**
Calcium (mmol/L)	2.3 ± 0.1 (2.0 – 2.5)	2.2 ± 0.1 (1.7 – 2.5)	**0.003**

Values supplied are mean ± standard deviation with range of values as minimum - maximum in parentheses.

Table 10.7 – Differences in plasma micronutrient levels and homocysteine at week 0 and 16, by treatment group

VARIABLE	SAMPLED	*POLYPILL* (*n* = 98, 49.25%)	PLACEBO (*n* = 101, 50.75%)	*P* (ANOVA)
Folate (nmol/L)	week 0	24.2 ± 8.3 (8.2 – 41.7)	23.0 ± 9.3 (4.7 – 40.4)	0.32
	week 16	33.7 ± 6.4 (14.4 – 45.3)	22.7 ± 9.3 (6.8 – 45.3)	**<0.0001**
Homocysteine (µmol/L)	week 0	8.1 ± 2.1 (4.6 – 16.5)	8.4 ± 2.4 (4.3 – 21.5)	0.41
	week 16	7.5 ± 2.0 (4.0 – 18.0)	8.5 ± 2.2 (5.1 – 17.0)	**0.0004**
Vitamin B_{12} (pmol/L)	week 0	314.5 ± 192.0 (71 – 1450)	287.6 ± 105.2 (102 – 686)	0.61
	week 16	394.7 ± 145.5 (81 – 1104)	304.4 ± 103.8 (119 – 627)	**<0.0001**
α-tocopherol (µmol/L)	week 0	30.2 ± 6.3 (17.4 – 48.0)	29.3 ± 7.0 (14.8 – 55.6)	0.32
	week 16	32.2 ± 7.0 (19.7 – 66.5)	29.0 ± 6.0 (16.1 – 46.3)	**0.001**
Retinol (µmol/L)	week 0	2.3 ± 0.6 (1.0 -4.5)	2.2 ± 0.5 (1.3 – 4.0)	0.16
	week 16	2.4 ± 0.6 (1.2 – 4.8)	2.2 ± 0.5 (1.3 – 4.2)	**0.005**
Niacin number $\left(\frac{\text{NAD}}{\text{NAD+NADP}} \times 100\right)$	week 0	155.6 ± 26.7 (89.0 – 250.8)	157.7 ± 28.3 (93.6 – 242.4)	0.64
	week 16	159.3 ± 28.3 (97.6 – 271.7)	157.5 ± 29.1 (104.2 – 231.6)	0.59
Calcium (mmol/L)	week 0	2.3 ± 0.1 (2.0 – 2.5)	2.3 ± 0.1 (2.0 – 2.4)	0.27
	week 16	2.2 ± 0.1 (1.7 – 2.5)	2.3 ± 0.1 (2.0 – 2.5)	0.20
Vitamin D (nmol/L)	week 0	79.9 ± 24.6 (33 – 158)	77.9 ± 24.8 (30 – 190)	0.74
	week 16	54.9 ± 20.9 (23 – 141)	58.5 ± 22.0 (24 – 131)	0.19

Mg (µmol/L)	week 0	781.7 ± 53.3 (668.9 – 929.2)	779.3 ± 57.6 (588.3 – 925.0)	0.29
	week 16	769.5 ± 54.0 (604.2 – 937.1)	778.8 ± 47.0 (632.1 – 874.9)	0.48
Se (µmol/L)	week 0	1.4 ± 0.2 (0.9 – 1.9)	1.4 ± 0.2 (1.0 – 2.0)	0.48
	week 16	1.4 ± 0.2 (0.9 – 1.9)	1.4 ± 0.2 (1.1 – 2.1)	0.31
Zn (µmol/L)	week 0	13.3 ± 1.5 (9.0 – 16.5)	13.4 ± 1.7 (9.7 – 19.6)	0.69
	week 16	12.8 ± 1.5 (9.2 – 16.5)	13.1 ± 1.7 (9.9 – 18.5)	0.28

Values supplied are mean ± standard deviation with range of values as minimum - maximum in parentheses.

Table 10.8 – Demographic and anthropometric descriptives and telomere length of the study population, by telomere length trajectory from week 0 to week 16

VARIABLE	SHORTENED >10% LOSS	MAINTAINED ± 10% CHANGE	LENGTHENED >10% GAIN	*P* (ANOVA OR χ^2)
Number [% cases]	63 [31.7]	64 [32.2]	72 [36.2]	0.63
Age (y)	45.3 ± 8.4	44.1 ± 7.3	44.1 ± 8.7	0.62
Gender: males [%]	24 [38.1]	26 [40.6]	36 [50.0]	0.33
BMI (kg/m^2)	27.7 ± 6.1	25.9 ± 4.3	27.0 ± 5.2	0.14
Obese [%]	19 [30.2]	15 [23.4]	21 [29.2]	0.65
Maternal age (y)	27.2 ± 5.7	28.3 ± 5.6	27.9 ± 5.5	0.48
Paternal age (y)	30.1 ± 6.3	31.1 ± 7.6	30.9 ± 7.4	0.80
TL at week 0 (kb)	140.5 ± 62.8	115.8 ± 44.3	81.6 ± 29.7	**<0.001**

NB participants from Polypill and placebo treatment groups have been pooled.

Table 10.9 – Change in micronutrient levels by telomere length trajectory

VARIABLE	SHORTENED (*n* = 63, 31.66%)	MAINTAINED (*n* = 64, 32.16%)	LENGTHENED (*n* = 72, 36.18%)	*P* (ANOVA)
Δ_{0-16}Folate (nmol/L)	4.5	5.3	3.8	0.62
Δ_{0-16}Homocysteine (µmol/L)	-0.25	-0.31	-0.10	0.63
Δ_{0-16}Vitamin B_{12} (pmol/L)	56.4	38.9	48.9	0.72
Δ_{0-16}Alpha-tocopherol (µmol/L)	0.75	0.90	0.84	0.98
Δ_{0-16}Retinol (µmol/L)	-0.02	0.05	0.06	0.29
Δ_{0-16}Niacin number $\left(\frac{\text{NAD}}{\text{NAD} + \text{NADP}} \times 100\right)$	1.5	2.2	1.4	0.88
Δ_{0-16}Calcium (mmol/L)	-0.03	-0.02	-0.02	0.85
Δ_{0-16}Magnesium (µmol/L)	-4.1	-5.8	-7.8	0.92
Δ_{0-16}Selenium (µmol/L)	-0.01	-0.04	-0.02	0.44
Δ_{0-16}Zinc (µmol/L)	-0.89	-0.25	-0.10	**0.006**[a]
Δ_{0-16}Vitamin D (µmol/L)	-22.3	-23.7	-20.7	0.63

Where negative, there was a reduction in the micronutrient level; where positive, there was an increase in the micronutrient level

NB participants from Polypill and placebo treatment groups have been pooled.

[a] Bonferroni post hoc multiple comparisons revealed a near significant difference between telomere length in shortened and maintained trajectories (P = 0.051); significant difference between telomere length in shortened and lengthened trajectories (P = 0.007); and no significant difference between maintained and lengthened trajectories (P = 1.0).

Table 10.10 – Bivariate correlation matrix of telomere length and change in telomere length with change in plasma micronutrient status from week 0 to week 16

		TL @ week 16	ΔTL	ΔFOL	ΔHCY	ΔB_{12}	ΔαTOC	ΔRET	ΔNIA	ΔCa	ΔMg	ΔSe	ΔZn	ΔVITD
TL at week 16	*R*	1	0.172	-0.003	-0.044	-0.003	0.021	0.024	-0.061	-0.073	-0.076	-0.044	0.040	-0.016
	P	-	**0.015**	0.964	0.539	0.964	0.771	0.740	0.389	0.310	0.286	0.537	0.578	0.818
	n	199	199	199	199	199	199	199	199	198	198	198	198	199
ΔTelomere length	*R*		1	-0.063	0.035	-0.076	-0.015	0.057	-0.083	-0.033	-0.039	-0.034	0.131	0.046
	P	-	-	0.378	0.624	0.285	0.831	0.427	0.243	0.648	0.586	0.631	0.066	0.520
	n		199	199	199	199	199	199	199	198	198	198	198	199
ΔFolate	*R*			1	-0.452	0.284	0.173	0.064	0.114	-0.094	-0.109	-0.073	0.024	-0.119
	P	-	-	-	**<0.0001**	**<0.0001**	**0.015**	0.368	0.110	0.190	0.126	0.308	0.736	0.095
	n			199	199	199	199	199	199	198	198	198	198	199
ΔHomocysteine	*R*				1	-0.202	-0.002	0.070	-0.123	0.131	0.123	0.124	0.096	0.042
	P	-	-	-	-	**0.004**	0.980	0.325	0.082	0.066	0.084	0.082	0.177	0.560
	n				199	199	199	199	199	198	198	198	198	199
ΔVitamin B_{12}	*R*					1	0.126	0.057	0.113	0.047	0.004	-0.007	0.092	-0.022
	P	-	-	-	-	-	0.077	0.426	0.111	0.514	0.958	0.923	0.195	0.755
	n					199	199	199	199	198	198	198	198	199
ΔAlpha-tocopherol	*R*						1	0.425	-0.001	0.033	0.060	0.162	0.107	-0.100
	P	-	-	-	-	-	-	**<0.0001**	0.988	0.641	0.400	**0.023**	0.135	0.160
	n						199	199	199	198	198	198	198	199

ΔRetinol	*R*							1	0.055	0.039	0.068	0.053	0.079	-0.065
	P	-	-	-	-	-	-	-	0.439	0.583	0.339	0.460	0.270	0.362
	n							199	199	198	198	198	198	199
ΔNiacin number	*R*								1	-0.121	-0.207	-0.227	0.013	0.030
	P	-	-	-	-	-	-	-	-	0.90	**0.003**	**0.001**	0.852	0.676
	n								199	198	198	198	198	199
ΔCalcium	*R*									1	0.656	0.564	0.529	0.136
	P	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.056
	n									198	198	198	198	198
ΔMagnesium	*R*										1	0.544	0.446	0.059
	P	-	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	0.412
	n										198	198	198	198
ΔSelenium	*R*											1	0.422	0.061
	P	-	-	-	-	-	-	-	-	-	-	-	**<0.0001**	0.390
	n											198	198	198
ΔZinc	*R*												1	0.152
	P	-	-	-	-	-	-	-	-	-	-	-	-	**0.032**
	n												198	198
ΔVitamin D	*R*													1
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
	n													199

R, Pearson correlation coefficient
To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00064
NB participants from Polypill and placebo treatment groups have been pooled.

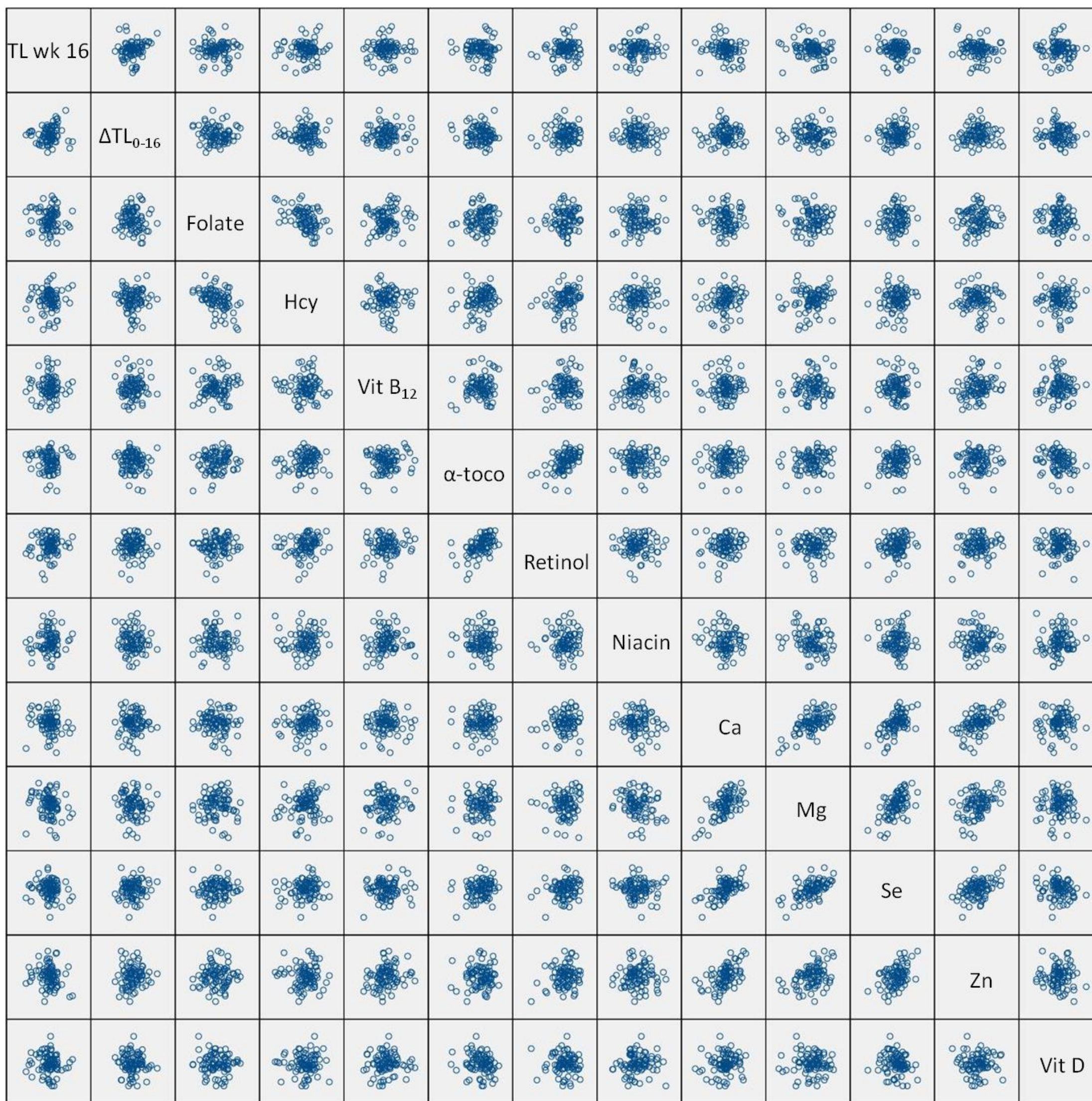

Figure 10.1 – Scatterplot matrix of bivariate correlation associations reported in Table 5.8

