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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
ABSTRACT

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

Key words: DNA methylation, Riboflavin, MTHFR C677T polymorphism, one-carbon metabolism
1. INTRODUCTION

DNA methylation involves the addition of a methyl group to the 5’ position of a cytosine and usually occurs at CpG dinucleotides. Global methylation influences genome stability while gene-specific methylation leads to transcription changes influencing gene expression and phenotypes [1,2]. Differential methylation has also been shown to occur at CpG island shores (~ 2kb outwards from CpG islands) and shelves (~ 2kb outwards from island shores) [3]. Alterations in methylation at these key regulatory regions influence phenotypes and contribute to disease risk [4]. Variations in DNA methylation can occur throughout the lifetime of an individual and have important consequences for health and disease [5–7]. DNA methylation is responsive to environmental changes [8] such as alterations in diet and this provides a mechanism through which epigenetic modulation can influence health outcomes.

One-carbon metabolism (Figure 1), is the main metabolic pathway through which nutrients, mainly folate and related B-vitamins, interact to modulate DNA methylation [9–13]. Factors influencing intake or metabolism of these nutrients including common polymorphisms within genes that influence the one-carbon pathway may therefore impact methylation reactions [14]. Riboflavin in the form of flavin adenine dinucleotide (FAD) is a cofactor for MTHFR, a critical enzyme in one-carbon metabolism and thus for the production of S-adenosylmethionine (SAM), the universal methyl donor. Riboflavin has been largely overlooked in studies investigating B-vitamins in relation to DNA methylation. Of the few reports focusing on riboflavin, an observational study of pregnant Gambian women showed that riboflavin was a significant predictor of peripheral blood DNA methylation at six metastable epialleles (BOLA3, LOC654433, EXD3, ZFYVE28, RBM46, PARD6G and ZNF678) the offspring [15]. A recent cross-sectional study also reported an inverse association between dietary riboflavin intake and LINE-1 methylation in peripheral blood [16] while another study observed a positive correlation between daily intake of riboflavin
and LINE-1 methylation in white blood cells [17]. The latter studies relied on food frequency
questionnaires to estimate riboflavin intake which may not accurately reflect status and as
such, biomarker concentrations are a much more reliable indicator to investigate the
relationship between riboflavin status and DNA methylation [13,18]. Furthermore, limited
conclusions can be drawn from observational data which highlights the need for randomised
controlled trials to determine the effects of one-carbon metabolism nutrients on epigenetic
mechanisms.

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

### 2. MATERIALS AND METHODS

#### 2.1. Participants and Sample Selection

Samples for this study were accessed from stored buffy coat samples from participants who were screened for the *MTHFR* C677T polymorphism and had consented and participated in targeted double-blind randomised controlled trials previously conducted at the Nutrition Innovation Centre for food and Health (NICHE) at Ulster University, Northern Ireland. Samples were drawn from three cohorts namely, the Genetic and Vitamin study (Genovit - FCBMA-15-070), the Genetic and Vitamin ten year follow up study (GENOVIT10 - UUREC/12/0338) and the optimization of RIBOfavin Status in Hypertensive Adults with a Genetic predisposition to Elevated Blood pressure study (RIBOGENE - REC/12/0136) to enable the required number of age and sex matched samples from placebo and treatment groups to be accessed. Each of these studies were conducted using a standardised protocol. Furthermore, each study had identical inclusion and exclusion criteria which included history of gastrointestinal, hepatic, renal or haematological disorders, usage of B-vitamin supplements, anticonvulsant therapy or any other drugs known to interfere with folate or B-vitamin metabolism. Additional ethical approval was granted by Office of Research and Ethics Northern Ireland for the analysis reported in this current study. Data on lifestyle variables, anthropometry and blood samples were collected as part of all three studies.
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
DNA methylation. In order to determine appropriate locations of gene-specific pyrosequencing assays for *MTHFR* gene regulatory regions, we carried out an analysis of DNA methylation at the region surrounding the *MTHFR* transcription start site (Chr1: 11,868,000-11,862,000) in USCS genome browser (hg19) using publicly available Infinium HumanMethylation450 BeadChip methylation data from the ENCODE project [32]. This analysis showed that the north shore and south shelf *MTHFR* gene regulatory regions are variably methylated in five different human cell lines (Supplementary Figure 1) while the CpG island itself is largely unmethylated. The base pair resolution of these datasets allowed us to accurately target the chromosomal region likely to be susceptible to variable DNA methylation. Using the above information, we next experimentally investigated the chromosomal regions for methylation change by pyrosequencing analysis in our human samples.

