

**Riboflavin supplementation alters global and gene-specific DNA methylation in adults with the *MTHFR* 677TT genotype**

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**Short running head:** Riboflavin supplementation and DNA methylation in adults screened for the *MTHFR* C677T polymorphism

**Abbreviations:** EGRac, erythrocyte glutathione reductase activation coefficient; FAD, flavin adenine dinucleotide, FMN, flavin mononucleotide; LINE- 1, long interspersed nucleotide element 1; MTHFR, 5,10-methylenetetrahydrofolate reductase; RCT, randomised controlled trial

## 1 ABSTRACT

2 DNA methylation is important in regulating gene expression and genomic stability while  
3 aberrant DNA methylation is associated with disease. Riboflavin (FAD) is a cofactor for  
4 methylenetetrahydrofolate reductase (MTHFR), a critical enzyme in folate recycling, which  
5 generates methyl groups for homocysteine remethylation to methionine, the pre-cursor to the  
6 universal methyl donor S-adenosylmethionine (SAM). A polymorphism (C677T) in MTHFR  
7 results in decreased MTHFR activity and increased homocysteine concentration. Previous  
8 studies demonstrated that riboflavin modulates this phenotype in homozygous adults  
9 (*MTHFR* 677TT genotype), however, DNA methylation was not considered. This study  
10 examined DNA methylation, globally and at key *MTHFR* regulatory sites, in adults stratified  
11 by *MTHFR* genotype and the effect of riboflavin supplementation on DNA methylation in  
12 individuals with the 677TT genotype. Samples were accessed from participants, screened for  
13 the *MTHFR* C677T polymorphism, who participated in observational (n = 80) and targeted  
14 riboflavin (1.6mg/day) RCTs (n = 80). DNA methylation at LINE-1 and key regulatory  
15 regions of the *MTHFR* locus were analysed by pyrosequencing in peripheral blood  
16 leukocytes. LINE-1 (+1.6%; p = 0.011) and *MTHFR* south shelf (+4.7%, p < 0.001) were  
17 significantly hypermethylated in individuals with the *MTHFR* 677TT compared to CC  
18 genotype. Riboflavin supplementation resulted in decreased global methylation, albeit only  
19 significant at one CpG. A significant reduction in DNA methylation at the *MTHFR* north  
20 shore (-1.2%, p < 0.001) was also observed in TT adults following intervention with  
21 riboflavin. This provides the first RCT evidence that DNA methylation may be modulated by  
22 riboflavin in adults with the *MTHFR* 677TT genotype.

23 **Key words:** DNA methylation, Riboflavin, *MTHFR* C677T polymorphism, one-carbon  
24 metabolism

25

## 26 1. INTRODUCTION

27 DNA methylation involves the addition of a methyl group to the 5' position of a cytosine and  
28 usually occurs at CpG dinucleotides. Global methylation influences genome stability while  
29 gene-specific methylation leads to transcription changes influencing gene expression and  
30 phenotypes [1,2]. Differential methylation has also been shown to occur at CpG island shores  
31 (~ 2kb outwards from CpG islands) and shelves (~ 2kb outwards from island shores) [3].  
32 Alterations in methylation at these key regulatory regions influence phenotypes and  
33 contribute to disease risk [4]. Variations in DNA methylation can occur throughout the  
34 lifetime of an individual and have important consequences for health and disease [5–7]. DNA  
35 methylation is responsive to environmental changes [8] such as alterations in diet and this  
36 provides a mechanism through which epigenetic modulation can influence health outcomes.  
37 One-carbon metabolism (**Figure 1**), is the main metabolic pathway through which nutrients,  
38 mainly folate and related B-vitamins, interact to modulate DNA methylation [9–13]. Factors  
39 influencing intake or metabolism of these nutrients including common polymorphisms within  
40 genes that influence the one-carbon pathway may therefore impact methylation reactions  
41 [14]. Riboflavin in the form of flavin adenine dinucleotide (FAD) is a cofactor for MTHFR, a  
42 critical enzyme in one-carbon metabolism and thus for the production of S-  
43 adenosylmethionine (SAM), the universal methyl donor. Riboflavin has been largely  
44 overlooked in studies investigating B-vitamins in relation to DNA methylation. Of the few  
45 reports focusing on riboflavin, an observational study of pregnant Gambian women showed  
46 that riboflavin was a significant predictor of peripheral blood DNA methylation at six  
47 metastable epialleles (*BOLA3*, *LOC654433*, *EXD3*, *ZFYVE28*, *RBM46*, *PARD6G* and  
48 *ZNF678*) the offspring [15]. A recent cross-sectional study also reported an inverse  
49 association between dietary riboflavin intake and LINE-1 methylation in peripheral blood  
50 [16] while another study observed a positive correlation between daily intake of riboflavin

51 and LINE-1 methylation in white blood cells [17]. The latter studies relied on food frequency  
52 questionnaires to estimate riboflavin intake which may not accurately reflect status and as  
53 such, biomarker concentrations are a much more reliable indicator to investigate the  
54 relationship between riboflavin status and DNA methylation [13,18]. Furthermore, limited  
55 conclusions can be drawn from observational data which highlights the need for randomised  
56 controlled trials to determine the effects of one-carbon metabolism nutrients on epigenetic  
57 mechanisms.

58 The C677T polymorphism in the methylenetetrahydrofolate reductase (*MTHFR*) gene is one  
59 of the most widely studied polymorphisms in relation to one-carbon metabolism and health  
60 and disease [19]. It involves a C to T transition at position 677 which causes a substitution of  
61 alanine with valine resulting in a thermolabile MTHFR enzyme with decreased enzyme  
62 activity in individuals homozygous for the genotype [20] due to the loss of affinity for its  
63 cofactor FAD [21]. Polymorphisms and reduced enzyme activity of MTHFR are linked to  
64 various diseases [22–24] however only a small number of studies conducted in mice and  
65 humans have examined the MTHFR epigenetic landscape and gene expression [25,26].  
66 Aberrant hypermethylation of key regulatory regions surrounding the *MTHFR* CpG island  
67 have been uncovered in human paediatric astrocytomas [25]. In mice, reduced levels of  
68 MTHFR resulting from homozygous or heterozygous genetic deletion, resulted in decreased  
69 SAM levels or significantly increased S-adenosylhomocysteine (SAH) levels, or both, and  
70 global DNA hypomethylation. [27]. Therefore, other factors which potentially alter MTHFR  
71 levels, such as riboflavin supplementation, may also impact global and gene-specific DNA  
72 methylation. Our hypothesis was that DNA methylation differed in adults stratified by the  
73 *MTHFR* C667T genotype, could be modulated by supplementation with riboflavin, the  
74 MTHFR cofactor, in those with TT genotype. To test this hypothesis, we examined  
75 differences in global and gene-specific methylation at key regulatory sites at the *MTHFR*

76 locus in adults stratified by the *MTHFR* C677T genotype. Furthermore, we examined the  
77 effect of riboflavin supplementation on DNA methylation in adults with the *MTHFR* 677TT  
78 genotype.

