



DNA methylation of hypertension-related genes and effect of riboflavin supplementation in adults stratified by genotype for the MTHFR C677T polymorphism

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1 **DNA methylation of hypertension-related genes and effect of riboflavin**
2 **supplementation in adults stratified by genotype for the *MTHFR* C677T polymorphism**

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13 **Short running head:** DNA methylation of hypertension-related genes in adults screened for
14 the *MTHFR* C677T polymorphism

15 **Declarations of interest:** DLM, CPW, SDA, AM, CFH no conflicts of interest. MW, HN,
16 JJS hold an international patent on the use of riboflavin in the treatment of blood pressure.

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22 data; in the writing of the report; and in the decision to submit the article for publication.

23 **Abbreviations:** BP, blood pressure; CVD, cardiovascular disease; EGRac, erythrocyte
24 glutathione reductase activation coefficient; FAD, flavin adenine dinucleotide, FMN, flavin

- 25 mononucleotide; MTHFR, 5,10-methylenetetrahydrofolate reductase; RCT, randomised
- 26 controlled trial

27 **ABSTRACT**

28 **Background:** The interaction between genetic, epigenetic and environmental factors plays
29 an important role in the aetiology of hypertension. GWAS and observational studies link the
30 C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR) with hypertension,
31 while riboflavin, the MTHFR cofactor, has been shown to reduce blood pressure and global
32 DNA methylation in homozygous (TT genotype) individuals. It is currently unclear whether
33 riboflavin modulates DNA methylation of other hypertension-related genes.

34 **Objectives:** To compare DNA methylation of hypertension-related genes in adults stratified
35 by *MTHFR* genotype and effect of riboflavin intervention in adults with the variant *MTHFR*
36 677TT genotype.

37 **Methods:** Pyrosequencing was carried out for hypertension-related genes (*ACE*, *AGTRI*,
38 *GCK*, *GNAI2*, *IGF2*, *MMP9* and *NOS3*) in blood samples from participants in previous trials
39 (CC, n = 40; TT, n = 40). The effect of intervention with riboflavin (1.6mg/d for 16 weeks) or
40 placebo on DNA methylation was investigated in adults with the variant *MTHFR* 677TT
41 genotype (n=80).

42 **Results:** Individuals with the *MTHFR* 677TT v CC genotype had significantly higher average
43 DNA methylation at *NOS3* (+1.66%, $P = 0.044$). In response to riboflavin supplementation in
44 TT individuals, there was an increase in average DNA methylation at *IGF2* (+1.09%, $P =$
45 0.019) and a decrease at *ACE* (-0.44%, $P=0.021$) in females only. Specific CpG sites were
46 hypomethylated in *GNAI2* and hypermethylated in *AGTRI*.

47 **Conclusion:** This study provides the first RCT evidence that riboflavin alters DNA
48 methylation of hypertension-related genes in adults with the *MTHFR* 677TT genotype,
49 providing some insight into mechanisms linking hypertension with the genotype-specific
50 response of BP to riboflavin.

- 52 **Key words:** DNA methylation, Hypertension, *NOS3*, *AGTR1*, *IGF2*, *GNA12*, *MMP9*, *ACE*,
- 53 *MTHFR* C677T polymorphism, riboflavin, one-carbon metabolism

54

55 **1.0 INTRODUCTION**

56 Hypertension is a global health challenge and a major risk factor for cardiovascular diseases,
57 particularly stroke (1,2). Genetic variation contributes to the risk of developing high blood
58 pressure with multiple genetic factors accounting for 30-70% of blood pressure (BP)
59 variability in hypertension (3,4). It does not however account for all blood pressure
60 variability and therefore a number of additional hypotheses have been proposed, with
61 epigenetics emerging as a strong candidate (5). Evidence from both genome-wide association
62 studies (GWAS) (6,7) and epidemiological studies (8) implicates the gene encoding the
63 folate-metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR) in hypertension
64 (9). Previous randomised controlled trials (RCT) from our research group have confirmed
65 that the *MTHFR* C677T polymorphism is associated with higher blood pressure and have
66 demonstrated that the blood pressure phenotype can be lowered in individuals with the
67 variant *MTHFR* 677TT genotype by supplementation with riboflavin, the MTHFR co-factor
68 (10–12). The biological mechanisms linking this polymorphism with blood pressure, and the
69 blood pressure-lowering effect of riboflavin in affected individuals, are not well understood
70 (13), but may involve alterations in DNA methylation of specific genes involved in blood
71 pressure regulation. In support of this hypothesis, we have recently shown that global
72 methylation is higher in 677TT individuals than their CC counterparts and can be reduced by
73 riboflavin supplementation (14).

74 Evidence from the literature indicates that perturbation of DNA methylation leads to genomic
75 instability and transcriptional repression and thereby influencing disease aetiology (15,16).

76 These perturbations result from imbalances in the supply of nutrients in one-carbon
77 metabolism, the main metabolic pathway for generating methyl groups for biological
78 reactions including DNA methylation (17,18). Alterations in DNA methylation both globally

79 and at key gene loci, have also been implicated in hypertension (19). Furthermore,
80 cardiovascular disease (CVD) has been identified as an age-related condition linked to
81 epigenetic age acceleration in blood using the DNA methylation-based Phenotypic Age
82 measure (PhenoAge) (20), which also demonstrates a positive correlation between systolic
83 blood pressure and epigenetic age.