2.4.2. Genomic DNA extraction

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

2.4.3. Bisulphite Conversion of Genomic DNA

Subsequent bisulphite conversion of 500ng of genomic DNA was carried out according to the manufacturer’s instructions [34–36] using the EZ DNA methylation kit (Zymo Research Corporation, California).
2.4.4. Polymerase Chain Reaction and Pyrosequencing

DNA methylation at the Long Interspersed Nuclear Elements (LINE-1) was measured as a surrogate marker for global methylation. The LINE-1 (GenBank accession number X58075.1) assay covered 3 CpG sites. Three regions of the MTHFR gene (GenBank accession: NM_001330358.1), covering the north shore, south shelf and the CpG island promoter were examined in this study. Commercially available assays for LINE-1 (970042) and MTHFR CpG island (PM00000091) promoter from Qiagen UK were used for PCR of bisulphite treated DNA. Primers for MTHFR north shore (Chr1: 11867263-11867362) and south shelf (Chr1: 11862886-11862985) were designed using PyroMark Assay Design software 2.0. Assay regions were chosen to align with publicly available Illumina 450k array data deposited in UCSC Genome Browser which displayed varying levels of DNA methylation in various cell lines: Primers for the commercially available LINE-1 assay and MTHFR CpG island covered 3 CpGs each while in-house designed primers for the MTHFR north shore and south shelf covered only one CpG due to technical difficulties in primer design. MTHFR north shore forward: 5’ TTTGGGTAATTTAAGTAGTGAGTGGTTTG 3’ and MTHFR north shore reverse: 5’ CCCTAAAACAAAAAATCAAAAAACATCTCT 3’; MTHFR south shelf forward: 5’ CCCTAAAACAAAAAATCAAAAAACATCTCT 3’ and MTHFR souths shelf reverse: 5’ TCCCCAAACACCACCACACT 3’. The PyroMark PCR kit (Qiagen UK) was used for generating amplicons. Each 25µl reaction mix consisted of 12.5µl master mix, 2.5µl coral load, 5.5µl nuclease-free water, 1.25µl each of 10µM forward and reverse primers (2.5µl for commercial primers) and 2µl each of bisulphite converted DNA. PCR was then carried out under the following conditions: initial hot start, 95°C for 15 minutes, followed by 45 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30s and a final elongation of 10 minutes at 72°C. The PCR products were subsequently
electrophoresed on a 1% (w/v) agarose gel electrophoresis to check the size of DNA fragments and also as a quality control measure to check samples for contamination. DNA methylation levels in samples were analysed using the PyroMark Q24 pyrosequencing instrument (Qiagen, UK). Enzymes, substrates and nucleotides from the PyroMark Gold Q24 kit (Qiagen UK) were used. Built in controls within assays to be analysed were used to verify bisulphite conversion. Levels of methylation at each CpG site were analysed using the PyroMark Q24 software [37,38]. The degree of methylation at each CpG site is expressed as the percentage of methylated cytosine over the sum of methylated and unmethylated cytosine. The degree of methylation is reported for each CpG analysed as well as the average percentage of methylation across CpG sites. To verify the accuracy of the analysis, control DNA from EpiTect PCR (Qiagen UK) containing human bisulphite converted fully methylated or unmethylated DNA were included as positive and negative controls in the pyrosequencing runs.