n ≥87 as 10 visible extreme outliers removed from n = 98
Abbreviations; α-toco; α-tocopherol; Ca, calcium; ΔTL_{0-16}, change in telomere length from week 0 to week 16; Hcy, homocysteine; Mg, magnesium; Se, selenium; TL, telomere length; vit, vitamin; Zn, zinc.

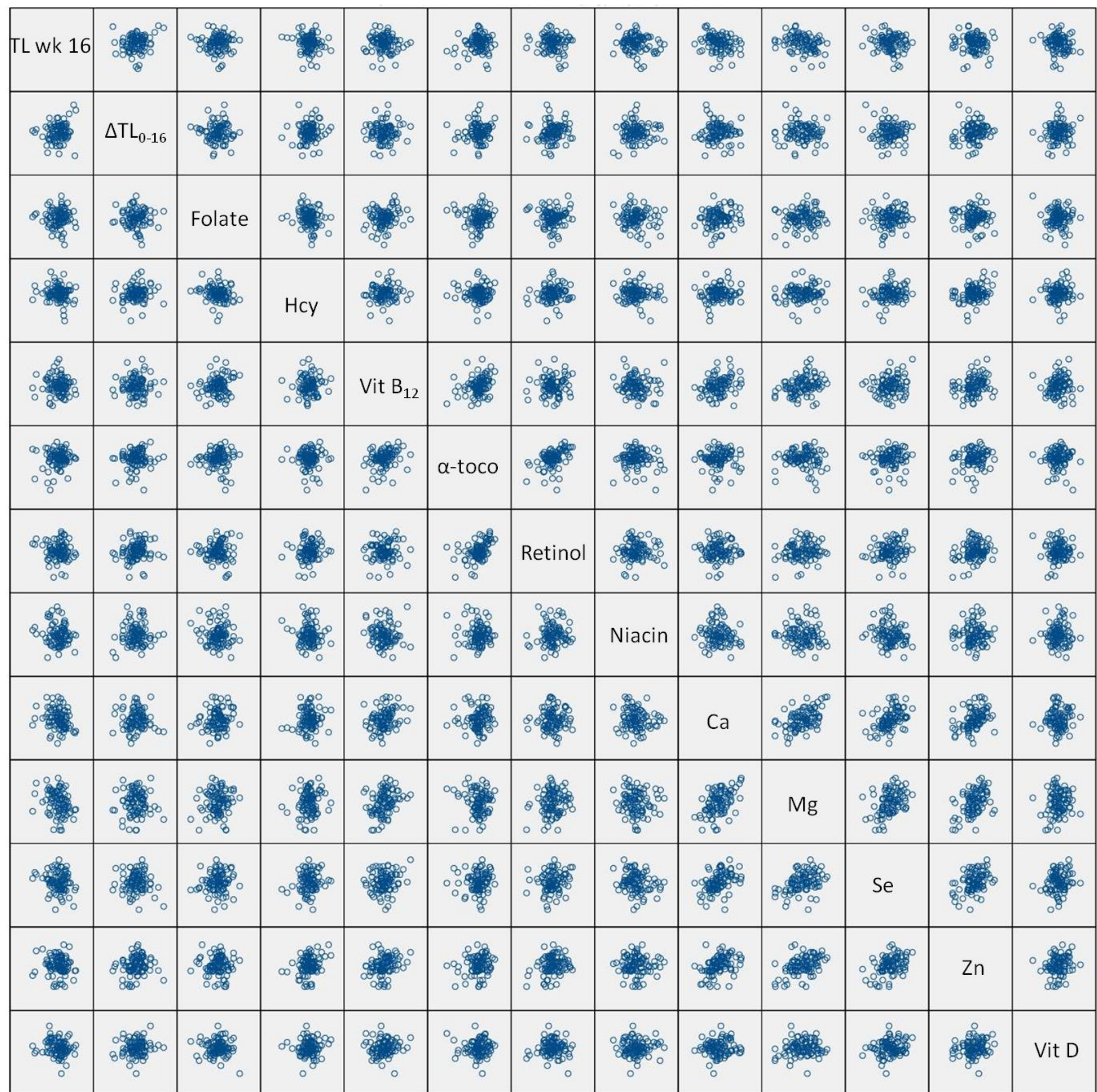

Figure 10.2 – Scatterplot matrix of bivariate correlation associations reported in Table 5.9

n = 92 as 9 extreme outliers removed from n = 101.
Abbreviations; α-toco; α-tocopherol; Ca, calcium; $\Delta TL_{0\text{-}16}$, change in telomere length from week 0 to week 16; Hcy, homocysteine; Mg, magnesium; Se, selenium; TL, telomere length; vit, vitamin; Zn, zinc.

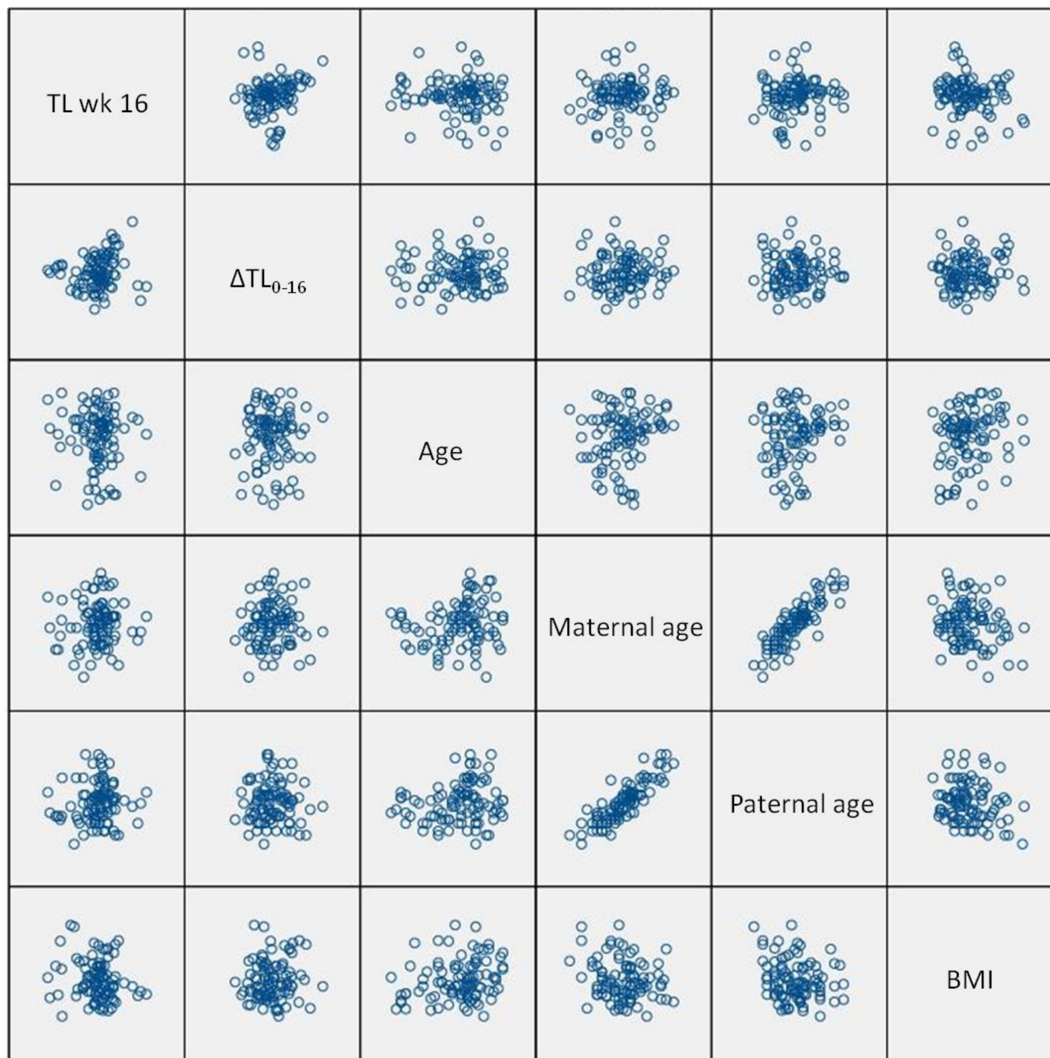

Figure 10.3 – Scatterplot matrix of bivariate correlation associations reported in Table 5.10

n ≥94 as three extreme outliers removed from n = 98.
Abbreviations; BMI, body mass index; $\Delta TL_{0\text{-}16}$; change in telomere length from week 0 to week 16; TL, telomere length; vit, vitamin.

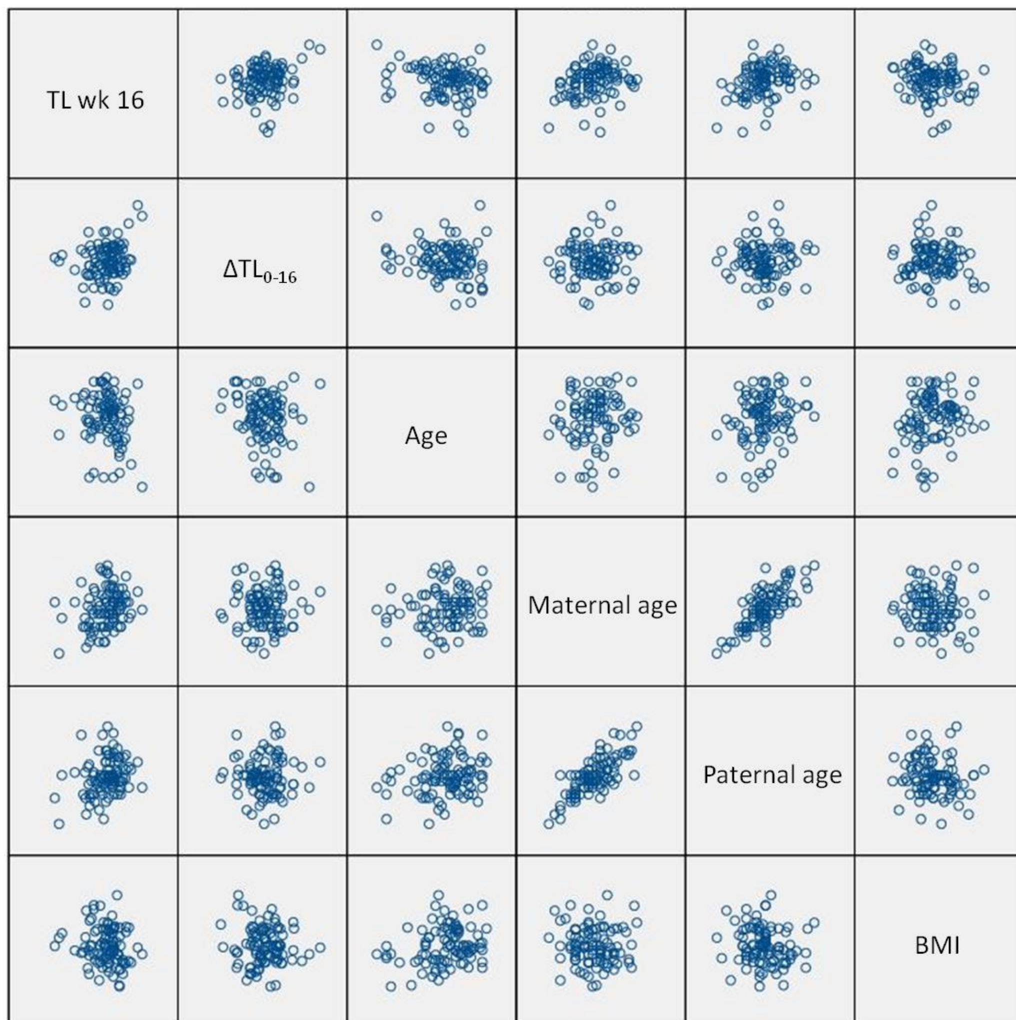

Figure 10.4 – Scatterplot matrix of bivariate correlation associations reported in Table 5.11

n = 98 as three extreme outliers removed from n = 101.
Abbreviations; BMI, body mass index; $\Delta TL_{0\text{-}16}$; change in telomere length from week 0 to week 16; TL, telomere length; vit, vitamin.