84 This study focuses on key genes implicated in hypertension including angiotensin I
85 converting enzyme (*ACE*), angiotensin receptor 1 (*AGTR1*), glucokinase (*GCK*), guanine
86 nucleotide-binding protein alpha-12 gene (*GNAI2*), insulin-like growth factor II (*IGF2*),
87 matrix metalloproteinase 9 (*MMP9*) and nitric oxide synthase 3 (*NOS3*). These genes are
88 involved in blood pressure regulation through their functions in the renin-angiotensin system,
89 smooth muscle cell regulation and endothelial function (21–23). We hypothesised that DNA
90 methylation of genes involved in hypertension-related pathways would differ by *MTHFR*
91 genotype and be modulated by riboflavin, the *MTHFR* cofactor, in individuals with the
92 variant *MTHFR* 677TT genotype. To explore this hypothesis, the aims of the current study
93 were to investigate DNA methylation of key hypertension-related genes in adults stratified by
94 *MTHFR* genotype, and to examine the effect of riboflavin supplementation on DNA
95 methylation of hypertension pathway loci specifically in individuals with the *MTHFR* 677TT
96 genotype.

97

98 **2.0 MATERIALS AND METHODS**

99 **2.1 Participants and study design**

100 Stored samples from participants pre-screened for the *MTHFR* C677T polymorphism, who
101 had previously consented and participated in targeted RCTs, investigating riboflavin as a
102 treatment for hypertension in individuals with the *MTHFR* 677TT genotype, were accessed
103 for the current investigation. Samples for the present analysis were drawn from three identical

104 cohorts namely, the Genetic and Vitamin follow up study (Genovit-FCBMA-15-070), the
105 Genetic and Vitamin ten year follow up study (GENOVIT10 -UUREC/12/0338) and the
106 optimisation of RIBOf flavin Status in Hypertensive Adults with a Genetic predisposition to
107 Elevated Blood pressure study (RIBOGENE - ORECNI/12/0136). Sampling from these three
108 trials facilitated access to the required number of age- and sex-matched samples from both
109 placebo and treatment groups. All studies were conducted at the Nutrition Innovation Centre
110 for food and Health (NICHE). Lifestyle data, blood pressure, anthropometry and blood
111 samples were collected as part of all three studies using identical standard operating
112 procedures. Riboflavin status, measured by the functional biomarker, erythrocyte glutathione
113 reductase activity coefficient (EGRac) was examined in all the samples (10,11). The EGRac
114 assay is a functional assay which measures the activity of the enzyme glutathione reductase in
115 washed red cells before and after in vitro reactivation with its prosthetic group FAD. EGRac
116 is calculated as a ratio of FAD-stimulated to unstimulated enzyme activity, with values <1.3
117 generally indicative of optimal riboflavin status was conducted using identical standard
118 operating procedures. Furthermore, each study utilised the same inclusion and exclusion
119 criteria. Participants were excluded if they had a history of gastrointestinal, hepatic, renal, or
120 haematological disorders, or were taking B-vitamin supplements, anticonvulsant therapy, or
121 any other drugs known to interfere with folate or B-vitamin metabolism (10–12). Ethical
122 approval was granted for each of the studies and was conducted in accordance with the
123 Declaration of Helsinki. All the participants provided informed consent. Additional ethical
124 approval was granted by University of Ulster Research Ethics Committee Northern Ireland
125 for the analysis reported in this current study.

126

127

128

129 2.2 Study design

130 DNA methylation analysis for this study was carried out in two phases: in an observational
131 phase, differences in gene-specific methylation were compared between the two *MTHFR*
132 C677T genotypes (i.e. CC, n = 40 versus TT, n = 40). In the intervention phase, changes in
133 gene-specific DNA methylation were examined in participants with the TT genotype only
134 (placebo, n = 40; riboflavin, n = 40) in response to intervention with riboflavin (1.6mg/d) or
135 placebo for 16weeks. The flow diagram of the study design is shown in **Supplementary**
136 **Figure 1**. Sample size calculations for the present analysis was carried out using the G Power
137 3.1.9.4 statistical power calculator (version 3) (24). Based on power calculations using data
138 from Bollati *et al* (25), it was estimated that 39 participants per group would be able to
139 discriminate differences of 3.4% in DNA methylation with a power of 80%, at $\alpha = 0.05$ and
140 effect size of 0.65.

141

142 2.3 DNA Methylation Analysis

143 2.3.1 Selection of candidate genes for DNA methylation analysis

144 A candidate gene approach focusing on hypertension pathway loci was used to select a set of
145 genes directly involved in blood pressure regulation or shown to be associated with
146 hypertension in the literature (**Supplementary Table 1**).

