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

Sophia D. Amenyah[1,2], Mary Ward[2], Amy McMahon[2], Jennifer Deane[1], Helene McNulty[2], Catherine Hughes[2], J.J. Strain[2], Geraldine Horigan[2], John Purvis[3], Colum P. Walsh[1], Diane J. Lees-Murdock[1].

Author Affiliations: [1]Genomic Medicine Research Group, [2]Nutrition Innovation Centre for Food and Health (NICHE), Ulster University, Coleraine, BT52 1SA, N. Ireland, UK. [3]Department of Cardiology, Altnagelvin Area Hospital, BT47 6SB, N. Ireland, UK.

Authors' last names: Amenyah, McMahon, Ward, Deane, McNulty, Hughes, Strain, Horigan, Purvis, Walsh, Lees-Murdock

Corresponding Author: Dr. Diane Lees-Murdock, School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine, N. Ireland, UK. BT52 1SA, Email: dj.lees@ulster.ac.uk

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

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

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

ABSTRACT

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

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

Methods: Pyrosequencing was carried out for hypertension-related genes (*ACE, AGTR1, GCK, GNA12, IGF2, MMP9* and *NOS3*) in blood samples from participants in previous trials (CC, n = 40; TT, n = 40). The effect of intervention with riboflavin (1.6mg/d for16 weeks) or placebo on DNA methylation was investigated in adults with the variant *MTHFR* 677TT genotype (n=80).

Results: Individuals with the *MTHFR* 677TT v CC genotype had significantly higher average DNA methylation at *NOS3* (+1.66%, $P = 0.044$). In response to riboflavin supplementation in TT individuals, there was an increase in average DNA methylation at *IGF2* (+1.09%, $P = 0.019$) and a decrease at *ACE* (-0.44%, $P=0.021$) in females only. Specific CpG sites were hypomethylated in *GNA12* and hypermethylated in *AGTR1*.

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

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

1.0 INTRODUCTION

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

Evidence from the literature indicates that perturbation of DNA methylation leads to genomic instability and transcriptional repression and thereby influencing disease aetiology (15). These perturbations result from imbalances in the supply of nutrients in one-carbon metabolism, the main metabolic pathway for generating methyl groups for biological reactions including DNA methylation (16,17). Alterations in DNA methylation both globally

and at key gene loci, have also been implicated in hypertension (18). Furthermore, cardiovascular disease (CVD) has been identified as an age-related condition linked to epigenetic age acceleration in blood using the DNA methylation-based Phenotypic Age measure (PhenoAge) (19), which also demonstrates a positive correlation between systolic blood pressure and epigenetic age.

This study focuses on key genes implicated in hypertension including angiotensin I converting enzyme (*ACE*), angiotensin receptor 1 (*AGTR1*), glucokinase (*GCK*), guanine nucleotide- binding protein alpha-12 gene (*GNA12*), insulin-like growth factor II (*IGF2*), matrix metalloproteinase 9 (*MMP9*) and nitric oxide synthase 3 (*NOS3*). These genes are involved in blood pressure regulation through their functions in the renin-angiotensin system, smooth muscle cell regulation and endothelial function (20–22). We hypothesised that DNA methylation of genes involved in hypertension-related pathways would differ by *MTHFR* genotype and be modulated by riboflavin, the MTHFR cofactor, in individuals with the variant *MTHFR* 677TT genotype. To explore this hypothesis, the aims of the current study were to investigate DNA methylation of key hypertension-related genes in adults stratified by *MTHFR* genotype, and to examine the effect of riboflavin supplementation on DNA methylation of hypertension pathway loci specifically in individuals with the *MTHFR* 677TT genotype.

2.0 MATERIALS AND METHODS

2.1 Participants and study design

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

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

2.2 Study design

DNA methylation analysis for this study was carried out in two phases: in an observational phase, differences in gene-specific methylation were compared between the two *MTHFR* C677T genotypes (i.e. CC, n = 40 versus TT, n = 40). In the intervention phase, changes in gene-specific DNA methylation were examined in participants with the TT genotype only (placebo, n = 40; riboflavin, n = 40) in response to intervention with riboflavin (1.6mg/d) or placebo for 16weeks. The flow diagram of the study design is shown in **Supplementary Figure 1.** Sample size calculations for the present analysis was carried out using the G Power 3.1.9.4 statistical power calculator (version 3) (23). Based on power calculations using data from Bollati *et al* (24), 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.