Table 10.11 – Bivariate correlation matrix of telomere length at week 16, change in telomere length from week 0 to week 16, participant age, maternal age, paternal age and BMI

GROUP		TL week 16	ΔTL	AGE	MAT AGE	PAT AGE	BMI
TL at week 16	*R*	1	0.172	-0.121	0.159	0.176	-0.192
	P	-	**0.015**	0.90	**0.025**	**0.013**	**0.007**
	n	199	199	199	198	196	199
ΔTelomere length	*R*		1	-0.099	-0.057	-0.064	-0.068
	P	-	-	0.163	0.426	0.373	0.342
	n		199	199	198	196	199
Age	*R*			1	0.140	0.183	0.201
	P	-	-	-	**0.048**	**0.010**	**0.004**
	n			199	198	196	199
Maternal age	*R*				1	0.827	-0.097
	P	-	-	-	-	**<0.0001**	0.174
	n				198	196	198
Paternal age	*R*					1	-0.138
	P	-	-	-	-	-	0.054
	n					196	196
BMI	*R*						1
	P	-	-	-	-	-	-
	n						199

R, Pearson correlation coefficient; To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.0033
NB participants from Polypill and placebo treatment groups have been pooled.

Table 10.12 – Partial correlation matrix of telomere length and change in telomere length with change in plasma micronutrient and homocysteine status from week 0 to week 16, with adjustment for participant age, gender, maternal age, paternal age and BMI

		TL week 16	ΔTL	ΔFOL	ΔHCY	ΔB_{12}	ΔαTOC	ΔRET	ΔNIA	ΔCa	ΔMg	ΔSe	ΔZn	ΔVITD
TL at week 16	*R*	1	0.188	-0.024	-0.001	-0.061	-0.004	-0.031	-0.053	-0.063	-0.047	-0.030	0.035	-0.005
	P	-	**0.009**	0.739	0.986	0.401	0.958	0.676	0.468	0.391	0.521	0.678	0.628	0.943
	df	0	188	188	188	188	188	188	188	188	188	188	188	188
ΔTelomere length	*R*		1	-0.069	0.034	-0.088	-0.021	0.064	-0.108	-0.028	-0.041	-0.023	0.125	0.042
	P	-	-	0.344	0.645	0.230	0.772	0.384	0.140	0.701	0.575	0.750	0.086	0.570
	df		0	188	188	188	188	188	188	188	188	188	188	188
ΔFolate	*R*			1	-0.447	0.260	0.166	0.035	0.085	-0.108	-0.106	-0.069	0.029	-0.130
	P	-	-	-	**<0.0001**	**<0.0001**	**0.022**	0.627	0.245	0.139	0.144	0.345	0.695	0.073
	df			0	188	188	188	188	188	188	188	188	188	188
ΔHomocysteine	*R*				1	-0.182	0.004	0.090	-0.121	0.122	0.094	0.092	0.077	0.030
	P	-	-	-	-	**0.012**	0.953	0.215	0.095	0.094	0.197	0.205	0.292	0.681
	df				0	188	188	188	188	188	188	188	188	188
ΔVitamin B_{12}	*R*					1	0.103	0.017	0.105	0.042	0.014	-0.006	0.080	-0.031
	P	-	-	-	-	-	0.156	0.819	0.148	0.568	0.847	0.932	0.270	0.672
	df					0	188	188	188	188	188	188	188	188
ΔAlpha-tocopherol	*R*						1	0.415	-0.007	0.023	0.060	0.161	0.082	-0.099
	P	-	-	-	-	-	-	**<0.0001**	0.922	0.748	0.410	**0.027**	0.260	0.174
	df						0	188	188	188	188	188	188	188

	R							1	0.041	0.030	0.094	0.044	0.045	-0.044
ΔRetinol	*P*	-	-	-	-	-	-	-	0.578	0.685	0.196	0.548	0.540	0.546
	df							0	188	188	188	188	188	188
	R								1	-0.135	-0.217	-0.233	0.012	0.026
ΔNiacin number	*P*	-	-	-	-	-	-	-	-	0.063	**0.003**	**0.001**	0.874	0.723
	df								0	188	188	188	188	188
	R									1	0.661	0.547	0.530	0.138
ΔCalcium	*P*	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	**<0.0001**	0.057
	df									0	188	188	188	188
	R										1	0.537	0.439	0.039
ΔMagnesium	*P*	-	-	-	-	-	-	-	-	-	-	**<0.0001**	**<0.0001**	0.592
	df										0	188	188	188
	R											1	0.410	0.052
ΔSelenium	*P*	-	-	-	-	-	-	-	-	-	-	-	**<0.0001**	0.479
	df											0	188	188
	R												1	0.156
ΔZinc	*P*	-	-	-	-	-	-	-	-	-	-	-	-	**0.032**
	df												0	188
	R													1
ΔVitamin D	*P*	-	-	-	-	-	-	-	-	-	-	-	-	-
	df													0

Partial correlation with adjustment for individual age, gender, BMI, maternal age and paternal age;
R, Pearson correlation coefficient; To penalise for multiple comparisons, Bonferroni-adjusted P threshold for statistical significance is 0.00064
NB participants from Polypill and placebo treatment groups have been pooled.

Table 10.13 – Micronutrient and homocysteine concentrations at weeks 0, 16 and 32 by season of blood sample collection

MICRONUTRIENT	WEEK	SEASON	*n* [% CASES]	MEAN ± SD	(RANGE)
Folate (nmol/L)	0	Summer	59 [28.0]	23.4 ± 8.5	(8.1 – 39.9)
		Autumn	152 [72.0]	23.6 ± 9.0	(4.7 – 41.7)
	16	Autumn	14 [6.9]	28.4 ± 10.3	(12.3 – 41.3)
		Winter	187 [92.6]	28.2 ± 9.6	(6.8 – 45.3)
		Spring	1 [0.5]	13.1	-
	32	Summer	41 [21.1]	29.3 ± 8.0	(12.6 – 40.2)
		Winter	1 [0.5]	37	-
		Spring	152 [78.4]	32.2 ± 9.6	(9.9 – 45.3)
Homocysteine (µmol/L)	0	Summer	59 [28.0]	7.7 ± 1.7	(4.4 – 13.7)
		Autumn	152 [72.0]	8.45 ± 2.3	(4.3 – 21.5)
	16	Autumn	14 [6.9]	7.7 ± 2.2	(5.4 – 12.3)
		Winter	188 [92.6]	8.0 ± 2.2	(4.0 – 18.0)
		Spring	1 [0.5]	8.2	-
	32	Summer	41 [21.1]	7.3 ± 2.0	(4.0 – 14.2)
		Winter	1 [0.5]	6.6	-
		Spring	152 [78.4]	6.9 ± 1.8	(1.0 – 11.9)
Vitamin B_{12} (pmol/L)	0	Summer	59 [28.0]	288.9 ± 113.9	(72 – 584)
		Autumn	152 [72.0]	303.1 ± 163.1	(79 – 1450)

Vitamin B_{12} (pmol/L)	16	Autumn	14 [6.9]	366.6 ± 117.1	(189 – 601)
		Winter	188 [92.6]	349.4 ± 136.4	(81 – 1104)
		Spring	1 [0.5]	341	-
	32	Summer	41 [21.1]	316.4 ± 99.6	(154 – 596)
		Winter	1 [0.5]	689	-
		Spring	152 [78.4]	379.5 ± 143.4	(77 – 986)
α-tocopherol (µmol/L)	0	Summer	58 [28.6]	29.7 ± 8.3	(14.8 – 55.6)
		Autumn	145 [71.4]	29.7 ± 6.0	(15.4 – 47.9)
	16	Autumn	14 [6.9]	32.7 ± 12.5	(16.1 – 66.5)
		Winter	188 [92.6]	30.4 ± 6.1	(18.0 – 46.5)
		Spring	1 [0.5]	31.2	-
	32	Summer	41 [21.1]	31.0 ± 5.2	(20.2 – 43.4)
		Winter	1 [0.5]	34.9	-
		Spring	152 [78.4]	31.0 ± 7.0	(17.4 – 54.6)
Retinol (µmol/L)	0	Summer	58 [28.6]	2.0 ± 0.5	(1.0 – 3.9)
		Autumn	145 [71.4]	2.4 ± 0.6	(1.3 – 4.5)
	16	Autumn	14 [6.9]	2.1 ± 0.3	(1.6 – 2.5)
		Winter	188 [92.6]	2.3 ± 0.6	(1.2 – 4.8)
		Spring	1 [0.5]	2.1	-
	32	Summer	41 [21.1]	2.5 ± 0.6	(1.6 – 4.1)
		Winter	1 [0.5]	2.45	-
		Spring	152 [78.4]	2.3 ± 0.5	(1.2 – 4.5)

Niacin number $\left(\frac{NAD}{NAD+NADP} \times 100\right)$	0	Summer	58 [28.6]	160.5 ± 26.9	(107.0 – 218.3)
		Autumn	145 [71.4]	155.8 ± 27.9	(89.0 – 250.8)
	16	Autumn	14 [6.9]	173.1 ± 26.1	(137.6 – 218.3)
		Winter	188 [92.6]	157.9 ± 28.7	(97.6 – 271.7)
		Spring	1 [0.5]	138.4	-
	32	Summer	41 [21.1]	160.2 ± 26.9	(116.9 – 227.2)
		Winter	1 [0.5]	154.8	-
		Spring	152 [78.4]	159.9 ± 28.8	(88.7 – 271.0)
Calcium (mmol/L)	0	Summer	59 [27.8]	2.2 ± 0.1	(2.0 – 2.4)
		Autumn	153 [72.1]	2.3 ± 0.1	(2.0 – 2.5)
	16	Autumn	14 [6.9]	2.2 ± 0.1	(2.1 – 2.4)
		Winter	187 [92.6]	2.2 ± 0.1	(1.7 – 2.5)
		Spring	1 [0.5]	2.2	-
	32	Summer	41 [21.1]	2.3 ± 0.1	(2.0 – 2.4)
		Winter	1 [0.5]	2.4	-
		Spring	152 [78.4]	2.3 ± 0.1	(2.0 – 2.9)
Vitamin D (nmol/L)	0	Summer	59 [27.8]	77.4 ± 18.9	(34 – 199)
		Autumn	153 [72.1]	79.5 ± 26.4	(30 – 190)
	16	Autumn	14 [6.9]	67.6 ± 18.9	(34 – 96)
		Winter	188 [92.6]	56.0 ± 21.5	(23 – 141)
		Spring	1 [0.5]	60	-

	32	Summer	41 [21.1]	74.7 ± 26.2	(32 – 137)
		Winter	1 [0.5]	59	-
		Spring	152 [78.4]	65.0 ± 25.3	(22 – 146)
Magnesium (µmol/L)	0	Summer	59 [27.8]	784.3 ± 57.4	(588.3 – 929.2)
		Autumn	153 [72.1]	782.2 ± 54.7	(650.6 – 925.0)
	16	Autumn	14 [6.9]	770.7 ± 48.8	(701.9 – 846.7)
		Winter	187 [92.6]	775.7 ± 51.2	(604.2 – 937.1)
		Spring	1 [0.5]	732.9	-
	32	Summer	41 [21.1]	777.2 ± 45.7	(613.5 – 850.0)
		Winter	1 [0.5]	821.2	-
		Spring	152 [78.4]	783.8 ± 50.6	(680.4 – 957.5)
Selenium (µmol/L)	0	Summer	59 [27.8]	1.4 ± 0.1	(1.0 – 2.0)
		Autumn	153 [72.1]	1.4 ± 0.2	(0.9 – 1.9)
	16	Autumn	14 [6.9]	1.4 ± 0.2	(1.1 – 1.8)
		Winter	187 [92.6]	1.4 ± 0.2	(0.9 – 2.1)
		Spring	1 [0.5]	1.4	-
	32	Summer	41 [21.1]	1.4 ± 0.2	(1.2 – 1.8)
		Winter	1 [0.5]	1.9	-
		Spring	152 [78.4]	1.4 ± 0.2	(0.9 – 2.2)
Zinc (µmol/L)	0	Summer	59 [28.0]	13.3 ± 1.7	(10.1 – 17.0)
		Autumn	152 [72.0]	13.4 ± 1.6	(9.0 – 19.6)

