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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 RIBOflavin 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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100

2.2. Study Design

Analysis for this study was carried out in two stages: in an observational stage (n = 80), DNA

methylation differences were examined between the two *MTHFR* C677T genotypes (i.e.

677CC and 677TT) and in an intervention stage (n = 80). DNA methylation was examined in

response to supplementation with either riboflavin (1.6mg/d) or placebo for 16 weeks in

individuals with the *MTHFR* 677TT genotype only. Appropriate samples of intervention with

riboflavin in CC participants, were not available for the current analysis from the only study

[28] to date to have conducted a riboflavin intervention in all three *MTHFR* 677 genotype

groups. Participants were age- and sex-matched for both the observational and intervention

stages of the study (**Table 1**). The flow diagram of the study design is illustrated in **Figure 2**.

2.3. Biomarker Status

Blood samples were analysed by standard laboratory assays for total homocysteine and

riboflavin biomarker status as reported in previous studies [28,29]. Riboflavin status was

determined using the erythrocyte glutathione reductase coefficient (EGRac), a functional

assay which measures the activity of glutathione reductase before and after in vitro reaction

with its prosthetic group flavin adenine dinucleotide (FAD). EGRac is calculated as a ratio of

FAD-stimulated to -unstimulated enzyme activity with higher values indicative of lower

riboflavin status and is recognised as the gold standard. Values of EGRac at or above 1.3 are

generally indicative of suboptimal riboflavin status [30,31].

2.4. DNA Methylation Analysis

2.4.1. ENCODE dataset analysis

The Infinium 450K Bead Array and DNA methylation data from the ENCODE consortium

available as user tracks in UCSC genome browser were utilized in this study [32]. The

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' CCCTAAAACAAAAAATCAAAAAACATCTCT 3';

166 *MTHFR* south shelf forward: 5' CCCTAAAACAAAAAATCAAAAAACATCTCT 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, 95ºC for 15

172 minutes, followed by 45 cycles of 94ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 30s

173 and a final elongation of 10 minutes at 72ºC. 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 × 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 × 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 ± 0.01) compared to placebo

258   (0.02 ± 0.01). Furthermore, there was a significant reduction in homocysteine concentrations

259   ($P = 0.001$) in the group supplemented with riboflavin (-1.79 ± 3.50 μmol/L) compared to

260   placebo (-0.42 ± 3.10 μmol/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% ± 0.91% vs. Placebo: -0.32% ± 0.69%, $P = 0.018$) which remained

266   significant following Bonferroni correction at CpG 2 (Riboflavin: -1.49% ± 0.72% vs.

267   Placebo : 1.23% ± 0.62%, $P = 0.006$). The *MTHFR* north shore was significantly

268   hypomethylated (-1.24% ± 0.50% vs. 0.90% ± 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.

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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)

	MTHFR Genotype		
	MTHFR 677 CC (n 40)	*MTHFR* 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)	**0.024**
BMI (kg/m^2)	29.5(4.8)	29.8(4.8)	0.769
B-vitamin biomarker status			
EGRac	1.34(0.17)	1.34(0.12)	0.945
Homocysteine (µmol/L)	10.6(3.5)	13.8(4.8)	**0.002**

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**
LINE-1			
CpG1	74.26(4.10)	74.39(3.84)	0.506
CpG2	65.82(4.02)	68.34(3.28)	**0.002**
CpG3	66.51(3.70)	68.59(5.06)	**0.033**
Average	68.86(2.71)	70.44(3.41)	**0.011**
***MTHFR* north shore**			
CpG1	95.63(1.98)	95.64(2.51)	0.338
***MTHFR* south shelf**			
CpG1	35.19(5.16)	40.04(3.95)	**< 0.001**
***MTHFR* CpG island**			
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
Average	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)