147 2.3.2 Genomic DNA extraction

148 For the current analysis, genomic DNA was extracted from 200 μ l of stored peripheral blood
149 leukocyte samples using the Qiagen QIAamp DNA blood mini kit (Qiagen, UK) according to
150 the manufacturer's protocol (26). Genomic DNA samples were electrophoresed on a 1%
151 (w/v) agarose gel to examine quality. The purity of the samples was evaluated, and
152 concentrations quantified using the NanodropND1000 spectrophotometer (Labtech
153 International, Ringmer, UK).

154 2.3.3 Bisulphite Conversion of Genomic DNA

155 500ng of genomic DNA was subsequently bisulphite converted using the EZ DNA
156 methylation kit (Zymo Research Corporation, California) according to manufacturer's
157 protocol (27) using the EZ DNA methylation kit.

158 2.3.4 Pyrosequencing

159 Commercially available predesigned methylation assays from Qiagen UK were used for
160 bisulphite PCR and pyrosequencing for the following loci: *ACE* (PM00181398), *AGTRI*
161 (PM00014875), *GNAI2* (PM00127925), *MMP9* (PM00079191) and *NOS3* (PM00129220)
162 while assays for *IGF2* and *GCK* were based on previously published primer sets from
163 previous studies which have examined these specific regions (28–30). Details of the assays,
164 chromosomal location and number of CpGs examined are provided in **Supplementary Table**
165 **2**. After bisulphite conversion, DNA amplicons were amplified by PCR using the PyroMark
166 PCR kit (Qiagen, UK) according to manufacturer's protocol. Each 25µl PCR reaction mix
167 consisted of 12.5µl master mix, 2.5µl coral load, 5.5µl nuclease-free water, 2.5µl each of
168 10µM primer set and 2µl each of bisulphite converted DNA. PCR was then carried out under
169 the following conditions: hot start of 95°C for 15 minutes, followed by 45 cycles of 94°C for
170 30 seconds, 56°C for 30 seconds, 72°C for 30s and a final elongation of 10 minutes at 72°C.
171 The PCR products were subsequently electrophoresed on a 1% (w/v) agarose gel to check the
172 integrity of PCR products. DNA methylation in samples was analysed using the PyroMark
173 Q24 pyrosequencing instrument (Qiagen, UK). Enzymes, substrates and nucleotides from the
174 PyroMark Gold Q24 kit (Qiagen UK) were used. Levels of methylation at each CpG site
175 were analysed using the PyroMark Q24 software (31). As an additional control, bisulphite
176 DNA controls from EpiTect PCR Control DNA (Qiagen, UK) that contained fully methylated
177 as well as fully unmethylated DNA was included in the analysis to ensure that the instrument
178 detected the full range of methylation values.

179 **2.4 Statistical analyses**

180 Statistical Package for the Social Sciences (SPSS) IBM Statistics (version 25, SPSS UK Ltd
181 Chertsey, UK) was used to statistically analyse the data obtained from the pyrosequencing
182 analysis. QQ-plots and the Kolmogorov-Smirnov test were used to verify the normality of
183 continuous variables. Chi-square tests were used for analysing baseline categorical data while
184 continuous variables were analysed using independent t-tests. Baseline differences in gene-
185 specific methylation between the two *MTHFR* C677T genotypes were analysed using one-
186 way analysis of covariance (ANCOVA) adjusting for age, sex, smoking status and study
187 cohort. The treatment effect of supplementation with riboflavin or placebo over time on
188 riboflavin biomarker status, blood pressure and gene-specific methylation in participants with
189 the *MTHFR* 677TT genotype only was analysed using mixed between-within analysis of
190 variance adjusting for age, sex, smoking status and study cohort as covariates. The between-
191 patient factor was the intervention group (placebo versus riboflavin), and the within-patient
192 factor was time (pre and post-intervention). Furthermore, multiple linear regression adjusting
193 for covariates was carried out to identify the determinants of gene-specific methylation. All
194 statistical tests were carried out at the 95% confidence interval and in all analyses p-values
195 less than 0.05 were considered statistically significant.

196

197

198 **3.0 RESULTS**

199 **3.1 Baseline characteristics of participants**

200 Age- and sex-matched participant samples were accessed for the observational (n = 80) and
201 intervention (n = 80) phases of this study. Demographic characteristics showed that the
202 average age of participants was 57 years and that baseline characteristic including age, sex,
203 BMI and smoking status were not different between *MTHFR* 677CC and TT individuals

204 **(Table 1)**. In the observational stage of the study, as expected, individuals with the TT
205 genotype had significantly higher systolic (+11.1 mmHg; $P = 0.005$) and diastolic blood
206 pressure (+5.1 mmHg; $P = 0.022$) compared to participants with CC genotype (10,11).
207 In individuals with the *MTHFR* 677TT genotype, who were supplemented with riboflavin,
208 biomarker status (EGRac), systolic and diastolic blood pressure were not significantly
209 different between treatment groups, prior to intervention. Following intervention, riboflavin
210 biomarker status improved as expected (indicated by a significant decrease (-0.10 ± 0.01 , $P <$
211 0.001) in EGRac) compared to no change in the placebo group. Furthermore, consistent with
212 previous studies which contributed these convenience samples (10,11), supplementation with
213 riboflavin resulted in significant decreases in systolic (-7.9 mmHg; $P < 0.001$) and diastolic ($-$
214 3.8 mmHg; $P < 0.019$) blood pressure in adults with the *MTHFR* 677TT in this combined
215 cohort (results not shown).