2.5. Statistical Analysis

For the current analysis, power calculations to determine sample size were carried out using the G Power 3.1.9.4 software (version 3) [39] statistical power calculator. Based on power calculations using data from Bollati and colleagues [40], it was estimated that 39 participants per group would be able to discriminate differences of 3.4% in DNA methylation with a power of 80%, at $\alpha = 0.05$ and effect size of 0.65. This sample size is similar to that reported in previous studies investigating folic acid and vitamin B-12 supplementation and DNA methylation [41,42]. Statistical analysis of data was conducted using SPSS IBM Statistics (version 25, SPSS UK Ltd Chertsey, UK). The normality of continuous variables was confirmed using QQ-plots and the Kolmogorov-Smirnov test. All tests were carried out at the 95% confidence interval and in all analyses $p < 0.05$ was considered statistically significant.
Methylation values are shown for all loci analysed and an average methylation for the three CpG sites analysed for the LINE-1 and MTHFR CpG island assay. The assays for the MTHFR north shore and south shelf contained one CpG. Change in methylation in response to riboflavin supplementation was calculated as the difference between post-intervention and baseline methylation values for each CpG analysed and the average.

Chi-square tests for independence were used for comparing categorical variables such as sex, smoking and hypertensive status. Continuous variables including age and body mass index (BMI), were analysed using independent t-tests. One-way analysis of covariance (ANCOVA) adjusted for age, sex, smoking status and study cohort was used to analyse DNA methylation stratified by MTHFR C677T genotypes at baseline. Biomarker (EGRac and homocysteine) responses to intervention with riboflavin were examined using mixed between-within repeated measures ANOVA. The time × treatment interaction was used to assess the effect of treatment versus placebo over time. The between-patient factor was the intervention group (placebo versus riboflavin), and the within-patient factor was time (pre- and post-supplementation). Mixed between-within repeated measures ANCOVA was used to analyse the effect of riboflavin supplementation on DNA methylation in individuals with the MTHFR 677TT genotype. The time × treatment interaction was used to assess the effect of treatment versus placebo over time. The between factor was the intervention group (riboflavin versus placebo) with time (pre- and post-intervention) as the within factor. The mixed between-within analysis tests whether there are main effects for each independent variable and whether the interaction between the two variables is significant. The analysis was further adjusted as appropriate for confounders previously reported to influence DNA methylation such as age, sex, smoking status and study cohort. To account for multiple testing, the level of significance ($P < 0.05$) was adjusted for Bonferroni correction at the assay level ($n = 4$ for LINE-1, $n = 6$ for MTHFR north shore, south shelf and CpG island DNA methylation).
therefore $P < 0.0125$ or $P < 0.008$ was considered statistically significant where appropriate.

Pearson’s bivariate correlation coefficient ($r$), was used to estimate correlations between riboflavin biomarker and DNA methylation in individuals with the TT genotype in the intervention study stratified by treatment groups.

3. RESULTS

3.1. General Characteristics of Participants

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

3.2. Differences in DNA methylation in individuals stratified by $MTHFR$ C677T genotype

There was a general trend towards higher methylation both globally and at sites assayed across the $MTHFR$ locus in individuals with the $MTHFR$ 677TT genotype in comparison to the CC genotype group (Table 2). Perhaps surprisingly, global DNA methylation measured by LINE-1 was significantly higher ($+1.6\%; p = 0.011$) in participants with the $MTHFR$ 677TT genotype compared to those with the CC genotype at baseline. Additionally, significant hypermethylation was detected in individuals with the $MTHFR$ 677TT genotype at
the $MTHFR$ south shelf (+4.85%, $p < 0.001$) compared to CC individuals. DNA methylation at the $MTHFR$ north shore and CpG island were however not significantly different between genotype groups at baseline, although there was again a trend for higher methylation in individuals with the $MTHFR$ 677TT genotype.