79

## 80 **2. MATERIALS AND METHODS**

### 81 **2.1. Participants and Sample Selection**

82 Samples for this study were accessed from stored buffy coat samples from participants who  
83 were screened for the *MTHFR* C677T polymorphism and had consented and participated in  
84 targeted double-blind randomised controlled trials previously conducted at the Nutrition  
85 Innovation Centre for food and Health (NICHE) at Ulster University, Northern Ireland.

86 Samples were drawn from three cohorts namely, the Genetic and Vitamin study (Genovit -  
87 FCBMA-15-070), the Genetic and Vitamin ten year follow up study (GENOVIT10 -  
88 UUREC/12/0338) and the optimization of RIBOf flavin Status in Hypertensive Adults with a  
89 Genetic predisposition to Elevated Blood pressure study (RIBOGENE - REC/12/0136) to  
90 enable the required number of age and sex matched samples from placebo and treatment  
91 groups to be accessed. Each of these studies were conducted using a standardised protocol.

92 Furthermore, each study had identical inclusion and exclusion criteria which included history  
93 of gastrointestinal, hepatic, renal or haematological disorders, usage of B-vitamin  
94 supplements, anticonvulsant therapy or any other drugs known to interfere with folate or B-  
95 vitamin metabolism. Additional ethical approval was granted by Office of Research and  
96 Ethics Northern Ireland for the analysis reported in this current study. Data on lifestyle  
97 variables, anthropometry and blood samples were collected as part of all three studies.

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## 101 **2.2. Study Design**

102 Analysis for this study was carried out in two stages: in an observational stage (n = 80), DNA  
103 methylation differences were examined between the two *MTHFR* C677T genotypes (i.e.  
104 677CC and 677TT) and in an intervention stage (n = 80). DNA methylation was examined in  
105 response to supplementation with either riboflavin (1.6mg/d) or placebo for 16 weeks in  
106 individuals with the *MTHFR* 677TT genotype only. Appropriate samples of intervention with  
107 riboflavin in CC participants, were not available for the current analysis from the only study  
108 [28] to date to have conducted a riboflavin intervention in all three *MTHFR* 677 genotype  
109 groups. Participants were age- and sex-matched for both the observational and intervention  
110 stages of the study (**Table 1**). The flow diagram of the study design is illustrated in **Figure 2**.

## 111 **2.3. Biomarker Status**

112 Blood samples were analysed by standard laboratory assays for total homocysteine and  
113 riboflavin biomarker status as reported in previous studies [28,29]. Riboflavin status was  
114 determined using the erythrocyte glutathione reductase coefficient (EGRac), a functional  
115 assay which measures the activity of glutathione reductase before and after in vitro reaction  
116 with its prosthetic group flavin adenine dinucleotide (FAD). EGRac is calculated as a ratio of  
117 FAD-stimulated to -unstimulated enzyme activity with higher values indicative of lower  
118 riboflavin status and is recognised as the gold standard. Values of EGRac at or above 1.3 are  
119 generally indicative of suboptimal riboflavin status [30,31].

## 120 **2.4. DNA Methylation Analysis**

### 121 *2.4.1. ENCODE dataset analysis*

122 The Infinium 450K Bead Array and DNA methylation data from the ENCODE consortium  
123 available as user tracks in UCSC genome browser were utilized in this study [32]. The  
124 *MTHFR* genomic region “Chr1:11,868,000-11,862,000” (hg19) was inspected for differential

125 DNA methylation. In order to determine appropriate locations of gene-specific  
126 pyrosequencing assays for *MTHFR* gene regulatory regions, we carried out an analysis of  
127 DNA methylation at the region surrounding the *MTHFR* transcription start site (Chr1:  
128 11,868,000-11,862,000) in USCS genome browser (hg19) using publicly available Infinium  
129 HumanMethylation450 BeadChip methylation data from the ENCODE project [32]. This  
130 analysis showed that the north shore and south shelf *MTHFR* gene regulatory regions are  
131 variably methylated in five different human cell lines (**Supplementary Figure 1**) while the  
132 CpG island itself is largely unmethylated. The base pair resolution of these datasets allowed  
133 us to accurately target the chromosomal region likely to be susceptible to variable DNA  
134 methylation. Using the above information, we next experimentally investigated the  
135 chromosomal regions for methylation change by pyrosequencing analysis in our human  
136 samples.

#### 137 *2.4.2. Genomic DNA extraction*

138 Genomic DNA was extracted from 200µl of stored peripheral blood leukocyte samples using  
139 the Qiagen QIAamp DNA blood mini kit (Qiagen, UK). The process was carried out  
140 according to the manufacturer's protocol [33]. The extracted genomic DNA samples were  
141 electrophoresed on a 1% (w/v) agarose gel to examine their quality. The purity, and  
142 concentration of DNA samples was quantified using the NanodropND1000  
143 spectrophotometer (Labtech International, Ringmer, UK).

#### 144 *2.4.3. Bisulphite Conversion of Genomic DNA*

145 Subsequent bisulphite conversion of 500ng of genomic DNA was carried out according to the  
146 manufacturer's instructions [34–36] using the EZ DNA methylation kit (Zymo Research  
147 Corporation, California).

148

149

150 2.4.4. Polymerase Chain Reaction and Pyrosequencing

151 DNA methylation at the Long Interspersed Nuclear Elements (LINE-1) was measured as a  
152 surrogate marker for global methylation. The LINE-1 (GenBank accession number  
153 X58075.1) assay covered 3 CpG sites. Three regions of the *MTHFR* gene (GenBank  
154 accession: NM\_001330358.1), covering the north shore, south shelf and the CpG island  
155 promoter were examined in this study. Commercially available assays for LINE-1 (970042)  
156 and *MTHFR* CpG island (PM00000091) promoter from Qiagen UK were used for PCR of  
157 bisulphite treated DNA. Primers for *MTHFR* north shore (Chr1: 11867263-11867362) and  
158 south shelf (Chr1: 11862886-11862985) were designed using PyroMark Assay Design  
159 software 2.0. Assay regions were chosen to align with publicly available Illumina 450k array  
160 data deposited in UCSC Genome Browser which displayed varying levels of DNA  
161 methylation in various cell lines: Primers for the commercially available LINE-1 assay and  
162 *MTHFR* CpG island covered 3CpGs each while in-house designed primers for the *MTHFR*  
163 north shore and south shelf covered only one CpG due to technical difficulties in primer  
164 design. *MTHFR* north shore forward: 5' TTTGGGTAATTAAAGTAGTGAGTGGTTTG 3'  
165 and *MTHFR* north shore reverse: 5' CCCTAAAACAAAAAATCAAAAACATCTCT 3';  
166 *MTHFR* south shelf forward: 5' CCCTAAAACAAAAAATCAAAAACATCTCT 3' and  
167 *MTHFR* souths shelf reverse: 5 'TCCCCAAACACCACCACT 3'. The PyroMark PCR kit  
168 (Qiagen UK) was used for generating amplicons. Each 25µl reaction mix consisted of 12.5µl  
169 master mix, 2.5µl coral load, 5.5µl nuclease-free water, 1.25µl each of 10µM forward and  
170 reverse primers (2.5µl for commercial primers) and 2µl each of bisulphite converted DNA.  
171 PCR was then carried out under the following conditions: initial hot start, 95°C for 15  
172 minutes, followed by 45 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30s  
173 and a final elongation of 10 minutes at 72°C. The PCR products were subsequently