2.3 DNA Methylation Analysis

2.3.1 Selection of candidate genes for DNA methylation analysis

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

2.3.2 Genomic DNA extraction

For the current analysis, genomic DNA was extracted from 200µl of stored peripheral blood leukocyte samples using the Qiagen QIAamp DNA blood mini kit (Qiagen, UK) according to the manufacturer's protocol (25). Genomic DNA samples were electrophoresed on a 1% (w/v) agarose gel to examine quality. The purity of the samples was evaluated, and concentrations quantified using the NanodropND1000 spectrophotometer (Labtech International, Ringmer, UK).

2.3.3 Bisulphite Conversion of Genomic DNA

500ng of genomic DNA was subsequently bisulphite converted using the EZ DNA methylation kit (Zymo Research Corporation, California) according to manufacturer's protocol (26) using the EZ DNA methylation kit.

2.3.4 Pyrosequencing

Commercially available predesigned methylation assays from Qiagen UK were used for bisulphite PCR and pyrosequencing for the following loci: *ACE* (PM00181398), *AGTR1* (PM00014875), *GNA12* (PM00127925), *MMP9* (PM00079191) and *NOS3* (PM00129220) while assays for *IGF2* and *GCK* were based on previously published primer sets from previous studies which have examined these specific regions (27–29). Details of the assays, chromosomal location and number of CpGs examined are provided in **Supplementary Table 2**. After bisulphite conversion, DNA amplicons were amplified by PCR using the PyroMark PCR kit (Qiagen, UK) according to manufacturer's protocol. Each 25µl PCR reaction mix consisted of 12.5µl master mix, 2.5µl coral load, 5.5µl nuclease-free water, 2.5µl each of 10µM primer set and 2µl each of bisulphite converted DNA. PCR was then carried out under the following conditions: hot start of 95^0C for 15 minutes, followed by 45 cycles of 94^0C for 30 seconds, 56^0C for 30 seconds, 72^0C for 30s and a final elongation of 10 minutes at 72^0C. The PCR products were subsequently electrophoresed on a 1% (w/v) agarose gel to check the integrity of PCR products. DNA methylation in samples was analysed using the PyroMark Q24 pyrosequencing instrument (Qiagen, UK). Enzymes, substrates and nucleotides from the PyroMark Gold Q24 kit (Qiagen UK) were used. Levels of methylation at each CpG site were analysed using the PyroMark Q24 software (30). As an additional control, bisulphite DNA controls from EpiTect PCR Control DNA (Qiagen, UK) that contained fully methylated as well as fully unmethylated DNA was included in the analysis to ensure that the instrument detected the full range of methylation values.

2.4 Statistical analyses

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

3.0 RESULTS

3.1 Baseline characteristics of participants

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

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

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

DNA methylation analysis of the candidate hypertension-related genes indicate an overall trend toward hypermethylation at several loci including *ACE*, *AGTR1*, *GCK*, *MMP9* and *NOS3* in individuals with the *MTHFR* 677TT genotype compared to the CC genotype (**Table 2**). Average DNA methylation levels were significantly higher at *NOS3* (1.66%, $P = 0.044$) in the TT genotype compared to individuals with the CC genotype after adjusting for age, sex, smoking status and study cohort. Significant CpG site-specific differences were observed at CpG2 of *AGTR1* and CpG1 of *GNA12*. Examination of sex-specific differences in methylation between the *MTHFR* genotypes showed that methylation differences observed at *NOS3* was marginally significant in females but not in males (**Table 2**). Multiple linear regression adjusting for covariates was used to identify the determinants of gene-specific methylation in adults with the *MTHFR* C677T polymorphism (CC and TT genotypes) at

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

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

Supplementation with riboflavin, resulted in increased overall methylation at *IGF2* (+1.08%, $P = 0.019$) compared with placebo. Increased methylation was observed at CpG1 of *AGTR1*, however, methylation decreased at CpG2 and CpG4 of *GNA12* in TT participants receiving riboflavin compared to placebo. Stratification of the analysis by sex, indicated increased methylation in response to riboflavin supplementation at *IGF2* (+1.44%; $P = 0.017$) compared with placebo in males but not females. However, decreased methylation was observed at *ACE* (-0.44%; $P = 0.021$) in females but not males (**Table 4**). Multiple linear regression analysis, focused specifically on individuals with the *MTHFR* 677TT genotype in the intervention stage of the study, showed that riboflavin treatment was a determinant of *IGF2* methylation ($\beta = 0.265$, $P = 0.021$, $R^2 = 0.106$). No other genes showed any significant interaction with any of the baseline determinants.