216 **3.2 Differences in DNA methylation in individuals stratified by *MTHFR* C677T** 217 **genotype**

218 DNA methylation analysis of the candidate hypertension-related genes indicate an overall
219 trend toward hypermethylation at several loci including *ACE*, *AGTR1*, *GCK*, *MMP9* and
220 *NOS3* in individuals with the *MTHFR* 677TT genotype compared to the CC genotype (**Table**
221 **2**). Average DNA methylation levels were significantly higher at *NOS3* (1.66%, $P = 0.044$) in
222 the TT genotype compared to individuals with the CC genotype after adjusting for age, sex,
223 smoking status and study cohort. Significant CpG site-specific differences were observed at
224 CpG2 of *AGTR1* and CpG1 of *GNAI2*. Examination of sex-specific differences in
225 methylation between the *MTHFR* genotypes showed that methylation differences observed at
226 *NOS3* was marginally significant in females but not in males (**Table 2**). Multiple linear
227 regression adjusting for covariates was used to identify the determinants of gene-specific
228 methylation in adults with the *MTHFR* C677T polymorphism (CC and TT genotypes) at

229 baseline in the observational stage of this analysis (**Table 3**). *MTHFR* genotype was
230 significantly associated with methylation at *NOS3* ($\beta = 0.256$, $P = 0.031$, $R^2 = 0.102$) and
231 *AGTR1* ($\beta = 0.264$, $P = 0.026$, $R^2 = 0.096$), while methylation at *GCK* loci was significantly
232 associated with age ($\beta = 0.321$, $P = 0.004$, $R^2 = 0.161$) and sex ($\beta = 0.224$, $P = 0.047$, $R^2 =$
233 0.161). No significant associations with baseline determinants were demonstrated for other
234 locations.

235 **3.3 Effect of riboflavin supplementation on gene-specific methylation in adults with the** 236 ***MTHFR* 677TT genotype**

237 Supplementation with riboflavin, resulted in increased overall methylation at *IGF2* (+1.08%,
238 $P = 0.019$) compared with placebo. Increased methylation was observed at CpG1 of *AGTR1*,
239 however, methylation decreased at CpG2 and CpG4 of *GNAI2* in TT participants receiving
240 riboflavin compared to placebo. Stratification of the analysis by sex, indicated increased
241 methylation in response to riboflavin supplementation at *IGF2* (+1.44%; $P = 0.017$)
242 compared with placebo in males but not females. However, decreased methylation was
243 observed at *ACE* (-0.44%; $P = 0.021$) in females but not males (**Table 4**). Multiple linear
244 regression analysis, focused specifically on individuals with the *MTHFR* 677TT genotype in
245 the intervention stage of the study, showed that riboflavin treatment was a determinant of
246 *IGF2* methylation ($\beta = 0.265$, $P = 0.021$, $R^2 = 0.106$). No other genes showed any significant
247 interaction with any of the baseline determinants.

248

249

250 **4.0 DISCUSSION**

251 This study is the first to show that DNA methylation is altered by intervention with riboflavin
252 at a number of important candidate genes related to hypertension in adults with the *MTHFR*

253 677TT genotype using samples from previously conducted RCTs. The results show that
254 riboflavin supplementation compared with placebo resulted in significant increases in average
255 *IGF2* methylation and CpG site-specific alterations in methylation at *AGTR1* and *GNA12* loci
256 in adults with the TT genotype. Additionally, at baseline, significantly higher methylation in
257 TT compared to CC individuals at *NOS3* was observed with significant sex differences
258 appearing to indicate that this difference is driven by females.

259 Riboflavin supplementation compared with placebo in individuals with the *MTHFR* 677TT
260 genotype, showed increased average methylation at *IGF2*, which was also demonstrated in
261 the linear regression model which showed riboflavin treatment as the sole determinant of
262 methylation of *IGF2*. Although no other study, to our knowledge, has investigated the role of
263 riboflavin in modulating DNA methylation at *IGF2*, studies investigating the epigenetic
264 effects of other B-vitamins, mainly folic acid and vitamin-B12, in various populations report
265 significant increases in *IGF2* methylation in response to supplementation (28,32) supporting
266 the findings of this study. *IGF2* is a paternally expressed imprinted gene with well-
267 established physiological roles in growth and development. Polymorphisms of *IGF2* have
268 been related to vascular risk factors and hypertension (33,34). Furthermore, *IGF2* functions
269 as part of the insulin-like growth factor (IGF) system which plays complex roles in nutrient-
270 sensitive pathways and may indirectly influence blood pressure through the regulation of
271 cardiac muscles (35). Alterations in methylation could therefore potentially impact *IGF2*
272 expression with implications for blood pressure regulation. Although significant, the changes
273 in methylation observed at *IGF2* are very small, however, the magnitude of change is in
274 agreement with previous studies showing that small changes in methylation can result in
275 transcriptional alterations including at imprinted genes (36). Further functional studies are
276 required to investigate the implications of our findings on gene expression. Apart from
277 overall changes in average methylation, we observed significant decreases at specific CpG

278 sites within the *GNAI2* loci. Similarly, in an RCT investigating supplementation of folic acid
279 and vitamin B-12 on genome-wide methylation, differential methylation was observed at the
280 *GNAI2* locus, with methylation shown to decrease in response to supplementation with folic
281 acid and vitamin B12 in comparison to placebo in adults (37).