174 electrophoresed on a 1% (w/v) agarose gel electrophoresis to check the size of DNA  
175 fragments and also as a quality control measure to check samples for contamination.  
176 DNA methylation levels in samples were analysed using the PyroMark Q24 pyrosequencing  
177 instrument (Qiagen, UK). Enzymes, substrates and nucleotides from the PyroMark Gold Q24  
178 kit (Qiagen UK) were used. Built in controls within assays to be analysed were used to verify  
179 bisulphite conversion. Levels of methylation at each CpG site were analysed using the  
180 PyroMark Q24 software [37,38]. The degree of methylation at each CpG site is expressed as  
181 the percentage of methylated cytosine over the sum of methylated and unmethylated cytosine.  
182 The degree of methylation is reported for each CpG analysed as well as the average  
183 percentage of methylation across CpG sites. To verify the accuracy of the analysis, control  
184 DNA from EpiTect PCR (Qiagen UK) containing human bisulphite converted fully  
185 methylated or unmethylated DNA were included as positive and negative controls in the  
186 pyrosequencing runs.

187

## 188 **2.5. Statistical Analysis**

189 For the current analysis, power calculations to determine sample size were carried out using  
190 the G Power 3.1.9.4 software (version 3) [39] statistical power calculator. Based on power  
191 calculations using data from Bollati and colleagues [40], it was estimated that 39 participants  
192 per group would be able to discriminate differences of 3.4% in DNA methylation with a  
193 power of 80%, at  $\alpha = 0.05$  and effect size of 0.65. This sample size is similar to that reported  
194 in previous studies investigating folic acid and vitamin B-12 supplementation and DNA  
195 methylation [41,42]. Statistical analysis of data was conducted using SPSS IBM Statistics  
196 (version 25, SPSS UK Ltd Chertsey, UK). The normality of continuous variables was  
197 confirmed using QQ-plots and the Kolmogorov-Smirnov test. All tests were carried out at the  
198 95% confidence interval and in all analyses  $p < 0.05$  was considered statistically significant.

199 Methylation values are shown for all loci analysed and an average methylation for the three  
200 CpG sites analysed for the LINE-1 and *MTHFR* CpG island assay. The assays for the  
201 *MTHFR* north shore and south shelf contained one CpG. Change in methylation in response  
202 to riboflavin supplementation was calculated as the difference between post-intervention and  
203 baseline methylation values for each CpG analysed and the average.

204 Chi-square tests for independence were used for comparing categorical variables such as sex,  
205 smoking and hypertensive status. Continuous variables including age and body mass index  
206 (BMI), were analysed using independent t-tests. One-way analysis of covariance (ANCOVA)  
207 adjusted for age, sex, smoking status and study cohort was used to analyse DNA methylation  
208 stratified by *MTHFR* C677T genotypes at baseline. Biomarker (EGRac and homocysteine)  
209 responses to intervention with riboflavin were examined using mixed between-within  
210 repeated measures ANOVA. The time  $\times$  treatment interaction was used to assess the effect of  
211 treatment versus placebo over time. The between-patient factor was the intervention group  
212 (placebo versus riboflavin), and the within-patient factor was time (pre- and post-  
213 supplementation). Mixed between-within repeated measures ANCOVA was used to analyse  
214 the effect of riboflavin supplementation on DNA methylation in individuals with the *MTHFR*  
215 677TT genotype. The time  $\times$  treatment interaction was used to assess the effect of treatment  
216 versus placebo over time. The between factor was the intervention group (riboflavin versus  
217 placebo) with time (pre- and post- intervention) as the within factor. The mixed between-  
218 within analysis tests whether there are main effects for each independent variable and  
219 whether the interaction between the two variables is significant. The analysis was further  
220 adjusted as appropriate for confounders previously reported to influence DNA methylation  
221 such as age, sex, smoking status and study cohort. To account for multiple testing, the level  
222 of significance ( $P < 0.05$ ) was adjusted for Bonferroni correction at the assay level ( $n = 4$  for  
223 LINE-1,  $n = 6$  for *MTHFR* north shore, south shelf and CpG island DNA methylation),

224 therefore  $P < 0.0125$  or  $P < 0.008$  was considered statistically significant where appropriate.  
225 Pearson's bivariate correlation coefficient ( $r$ ), was used to estimate correlations between  
226 riboflavin biomarker and DNA methylation in individuals with the TT genotype in the  
227 intervention study stratified by treatment groups.

### 228 **3. RESULTS**

#### 229 **3.1. General Characteristics of Participants**

230 A total of 80 participant samples were analysed in the observational stage of the study  
231 examining both global methylation and gene-specific methylation, the latter at the *MTHFR*  
232 north shore, south shelf and CpG island, in individuals stratified by *MTHFR* C677T  
233 genotype. Individuals with the CC genotype were age- and sex-matched to individuals with  
234 the *MTHFR* 677TT genotype (**Figure 2**). The characteristics of participants in the  
235 observation study are described in **Table 1**. Generally, participants were on average 57 years  
236 old and no statistically significant differences were observed between *MTHFR* C677T  
237 genotype groups in relation to baseline characteristics such as age, sex, BMI and smoking  
238 status. Riboflavin biomarker status was not significantly different between treatment groups  
239 prior to intervention.

#### 240 **3.2. Differences in DNA methylation in individuals stratified by *MTHFR* C677T** 241 **genotype**

242 There was a general trend towards higher methylation both globally and at sites assayed  
243 across the *MTHFR* locus in individuals with the *MTHFR* 677TT genotype in comparison to  
244 the CC genotype group (**Table 2**). Perhaps surprisingly, global DNA methylation measured  
245 by LINE-1 was significantly higher (+1.6%;  $p = 0.011$ ) in participants with the *MTHFR*  
246 677TT genotype compared to those with the CC genotype at baseline. Additionally,  
247 significant hypermethylation was detected in individuals with the *MTHFR* 677TT genotype at

248 the *MTHFR* south shelf (+4.85%,  $p < 0.001$ ) compared to CC individuals. DNA methylation  
249 at the *MTHFR* north shore and CpG island were however not significantly different between  
250 genotype groups at baseline, although there was again a trend for higher methylation in  
251 individuals with the *MTHFR* 677TT genotype.