4.0 DISCUSSION

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

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

Riboflavin supplementation compared with placebo in individuals with the *MTHFR* 677TT genotype, showed increased average methylation at *IGF2*, which was also demonstrated in the linear regression model which showed riboflavin treatment as the sole determinant of methylation of *IGF2*. Although no other study, to our knowledge, has investigated the role of riboflavin in modulating DNA methylation at *IGF2*, studies investigating the epigenetic effects of other B-vitamins, mainly folic acid and vitamin-B12, in various populations report significant increases in *IGF2* methylation in response to supplementation (27,31) supporting the findings of this study. *IGF2* is a paternally expressed imprinted gene with well-established physiological roles in growth and development. Polymorphisms of *IGF2* have been related to vascular risk factors and hypertension (32,33). Furthermore, *IGF2* functions as part of the insulin-like growth factor (IGF) system which plays complex roles in nutrient-sensitive pathways and may indirectly influence blood pressure through the regulation of cardiac muscles (34). Alterations in methylation could therefore potentially impact *IGF2* expression with implications for blood pressure regulation. Although significant, the changes in methylation observed at *IGF2* are very small however the magnitude of change is in agreement with previous studies showing that small changes in methylation can result in transcriptional alterations including at imprinted genes(35). Further functional studies are required to investigate the implications of our findings on gene expression. Apart from overall changes in average methylation, we observed significant decreases at specific CpG

sites within the *GNA12* loci. Similarly, in an RCT investigating supplementation of folic acid and vitamin B-12 on genome-wide methylation, differential methylation was observed at the *GNA12* locus, with methylation shown to decrease in response to supplementation with folic acid and vitamin B12 in comparison to placebo in adults (36).

While associations between polymorphisms in the *NOS3* gene and cardiovascular disease have been widely studied, methylation at *NOS3* in individuals with the *MTHFR* C677T has not been extensively investigated. It is widely accepted that CpG islands at promoters of housekeeping genes are usually unmethylated allowing transcription. Hypermethylation at the *NOS3* loci as observed in individuals with the *MTHFR* 677TT genotype has the potential to inhibit the expression of this gene and thereby influencing its function in regulating blood pressure. *NOS3* is a key regulator of vasotone, platelet aggregation and blood pressure (20,21,37). Furthermore, mendelian randomisation studies in stroke patients indicate that genetic variation in the nitric oxide synthase pathway affects stroke risk via variations in blood pressure (38). Surprisingly, there were no changes in *NOS3* methylation in response to riboflavin supplementation suggesting that other mechanisms in addition to methylation may be modulating the effect of riboflavin on blood pressure in adults with the *MTHFR* 677TT genotype. For, example it has been postulated that endothelial nitric oxide synthase (eNOS) may provide a link between *MTHFR* 677TT genotype and blood pressure (39).

Consistent with findings of the present study, several studies have reported sex- and age-specific differences in methylation at several gene loci (40,41). These sex-specific differences could be owing to different mechanisms and pathogenic processes involved in blood pressure regulation by these genes in males and females. These findings are in general agreement with studies investigating blood pressure which also showed that metabolic and haemodynamic abnormalities associated with hypertension differed markedly between sexes (42). For example, while a cardiac phenotype was associated with elevated blood pressure and

hypertension in males, a vascular phenotype characterised by elevated peripheral vascular resistance was more prominent in females (42). Furthermore, similar to findings from Xu and colleagues (29) who reported significant correlations between *GCK* gene body methylation and aging, multiple linear regression in the present study identified age and sex as determinants of methylation at the *GCK* locus although no significant differences were observed between *MTHFR* genotypes. Changes in methylation have been shown to correlate with age providing a biological marker for ageing (43) and these sites could play important roles in disease such as hypertension. It must be noted that although overall changes may not be seen across all CpGs within a gene, site-specific alterations may still occur, and these site-specific alterations indicate biologically relevant heterogeneity in DNA methylation and are still relevant in the aetiology of disease (44). Additionally, methylation of a particular CpG position may have a strong influence on transcriptional suppression or expression while methylation at other CpG sites may have little influence (45). For example, surprisingly methylation at the *ACE* locus was reduced in response to riboflavin supplementation, which would suggest increased gene expression which might lead to high blood pressure however the effect on blood pressure may involve a complex interplay with other genes and warrants further investigation. Although the present study demonstrates significant methylation differences of hypertension pathway genes following supplementation with riboflavin in *MTHFR* 677TT individuals, further investigations are required to better understand the interconnections and interactions between these genes and the resulting effects on blood pressure.