282 While associations between polymorphisms in the *NOS3* gene and cardiovascular disease
283 have been widely studied, methylation at *NOS3* in individuals with the *MTHFR* C677T has
284 not been extensively investigated. It is widely accepted that CpG islands at promoters of
285 housekeeping genes are usually unmethylated allowing transcription. Hypermethylation at the
286 *NOS3* loci as observed in individuals with the *MTHFR* 677TT genotype has the potential to
287 inhibit the expression of this gene and thereby influencing its function in regulating blood
288 pressure. *NOS3* is a key regulator of vasotone, platelet aggregation and blood pressure
289 (21,22,38). Furthermore, mendelian randomisation studies in stroke patients indicate that
290 genetic variation in the nitric oxide synthase pathway affects stroke risk via variations in
291 blood pressure (39). Surprisingly, there were no changes in methylation at the CpGs analysed
292 at the *NOS3* locus in this study in response to riboflavin supplementation. It is, however,
293 likely that other CpGs at this locus were altered by intervention with riboflavin, indicated by
294 preliminary data from an ongoing epigenome-wide study. This further highlights the
295 limitation of the candidate gene approach of this study. Furthermore, it also suggests that
296 other mechanisms in addition to methylation may be modulating the effect of riboflavin on
297 blood pressure in adults with the *MTHFR* 677TT genotype. For, example it has been
298 postulated that endothelial nitric oxide synthase (eNOS) may provide a link between *MTHFR*
299 677TT genotype and blood pressure (40).

300 Consistent with findings of the present study, several studies have reported sex- and age-
301 specific differences in methylation at several gene loci (41,42). These sex-specific differences
302 could be owing to different mechanisms and pathogenic processes involved in blood pressure

303 regulation by these genes in males and females. These findings are in general agreement with
304 studies investigating blood pressure which also showed that metabolic and haemodynamic
305 abnormalities associated with hypertension differed markedly between sexes (43). For
306 example, while a cardiac phenotype was associated with elevated blood pressure and
307 hypertension in males, a vascular phenotype characterised by elevated peripheral vascular
308 resistance was more prominent in females (43). Furthermore, similar to findings from Xu and
309 colleagues (30) who reported significant correlations between *GCK* gene body methylation
310 and aging, multiple linear regression in the present study identified age and sex as
311 determinants of methylation at the *GCK* locus although no significant differences were
312 observed between *MTHFR* genotypes. Changes in methylation have been shown to correlate
313 with age providing a biological marker for ageing (44) and these sites could play important
314 roles in disease such as hypertension. It must be noted that although overall changes may not
315 be seen across all CpGs within a gene, site-specific alterations may still occur, and these site-
316 specific alterations indicate biologically relevant heterogeneity in DNA methylation and are
317 still relevant in the aetiology of disease (45). Additionally, methylation of a particular CpG
318 position may have a strong influence on transcriptional suppression or expression while
319 methylation at other CpG sites may have little influence (46). Surprisingly, methylation at the
320 *ACE* locus was reduced in response to riboflavin supplementation, which may be expected to
321 increase gene expression and thereby increase blood pressure, however the effects on
322 transcription were not analysed in this study and the effect on blood pressure may involve a
323 complex interplay with other genes and warrants further investigation. Although the present
324 study demonstrates significant methylation differences of hypertension pathway genes
325 following supplementation with riboflavin in *MTHFR* 677TT individuals, further
326 investigations are required to better understand the interconnections and interactions between
327 these genes and the resulting effects on blood pressure.

328 The main strength of this study is that it draws on samples from randomised controlled trials,
329 providing a rigorous tool to examine the effects of riboflavin supplementation on DNA
330 methylation and enabled us to examine specific changes at CpG sites of interest.
331 Additionally, our investigation used a robust biomarker, EGRac, to evaluate riboflavin status
332 in participants, which is rarely reported due to lack of accessible laboratory methods and
333 labour-intensive pre-analysis sample preparation; our laboratory is one of the few worldwide
334 to routinely measure EGRac. Biomarker status offers many advantages over estimated dietary
335 intake which is widely reported to be inherently flawed (47). Furthermore, we adjusted for
336 several variables in the statistical analysis of the methylation data ensuring that the findings
337 were not masked by confounding factors. A limitation, however, is that although the
338 candidate gene approach employed investigated specific hypertension-related genes, further
339 relevant genes and CpG sites may have been omitted (48) and could potentially be influenced
340 by riboflavin supplementation. Despite the limitations of the candidate approach this study
341 has yielded some interesting results to direct further studies. Further as DNA methylation was
342 examined in blood we cannot exclude the possibility that methylation patterns identified may
343 represent an overall effect contributed from the different cell fractions.