252

### 253 **3.3. Effect of riboflavin supplementation on biomarker status**

254 The biomarker responses to riboflavin intervention are shown in **Table 3**. As expected,  
255 riboflavin biomarker status in adults with the *MTHFR* 677TT genotype improved in response  
256 to riboflavin supplementation ( $P < 0.001$ ), as indicated by a mean decrease in the functional  
257 biomarker EGRac in participants who received riboflavin ( $-0.10 \pm 0.01$ ) compared to placebo  
258 ( $0.02 \pm 0.01$ ). Furthermore, there was a significant reduction in homocysteine concentrations  
259 ( $P = 0.001$ ) in the group supplemented with riboflavin ( $-1.79 \pm 3.50 \mu\text{mol/L}$ ) compared to  
260 placebo ( $-0.42 \pm 3.10 \mu\text{mol/L}$ ).

### 261 **3.4. Effect of riboflavin supplementation on global and gene-specific methylation in**

#### 262 ***MTHFR* 677TT participants**

263 Investigation of the effect of riboflavin supplementation on DNA methylation in individuals  
264 with the *MTHFR* 677TT genotype indicated decreased average methylation at LINE-1  
265 (Riboflavin:  $-3.16\% \pm 0.91\%$  vs. Placebo:  $-0.32\% \pm 0.69\%$ ,  $P = 0.018$ ) which remained  
266 significant following Bonferroni correction at CpG 2 (Riboflavin:  $-1.49\% \pm 0.72\%$  vs.  
267 Placebo :  $1.23\% \pm 0.62\%$ ,  $P = 0.006$ ). The *MTHFR* north shore was significantly  
268 hypomethylated ( $-1.24\% \pm 0.50\%$  vs.  $0.90\% \pm 0.50\%$ ,  $P = 0.001$ ) in participants  
269 supplemented with riboflavin compared to placebo respectively. Methylation at the *MTHFR*  
270 south shelf and CpG island in individuals with the *MTHFR* 677TT genotype group was not  
271 influenced by supplementation with riboflavin or placebo. Furthermore, we observed a non-

272 significant trend for a positive correlation between riboflavin biomarker status and LINE-1  
273 DNA methylation in the riboflavin group compared to the placebo however this was not  
274 significant (**Figure 3**). A similar non-significant correlation was observed for riboflavin  
275 biomarker and *MTHFR* north shore methylation (data not shown).

276

#### 277 **4. DISCUSSION**

278 The current study provides the first RCT evidence that supplementation with riboflavin  
279 results in decreased global and *MTHFR* north shore methylation in individuals with the  
280 *MTHFR* 677TT genotype. Consistent with these findings, higher homocysteine levels,  
281 indicative of perturbed B-vitamin status, were significantly reduced and riboflavin status  
282 improved following riboflavin supplementation. This provides some evidence for a  
283 mechanism in which supplementation with riboflavin influenced metabolite levels, and thus  
284 DNA methylation potential. In addition, at baseline, significant hypermethylation was  
285 observed in LINE-1 and *MTHFR* south shelf methylation in individuals with the *MTHFR*  
286 677TT genotype compared to individuals with the CC genotype.

287 In comparison to folate, one of the main substrates used for generation of methyl groups in  
288 one-carbon metabolism, which has been studied extensively in relation to DNA methylation  
289 [43] the role of riboflavin has been largely overlooked. The evidence regarding the role of  
290 folate on DNA methylation is not entirely consistent. Some previous studies [44–46],  
291 reported that supplementation with folic acid or improved folate status increased global DNA  
292 methylation across a range of tissues, including whole blood, leukocytes and colonic mucosa,  
293 while more recent studies [42,47–49] examining both global, LINE-1 and genome-wide  
294 methylation, including from our own labs, indicate that increased folic acid intake results in  
295 lower DNA methylation. DNA methylation was assessed in whole blood, leukocyte samples  
296 and cord blood in the studies above showing that the findings of inverse association were

297 present irrespective of the tissue examined. The results of these recent studies are similar to  
298 our findings which demonstrate an inverse relationship between riboflavin and DNA  
299 methylation in leukocytes as indicated by decreases in both LINE-1 and *MTHFR* north shore  
300 methylation in response to riboflavin supplementation. Additionally, in general agreement  
301 with the results of this study, Van den Donk *et al* [50], reported higher dietary folate intake  
302 was associated with lower methylation in whole blood and adenoma tissue in individuals with  
303 the *MTHFR* 677TT genotype.

304 Concordant with the findings of the present work, a recent genome-wide methylation study  
305 by Chamberlain *et al.* [16] reported an inverse association between dietary intake of  
306 riboflavin and LINE-1 methylation in blood samples. The authors did not however measure  
307 biomarker status of riboflavin thus the results should be interpreted with caution. The study  
308 also showed low riboflavin intake to be associated with higher CpG site-specific methylation  
309 at the first exon of the *PROM1* locus although no significant associations were observed for  
310 other nutrients involved in one-carbon metabolism including folate, vitamin B-12 and vitamin  
311 B-6 or the methyl donor index, [16]. The “methyl donor index” was calculated as the sum of  
312 the standardised intake values on the log scale [(value – mean)/SD] across 7 individual  
313 nutrients namely riboflavin, vitamin B6, folate, vitamin B12, choline, betaine, and  
314 methionine which are considered to contribute to DNA methylation.

315 Several factors could account for the inverse relationship between riboflavin biomarker  
316 concentration and both LINE-1 and *MTHFR* north shore DNA methylation. Methyl groups  
317 generated from one-carbon metabolism are used in a wide range of biological processes and  
318 the complexity in the interactions of these systems implies that there may not necessarily  
319 exist a linear relationship between nutrients involved in one-carbon metabolism and DNA  
320 methylation [43,51]. For example, simple correlations such as high riboflavin status leading  
321 to increased DNA methylation are unlikely to broadly apply and may differ based on cellular

322 conditions, dose of riboflavin administered and health status of participants. Secondly,  
323 channelling of methyl groups into DNA methylation is dependent on DNA methyltransferase  
324 enzymes (*DNMTs*) which tightly regulate the process [52–55]; therefore, an abundance of  
325 methyl groups available for DNA methylation does not necessarily result in increased DNA  
326 methylation. Methyl groups may be directed towards other methylation pathways such as  
327 RNA and histone methylation based on prioritization of cellular conditions and requirements.  
328 A small fraction may also be diverted to non-CG methylation (mostly CpH where H = A,C or  
329 T) which has been detected in almost all tissues tested to date [56]. Similarly, interplay  
330 between *DNMTs* and transcription factors may potentially influence DNA methylation [57].  
331 Through interaction with *DNMTs*, transcription factors influence the establishment and  
332 maintenance of DNA methylation [58,59]. Furthermore, regulation of DNA methylation by  
333 *DNMTs* is highlighted in a recent study of polymorphisms in genes involved in one-carbon  
334 metabolism which revealed a significant association between functional polymorphisms of  
335 *DNMT3B* and *MTHFR* methylation [60]. In addition, a study of 2,453 individuals from eight  
336 European countries, investigating variables that may have potential impact on *DNMT*  
337 expression, reported associations between intake of dairy foods (which are a rich source of  
338 riboflavin) and *DNMT1* expression [61], suggesting an additional pathway through which  
339 riboflavin can modulate DNA methylation.