Zinc (µmol/L)	16	Autumn	14 [6.9]	12.7 ± 1.7	(10.1 – 15.0)
		Winter	187 [92.6]	13.0 ± 1.6	(9.2 – 18.5)
		Spring	1 [0.5]	13.5	-
	32	Summer	41 [21.1]	13.4 ± 1.9	(9.9 – 17.8)
		Winter	1 [0.5]	14.4	-
		Spring	152 [78.4]	13.1 ± 1.5	(9.7 – 22.5)

NB participants from Polypill and placebo treatment groups have been pooled here, Table 10.14 reports values by treatment group

Table 10.14 – Micronutrient and homocysteine concentrations at week 0, 16 and 32 by season of blood collection, and treatment group

MICRONUTRIENT	WEEK	SEASON	NO TREATMENT (WK 0) OR PLACEBO (WK 16, 32)			*POLYPILL* TREATMENT (WK 16, 32)		
			n [% CASES]	MEAN ± SD	(RANGE)	*n* [% CASES]	MEAN ± SD	(RANGE)
Folate (nmol/L)	0	Summer	59 [28.0]	23.4 ± 8.5	(8.1 – 39.9)	-	-	-
		Autumn	152 [72.0]	23.6 ± 9.0	(4.7 – 41.7)			
	16	Autumn	5 [5.0]	23.6 ± 12.4	(12.3 – 41.3)	9 [9.2]	31.1 ± 8.5	(14.4 – 40.7)
		Winter	95 [94.0]	22.8 ± 9.2	(6.8 – 45.3)	89 [90.8]	34.0 ± 6.2	(18.3 – 45.3)
		Spring	1 [1.0]	13.1	-	-	-	-
	32	Summer	11 [73.3]	25.2 ± 7.8	(12.6 – 36.8)	30 [17.1]	30.8 ± 7.7	(13.0 – 40.2)
		Winter	-	-	-	1 [0.6]	37.0	-
		Spring	4 [26.7]	21.6 ± 10.2	(14.4 – 36.6)	144 [82.3]	32.5 ± 9.6	(9.9 – 45.3)
Homocysteine (µmol/L)	0	Summer	59 [28.0]	7.7 ± 1.7	(4.4 – 13.7)	-	-	-
		Autumn	152 [72.0]	8.45 ± 2.3	(4.3 – 21.5)			
	16	Autumn	5 [5.0]	8.0 ± 2.2	(5.4 – 10.8)	9 [9.2]	7.5 ± 2.2	(5.5 – 12.3)
		Winter	95 [94.0]	8.6 ± 2.3	(5.1 – 17.0)	89 [90.8]	7.5 ± 2.0	(4.0 – 18.0)
		Spring	1 [1.0]	8.2	-	-	-	-
	32	Summer	11 [73.3]	7.4 ± 1.8	(4.0 - 10.0)	30 [17.1]	7.3 ± 2.1	(4.2 – 14.2)
		Winter	-	-	-	1 [0.6]	6.6	-
		Spring	4 [26.7]	8.6 ± 2.1	(6.9 – 11.7)	144 [82.3]	6.9 ± 1.8	(1.0 – 11.9)
Vitamin B_{12} (pmol/L)	0	Summer	59 [28.0]	288.9 ± 113.9	(72 – 584)	-	-	-
		Autumn	152 [72.0]	303.1 ± 163.1	(79 – 1450)			

Vitamin B_{12} (pmol/L)	16	Autumn	5 [5.0]	319.2 ± 60.2	(242 – 403)	9 [9.2]	393.0 ± 135.5	(189 – 601)
		Winter	95 [94.0]	303.3 ± 106.2	(119 – 627)	89 [90.8]	394.9 ± 147.2	(81 – 1104)
		Spring	1 [1.0]	341	-	-	-	-
	32	Summer	11 [73.3]	314.2 ± 81.3	(213 – 458)	30 [17.1]	317.2 ± 106.8	(154 – 596)
		Winter	-	-	-	1 [0.6]	689	-
		Spring	4 [26.7]	257.5 ± 28.9	(224 – 292)	144 [82.3]	381 ± 143.4	(77 – 986)
α-tocopherol (µmol/L)	0	Summer	58 [28.6]	29.7 ± 8.3	(14.8 – 55.6)	-	-	-
		Autumn	145 [71.4]	29.7 ± 6.0	(15.4 – 47.9)			
	16	Autumn	5 [5.0]	27.7 ± 10.2	(16.2 – 42.6)	9 [9.2]	35.5 ± 13.3	(22.7 – 66.5)
		Winter	95 [94.0]	29.1 ± 5.8	(18.0 – 46.3)	89 [90.8]	31.8 ± 6.1	(19.7 – 46.5)
		Spring	1 [1.0]	31.2	-	-	-	-
	32	Summer	11 [73.3]	31.1 ± 5.0	(23.0 – 39.0)	30 [17.1]	31.0 ± 5.4	(20.2 – 43.4)
		Winter	-	-	-	1 [0.6]	34.9	-
		Spring	4 [26.7]	31.0 ± 4.5	(25.4 – 36.2)	144 [82.3]	31.0 ± 7.1	(17.4 – 54.6)
Retinol (µmol/L)	0	Summer	58 [28.6]	2.0 ± 0.5	(1.0 – 3.9)	-	-	-
		Autumn	145 [71.4]	2.4 ± 0.6	(1.3 – 4.5)			
	16	Autumn	5 [5.0]	1.9 ± 0.3	(1.6 – 2.4)	9 [9.2]	2.2 ± 0.2	(1.9 – 2.5)
		Winter	95 [94.0]	2.2 ± 0.5	(1.3 – 4.2)	89 [90.8]	2.4 ± 0.6	(1.2 – 4.8)
		Spring	1 [1.0]	2.1		-	-	-
	32	Summer	11 [73.3]	2.5 ± 0.6	(1.8 – 3.8)	30 [17.1]	2.5 ± 0.6	(1.6 – 4.1)
		Winter	-	-	-	1 [0.6]	2.5	-
		Spring	4 [26.7]	2.3 ± 0.3	(2.0 – 2.7)	144 [82.3]	2.3 ± 0.6	(1.2 – 4.5)

Niacin number $\left(\frac{\mathrm{NAD}}{\mathrm{NAD} + \mathrm{NADP}} \times 100\right)$	0	Summer	58 [28.6]	160.5 ± 26.9	(107.0 – 218.3)	-	-	-
		Autumn	145 [71.4]	155.8 ± 27.9	(89.0 – 250.8)			
	16	Autumn	5 [5.0]	190.9 ± 19.7	(168.5 – 218.3)	9 [9.2]	163.2 ± 24.7	(137.6 – 209.6)
		Winter	95 [94.0]	155.9 ± 28.6	(104.2 – 231.6)	89 [90.8]	158.9 ± 28.7	(97.6 – 271.7)
		Spring	1 [1.0]	138.4	-	-	-	-
	32	Summer	11 [73.3]	156.0 ± 35.1	(120.0 – 227.2)	30 [17.1]	161.7 ± 23.7	(116.9 – 213.1)
		Winter	-	-	-	1 [0.6]	154.8	-
		Spring	4 [26.7]	137.0 ± 20.7	(122.6 – 167.7)	144 [82.3]	159.7 ± 28.6	(88.7 – 271.0)
Calcium (mmol/L)	0	Summer	59 [27.8]	2.2 ± 0.1	(2.0 – 2.4)	-	-	-
		Autumn	153 [72.1]	2.3 ± 0.1	(2.0 – 2.5)			
	16	Autumn	5 [5.0]	2.2 ± 0.1	(2.1 – 2.4)	9 [9.2]	2.2 ± 0.1	(2.1 – 2.4)
		Winter	95 [94.0]	2.3 ± 0.1	(2.0 – 2.5)	89 [90.8]	2.2 ± 0.1	(1.7 – 2.5)
		Spring	1 [1.0]	2.2	-	-	-	-
	32	Summer	11 [73.3]	2.3 ± 0.1	(2.0 – 2.4)	30 [17.1]	2.3 ± 0.1	(2.1 – 2.4)
		Winter	-	-	-	1 [0.6]	2.4	-
		Spring	4 [26.7]	2.3 ± 0.04	(2.2 – 2.3)	144 [82.3]	2.3 ± 0.1	(2.0 – 2.9)
Vitamin D (nmol/L)	0	Summer	59 [27.8]	77.4 ± 18.9	(34 – 199)	-	-	-
		Autumn	153 [72.1]	79.5 ± 26.4	(30 – 190)			
	16	Autumn	5 [5.0]	57.8 ± 15.9	(34 – 77)	9 [9.2]	73.0 ± 19.0	(41 – 96)
		Winter	95 [94.0]	58.5 ± 22.5	(24 – 131)	89 [90.8]	53.1 ± 20.3	(23 – 141)
		Spring	1 [1.0]	60	-	-	-	-

Vitamin D (nmol/L)	32	Summer	11 [73.3]	92.7 ± 29.7	(52 – 137)	30 [17.1]	68.0 ± 21.7	(32 – 124)
		Winter	-	-	-	1 [0.6]	59	-
		Spring	4 [26.7]	52.5 ± 29.0	(25 – 80)	144 [82.3]	65.6 ± 25.3	(22-146)
Magnesium (µmol/L)	0	Summer	59 [27.8]	784.3 ± 57.4	(588.3 – 929.2)	-	-	-
		Autumn	153 [72.1]	782.2 ± 54.7	(650.6 – 925.0)			
	16	Autumn	5 [5.0]	783.5 ± 48.3	(722.1 – 842.1)	9 [9.2]	763.5 ± 50.5	(701.9 – 846.7)
		Winter	95 [94.0]	779.0 ± 47.2	(632.1 – 874.9)	89 [90.8]	770.1 ± 54.5	(604.2 – 937.1)
		Spring	1 [1.0]	732.9	-	-	-	-
	32	Summer	11 [73.3]	774.8 ± 61.1	(613.5 – 837.4)	30 [17.1]	778.1 ± 39.9	(704.0 – 850.0)
		Winter	-	-	-	1 [0.6]	821.2	-
		Spring	4 [26.7]	764.9 ± 39.5	(712.6 – 806.7)	144 [82.3]	783.5 ± 51.0	(680.4 – 957.5)
Selenium (µmol/L)	0	Summer	59 [27.8]	1.4 ± 0.1	(1.0 – 2.0)	-	-	-
		Autumn	153 [72.1]	1.4 ± 0.2	(0.9 – 1.9)			
	16	Autumn	5 [5.0]	1.3 ± 0.2	(1.1 – 1.7)	9 [9.2]	1.5 ± 0.2	(1.2 – 1.8)
		Winter	95 [94.0]	1.4 ± 0.2	(1.1 – 2.1)	89 [90.8]	1.4 ± 0.2	(0.9 – 1.9)
		Spring	1 [1.0]	1.4	-	-	-	-
	32	Summer	11 [73.3]	1.4 ± 0.1	(1.2 – 1.6)	30 [17.1]	1.4 ± 0.2	(1.2 – 1.8)
		Winter	-	-	-	1 [0.6]	1.9	-
		Spring	4 [26.7]	1.4 ± 0.1	(1.3 – 1.5)	144 [82.3]	1.4 ± 0.2	(1.0 – 2.2)
Zinc (µmol/L)	0	Summer	59 [28.0]	13.3 ± 1.7	(10.1 – 17.0)	-	-	-
		Autumn	152 [72.0]	13.4 ± 1.6	(9.0 – 19.6)			

Zinc (µmol/L)	16	Autumn	5 [5.0]	13.0 ± 1.4	(11.6 – 14.7)	9 [9.2]	12.6 ± 1.9	(10.1 – 15.0)
		Winter	95 [94.0]	13.1 ± 1.8	(9.9 – 18.5)	89 [90.8]	12.8 ± 1.5	(9.2 – 16.5)
		Spring	1 [1.0]	13.5	-	-	-	-
	32	Summer	11 [73.3]	12.6 ± 1.3	(11.1 – 14.8)	30 [17.1]	13.7 ± 2.0	(9.9 – 17.8)
		Winter	-	-	-	1 [0.6]	14.4	-
		Spring	4 [26.7]	13.5 ± 1.9	(11.4 – 15.3)	144 [82.3]	13.0 ± 1.5	(9.7 – 22.5)