3.3. Effect of riboflavin supplementation on biomarker status

The biomarker responses to riboflavin intervention are shown in Table 3. As expected, riboflavin biomarker status in adults with the $MTHFR$ 677TT genotype improved in response to riboflavin supplementation ($p < 0.001$), as indicated by a mean decrease in the functional biomarker EGRac in participants who received riboflavin (-0.10 ± 0.01) compared to placebo (0.02 ± 0.01). Furthermore, there was a significant reduction in homocysteine concentrations ($p = 0.001$) in the group supplemented with riboflavin (-1.79 ± 3.50 μmol/L) compared to placebo (-0.42 ± 3.10 μmol/L).

3.4. Effect of riboflavin supplementation on global and gene-specific methylation in $MTHFR$ 677TT participants

Investigation of the effect of riboflavin supplementation on DNA methylation in individuals with the $MTHFR$ 677TT genotype indicated decreased average methylation at LINE-1 (Riboflavin: -3.16% ± 0.91% vs. Placebo: -0.32% ± 0.69%, $p = 0.018$) which remained significant following Bonferroni correction at CpG 2 (Riboflavin: -1.49% ± 0.72% vs. Placebo: 1.23% ± 0.62%, $p = 0.006$). The $MTHFR$ north shore was significantly hypomethylated (-1.24% ± 0.50% vs. 0.90% ± 0.50%, $p = 0.001$) in participants supplemented with riboflavin compared to placebo respectively. Methylation at the $MTHFR$ south shelf and CpG island in individuals with the $MTHFR$ 677TT genotype group was not influenced by supplementation with riboflavin or placebo. Furthermore, we observed a non-
significant trend for a positive correlation between riboflavin biomarker status and LINE-1 DNA methylation in the riboflavin group compared to the placebo however this was not significant (Figure 3). A similar non-significant correlation was observed for riboflavin biomarker and MTHFR north shore methylation (data not shown).

4. DISCUSSION

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

In comparison to folate, one of the main substrates used for generation of methyl groups in one-carbon metabolism, which has been studied extensively in relation to DNA methylation [43] the role of riboflavin has been largely overlooked. The evidence regarding the role of folate on DNA methylation is not entirely consistent. Some previous studies [44–46], reported that supplementation with folic acid or improved folate status increased global DNA methylation across a range of tissues, including whole blood, leukocytes and colonic mucosa, while more recent studies [42,47–49] examining both global, LINE-1 and genome-wide methylation, including from our own labs, indicate that increased folic acid intake results in lower DNA methylation. DNA methylation was assessed in whole blood, leukocyte samples and cord blood in the studies above showing that the findings of inverse association were
present irrespective of the tissue examined. The results of these recent studies are similar to
our findings which demonstrate an inverse relationship between riboflavin and DNA
methylation in leukocytes as indicated by decreases in both LINE-1 and MTHFR north shore
methylation in response to riboflavin supplementation. Additionally, in general agreement
with the results of this study, Van den Donk et al [50], reported higher dietary folate intake
was associated with lower methylation in whole blood and adenoma tissue in individuals with
the MTHFR 677TT genotype.