340 Furthermore, the production of methyl groups for methylation is also dependent on other  
341 enzymes and one-carbon metabolism nutrients such vitamin B-12 and folate. For example,  
342 vitamin B-12 dependent methionine synthase enzyme functions in the remethylation of  
343 homocysteine to methionine and subsequently, the generation of SAM [62]. Therefore, while  
344 it is possible that the *MTHFR* enzyme may be stabilized by providing riboflavin [20], other  
345 nutrients and enzymes within one-carbon metabolism could impact the production and  
346 availability of methyl groups necessary for DNA methylation. As a first crucial step in

347 demonstrating that riboflavin modulates DNA methylation, we show that riboflavin resulted  
348 in decreased total homocysteine, providing important data to support a potential mechanism  
349 whereby riboflavin influences metabolite levels with potential effects on DNA methylation.  
350 Hypermethylation at the *MTHFR* south shelf in *MTHFR* 677TT individuals may reflect an  
351 increased demand for protein production to compensate for the reduced stability and activity  
352 of the enzyme [20] in the TT genotype. Methylation within gene bodies, where the south  
353 shelf is located, is associated with higher-level transcription generally. Though exact  
354 mechanisms are not yet clear, it may prevent aberrant transcription of short transcripts and  
355 thereby direct translation of full-length messenger RNAs [4]. Methylation at the *MTHFR*  
356 south shelf remains unchanged following riboflavin intervention and may therefore be  
357 important in preventing aberrant *MTHFR* transcript production in individuals with the TT  
358 genotype. Global hypermethylation observed in individuals with the *MTHFR* 677TT  
359 genotype in this study at LINE-1 repetitive elements is also observed in patients with diseases  
360 such as multiple sclerosis and Alzheimer's compared to healthy controls [63–65].  
361 While the differences observed in DNA methylation between the *MTHFR* genotypes in this  
362 study were small, they are comparable with those reported in other studies investigating  
363 LINE-1 methylation in peripheral blood in atherosclerosis, cancer and benzene exposure  
364 [40,66,67]. The changes in methylation elicited by riboflavin supplementation are also similar  
365 to findings of studies investigating other B-vitamins and DNA methylation [68,69]. It is  
366 postulated therefore that the small but significant changes observed may be able to mediate  
367 changes in gene expression and could be reflective of important alterations in the epigenome,  
368 especially in at-risk populations such as individuals with the *MTHFR* 677TT genotype. In  
369 support of this, it has been shown that drug treatment of cell lines which produced relatively  
370 small methylation changes resulted in transcription changes [70]. Further studies  
371 investigating gene expression and synthesis of the *MTHFR* protein are required to provide



372 further insight into the underlying biological mechanism. We observed decreased methylation  
373 at the *MTHFR* north shore following supplementation with riboflavin, which has important  
374 implications for gene expression as previous studies have shown that alterations in  
375 methylation at CpG sites within shores display higher correlation to gene expression  
376 compared to CpG islands [3]. As expected, methylation remained unchanged at the *MTHFR*  
377 CpG island or south shelf following intervention with riboflavin, highlighting the sensitivity  
378 of the *MTHFR* north shore to nutritional influences in comparison to the south shelf or CpG  
379 island and this could be a potential target for future epigenetic studies.

380 The major strength of the current study is the inclusion of samples from RCTs incorporating  
381 a parallel placebo group. Importantly, by measuring DNA methylation in the same  
382 individuals before and after supplementation, we can also minimise inter-individual variation  
383 as a source of observed changes in methylation. Furthermore, global and gene-specific  
384 methylation were analysed using the robust pyrosequencing method which has been shown to  
385 be very sensitive and reproducible. In a multicentre benchmarking study evaluating DNA  
386 methylation assays for clinical use, pyrosequencing of repetitive elements including LINE-1  
387 provided highly reproducible results and bisulphite pyrosequencing showed the best  
388 performance for assay sensitivity [71]. Our results however highlight the need for further  
389 work as DNA methylation was examined at a limited number of CpG sites. Therefore, it is  
390 likely that other regions of the genome which are also influenced by riboflavin require further  
391 investigation. Further, while DNA methylation in blood is reflective of methylation status in  
392 other tissues, blood consists of a mixed cell population and further work is required to  
393 completely exclude the possibility that this contributed to the changes in methylation  
394 observed here. We acknowledge that the current study does not allow us to determine tissue-  
395 specific effects of riboflavin supplementation on DNA methylation that may be present, the  
396 technique used for assessing DNA methylation does not allow us they may be undetected in

397 the current study. The present study was confined to investigating the effect of riboflavin on  
398 DNA methylation in adults with the variant TT genotype; future studies should include  
399 individuals with the CC genotype to confirm that the effects observed are genotype driven  
400 which would help to provide some additional mechanistic insights into the role of this gene-  
401 nutrient interaction in modifying DNA methylation.

402 In conclusion, this study is the first to provide RCT evidence demonstrating a novel role for  
403 riboflavin in modulating DNA methylation in adults with the *MTHFR* 677TT genotype.

404 Supplementation with riboflavin resulted in decreased global and *MTHFR* north shore  
405 methylation in TT individuals. Further studies of genome-wide DNA methylation in both TT  
406 and non-TT genotypes, as well as gene expression analysis are required to fully elucidate the  
407 role of riboflavin in modulating the epigenome.

408

409

410 **Authors' Contributions were as follows:**

411 DLM and MW planned and designed the research, with contributions from CPW on assay  
412 design. SDA, AM and JD conducted the epigenetic laboratory work and SDA performed the  
413 statistical analysis of the data. AM, GH conducted the original vitamin trials under the  
414 supervision of MW, CFH, HM, JP and JJS. SDA, CFH, MW and DLM wrote the initial draft  
415 of the manuscript and all authors provided important revisions. HM, JJS and CPW carried out  
416 critical revision for important intellectual content. DLM had primary responsibility for the  
417 final content. All authors read and approved the final version of the manuscript.

418 **Declaration of competing interest**

419 DLM, CPW, SDA, AM, CFH no conflicts of interest. MW, HN, JJS hold an international  
420 patent on the use of riboflavin in the treatment of blood pressure.

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**Table 1.**

General characteristics of participants for observational study grouped according to the *MTHFR* C677T genotype at baseline (n 80)

	<i>MTHFR</i> Genotype		
	<i>MTHFR</i> 677 CC (n 40)	<i>MTHFR</i> 677 TT (n 40)	p-value
Age (yr)	58.3(3.9)	56.8(6.9)	0.215
Male n (%)	22(55.5)	24(60.0)	0.651
Smoker n (%)	5(12.5)	6(15.0)	0.745
Alcohol (%)	28(70.0)	26(65.0)	0.633
Hypertensive BP n (%)	12(30.0)	22(55.0)	<b>0.024</b>
BMI (kg/m <sup>2</sup> )	29.5(4.8)	29.8(4.8)	0.769
<b>B-vitamin biomarker status</b>			
EGRac	1.34(0.17)	1.34(0.12)	0.945
Homocysteine (µmol/L)	10.6(3.5)	13.8(4.8)	<b>0.002</b>

Data are expressed as mean (SD) for continuous variables and frequency (%) for categorical variables. Categorical variables analysed using chi square statistics and continuous data were analysed using independent t-tests with,  $p < 0.05$  considered statistically significant (significant p-values shown in boldface). Hypertension (baseline) defined as BP readings (systolic/diastolic) 140mmHg and/or 90mmHg or greater.