344

345 **5.0 Conclusion**

346 The findings of this study demonstrate that supplementation with riboflavin (the MTHFR co-
347 factor) in adults with the *MTHFR* 677TT genotype modulates DNA methylation at key
348 hypertension-related genes including *IGF2* and *GNAI2*. Furthermore, we observed
349 significant differences in DNA methylation at *NOS3* and *GNAI2* between individuals with
350 CC and TT genotypes for this polymorphism. The results from this study provide some
351 preliminary data to indicate that methylation of hypertension related genes may be implicated

352 in the mechanism linking MTHFR with blood pressure however further investigations are
353 required to understand the complex mechanism. Furthermore, this study highlights the
354 interaction between genetic, epigenetic and environmental factors which could play a
355 potential role in the prediction of vascular events and in the development of therapeutic
356 options for the treatment of high blood pressure. Replication of our findings in larger
357 independent cohorts using a genome-wide approach is required to understand the complex
358 mechanisms linking this common polymorphism with higher blood pressure and the DNA
359 methylation response to riboflavin intervention in individuals with the variant *MTHFR*
360 677TT genotype.

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

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

369

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Table 1: General characteristics of participants for observational study grouped according to *MTHFR* C677T genotype (n 80)

	<i>MTHFR</i> C677T Genotype		
	<i>MTHFR</i> 677CC (n 40)	<i>MTHFR</i> 677TT (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
Blood pressure (mmHg)			
Systolic BP	132.4(18.3)	143.5(16.0)	0.005
Diastolic BP	78.3(9.5)	83.4(9.9)	0.022
Riboflavin biomarker status (EGRac)	1.34(0.17)	1.34(0.12)	0.945

Data expressed as mean (SD) for continuous variables and frequency (%) for categorical variables. $P < 0.05$ considered statistically significant. Categorical variables analysed using chi square statistics, independent t-test used for analysing continuous data, Hypertensive status (baseline) defined as blood pressure readings (systolic/diastolic) 140 mmHg and or 90 mmHg or greater.

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

Table 2: Baseline DNA methylation in hypertension-related genes stratified by *MTHFR* C677T genotype (n 80)

DNA methylation (%)				
	Genomic location	<i>MTHFR</i> 677CC (n 40)	<i>MTHFR</i> 677TT (n 40)	P-value
<i>ACE</i>				
	Promoter			
CpG1		1.23(0.07)	1.61(0.23)	0.180
CpG2		1.18(0.05)	1.42(0.15)	0.276
CpG3		1.17(0.06)	1.07(0.12)	0.518
Average		1.19(0.04)	1.37(0.14)	0.351
<i>Male</i>		1.21(0.06)	1.41(0.20)	0.607
<i>Female</i>		1.17(0.06)	1.31(0.17)	0.311
<i>AGTRI</i>				
	Promoter			
CpG1		1.23(0.09)	3.45(0.09)	0.572
CpG2		3.45(0.11)	4.28(0.29)	0.048
CpG3		3.73(0.11)	3.99(0.22)	0.463
Average		2.80(0.08)	3.20(0.16)	0.102
<i>Male</i>		2.87(0.11)	3.27(0.25)	0.214
<i>Female</i>		2.72(0.13)	3.10(0.10)	0.327
<i>GCK</i>				
	Gene body			
CpG1		46.21(1.16)	46.45(1.02)	0.398
CpG2		40.49(1.42)	38.37(1.23)	0.439
CpG3		52.41(1.30)	52.83(1.31)	0.577
CpG4		41.68(1.30)	43.20(1.12)	0.309
Average		45.20(1.08)	45.22(1.00)	0.653
<i>Male</i>		43.59(1.12)	44.47(1.24)	0.387
<i>Female</i>		47.16(1.91)	46.33(1.69)	0.642
<i>GNAI2</i>				
	Promoter			
CpG1		0.26(0.04)	0.42(0.04)	0.006
CpG2		0.67(0.05)	0.72(0.05)	0.651
CpG3		1.02(0.09)	1.06(0.14)	0.901
CpG4		0.43(0.04)	0.53(0.05)	0.125
CpG5		0.35(0.03)	0.49(0.07)	0.118
CpG6		0.73(0.05)	0.78(0.09)	0.701
CpG7		1.03(0.06)	1.13(0.20)	0.487
Average		0.64(0.05)	0.73(0.07)	0.366
<i>Male</i>		0.64(0.07)	0.75(0.11)	0.369
<i>Female</i>		0.65(0.05)	0.71(0.07)	0.869
<i>IGF2</i>				
	DMR2			
CpG1		38.11(1.07)	36.02(1.47)	0.287
CpG2		37.11(0.74)	37.12(1.12)	0.708