Table 10.15 – Supplementary data from Chapter 7: cell count data, population doublings and produced homocysteine

FA (nM)	dUTP (µM)	TOTAL CELLS	LIVE CELLS	CELL VIABILITY (%)	POPULATION DOUBLINGS	HCY PRODUCED (µmol/10^6 TOTAL CELLS)
30	0	7.2 ± 0.75 $\times 10^5$	6.5 ± 0.68 $\times 10^5$	90.2 ± 0.3	3.8 ± 0.16	8.2
	15	5.3 ± 0.20 $\times 10^5$	4.7 ± 0.20 $\times 10^5$	88.5 ± 0.5	3.4 ± 0.05	11.0
	150	4.2 ± 0.21 $\times 10^5$	3.6 ± 0.26 $\times 10^5$	85.3 ± 2.0	3.1 ± 0.07	12.4
300	0	2.0 ± 0.14 $\times 10^6$	1.8 ± 0.15 $\times 10^6$	92.6 ± 1.4	5.3 ± 0.10	4.7
	15	1.6 ± 0.09 $\times 10^6$	1.4 ± 0.07 $\times 10^6$	88.8 ± 1.5	5.0 ± 0.08	6.2
	150	1.4 ± 0.04 $\times 10^6$	1.3 ± 0.04 $\times 10^6$	89.3 ± 0.6	4.8 ± 0.04	7.3
3000	0	2.9 ± 0.14 $\times 10^6$	2.6 ± 0.16 $\times 10^6$	88.4 ± 1.2	5.9 ± 0.07	2.6
	15	2.5 ± 0.07 $\times 10^6$	2.2 ± 0.08 $\times 10^6$	85.5 ± 1.3	5.7 ± 0.04	3.7
	150	2.3 ± 0.09 $\times 10^6$	1.9 ± 0.07 $\times 10^6$	84.2 ± 0.6	5.5 ± 0.06	5.0

Abbreviations: FA, folic acid; dUTP, 2´-Deoxyuridine, 5´-Triphosphate; HCY, homocysteine
Data are mean (± SEM) for n = 3; homocysteine in spent media was measured from n = 6 pooled samples (n = 1 measure per treatment group)

Table 10.16 – Supplementary data from Chapter 7: telomere length, USER ΔC_T and uracil per kb telomere sequence

FA (nM)	dUTP (µM)	TELOMERE LENGTH (KB/DIPLOID GENOME)	USER ΔC_T (DIGESTED – UNDIGESTED)	URACIL/KB
30	0	49.7 ± 1.4	2.0 ± 0.1	5.7 ± 0.5
	15	55.2 ± 1.7	1.7 ± 0.2	4.4 ± 0.8
	150	51.5 ± 1.7	1.7 ± 0.2	4.6 ± 0.7
300	0	53.7 ± 1.7	1.9 ± 0.2	5.4 ± 0.8
	15	55.2 ± 1.1	1.5 ± 0.1	4.0 ± 0.6
	150	55.6 ± 1.7	2.1 ± 0.4	5.6 ± 1.4
3000	0	50.4 ± 1.6	1.9 ± 0.3	5.0 ± 1.1
	15	54.1 ± 2.1	1.7 ± 0.2	4.8 ± 0.6
	150	54.3 ± 2.1	1.6 ± 0.2	4.2 ± 0.8

Abbreviations: FA, folic acid; dUTP, 2´-Deoxyuridine, 5´-Triphosphate; HCY, homocysteine
Data are mean (± SEM) for n = 6

Table 10.17 – Supplementary data from Chapter 8: cell count data, population doublings and produced homocysteine – experiment one

FA (nM)	dUTP (µM)	SAM	TOTAL CELLS	LIVE CELLS	CELL VIABILITY (%)	POPULATION DOUBLINGS	HCY PRODUCED (µmol/10^6 TOTAL CELLS)
30	0	0	$1.5 \pm 0.01 \times 10^6$	$8.5 \pm 0.45 \times 10^5$	58.3 ± 2.7	4.9	2.9
		5	$1.3 \pm 0.03 \times 10^6$	$7.3 \pm 0.38 \times 10^5$	54.7 ± 3.8	4.7	1.9
		10	$1.4 \pm 0.13 \times 10^6$	$8.3 \pm 1.07 \times 10^5$	60.7 ± 2.9	4.8	2.3
		50	$1.4 \pm 0.14 \times 10^6$	$6.9 \pm 0.93 \times 10^5$	48.0 ± 2.6	4.8	4.3
	15	0	$1.3 \pm 0.05 \times 10^6$	$7.5 \pm 0.19 \times 10^5$	59.3 ± 3.5	4.7	3.1
		5	$1.2 \pm 0.08 \times 10^6$	$6.6 \pm 0.86 \times 10^5$	52.7 ± 3.5	4.6	1.4
		10	$8.9 \pm 2.30 \times 10^5$	$5.0 \pm 1.6 \times 10^5$	54.3 ± 4.1	4.2	3.6
		50	$1.1 \pm 0.09 \times 10^6$	$6.1 \pm 0.42 \times 10^5$	54.3 ± 1.3	4.5	5.6
	150	0	$1.5 \pm 0.09 \times 10^6$	$1.0 \pm 0.03 \times 10^6$	67.0 ± 2.9	4.9	1.9
		5	$1.1 \pm 0.09 \times 10^6$	$6.4 \pm 0.70 \times 10^5$	59.0 ± 2.9	4.4	3.1
		10	$1.1 \pm 0.02 \times 10^6$	$6.7 \pm 0.24 \times 10^5$	60.0 ± 2.0	4.5	2.4
		50	$1.5 \pm 0.14 \times 10^6$	$7.9 \pm 0.71 \times 10^5$	54.3 ± 0.3	4.9	4.7

300	0	0	$1.9 \pm 0.17 \times 10^{6}$	$1.0 \pm 0.08 \times 10^{6}$	54.7 ± 3.8	5.3	3.9
		5	$1.7 \pm 0.14 \times 10^{6}$	$7.6 \pm 0.50 \times 10^{5}$	44.0 ± 1.0	5.1	6.0
		10	$2.1 \pm 0.12 \times 10^{6}$	$8.8 \pm 0.35 \times 10^{5}$	43.3 ± 0.9	5.4	5.5
		50	$1.6 \pm 0.09 \times 10^{6}$	$8.2 \pm 0.57 \times 10^{5}$	52.7 ± 1.8	5.0	6.8
	15	0	$1.7 \pm 0.12 \times 10^{6}$	$8.9 \pm 1.01 \times 10^{5}$	52.3 ± 2.3	5.1	5.2
		5	$1.7 \pm 0.16 \times 10^{6}$	$7.7 \pm 0.95 \times 10^{5}$	44.3 ± 2.9	5.1	6.1
		10	$2.0 \pm 0.21 \times 10^{6}$	$9.4 \pm 1.03 \times 10^{5}$	46.3 ± 0.7	5.3	5.3
		50	$2.0 \pm 0.24 \times 10^{6}$	$1.1 \pm 0.16 \times 10^{6}$	54.7 ± 2.6	5.3	5.2
	150	0	$1.7 \pm 0.18 \times 10^{6}$	$8.6 \pm 1.31 \times 10^{5}$	51.7 ± 2.6	5.0	5.8
		5	$1.5 \pm 0.11 \times 10^{6}$	$6.2 \pm 0.49 \times 10^{5}$	42.0 ± 3.5	4.9	7.4
		10	$1.9 \pm 0.16 \times 10^{6}$	$9.1 \pm 0.54 \times 10^{5}$	52.8 ± 6.0	5.3	4.4
		50	$1.3 \pm 0.09 \times 10^{6}$	$6.5 \pm 0.87 \times 10^{5}$	51.7 ± 2.6	4.7	8.6

3000	0	0	2.2 ± 0.03 ×10^{6}	1.0 ± 0.03 ×10^{6}	46.7 ± 1.8	5.4	2.6
		5	2.1 ± 0.02 ×10^{6}	9.9 ± 0.59 ×10^{5}	47.7 ± 2.9	5.4	2.6
		10	1.9 ± 0.14 ×10^{6}	9.6 ± 1.13 ×10^{5}	51.0 ± 3.6	5.2	3.5
		50	9.2 ± 2.34 ×10^{5}	8.0 ± 2.32 ×10^{5}	82.3 ± 7.1	4.2	7.0
	15	0	1.6 ± 0.24 ×10^{6}	1.2 ± 0.22 ×10^{6}	75.0 ± 2.5	5.0	2.9
		5	1.8 ± 0.12 ×10^{6}	1.4 ± 0.09 ×10^{6}	78.3 ± 3.7	5.2	3.0
		10	1.8 ± 0.14 ×10^{6}	1.4 ± 0.10 ×10^{6}	79.7 ± 1.5	5.1	3.5
		50	1.2 ± 0.09 ×10^{5}	3.9 ± 2.14 ×10^{4}	30.3 ± 15.1	1.3	72.5
	150	0	2.2 ± 0.17 ×10^{6}	1.4 ± 0.13 ×10^{6}	65.0 ± 1.0	5.4	2.5
		5	1.5 ± 0.24 ×10^{6}	9.5 ± 1.56 ×10^{5}	62.0 ± 2.5	4.9	4.3
		10	1.9 ± 0.06 ×10^{6}	1.4 ± 0.10 ×10^{6}	71.3 ± 3.4	5.3	3.3
		50	1.1 ± 0.28 ×10^{5}	3.7 ± 0.34 ×10^{4}	39.7 ± 11.4	1.1	78.5

Abbreviations: FA, folic acid; dUTP, 2´-Deoxyuridine, 5´-Triphosphate; HCY, homocysteine

Data are mean (± SEM) for n = 3; homocysteine in spent media was measured from n = 6 pooled samples (n = 1 measure per treatment group)

Table 10.18 – Supplementary data from Chapter 8: cell count data, population doublings and produced homocysteine – experiment two

FA (nM)	dUTP (µM)	SAM	TOTAL CELLS	LIVE CELLS	CELL VIABILITY (%)	POPULATION DOUBLINGS	HCY PRODUCED (µmol/10^6 TOTAL CELLS)
30	0	0	$9.7 \pm 0.51 \times 10^5$	$7.2 \pm 0.35 \times 10^5$	75.3 ± 4.6	4.3	5.3
		5	$8.4 \pm 0.61 \times 10^5$	$5.4 \pm 0.29 \times 10^5$	68.3 ± 4.9	4.1	6.1
		10	$9.6 \pm 1.05 \times 10^5$	$6.6 \pm 0.62 \times 10^5$	68.3 ± 1.9	4.3	5.7
		50	$1.1 \pm 0.03 \times 10^6$	$7.8 \pm 0.36 \times 10^5$	70.7 ± 1.2	4.5	6.3
	15	0	$8.4 \pm 1.07 \times 10^5$	$6.9 \pm 0.89 \times 10^5$	82.7 ± 1.8	4.1	5.9
		5	$7.2 \pm 0.22 \times 10^5$	$5.1 \pm 0.13 \times 10^5$	71.3 ± 0.7	3.8	5.9
		10	$9.9 \pm 0.67 \times 10^5$	$6.0 \pm 0.59 \times 10^5$	60.0 ± 2.0	4.3	3.8
		50	$1.1 \pm 0.07 \times 10^6$	$7.2 \pm 0.59 \times 10^5$	66.7 ± 4.2	4.4	8.3
	150	0	$8.4 \pm 0.46 \times 10^5$	$7.0 \pm 0.38 \times 10^5$	83.7 ± 0.3	4.1	6.4
		5	$9.3 \pm 0.45 \times 10^5$	$6.3 \pm 0.56 \times 10^5$	68.5 ± 6.6	4.2	5.5
		10	$9.6 \pm 1.05 \times 10^5$	$5.4 \pm 0.41 \times 10^5$	57.8 ± 4.5	4.3	4.4
		50	$1.5 \pm 0.06 \times 10^6$	$9.5 \pm 0.27 \times 10^5$	65.3 ± 0.9	4.9	6.3