**Abbreviations:** BMI, body mass index; BP, blood pressure; EGRac, erythrocyte glutathione reductase coefficient.

**Table 2.**

Baseline global and *MTHFR* gene methylation stratified by the *MTHFR* C677T genotype (n 80)

	DNA methylation (%)		
	CC (n 40)	TT (n 40)	p-value
<b>LINE-1</b>			
CpG1	74.26(4.10)	74.39(3.84)	0.506
CpG2	65.82(4.02)	68.34(3.28)	<b>0.002</b>
CpG3	66.51(3.70)	68.59(5.06)	<b>0.033</b>
<b>Average</b>	68.86(2.71)	70.44(3.41)	<b>0.011</b>
<b><i>MTHFR</i> north shore</b>			
CpG1	95.63(1.98)	95.64(2.51)	0.338
<b><i>MTHFR</i> south shelf</b>			
CpG1	35.19(5.16)	40.04(3.95)	<b>&lt; 0.001</b>
<b><i>MTHFR</i> CpG island</b>			
CpG1	1.17(0.36)	1.64(1.50)	0.120
CpG2	0.66(0.31)	0.88(0.60)	0.020
CpG3	0.57(0.38)	0.86(1.10)	0.241
<b>Average</b>	0.80(0.32)	1.13(1.04)	0.107

Data are expressed as mean (SD). Data analysed using one-way ANCOVA adjusting for age, sex, smoking status and study cohort with  $p < 0.05$  considered statistically significant.

**Abbreviations:** LINE-1, long interspersed nuclear element; *MTHFR*, methylenetetrahydrofolate reductase.



**Table 3.**

Biomarker response to riboflavin intervention in adults with the *MTHFR* 677TT genotype (n 80)

<b>Response indicator</b>	<b>Placebo (n 40)</b>	<b>Riboflavin (n 40)</b>	<b><i>P</i>-value*</b>	<b><i>P</i>-value†</b>
<b>EGRac</b>				
Pre-intervention	1.35(0.12)	1.32(0.19)		
Post-intervention	1.37(0.13)	1.22(0.08)	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
Change	0.02(0.08)	-1.02(0.08)		
<b>Homocysteine (µmol/L)</b>				
Pre-intervention	15.8(6.6)	13.5(6.2)		
Post intervention	15.4(6.4)	11.7(3.0)	0.068	<b>0.001</b>
Change	-0.4(3.1)	-1.8(3.5)		

Data expressed as mean (SD). EGRac, biomarker of riboflavin status; a higher value indicates lower status. \**P*-values refer to the time×treatment interaction of the mixed between-within repeated measures ANOVA, comparing the effect of treatment vs placebo over time. †*P*-values refer to the time×treatment interaction of the repeated measures ANOVA, comparing the effect of treatment vs placebo over time with adjustment for baseline homocysteine. *P* < 0.05 considered statistically significant are shown in bold.

**Abbreviations:** EGRac, erythrocyte glutathione reductase activation coefficient.

**Table 4.**Effect of riboflavin supplementation on global and *MTHFR* gene DNA methylation in participants with the *MTHFR* 677TT genotype (n 80)

	DNA methylation (%)						<i>P</i> -value
	Placebo (n 40)			Riboflavin (n 40)			
	<i>Pre-intervention</i>	<i>Post-intervention</i>	<i>Change<sup>a</sup></i>	<i>Pre - intervention</i>	<i>Post-intervention</i>	<i>Change<sup>a</sup></i>	
<b>LINE-1</b>							
CpG1	73.61(3.61)	73.53(4.72)	0.08(0.85)	73.43(4.52)	72.12(5.10)	-1.31(1.10)	0.397
CpG2	67.89(3.22)	69.13(2.26)	1.23(0.62)	68.77(2.98)	67.28(3.07)	-1.49(0.72)	<b>0.006†</b>
CpG3	67.97 (4.92)	65.85(4.40)	-2.10(1.01)	68.61(4.07)	61.93(7.95)	-6.68(1.45)	0.014
Average	69.82(3.27)	69.51(3.20)	-0.32(0.69)	70.27(3.19)	67.11(4.62)	-3.16(0.91)	0.018
<b><i>MTHFR</i> north shore</b>							
CpG1	94.70(2.54)	95.61(1.77)	0.90(0.50)	96.40(2.20)	95.17(1.97)	-1.24(0.50)	<b>0.001*</b>
<b><i>MTHFR</i> south shelf</b>							
CpG1	39.13(4.03)	39.39(6.03)	0.25(0.70)	39.65(4.11)	38.84(3.67)	-0.81(0.70)	0.302
<b><i>MTHFR</i> CpG island</b>							
CpG1	1.55(0.71)	1.30(0.39)	-0.25(0.10)	1.55(1.50)	1.25(0.29)	-0.31(0.20)	0.824
CpG2	0.87(0.46)	0.66(0.38)	-0.22(0.09)	0.78(0.53)	0.68(0.17)	-0.10(0.09)	0.396
CpG3	1.05(0.82)	0.67(0.35)	-0.37(0.10)	0.72(1.09)	0.57(0.24)	-0.14(0.20)	0.293
Average	1.16(0.53)	0.88(0.35)	-0.28(0.09)	1.02(1.03)	0.83(0.19)	-0.18(0.20)	0.636

Data are expressed as mean (SD). Data analysis conducted using mixed between-within repeated measures of ANCOVA adjusting for age, sex, smoking status and study cohort as covariates. *P*-values represent time×treatment interaction comparing the effect of treatment vs placebo over time, with between factor as intervention group (riboflavin versus placebo) and within factor as time (pre and post- intervention). †*P* < 0.0125 or \**P* < 0.008, considered

statistically significant after adjusting for Bonferroni correction at assay level (n = 4 for LINE-1, n = 6 for *MTHFR* north shore, south shelf and CpG island). Significant *P*-values are shown in bold font. <sup>a</sup>Change in methylation in response to supplementation with riboflavin or placebo was calculated as the difference between post-intervention and baseline methylation values.

**Abbreviations:** LINE-1, long interspersed nuclear element; MTHFR, methylenetetrahydrofolate reductase.

## FIGURE LEGENDS

**Figure 1.** One-carbon metabolism pathway.

**Abbreviations:** BHMT, betaine-homocysteine s-methyltransferase; DMG, dimethylglycine; DNMT, DNA methyltransferase; FAD, flavin adenine dinucleotide (a form of riboflavin); FMN, flavin mononucleotide; MAT, methionine adenosyltransferase; PLP, Pyridoxal-5'-phosphate; SAH, S-adenosylhomocysteine

**Figure 2.** Flow diagram of study design investigating DNA methylation.

The observation component of the study (n = 80) compared DNA methylation between the TT and CC genotypes for the *MTHFR* C677T polymorphism. The intervention stage (n = 80) investigated alterations in DNA methylation in participants with the TT genotype in response to supplementation with 1.6mg/day of riboflavin or placebo for 16 weeks.