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

Several factors could account for the inverse relationship between riboflavin biomarker
concentration and both LINE-1 and MTHFR north shore DNA methylation. Methyl groups
generated from one-carbon metabolism are used in a wide range of biological processes and
the complexity in the interactions of these systems implies that there may not necessarily
exist a linear relationship between nutrients involved in one-carbon metabolism and DNA
methylation [43,51]. For example, simple correlations such as high riboflavin status leading
to increased DNA methylation are unlikely to broadly apply and may differ based on cellular
conditions, dose of riboflavin administered and health status of participants. Secondly,
channelling of methyl groups into DNA methylation is dependent on DNA methyltransferase
enzymes (DNMTs) which tightly regulate the process [52–55]; therefore, an abundance of
methyl groups available for DNA methylation does not necessarily result in increased DNA
methylation. Methyl groups may be directed towards other methylation pathways such as
RNA and histone methylation based on prioritization of cellular conditions and requirements.
A small fraction may also be diverted to non-CG methylation (mostly CpH where H = A,C or
T) which has been detected in almost all tissues tested to date [56]. Similarly, interplay
between DNMTs and transcription factors may potentially influence DNA methylation [57].
Through interaction with DNMTs, transcription factors influence the establishment and
maintenance of DNA methylation [58,59]. Furthermore, regulation of DNA methylation by
DNMTs is highlighted in a recent study of polymorphisms in genes involved in one-carbon
metabolism which revealed a significant association between functional polymorphisms of
DNMT3B and MTHFR methylation [60]. In addition, a study of 2,453 individuals from eight
European countries, investigating variables that may have potential impact on DNMT
expression, reported associations between intake of dairy foods (which are a rich source of
riboflavin) and DNMT1 expression [61], suggesting an additional pathway through which
riboflavin can modulate DNA methylation.
Furthermore, the production of methyl groups for methylation is also dependent on other
enzymes and one-carbon metabolism nutrients such vitamin B-12 and folate. For example,
vitamin B-12 dependent methionine synthase enzyme functions in the remethylation of
homocysteine to methionine and subsequently, the generation of SAM [62]. Therefore, while
it is possible that the MTHFR enzyme may be stabilized by providing riboflavin [20], other
nutrients and enzymes within one-carbon metabolism could impact the production and
availability of methyl groups necessary for DNA methylation. As a first crucial step in
demonstrating that riboflavin modulates DNA methylation, we show that riboflavin resulted in decreased total homocysteine, providing important data to support a potential mechanism whereby riboflavin influences metabolite levels with potential effects on DNA methylation. Hypermethylation at the MTHFR south shelf in MTHFR 677TT individuals may reflect an increased demand for protein production to compensate for the reduced stability and activity of the enzyme [20] in the TT genotype. Methylation within gene bodies, where the south shelf is located, is associated with higher-level transcription generally. Though exact mechanisms are not yet clear, it may prevent aberrant transcription of short transcripts and thereby direct translation of full-length messenger RNAs [4]. Methylation at the MTHFR south shelf remains unchanged following riboflavin intervention and may therefore be important in preventing aberrant MTHFR transcript production in individuals with the TT genotype. Global hypermethylation observed in individuals with the MTHFR 677TT genotype in this study at LINE-1 repetitive elements is also observed in patients with diseases such as multiple sclerosis and Alzheimer’s compared to healthy controls [63–65].

While the differences observed in DNA methylation between the MTHFR genotypes in this study were small, they are comparable with those reported in other studies investigating LINE-1 methylation in peripheral blood in atherosclerosis, cancer and benzene exposure [40,66,67]. The changes in methylation elicited by riboflavin supplementation are also similar to findings of studies investigating other B-vitamins and DNA methylation [68,69]. It is postulated therefore that the small but significant changes observed may be able to mediate changes in gene expression and could be reflective of important alterations in the epigenome, especially in at-risk populations such as individuals with the MTHFR 677TT genotype. In support of this, it has been shown that drug treatment of cell lines which produced relatively small methylation changes resulted in transcription changes [70]. Further studies investigating gene expression and synthesis of the MTHFR protein are required to provide
further insight into the underlying biological mechanism. We observed decreased methylation at the MTHFR north shore following supplementation with riboflavin, which has important implications for gene expression as previous studies have shown that alterations in methylation at CpG sites within shores display higher correlation to gene expression compared to CpG islands [3]. As expected, methylation remained unchanged at the MTHFR CpG island or south shelf following intervention with riboflavin, highlighting the sensitivity of the MTHFR north shore to nutritional influences in comparison to the south shelf or CpG island and this could be a potential target for future epigenetic studies.