CpG3		46.85(1.13)	46.24(0.91)	0.685
CpG4		43.58(1.01)	43.66(1.05)	0.831
CpG5		57.11(1.31)	60.89(1.68)	0.116
CpG6		42.82(1.07)	42.38(0.82)	0.881
CpG7		48.02(1.24)	48.93(0.87)	0.634
Average		44.80(0.97)	45.03(0.81)	0.909
<i>Male</i>		45.77(1.39)	44.84(1.15)	0.762
<i>Female</i>		43.61(1.33)	45.33(1.12)	0.606
<i>MMP9</i>	Promoter			
CpG1		5.98(0.36)	6.00(0.29)	0.895
CpG2		4.66(0.27)	4.98(0.27)	0.416
CpG3		2.08(0.14)	2.07(0.15)	0.949
CpG4		3.01(0.12)	3.36(0.47)	0.720
Average		3.94(0.20)	4.11(0.25)	0.753
<i>Male</i>		4.14(0.30)	4.11(0.37)	0.869
<i>Female</i>		3.68(0.24)	4.10(0.31)	0.454
<i>NOS3</i>	Promoter			
CpG1		11.74(0.65)	13.46(0.73)	0.248
CpG2		6.15(0.30)	8.50(0.53)	0.002
CpG3		3.80(0.28)	5.05(0.43)	0.051
CpG4		4.22(0.36)	5.56(0.52)	0.123
Average		6.48(0.36)	8.14(0.52)	0.044
<i>Male</i>		6.87(0.54)	7.75(0.50)	0.356
<i>Female</i>		6.00(0.44)	8.74(1.05)	0.052

The data are expressed as mean (SEM) with $P < 0.05$ considered statistically significant. Data was analysed using one-way ANCOVA adjusting for covariates: age, sex, smoking status and study cohort. *ACE*, Angiotensin I-converting enzyme; *AGTRI*, Angiotensin receptor 1; *GCK*, Glucokinase; *GNAI2*, Guanine nucleotide-binding protein, alpha-12; *IGF2*, Insulin-like growth factor II; *MMP9*, Matrix metalloproteinase 9; *NOS3*, Nitric oxide synthase 3.

Table 3: Determinants of baseline gene-specific methylation in adults stratified by the *MTHFR* C677T genotype (CC, n = 40; TT, n = 40)

	Gene-specific DNA methylation					
	<i>AGTR1</i>		<i>GCK</i>		<i>NOS3</i>	
	β	<i>P</i> -value	β	<i>P</i> -value	β	<i>P</i> -value
<i>MTHFR</i> C677T genotype	0.264	0.026	0.076	0.503	0.256	0.031
Age	0.009	0.936	0.321	0.004	-0.047	0.678
Sex	-0.070	0.546	0.224	0.047	-0.006	0.957
Smoker	-0.047	0.676	0.117	0.282	0.103	0.359
Hypertensive status	-0.094	0.432	-0.088	0.447	0.088	0.458
BMI	0.155	0.191	0.006	0.958	0.073	0.538

Multiple linear regression analysis was conducted with gene-specific DNA methylation as dependent variable. $P < 0.05$ was considered as statistically significant. Regression was performed for each gene with adjustment for significant covariates as appropriate. All genes were investigated; those showing significant relations are shown. BMI, body mass index

Table 4: DNA methylation in hypertension-related genes response to intervention with riboflavin in *MTHFR* 677TT genotype individuals (n 80)

DNA methylation (%)					
	Placebo (n 40)		Riboflavin (n 40)		P-value
	<i>Pre- intervention</i>	<i>Post- intervention</i>	<i>Pre - intervention</i>	<i>Post- intervention</i>	
<i>ACE</i>					
CpG1	1.35(0.08)	1.34(0.13)	1.75(0.24)	1.29(0.13)	0.109
CpG2	1.30(0.09)	1.43(0.12)	1.41(0.16)	1.18(0.10)	0.109
CpG3	1.21(0.10)	1.18(0.14)	1.06(0.16)	0.92(0.09)	0.723
Average	1.29(0.07)	1.32(0.12)	1.40(0.14)	1.13(0.08)	0.155
<i>Male</i>	1.36(0.11)	1.11(0.07)	1.37(0.20)	1.21(0.12)	0.705
<i>Female</i>	1.19(0.09)	1.64(0.27)	1.45(0.18)	1.01(0.09)	0.021
<i>AGTRI</i>					
CpG1	1.26(0.08)	1.32(0.08)	1.25(0.08)	1.76(0.19)	0.045
CpG2	4.10(0.22)	3.42(0.08)	3.81(0.22)	3.58(0.23)	0.268
CpG3	3.94(0.15)	3.95(0.11)	4.03(0.19)	4.21(0.28)	0.649
Average	3.10(0.09)	2.90(0.07)	3.03(0.15)	3.19(0.22)	0.231
<i>Male</i>	3.16(0.14)	2.92(0.08)	3.11(0.24)	3.25(0.32)	0.542
<i>Female</i>	3.01(0.10)	2.86(0.12)	2.89(0.08)	3.08(0.28)	0.360
<i>GCK</i>					
CpG1	46.80(1.08)	46.62(1.20)	45.57(0.81)	45.19(0.68)	0.701
CpG2	38.45(1.37)	38.30(1.41)	38.37(0.99)	38.03(0.88)	0.833
CpG3	54.21(1.39)	54.22(1.32)	52.39(1.16)	52.71(1.10)	0.677
CpG4	42.75(0.97)	42.95(1.02)	42.82(0.10)	42.23(0.64)	0.518
Average	45.55(1.03)	45.52(1.10)	44.79(0.80)	44.54(0.69)	0.749
<i>Male</i>	44.78(1.12)	44.49(1.08)	44.49(1.07)	44.04(0.73)	0.815
<i>Female</i>	46.71(1.98)	47.08(2.22)	45.71(1.24)	45.38(1.40)	0.995
<i>GNAI2</i>					
CpG1	0.44(0.04)	0.49(0.03)	0.38(0.04)	0.37(0.03)	0.348
CpG2	0.67(0.03)	0.77(0.29)	0.72(0.06)	0.63(0.04)	0.025
CpG3	0.96(0.08)	0.98(0.03)	1.06(0.14)	0.89(0.11)	0.360
CpG4	0.50(0.03)	0.68(0.06)	0.52(0.05)	0.43(0.04)	0.001
CpG5	0.47(0.05)	0.48(0.03)	0.45(0.06)	0.39(0.05)	0.463
CpG6	0.74(0.06)	0.71(0.04)	0.75(0.08)	0.65(0.07)	0.535
CpG7	1.06(0.05)	1.14(0.07)	1.09(0.09)	1.04(0.09)	0.372
Average	0.69(0.04)	0.75(0.04)	0.71(0.07)	0.63(0.06)	0.180
<i>Male</i>	0.69(0.05)	0.71(0.04)	0.77(0.11)	0.58(0.03)	0.129
<i>Female</i>	0.69(0.06)	0.81(0.07)	0.60(0.04)	0.71(0.15)	0.791