300	0	0	2.1 ± 0.11 ×10^{6}	1.1 ± 0.07 ×10^{6}	50.7 ± 2.2	5.4	4.6
		5	2.1 ± 0.06 ×10^{6}	9.8 ± 0.26 ×10^{5}	46.3 ± 0.3	5.4	4.1
		10	2.3 ± 0.04 ×10^{6}	1.1 ± 0.04 ×10^{6}	46.0 ± 2.3	5.5	3.4
		50	2.2 ± 0.08 ×10^{6}	1.2 ± 0.07 ×10^{6}	55.7 ± 3.4	5.5	4.4
	15	0	2.4 ± 0.06 ×10^{6}	1.3 ± 0.12 ×10^{6}	55.3 ± 3.5	5.6	4.3
		5	2.2 ± 0.11 ×10^{6}	1.1 ± 0.03 ×10^{6}	48.7 ± 2.2	5.5	3.9
		10	2.1 ± 0.08 ×10^{6}	1.0 ± 0.05 ×10^{6}	48.0 ± 2.1	5.4	4.2
		50	2.0 ± 0.04 ×10^{6}	1.0 ± 0.03 ×10^{6}	51.0 ± 1.0	5.3	4.8
	150	0	2.2 ± 0.12 ×10^{6}	1.2 ± 0.04 ×10^{6}	56.0 ± 2.0	5.5	4.5
		5	2.4 ± 0.11 ×10^{6}	1.1 ± 0.08 ×10^{6}	46.0 ± 3.5	5.6	4.2
		10	2.0 ± 0.03 ×10^{6}	8.8 ± 0.39 ×10^{5}	44.3 ± 1.9	5.3	4.7
		50	2.2 ± 0.12 ×10^{6}	1.1 ± 0.03 ×10^{6}	49.0 ± 1.7	5.5	4.8

		0	2.4 ± 0.14 $\times10^6$	1.4 ± 0.18 $\times10^6$	56.0 ± 3.5	5.6	2.9
	0	5	2.6 ± 0.05 $\times10^6$	1.3 ± 0.03 $\times10^6$	51.3 ± 1.3	5.7	3.0
		10	2.4 ± 0.11 $\times10^6$	1.3 ± 0.04 $\times10^6$	54.3 ± 2.7	5.6	3.4
		50	8.5 ± 4.01 $\times10^5$	5.0 ± 2.21 $\times10^5$	66.3 ± 10.3	4.1	15.6
		0	2.6 ± 0.12 $\times10^6$	1.4 ± 0.06 $\times10^6$	52.7 ± 3.7	5.7	2.3
3000	15	5	2.7 ± 0.05 $\times10^6$	1.4 ± 0.06 $\times10^6$	50.0 ± 1.5	5.8	2.3
		10	2.5 ± 0.08 $\times10^6$	1.2 ± 0.06 $\times10^6$	47.0 ± 1.0	5.7	2.9
		50	1.1 ± 0.35 $\times10^6$	9.0 ± 2.67 $\times10^5$	85.2 ± 2.9	4.5	9.9
		0	2.7 ± 0.10 $\times10^6$	1.4 ± 0.12 $\times10^6$	50.0 ± 2.7	5.8	2.2
	150	5	2.8 ± 0.17 $\times10^6$	1.3 ± 0.07 $\times10^6$	48.0 ± 1.7	5.8	2.8
		10	2.4 ± 0.18 $\times10^6$	1.2 ± 0.13 $\times10^6$	51.3 ± 1.9	5.6	3.3
		50	1.1 ± 0.33 $\times10^6$	8.9 ± 2.76 $\times10^5$	67.8 ± 11.0	4.4	13.1

Abbreviations: FA, folic acid; dUTP, 2´-Deoxyuridine, 5´-Triphosphate; HCY, homocysteine

Data are mean (± SEM) for n = 3; homocysteine in spent media was measured from n = 6 pooled samples (n = 1 measure per treatment group)

10.2 Publications arising from this thesis

Moores, CJ, Fenech, M & O'Callaghan, NJ 2011 Telomere dynamics: the influence of folate and DNA methylation, *Annals of the New York Academy of Sciences,* 1229, 76-88. Journal Citation Reports® Science Edition (Thomson Reuters, 2013) Impact Factor: 4.375.

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Issue: *Nutrition and Physical Activity in Aging, Obesity, and Cancer*

Telomere dynamics: the influence of folate and DNA methylation

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Since the suggestion of their existence, a wealth of literature on telomere biology has emerged aimed at solving the DNA end-underreplication problem identified by Olovnikov in 1971. Telomere shortening/dysfunction is now recognized as increasing degenerative disease risk. Recent studies have suggested that both dietary patterns and individual micronutrients—including folate—can influence telomere length and function. Folate is an important dietary vitamin required for DNA synthesis, repair, and one-carbon metabolism within the cell. However, the potential mechanisms by which folate deficiency directly or indirectly affects telomere biology has not yet been reviewed comprehensively. The present review summarizes recent published knowledge and identifies the residual knowledge gaps. Specifically, this review addresses whether it is plausible that folate deficiency may (1) cause accelerated telomere shortening, (2) intrinsically affect telomere function, and/or (3) cause increased telomere-end fusions and subsequent breakage–fusion–bridge cycles in the cell.

Keywords: folate; telomeres; epigenetics; DNA methylation; diet

Folate

Folate, a water-soluble B group vitamin (vitamin B9), is required for the vital metabolic processes of DNA methylation and nucleotide biosynthesis, including conversion of dUMP to dTTP.[1,2] Such essential cellular pathways are affected by total dietary intake of folate and its metabolic cofactors, and certain genetic variants.[3] Folate can occur in various metabolic forms (Fig. 1); and some of these, such as 5-methyltetrahydrofolate (5-MeTHF) and 5,10-methylenetetrahydrofolate (5,10-MeTHF), can donate methyl groups to other molecules. In a folate-deficient state, a cell is limited in 5,10-MeTHF required for conversion of dUMP to dTTP by methylation, and hence the ratio of dUMP:dTTP increases, leading to inadvertent incorporation of uracil in the DNA of dividing cells.[4] 5-MeTHF is required to synthesize methionine from homocysteine, and this reaction requires vitamin B12 as a cofactor and zinc at the catalytic site to activate homocysteine.[5,6] Methionine is then converted to S-adenosyl methionine, the universal methyl donor employed for methylation of histones and cytosine in mammalian DNA[7] (Fig. 1). When folate is deficient, maintenance of methylation of histones and cytosine may be inadequate.[8]

Homocysteine accumulates in response to folate- and B12-deficient conditions, as there is insufficent 5-MeTHF to provide methyl groups to reform methionine. A high plasma level of homocysteine has been shown to increase risk of adverse cardiovascular events[9] and is associated with increased chromosome damage.[10] Periconceptional exposure to elevated folate has long been recognized to prevent neural tube defects in the developing fetus,[11,12] and use of a folate supplement before and during early pregancy is now considered "best practice" in the Western world.[13] Additionally, suboptimal folate status has been associated with an increased risk of many cancer types such as colorectal cancer, adenoma, esophageal, and gastric cancer[14] as well as cardiovascular disease.[15]

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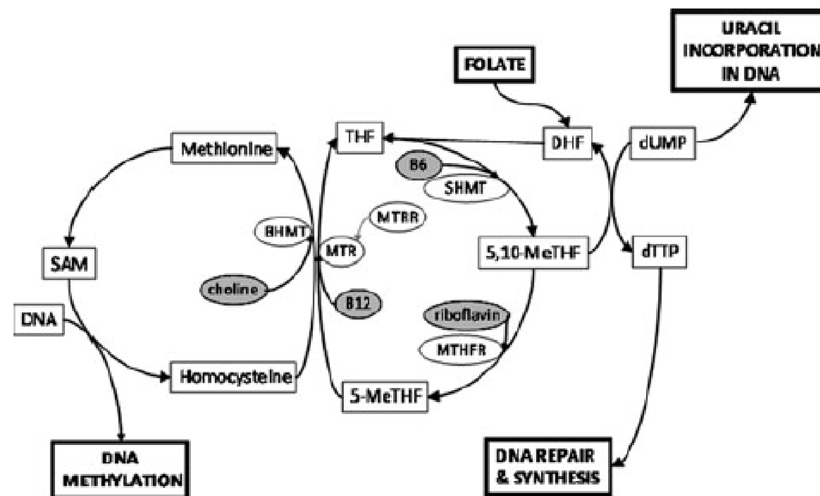

Figure 1. Folate metabolism: folate, along with choline, methionine, cobalamin, pyridoxine, and riboflavin, is involved in several essential metabolic processes within the cell, in particular DNA synthesis, repair, and methylation. Folate is also essential as a methyl donor in the maintenance of dUMP:dTTP ratios within the cell. When the ratio of dUMP:dTTP is increased, there is an increased incorporation of uracil into the DNA. Image modified from Ref. 78. 5-MeTHF, 5-methyltetrahydrofolate; 5,10-MeTHF, 5,10-methylenetetrahydrofolate; B6, pyridoxine; B12, cobalamin; BHMT, betaine homocysteine methyltransferase; DHF, dihydrofolate; DNA, deoxyribonucleic acid; dUMP, deoxyuridine monophosphate; dTTP, deoxythymidine triphosphate; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; SAM, S-adenosyl methionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate.

Telomeres and cellular aging

Telomeres are repeats of the hexamer sequence $(TTAGGG)_n$, which, with the associated shelterin protein complex, cap the end of all mammalian chromosomes. As the ends of the double-helical DNA of linear chromosomes are unable to be entirely replicated during each cycle of nuclear division, telomeric DNA is sacrificed to ensure that the coding genetic sequence is not lost from the extremities of the chromosome, known as the end-replication problem or marginotomy.[16–18] As a consequence of the end-replication problem, telomeric sequence repeats abridge by approximately 30–200 bp during each cycle of cell division in most somatic cells.[19] Telomere attrition occurs naturally during the aging process;[20,21] however, it is accelerated in certain premature aging syndromes such as ataxia telangiectasia (Fig. 2). The length of the telomeric sequence declines with age until the telomere becomes critically short, typically signaling cellular senescence and resulting in programmed cell death.[22–25] Telomere length is regarded as an indicator of the biological age of a cell or its ability to undergo additional mitotic divisions.[26] Since telomere length changes are also known to be induced by various environmental, physiological, and psychological stressors,[27] such variations in telomere length may suggest that aside from the effect of the end-replication problem, other factors may contribute to telomere shortening.

Telomeres and disease

Short telomeres have been widely investigated for use as a potential biomarker in determining increased risk of many diseases, particularly those with age-associated manifestations. Studies have determined that truncated telomeric repeats are associated with increased risk of cancer,[28] Alzheimer's disease,[29] Parkinson's disease,[30] rheumatoid arthritis,[31] cardiovascular disease,[32] and progression of liver cirrhosis.[33] Telomere length dynamics, however, appear to be complex, with long telomere length not consistently a sign of healthy cellular status. Recently Svenson *et al.* reported that an increased telomere length in peripheral blood cells was associated with negative prognosis in breast cancer patients.[34] Furthermore, it has been observed that offspring born to older parents have a longer telomere length[35–38] and, in isolation, that increased parental age at birth can increase the risk of adult-onset non-Hodgkin's

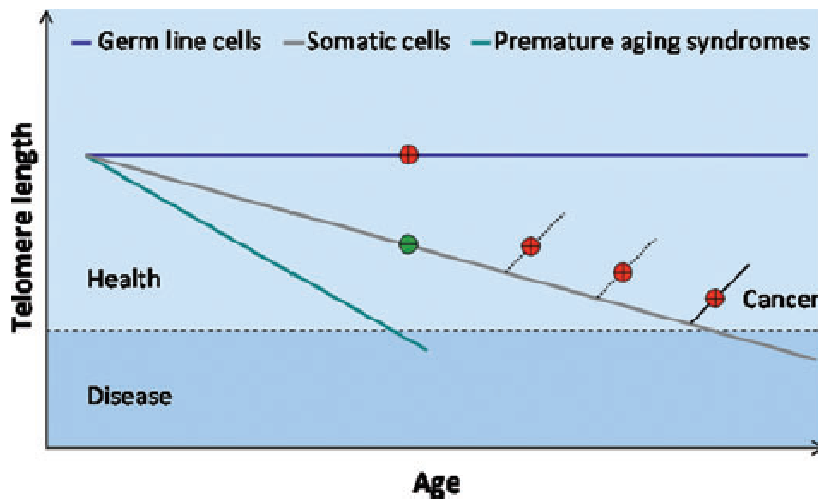

Figure 2. Telomere attrition with aging: the erosion of telomeric sequence accumulates with age in normal somatic cells; however, telomere attrition is greatly accelerated in premature aging syndromes such as ataxia telangiectasia. The length of telomeres in germ cells is stable due to the activity of telomerase (+). Somatic cells express basal levels of telomerase (−) unless they become transformed, cancerous (+). Image modified from Ref. 154.

lymphoma,[39] and breast[40–42] and prostate[43] cancers in the progeny. These observations suggest that the telomere length alone may not be an adequate predictor of degenerative disease risk and that, perhaps, the quality and functionality of telomeres should also be an important consideration.