<sup>1</sup>Samples were drawn from the following studies: Genetic and Vitamin study (Genovit) n = 14; the Genetic and Vitamin ten year follow up study (GENOVIT10) n = 19; and the optimization of RIBOfLavin Status in Hypertensive Adults with a Genetic predisposition to Elevated Blood pressure study (RIBOGENE), n = 87.

**Figure 3.** Correlation between riboflavin biomarker status (EGRac) and LINE-1 DNA methylation stratified by treatment groups. A lower EGRac value indicates improved riboflavin biomarker status. Correlations were estimated using Pearson's bivariate correlation coefficient (r), with p-value < 0.05 considered statistically significant.

**Abbreviations:** EGRac, Erythrocyte glutathione reductase activation coefficient; LINE-1, long interspersed nuclear element

**Supplementary Figure 1.** UCSC genome browser representation of the 5' region of the *MTHFR* RefSeq gene present in human chromosome 1.

A) Chromosome ideogram of chromosome 1 showing the location of the *MTHFR* gene. B) Expanded view of the *MTHFR* locus on chromosome 1 (p36.22). *MTHFR* regions analysed by pyrosequencing are represented by solid black horizontal bars. *MTHFR* Ref Seq gene shown in dark blue, exons are indicated by solid blue boxes and introns by the blue line with arrows. The CpG island present at the 5' *MTHFR* region is shown as a green horizontal bar. C) ENCODE 450K array datasets indicate variable methylation in the *MTHFR* north shore and south shelf CpGs in various human cell lines, while those in the CpG island are largely unmethylated. (GM12878 B lymphocyte; H1-hESC embryonic stem cell; K562 lymphoblast chronic myeloid leukaemia; HeLa S3 cervical cancer; HepG2 liver cancer; HUVEC umbilical epithelial cells). The CpG positions assayed by these methods are represented as vertical bars coloured according to their methylation status; orange = fully methylated (beta value  $\geq 0.6$ ), purple = partially methylated ( $0.2 < \text{beta value} < 0.6$ ), blue = fully unmethylated (beta value  $\leq 0.2$ ).