The major strength of the current study is the inclusion of samples from RCTs incorporating a parallel placebo group. Importantly, by measuring DNA methylation in the same individuals before and after supplementation, we can also minimise inter-individual variation as a source of observed changes in methylation. Furthermore, global and gene-specific methylation were analysed using the robust pyrosequencing method which has been shown to be very sensitive and reproducible. In a multicentre benchmarking study evaluating DNA methylation assays for clinical use, pyrosequencing of repetitive elements including LINE-1 provided highly reproducible results and bisulphite pyrosequencing showed the best performance for assay sensitivity [71]. Our results however highlight the need for further work as DNA methylation was examined at a limited number of CpG sites. Therefore, it is likely that other regions of the genome which are also influenced by riboflavin require further investigation. Further, while DNA methylation in blood is reflective of methylation status in other tissues, blood consists of a mixed cell population and further work is required to completely exclude the possibility that this contributed to the changes in methylation observed here. We acknowledge that the current study does not allow us to determine tissue-specific effects of riboflavin supplementation on DNA methylation that may be present, the technique used for assessing DNA methylation does not allow us they may be undetected in
the current study. The present study was confined to investigating the effect of riboflavin on DNA methylation in adults with the variant TT genotype; future studies should include individuals with the CC genotype to confirm that the effects observed are genotype driven which would help to provide some additional mechanistic insights into the role of this gene-nutrient interaction in modifying DNA methylation.

In conclusion, this study is the first to provide RCT evidence demonstrating a novel role for riboflavin in modulating DNA methylation in adults with the *MTHFR 677TT* genotype. Supplementation with riboflavin resulted in decreased global and *MTHFR* north shore methylation in TT individuals. Further studies of genome-wide DNA methylation in both TT and non-TT genotypes, as well as gene expression analysis are required to fully elucidate the role of riboflavin in modulating the epigenome.

**Authors’ Contributions were as follows:**

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

**Declaration of competing interest**

DLM, CPW, SDA, AM, CFH no conflicts of interest. MW, HN, JJS hold an international patent on the use of riboflavin in the treatment of blood pressure.
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F.F. Zhang, R. Cardarelli, J. Carroll, K.G. Fulda, M. Kaur, K. Gonzalez, J.K.


Table 1.

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

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>MTHFR 677 CC (n 40)</th>
<th>MTHFR 677 TT (n 40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>58.3(3.9)</td>
<td>56.8(6.9)</td>
<td>0.215</td>
</tr>
<tr>
<td>Male n (%)</td>
<td>22(55.5)</td>
<td>24(60.0)</td>
<td>0.651</td>
</tr>
<tr>
<td>Smoker n (%)</td>
<td>5(12.5)</td>
<td>6(15.0)</td>
<td>0.745</td>
</tr>
<tr>
<td>Alcohol (%)</td>
<td>28(70.0)</td>
<td>26(65.0)</td>
<td>0.633</td>
</tr>
<tr>
<td>Hypertensive BP n (%)</td>
<td>12(30.0)</td>
<td>22(55.0)</td>
<td><strong>0.024</strong></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.5(4.8)</td>
<td>29.8(4.8)</td>
<td>0.769</td>
</tr>
</tbody>
</table>

B-vitamin biomarker status

<table>
<thead>
<tr>
<th></th>
<th>MTHFR 677 CC (n 40)</th>
<th>MTHFR 677 TT (n 40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGRac</td>
<td>1.34(0.17)</td>
<td>1.34(0.12)</td>
<td>0.945</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>10.6(3.5)</td>
<td>13.8(4.8)</td>
<td><strong>0.002</strong></td>
</tr>
</tbody>
</table>

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)

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

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)

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

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)

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

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 timex 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). iP < 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. *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.

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 \textit{MTHFR} gene. B) Expanded view of the \textit{MTHFR} locus on chromosome 1 (p36.22). \textit{MTHFR} regions analysed by pyrosequencing are represented by solid black horizontal bars. \textit{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 \textit{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 < beta value < 0.6), blue = fully unmethylated (beta value $\leq$ 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.

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

- **MTHFR 677CC genotype** (n 40)
- **MTHFR 677TT genotype** (n 40)

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

- **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

\[ r = 0.173, \ p = 0.125, \ n = 80 \]
Supplementary Figure 1

chr1 (p36.22) expanded view pyrosequencing assay locations

GM12878
H1-hESC
K562
HeLa-S3
HepG2
HUVEC

450K arrays in human cell lines

* CpGs included in pyroassays