Telomere structure and telomeric dysfunction

The terminal end of the telomere consists of both a double stranded and single stranded DNA region, which loops back on itself to form a cap at the end of the chromosome.[44] This lariat structure contains a larger, double-stranded telomere-duplex loop (t-loop) into which the long 3′ G-rich single-strand overhang, the displacement loop (D-loop), is inserted.[44,45] The D-loop-t-loop lariat and associated proteins, including the shelterin complex, serve to eliminate "free ends" of DNA that may be processed as double- or single-stranded DNA breaks. The structure prevents the triggering of an apoptotic response within the cell that is otherwise induced at broken chromosome loci.[46] Whether the deficiency of folate or other dietary methyl donors might affect telomeric structure and cause chromosomal dysfunction within the cell has been suggested previously but remains indefinite.[47] It is a possibility that folate deficiency and resulting uracil incorporation, DNA hypomethylation, or both, could affect the assembly of the D-loop-t-loop structure at the telomere end and/or the binding of telomere-associated proteins.

The shelterin complex and chromosomal stability

Telomeres and their shelterin complex additionally serve to prevent chromosome end-to-end fusions or other chromosomal rearrangements.[48] The mammalian shelterin complex consists of six protein subunits (TRF1, TRF2, POT1, Rap1, TIN2, and TPP1) that are in part responsible for telomere length maintenance and shaping the telomere cap.[49–51] Telomere binding proteins may reduce the likelihood of nucleolytic degradation and increase chromosomal stability as well as influence the access of the telomerase enzyme to the telomeric sequence.[52] Furthermore, the core protein components of the shelterin complex are involved in an assortment of intracellular signaling pathways.[53] Probing the interaction networks of the shelterin compartments and their activities has permitted the construction of an intricate shelterin interactome.[50,53] The composition and localization of the shelterin complex serves to prevent the recognition of telomeric DNA termini as double-stranded breaks and, as such, vetoing DNA damage response pathways.[49] However,

DNA damage response proteins have been observed at telomeres and appear to be essential for telomere replication, protection, and function.[54,55]

Telomere dysfunction within a cell has been measured as telomere end-to-end associations, telomere aggregates, telomere doublets, nucleoplasmic bridges (NPB), telomere clusters, or telomeric chromatid concatenates, and these have been associated with disease states or predisease conditions. Recent evidence is emerging to show that such telomeric dysfunction is increased under conditions of tumourigenesis and oxidative stress.[56,57] Telomere aggregates can be visualized using 2-D fluorescence microscopy and may represent telomere-end fusions that later could potentially become involved in breakage–fusion–bridge (BFB) cycles.[58] Folate deficiency has been shown to induce NPB formation;[59,60] however, it is not clear whether these events are caused by telomere end fusion, misrepair of DNA breaks in telomeres or anywhere else along the chromosome.[61] Telomere doublets or telomeric DNA-containing double-minute chromosomes (TDMs) are characterized by multiple telomere signals at a lone chromatid end.[62] These TDMs are postulated to be formed through recombination events between telomeric sequence and interstitial telomere-like sequences,[63] such as those located in subtelomeric regions.[64]

Cellular senescence, crisis, and immortality

The *in vitro* culture of normal somatic cells provides evidence of a limited replicative potential, whereby cells enter a state of senescence, mortality stage 1 (M1), or the Hayflick limit.[65] The Hayflick limit of cellular division is the number of population doublings determined by continued and accumulative telomere erosion.[66,67] Continued cellular proliferation following bypass or inactivation of the M1 mechanism induces the mortality stage 2 (M2) or crisis mechanism. *In vitro* escape from this M2 stage results in the emergence of an immortal cell line[68] by telomerase expression[69] and/or via the alternative lengthening of telomeres (ALT) mechanism.[70] A study by Counter *et al.* showed that unlike M1 cells, M2 cells had a reduced mean telomere length (1.5 kb content) and an increased number of dicentric chromosomes due to telomere end-fusions, resulting in greater genomic instability.[69]

For some time it has been recognized that the telomere length is polymorphic or heterogeneous across chromosomes, with senescence, likely the result of a critical telomere loss to just one or a few chromosomes.[71] The chromosome 17p telomere is noted as the critical telomere in humans because of its comparatively short-telomeric sequence.[72] High frequency loss of heterozygosity of 17p—the arm that houses p53 and potentially other tumor suppressor genes such as *HIC1*[73–76]—is one of the most common genetic modifications in cancer.[77] As folate is required for DNA repair, nucleotide biosynthesis, and conversion of dUMP to dTTP, it is undoubtedly important for accurate nuclear division and cellular proliferation. It is not known whether folate deficiency or excess affects 17p-specific telomere loss. Even so, given the tendency for folate deficiency to cause chromosomal breaks, it is plausible that insufficient folate could cause global telomere attrition, including at 17p by DNA strand break induction, while surplus folate could accelerate cellular proliferation,[78] with both scenarios resulting in "unexclusive" telomere loss.

Telomerase and the ALT mechanism

Unlike somatic cells that express very low or basal levels of telomerase transcripts, stem and germ cells have greatly upregulated telomerase expression in order to maintain telomere length. Somatic cells become immortal by activating expression of the human telomerase reverse transcriptase (hTERT) enzyme or by an ALT recombination-based mechanism of telomere elongation. hTERT adds *de novo* $(TTAGGG)_n$ hexamer repeats to the 3′ G-rich end of the telomere[79] and appears to operate, such that short telomeric sequences are preferentially elongated.[80] Both telomerase and ALT recombination-based[81–83] mechanisms of telomere elongation are dysregulated during tumorigenesis. A large majority of tumors express telomerase, while approximately 10–15% of tumors are ALT positive.[81] Epidemiologic evidence suggests that deficiency of folate is potentially procarcinogenic,[84] perhaps as increased cellular dUMP:dTTP ratios in low-folate conditions cause a halt or collapse of DNA replication forks, as well as intermediates of base excision repair, and subsequently DNA strand breaks.[85] The processing of these DNA double-stranded breaks by homologous recombination can induce a variety of changes in the genetic material.[85]

While folate deficiency is purported to cause increased recombination in the cell for this reason, whether folate could influence ALT-associated recombination is unknown. It is also plausible that low dTTP in folate-deficient cells could influence the action and/or efficiency of the *de novo* addition of $(TTAGGG)_n$ to the chromosome ends by hTERT. In addition, the recent observation that methionine restriction causes a reversible induction of ALT-associated promyelocytic leukemia bodies (APBs) in the nucleus suggests that it may be possible that deficiency in folate, which is required for methionine synthesis, may indirectly influence ALT mechanisms of telomere lengthening in ALT-positive cells.[86]

The Breakage–Fusion–Bridge cycle

Along with telomere loss, BFB cycles are a central mechanism in carcinogenesis.[87] BFB cycles may ensue when a dicentric chromosome is formed either through an incorrect repair of DNA double-stranded breaks, or when fusion occurs between telomeric regions. During the anaphase, spindle fibers may guide this dicentric chromosome to opposite poles of the cell, which results in asymmetrical and unordered breakage of the chromosome; thus daughter cells might contain deletions or duplications of the chromosomal complement. Chromatids that lack telomere sequence after dicentric chromosome breakage that is likely fuse again following DNA replication during the S-phase, forming a dicentric chromosome and further perpetuating the BFB cycle.[88–90] These BFB cycles contribute to deletions, gene amplification, and nonreciprocal translocations.[57] Deficiency in dietary methyl donor folate induces NPB formation[60] and is consistent with observations that loss of DNA methylation results in increased telomeric recombination.[91] Though substantial progress has been made in understanding the molecular mechanisms behind NPB formation,[61] the influence of folate deficiency still requires definition. It is unresolved whether NPBs in folate-deficient cells are induced by misrepair of DNA-stranded breaks or by dysfunctional telomeres that could cause telomere sequence amplification and telomere-end fusions.

Epigenetics, DNA methylation, and folate

While telomerase and ALT mechanisms actively govern telomere length, epigenetic modification at or near the telomere also influences the length of the repeat.[92–96] The epigenetic state of a DNA sequence is both heritable and reversible, involving higher order control of gene transcription while the underlying DNA sequence remains unchanged.[97] Epigenetic mechanisms of gene expression control include histone modifications, DNA methylation, and small noncoding RNA sequences. The former two mechanisms contribute to the remodeling of chromatin into an active or inactive state, while the latter modulates gene expression by binding target RNA transcripts (mRNA) and negatively regulating efficiency of protein translation and stability of these translation products.[98]

Perhaps the most important function of dietary folate in genome integrity maintenance is its role as a methyl donor for epigenetic control of gene expression by DNA methylation. Vertebrate DNA methylation is the addition of a methyl group (CH_3) to cytosine residues resulting in the modified base 5-methylcytosine (m^5C). A family of DNA methyltransferase (DNMT) enzymes is responsible for catalyzing methylation of cytosine bases in mammals. DNMT3A and DNMT3B enzymes catalyze primarily *de novo* methylation,[99] while DNMT1 is the principal enzyme involved in maintenance methylation of hemimethylated DNA following semiconservative DNA replication.[100] It is purported that approximately 4% of cytosine bases are methylated in vertebrates,[101] with these modified residues largely occurring at CpG dinucleotides located within promoter regions.[102] As gene promoter regions are typically rich in these CpG dinucleotides, they are known as CpG islands. CpG islands are also in large abundance in human gene exons. Gene-specific DNA methylation can cause gene silencing and expression via hyper- or hypomethylation, respectively. Both global (genome-wide) and local (gene-specific) DNA methylation patterns are modified by micronutrients such as folate, and these methylation patterns are often altered in cancer cells.[103]

Subtelomere DNA methylation and telomere length

Unlike the repeat unit of the telomere, which is highly conserved, the subtelomeric region (refer to Fig. 3 for schematic diagram) of the chromosome is variable,[104,105] with human subtelomere assemblies differing across all chromosome ends.[64,106,107] These subtelomeric assemblies contain segmental tandem repeats sourced from other sequences

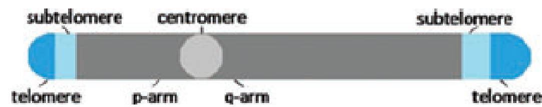

Figure 3. Schematic image of a chromosome: the mammalian chromosome includes the following distinct regions, from left to right: the telomere region (blue) contains tandem repeats of the hexamer $(TTAGGG)_n$ in humans; the subtelomere (light blue); chromosomal coding DNA (gray); the centromere (light gray). N.B.: schematic not to scale.

within the genome,[108] $(TTAGGG)_n$-like sequences, single-copy regions, and subtelomeric repeat sequences.[64,107,109] While mammalian telomeric hexamer repeats are devoid of DNA methylation substrates (CpG), the subtelomere contains a high proportion of CpG dinucleotides, which are methylated in human somatic cells.[104,110,111] Analogous subtelomeric regions in mice have high-CpG methylation that has been shown to taper when DNMT is abrogated.[91] Since global hypomethylation is a hallmark of cancer cells, it was hypothesized that reduced methylation may mediate telomere elongation.[7] Inadequate folate in the diet *in vivo*, as well as in *in vitro* models, has been consistently shown to be result in reduced DNA methylation.[112]

Maintenance (DNMT1) and *de novo* (DNMT3A and DNMT3B) DNA methyltransferases negatively regulate telomere length[91] through methylation of cytosine on a global scale, including at subtelomeric regions. Increased events of telomeric sister chromatid exchanges—a hallmark of ALT-positive cells[113,114]—occur when DNA methylation is lost by DNMT deficiency.[91] Additional ALT cell features—heterogeneous telomere length and ALT-associated APBs[83]—are displayed in these DNMT-deficient cells, showing that loss of DNA methylation occurs together with elevated telomeric recombination.[91] While subtelomeric hypomethylation is suggested to permit ALT activity,[91] it is not mandatory that all subtelomeric regions be hypomethylated for ALT recombination to occur.[115]

Although a hypomethylated subtelomere appears to elicit characteristics observed in ALT-positive cells,[91] telomerase-positive cells have hypermethylated subtelomeric regions.[115] Although the extent of subtelomeric CpG methylation in healthy cells is not known, it appears that the epigenetic status of the subtelomere is altered in both ALT- and telomerase-positive cells. That increase in telomere length—caused by *DNMT* knockdown leading to hypomethylated subtelomeres—occurred while other telomeric heterochromatic marks such as histone methylation were unchanged suggests that DNA methylation exerts higher order control of the telomere length.[91] As the subtelomeric assembly of each chromosome arm is variable, with subtelomeric repeat sequences ranging from <10 kb to >300 kb,[64] the number of CpG and, hence, the extent of subtelomeric methylation is likely to be different among human chromosomes. Currently, subtelomeric methylation is determined by methylation-specific PCR;[116] however, this method is limited in that the amplicon size is very small (< 200 bp), and so the result reflects the methylation status of few cytosine residues in the possible 500 kb subtelomeric region.[64] The conception of optimal methods that would allow for the definitive subtelomeric methylation status across each chromosome arm, and comparison of this status with chromosome arm–specific telomere length[117] remain important goals.