Response indicator	Placebo (n 40)	Riboflavin (n 40)	*P*-value*	*P*-value‡
EGRac				
Pre-intervention	1.35(0.12)	1.32(0.19)		
Post-intervention	1.37(0.13)	1.22(0.08)	**< 0.001**	**< 0.001**
Change	0.02(0.08)	-1.02(0.08)		
Homocysteine (μmol/L)				
Pre-intervention	15.8(6.6)	13.5(6.2)		
Post intervention	15.4(6.4)	11.7(3.0)	0.068	**0.001**
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 (%)						
	Placebo **(n 40)**			**Riboflavin** **(n 40)**			
	Pre-intervention	*Post-intervention*	*Change[a]*	*Pre - intervention*	*Post-intervention*	*Change[a]*	**P-value**
LINE-1							
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)	**0.006ǂ**
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
***MTHFR* north shore**							
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)**	**0.001***
***MTHFR* south shelf**							
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
***MTHFR* CpG island**							
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). ǂ*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. [a]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.

[1]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 ≥ 0.6), purple = partially methylated ($0.2 <$ beta value < 0.6), blue = fully unmethylated (beta value ≤ 0.2).
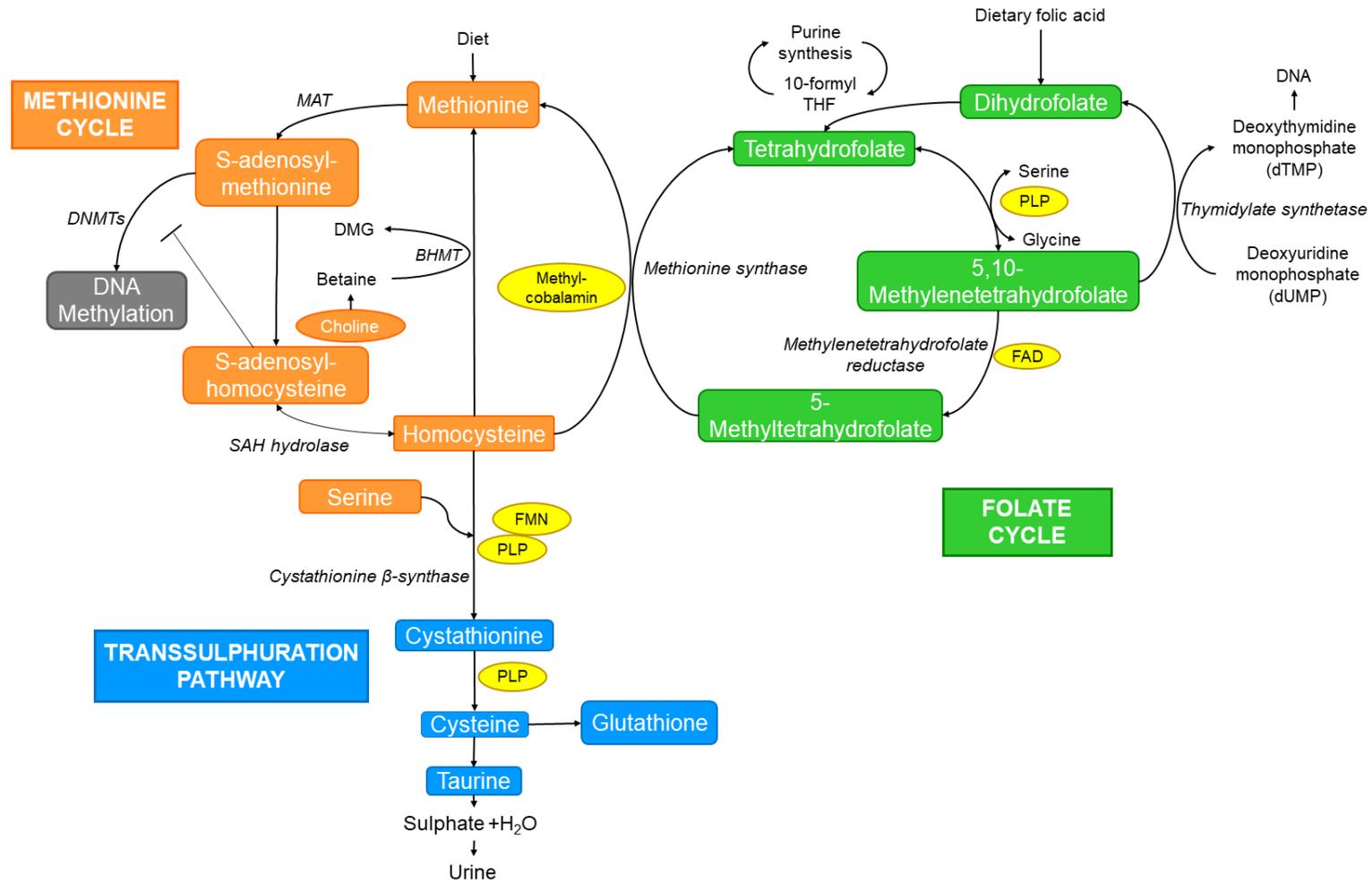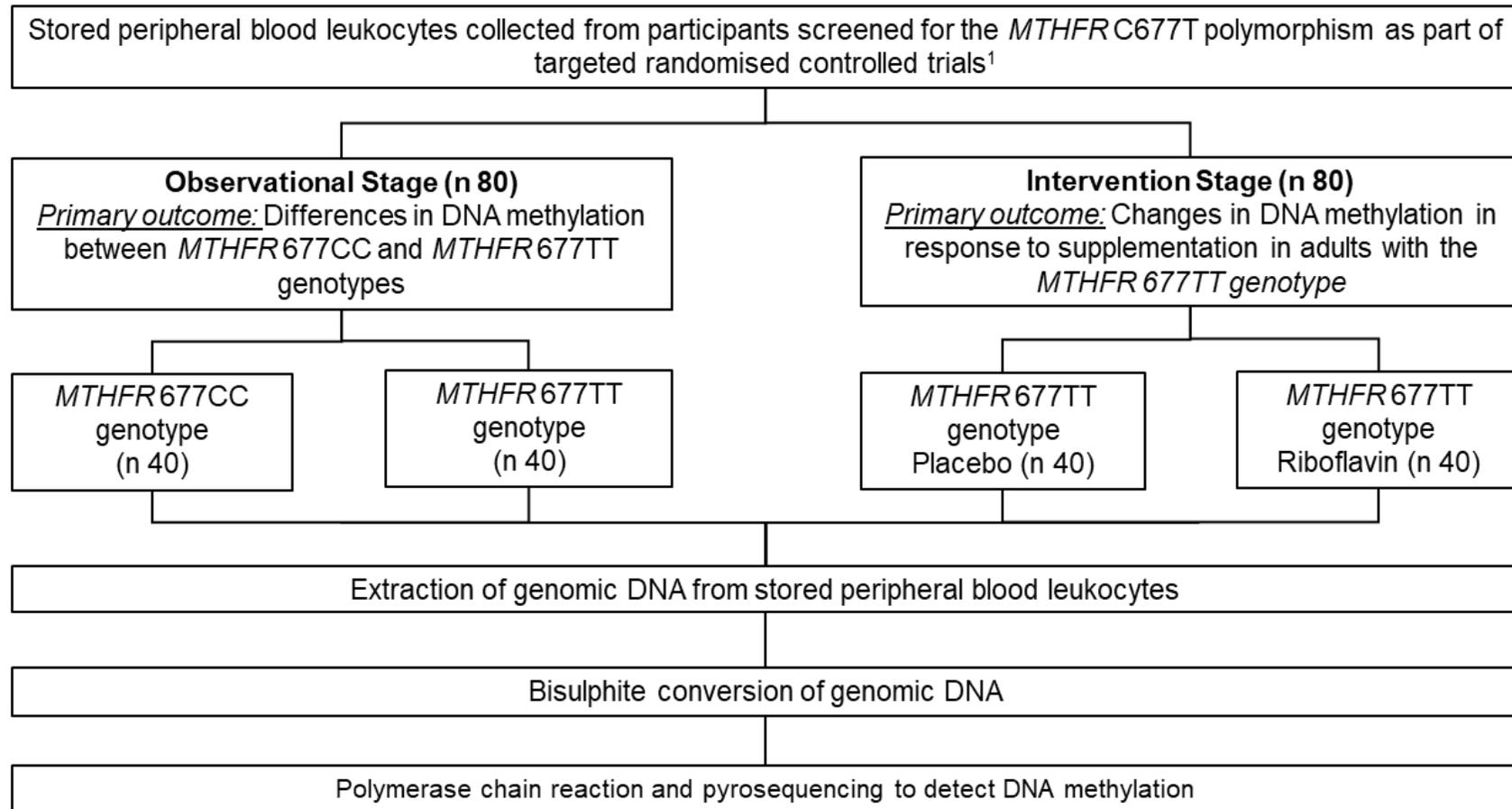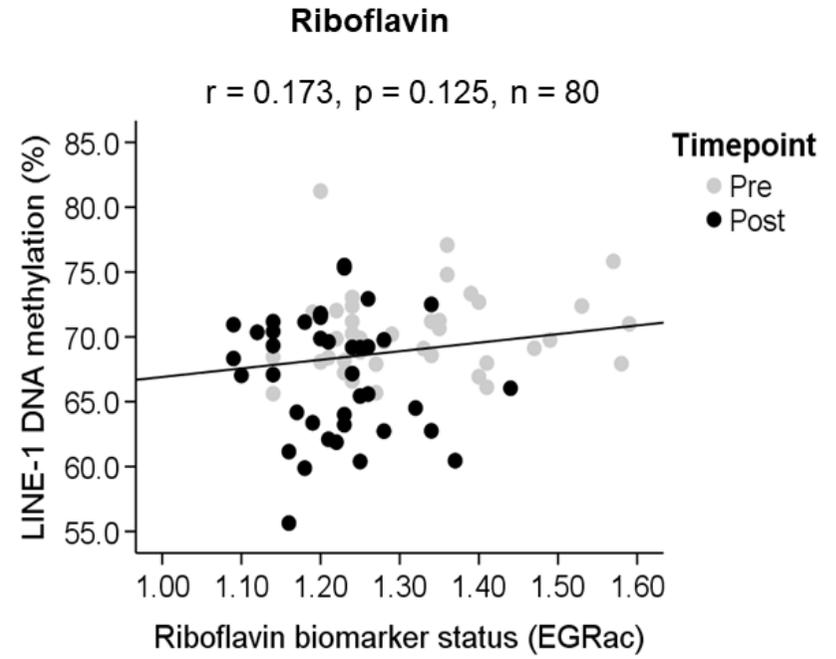
Figure 1

Figure 2

Stored peripheral blood leukocytes collected from participants screened for the *MTHFR* C677T polymorphism as part of targeted randomised controlled trials[1]

Observational Stage (n 80)
Primary outcome: Differences in DNA methylation between *MTHFR* 677CC and *MTHFR* 677TT genotypes

Intervention Stage (n 80)
Primary outcome: Changes in DNA methylation in response to supplementation in adults with the *MTHFR* 677TT genotype

MTHFR 677CC genotype (n 40)

MTHFR 677TT genotype (n 40)

MTHFR 677TT genotype Placebo (n 40)

MTHFR 677TT genotype Riboflavin (n 40)

Extraction of genomic DNA from stored peripheral blood leukocytes

Bisulphite conversion of genomic DNA

Polymerase chain reaction and pyrosequencing to detect DNA methylation

Figure 3

Placebo — r = -0.082, p = 0.468, n = 80. Riboflavin — r = 0.173, p = 0.125, n = 80. X-axis: Riboflavin biomarker status (EGRac). Y-axis: LINE-1 DNA methylation (%). Timepoint: Pre, Post.

Supplementary Figure 1