Figure 1

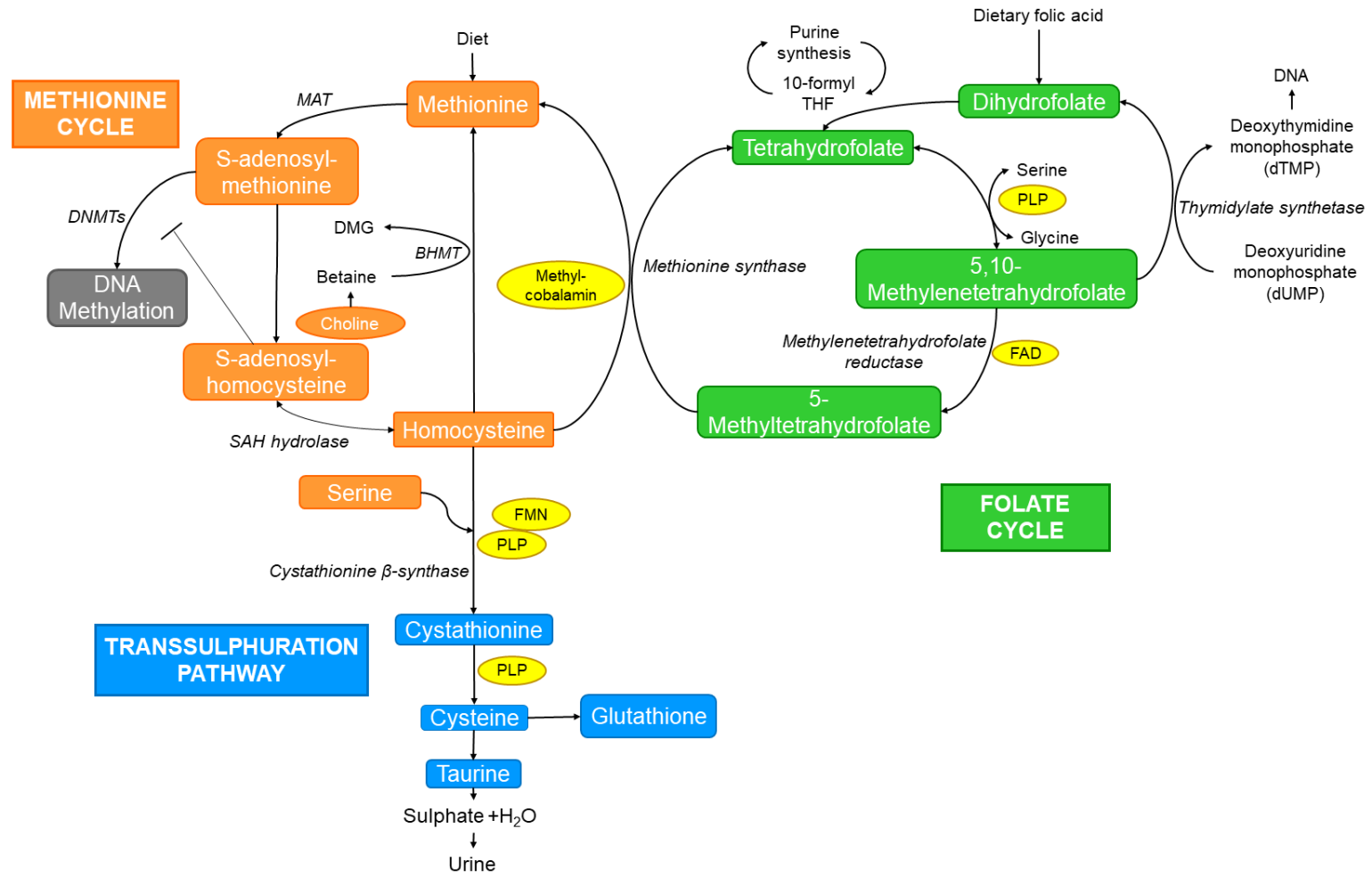


Figure 2

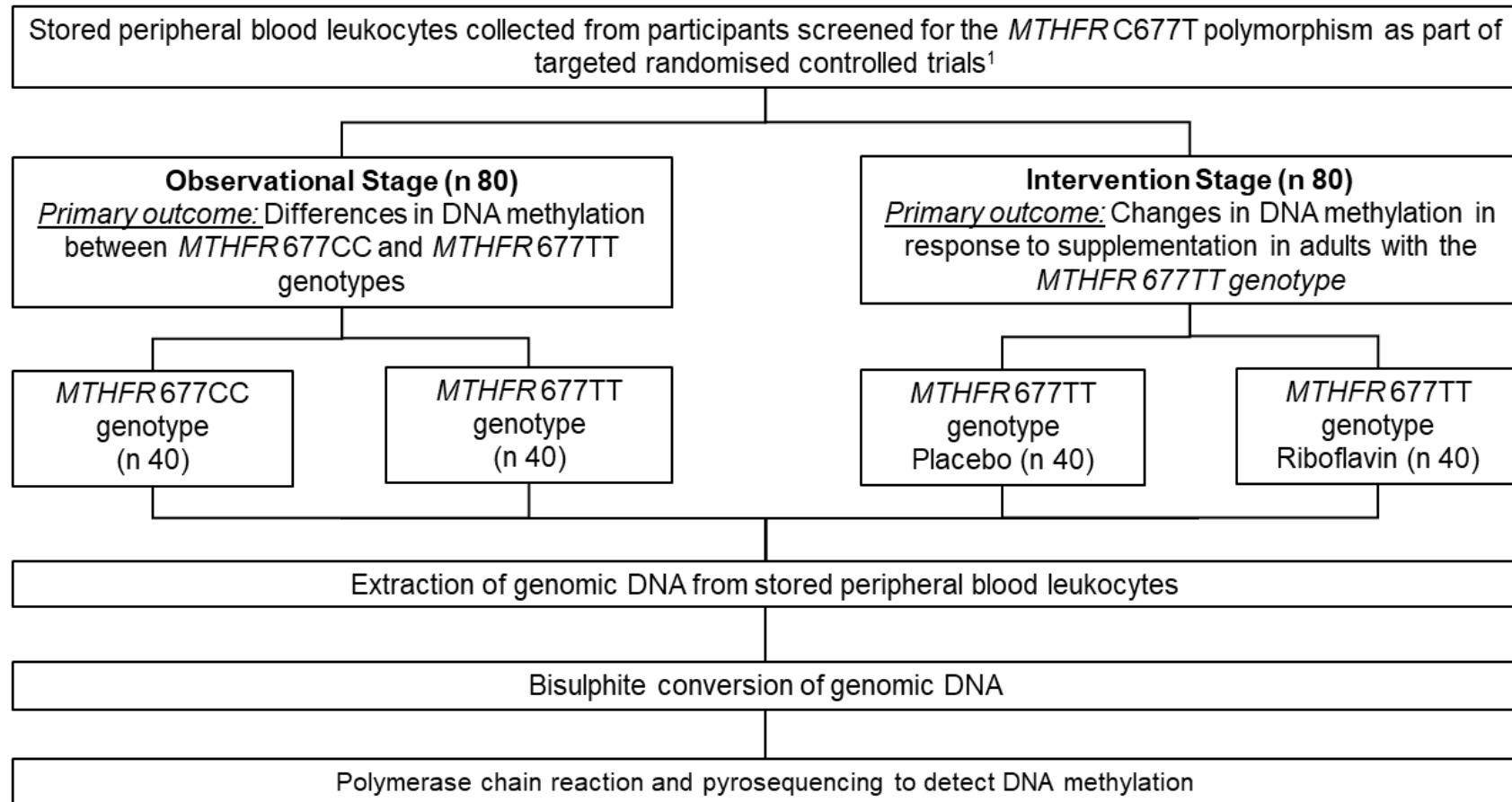
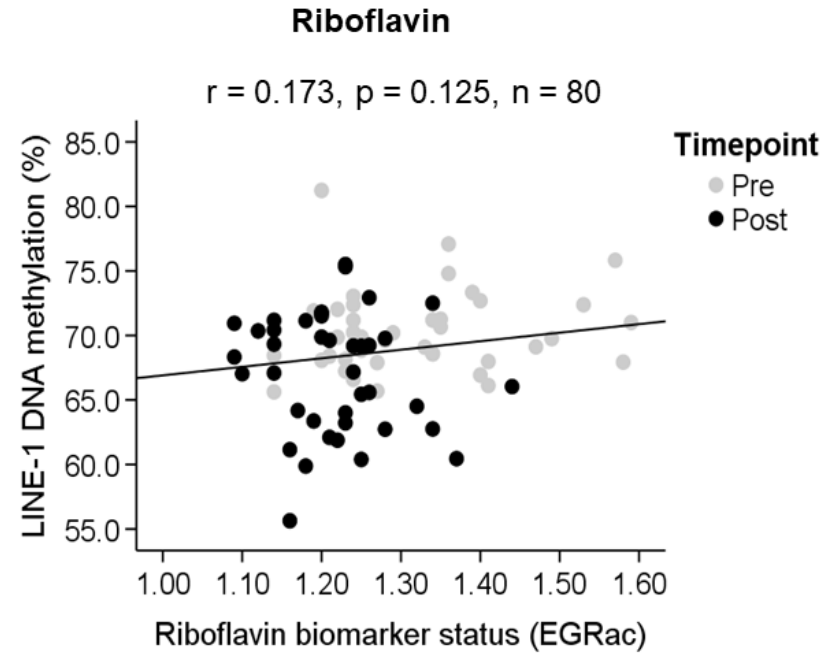
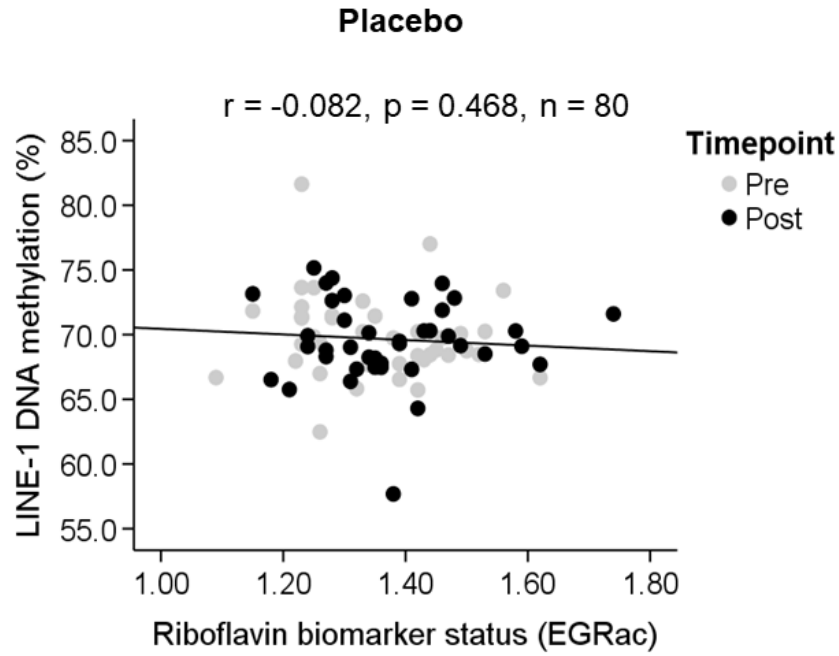


Figure 3





## Supplementary Figure 1