It was initially believed that telomeric sequences were transcriptionally silent; however, telomeres encode telomeric repeat-containing RNA (TERRA)[118] known to localize at the telomere.[118,119] Noncoding TERRA contains telomeric and subtelomeric sequences,[120] and, recently, TERRA transcription was shown to be negatively regulated by cytosine methylation of its promoter, housed in the subtelomere.[115,121] Increased methylation of the subtelomere, a notable feature of telomerase-expressing cells, results in silencing of subtelomeric/telomeric transcription of TERRA, hence suggesting telomerase is inhibited by TERRA, and that this transcriptional silencing may be selected in cancer cells.[115] Whether the inhibition of telomerase by TERRA occurs primarily *in situ* or *in trans* is unknown,[115] and although TERRA transcription has been shown to be higher in ALT-positive cells than telomerase-positive cells, this is likely due to an increased TERRA signal by the generally longer telomeres characteristic of heterogeneous ALT cells.[115] Despite the importance of folate as a methyl donor, there are no published studies on the relationship of folate status with subtelomere methylation and/or TERRA expression.

Telomeric chromatin and histone modifications

The human genetic code is organized within the cell as chromatin in the form of DNA packages of

146 bp wound around a nucleosome comprising pairs of each histone, H2A, H2B, H3, and H4.[122] DNA methylation at CpG dinucleotides, along with histone acetylation, induces reversible changes on chromatin structure. Condensed and transcriptionally silent chromatin or heterochromatin largely contains methylated cytosine residues and histones that are deacetylated. Histone modifications typical of heterochromatic telomeres include increased trimethylation of histones H3 (lysine 9) and H4 (lysine 20) and lowered acetylation of histones H3 and H4.[123] Other modifications at H3 and H4 tails include phosphorylation, ubiquitination, and methylation.[122] The observation that induced changes in telomeric heterochromatin, via histone methyltransferase (HMTase) knockdown, imparted abnormal elongation of telomeric sequence, suggests involvement of histone modifications in telomere length control.[124] Another exemplar of histone modification affecting telomere function includes heterochromatin protein 1 (HP1) that is involved in telomere capping and telomeric function.[125] HP1 functions to cap the telomere by binding directly to the telomeric sequence, while telomere elongation and transcriptional regression of the telomere by HP1 can occur via interaction with histone H3 methylated at lysine 9.[126,127]

Telomere length, diet, and folate

It is generally recognized firsthand that those who have a healthy lifestyle appear to live longer. Since an *in vitro* study showed that vitamin C enrichment slows telomere attrition in a human endothelial cell line,[128] various *in vivo* studies have investigated the effect of dietary components on the telomere length in humans. These associative studies have shown that vitamin D[129] and vitamin E intake,[130] multivitamin use,[131] dietary fiber consumption,[132] and intake of marine omega-3 fatty acid[133] were correlated with longer telomeres; whereas processed meat consumption,[134] increased alcohol intake,[135] and low fruit and vegetable intake, as well as increased meat intake[136] were dietary patterns that were negatively associated with telomere length. The potential effects of additional nutrients on telomere mechanics have recently been published.[137]

Plasma homocysteine concentration—which increases when folate and B12 are deficient—has been shown to be inversely associated with telomere length in human cross-sectional studies comprising up to 1319 subjects.[138–140] The attrition of telomeric sequence induced by elevated homocysteine is possibly mediated by increased oxidative stress or inflammation,[138,140] or the increased demand for proliferation of certain cell types, in these cases hematopoietic stem cells.[140] This negative effect of homocysteine on the telomere length is purported to also be the effect of folate deficiency on the telomere, as the amino acid and vitamin are inversely correlated, and as such it is suggested that the effect would be mitigated by increased folate intake.[138,140] To date, constituents of the folate pathway have not been adequately studied for their effect on telomere length in humans. As yet, B_{12} has been reported to have no effect on the telomere length[7,138] even though plasma B_{12} is considered an important factor in folate and homocysteine metabolism and should be considered in statistical analyses involving these measures. The effect of folate on the telomere length appears to be complex, on the basis of the few studies that have explored the relationship. In one study, low levels of plasma folate were correlated with the shorter telomere length in the older male cohort, though no such effect was observed in corresponding older female participants or in younger adults.[138] Perplexingly, in another study, plasma folate was inversely correlated with the longer telomere length when folate levels were below the cohort median, yet once plasma levels were above this level, the relationship with the telomere length was positive.[7] In a recent review, it has been suggested that folate deficiency might induce telomere attrition and/or dysfunction by molecular mechanisms, including (1) the excision of increased uracil in the telomeric hexamer repeat that is known to generate abasic sites and DNA breaks; (2) the aberrant epigenetic state of the subtelomeric DNA; and (3) inefficient binding of the shelterin proteins to the telomeric DNA due to reduced affinity to uracil and/or abasic sites, resulting from excision repair of uracil in the telomere sequence.[47] Under conditions of folate deficiency, incorporation of uracil instead of thymine in DNA is increased.[4,141] Uracil might also arise in the telomeric hexamer repeat due to spontaneous deamination of cytosine. Excision repair of uracil in DNA has a range of molecular consequences, including generation of abasic sites through base excision repair pathways, which can cause single- or double-stranded DNA breaks and chromosomal aberrations.[4,142,143] A lowered

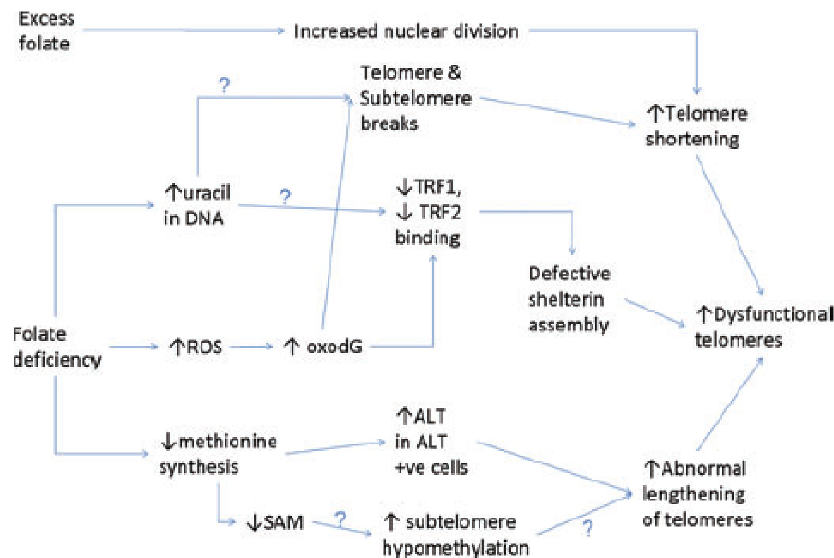

Figure 4. Possible mechanisms by which folate deficiency or excess may influence telomere structure and function: indentifies plausible but untested mechanisms.

synthesis of dTTP from dUMP has been suggested to accelerate telomere shortening,[143] while successive uracil misincorporation within the telomere could result in shorter telomeres as single-stranded breaks of the G-rich strand may not be repairable or cause degradation of the complementary C-rich strand.[143] However, continued investigation, both *in vitro* and *in vivo*, is required in order to validate or repudiate these plausible mechanisms and to better undstand the biological impact of folate deficiency on telomere function.

Knowledge gaps and conclusion

It is evident that dietary deficiency of micronutrients, including folate, may have an effect on the telomere length and function in humans. The development of a nutrient profile for optimal telomere function and maintenance in the general population is a clear goal in this field. Doing so on an individual level by employing principles of nutritional genomics and genetics, while arduous, could be a cost-effective approach to prevention of those chronic disease conditions that are often exacerbated by genomic disturbances caused by nutritional deficiencies.[47,144] In the case of folate, it is still unclear whether (1) deficiency causes increased uracil in the telomere and if, as a result, base excision and attempted repair causes telomere breaks and shortening; (2) low levels induce subtelomeric hypomethylation and whether the degree of this epigenetic modification can affect the length and function of the telomere; and (3) whether decreased levels of folate cause nucleoplasmic bridges or chromosome fusions that specifically involve telomeric and/or subtelomeric sequences. The effect of genetic polymorphisms that alter the activity of enzymes required in folate uptake, transport, and metabolism on the telomere length and function remains unexplored and should be thoroughly investigated for interactive effects with folate status.

It is apparent that there is not one distinct test for telomere integrity and function. Instead there are a suite of assays that individually measure, for example, telomere length (both absolute[145,146] and chromosome arm specific),[117,147] subtelomeric methylation[115,116] and TERRA RNA expression,[119] telomere dysfunction,[148] ALT mechanism presence,[149] and telomerase activity.[150] Despite the likely importance of telomere base sequence mutation, there do not appear to be any published methods that can detect DNA base damage within the telomere hexamer repeat. It has been shown that DNA damage repair within the telomeric region is less efficacious than repair to regions of the coding sequence,[151–153] with both the rate and degree of telomeric repair declining with age.[152] To be able to detect base damage within the telomeric sequence would be valuable, as the telomere is rich in guanine and thymine residues,

and, as such, it may be particularly vulnerable to guanine oxidation and increased incorporation of uracil under oxidative stress and folate deficiency conditions, respectively. In conclusion, as summarized in Figure 4, there are several potential mechanisms by which folate deficiency or excess may adversely affect telomere length, dynamics, and function. Several of these possible mechanisms need to be rigorously tested to obtain a deeper understanding of these fundamental processes that may be instrumental in refining decisions on folate requirements for maintenance of chromosomal stability.

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Conflicts of interest

The authors declare no conflicts of interest.

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10.3 Presentations at scientific meetings arising from this thesis

10.3.1 Oral

C Moores, N O'Callaghan, M Donoghoe, P Forder, B Armstrong & M Fenech. Micronutrient supplementation in a randomised controlled trial had no impact on telomere length in healthy, middle-aged South Australian men and women. The Australian Society for Medical Research South Australian Annual Scientific Meeting. Adelaide, South Australia, Australia. June 5, 2013.

C Moores, N O'Callaghan, M Donoghoe, P Forder, B Armstrong & M Fenech. The influence of plasma micronutrients and parental age on telomere length. Australian Telomere and DNA Repair Workshop. Sydney, New South Wales, Australia. October 29, 2010.

10.3.2 Oral/poster

C Moores, N O'Callaghan, M Donoghoe, P Forder, B Armstrong & M Fenech. The effect of a micronutrient supplement on telomere length. The Nutrition Societies of Australia and New Zealand Joint Annual Scientific Meeting. Queenstown, Otago, New Zealand. November 28 – December 2, 2011.

10.3.3 Poster

C Moores, N O'Callaghan, M Donoghoe, P Forder, B Armstrong & M Fenech, Short-term micronutrient supplementation has no effect on telomere length in healthy, middle-aged Australians, NuGO week "Nutrition, lifestyle and genes in the changing environment", Helsinki, Finland, August 28 – 31, 2012.

C Moores, N O'Callaghan, M Donoghoe, P Forder, B Armstrong & M Fenech. Nutritional influences on telomere length – plasma zinc and vitamin D are associated with telomere length. Keystone Symposia Changing Landscape of the Cancer Genome Conference, Boston, Massachusetts, United States of America, June 20 – 25, 2011.

C Moores, N O'Callaghan, M Donoghoe, P Forder, B Armstrong & M Fenech. The influence of plasma micronutrients and parental age on telomere length. The Nutrition Society of Australia Annual Scientific Meeting. Perth, Western Australia, Australia. November 30 – December 3, 2010. (Awarded Best Student Poster)

This poster was also presented at the following meetings:

- The Australian Society for Medical Research South Australian Annual Scientific Meeting. Adelaide, South Australia, Australia. June 9, 2010.
- CSIRO Nutritional Genomics Symposium. Adelaide, South Australia, Australia. July 30, 2010.

C Moores, N O'Callaghan, C Bull & M Fenech. Investigating the impact of folate and other methyl donors on telomere integrity and function. The Australian Society for Medical Research South Australian Annual Scientific Meeting. Adelaide, South Australia, Australia. June 2, 2009.

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