<i>IGF2</i>					
CpG1	38.19(1.33)	37.04(1.33)	35.66(1.33)	40.92(0.73)	< 0.001
CpG2	37.04(0.72)	37.97(0.66)	37.55(1.09)	38.29(0.94)	0.819
CpG3	46.52(0.88)	45.41(1.04)	46.79(0.84)	46.87(0.82)	0.302
CpG4	44.16(0.92)	43.39(1.05)	44.04(1.00)	45.71(0.82)	0.033
CpG5	59.13(1.54)	56.86(2.46)	59.22(1.39)	59.80(1.23)	0.237
CpG6	43.84(0.87)	41.81(1.11)	42.57(0.90)	42.14(1.10)	0.288
CpG7	49.78(0.77)	48.06(0.90)	48.42(0.98)	48.10(0.89)	0.216
Average	45.52(0.74)	44.36(0.91)	44.89(0.86)	45.98(0.70)	0.019
<i>Male</i>	45.63(0.87)	43.81(1.08)	44.86(1.23)	46.30(0.96)	0.017
<i>Female</i>	45.36(1.36)	45.18(1.63)	44.94(1.10)	45.44(1.00)	0.629
<i>MMP9</i>					
CpG1	5.82(0.33)	5.69(0.34)	6.11(0.26)	5.35(0.23)	0.117
CpG2	4.78(0.29)	4.73(0.25)	4.99(0.26)	4.57(0.20)	0.317
CpG3	2.00(0.13)	1.86(0.10)	2.12(0.16)	1.74(0.09)	0.226
CpG4	3.27(0.33)	2.60(0.09)	3.40(0.46)	2.67(0.13)	0.864
Average	3.97(0.23)	3.72(0.19)	4.16(0.25)	3.58(0.15)	0.321
<i>Male</i>	4.15(0.32)	3.58(0.24)	4.07(0.38)	3.40(0.18)	0.852
<i>Female</i>	3.70(0.32)	3.93(0.30)	4.31(0.18)	3.88(0.25)	0.061
<i>NOS3</i>					
CpG1	13.99(0.79)	14.21(0.82)	13.66(0.73)	13.40(0.71)	0.567
CpG2	8.25(0.49)	8.39(0.50)	8.42(0.43)	7.51(0.32)	0.150
CpG3	5.02(0.45)	5.31(0.38)	4.91(0.26)	4.50(0.25)	0.092
CpG4	5.73(0.56)	5.72(0.48)	5.20(0.34)	5.11(0.29)	0.918
Average	8.25(0.54)	8.41(0.51)	8.05(0.04)	7.63(0.37)	0.348
<i>Male</i>	7.81(0.54)	8.77(0.69)	8.03(0.53)	7.56(0.54)	0.116
<i>Female</i>	8.90(1.09)	7.87(0.73)	8.09(0.63)	7.74(0.43)	0.437

The data is expressed as mean (SEM), with $P < 0.05$ considered statistically significant. Data was analysed using mixed between-within repeated measures ANCOVA adjusting for covariates: age, sex, smoking status and study cohort as covariates. *ACE*, Angiotensin I-converting enzyme; *AGTR1*, Angiotensin receptor 1; *GCK*, Glucokinase; *GNAI2*, Guanine nucleotide-binding protein, alpha-12; *IGF2*, Insulin-like growth factor II; *MMP9*, Matrix metalloproteinase 9; *NOS3*, Nitric oxide synthase

3.