The main strength of this study is that it draws on samples from randomised controlled trials, providing a rigorous tool to examine the effects of riboflavin supplementation on DNA methylation. Additionally, our investigation used a robust biomarker, EGRac, to evaluate riboflavin status in participants, which is rarely reported due to lack of accessible laboratory

methods and labour-intensive pre-analysis sample preparation; our laboratory is one of the few worldwide to routinely measure EGRac. Biomarker status offers many advantages over estimated dietary intake which is widely reported to be inherently flawed (46). Furthermore, we adjusted for several variables in the statistical analysis of the methylation data ensuring that the findings were not masked by confounding factors. A limitation however is that the candidate gene approach employed means that although specific hypertension-related genes were investigated, further relevant genes and CpG sites essential to blood pressure regulation, that were not included in this analysis could potentially be influenced by riboflavin supplementation. Further as DNA methylation was examined in blood we cannot exclude the possibility that methylation patterns identified may represent an overall effect contributed from the different cell fractions.

5.0 Conclusion

The findings of this study demonstrate that supplementation with riboflavin (the MTHFR co-factor) in adults with the *MTHFR* 677TT genotype modulates DNA methylation at key hypertension-related genes including *IGF2* and *GNA12*. Furthermore, we observed significant differences in DNA methylation at *NOS3* and *GNA12* between individuals with CC and TT genotypes for this polymorphism. The results from this study provide some preliminary data to indicate that methylation of hypertension related genes may be implicated in the mechanism linking MTHFR with blood pressure however further investigations are required to understand the complex mechanism. Furthermore, this study highlights the interaction between genetic, epigenetic and environmental factors which could play a potential role in the prediction of vascular events and in the development of therapeutic options for the treatment of high blood pressure. Replication of our findings in larger

independent cohorts using a genome-wide approach is required to understand the complex mechanisms linking this common polymorphism with higher blood pressure and the DNA methylation response to riboflavin intervention in individuals with the variant *MTHFR* 677TT genotype.

Authors' Contributions were as follows:

DLM and MW planned and designed the research, with contributions from CPW on assay design. SDA 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.

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

	***MTHFR* C677T Genotype**		
	***MTHFR* 677CC (n 40)**	***MTHFR* 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	***MTHFR* 677CC (n 40)**	***MTHFR* 677TT (n 40)**	***P*-value**
ACE	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
Male		1.21(0.06)	1.41(0.20)	0.607
Female		1.17(0.06)	1.31(0.17)	0.311
AGTR1	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
Male		2.87(0.11)	3.27(0.25)	0.214
Female		2.72(0.13)	3.10(0.10)	0.327
GCK	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
Male		43.59(1.12)	44.47(1.24)	0.387
Female		47.16(1.91)	46.33(1.69)	0.642
GNA12	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
Male		0.64(0.07)	0.75(0.11)	0.369
Female		0.65(0.05)	0.71(0.07)	0.869
IGF2	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
Male		45.77(1.39)	44.84(1.15)	0.762
Female		43.61(1.33)	45.33(1.12)	0.606
MMP9	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
Male		4.14(0.30)	4.11(0.37)	0.869
Female		3.68(0.24)	4.10(0.31)	0.454
NOS3	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**
Male		6.87(0.54)	7.75(0.50)	0.356
Female		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; *AGTR1*, Angiotensin receptor 1; *GCK*, Glucokinase; *GNA12*, 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					
	AGTR1		***GCK***		***NOS3***	
	β	*P*-value	β	*P*-value	β	*P*-value
MTHFR 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**
	Pre-intervention	*Post-intervention*	*Pre -intervention*	*Post-intervention*	
ACE					
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
Male	1.36(0.11)	1.11(0.07)	1.37(0.20)	1.21(0.12)	0.705
Female	1.19(0.09)	1.64(0.27)	1.45(0.18)	1.01(0.09)	**0.021**
AGTR1					
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
Male	3.16(0.14)	2.92(0.08)	3.11(0.24)	3.25(0.32)	0.542
Female	3.01(0.10)	2.86(0.12)	2.89(0.08)	3.08(0.28)	0.360
GCK					
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
Male	44.78(1.12)	44.49(1.08)	44.49(1.07)	44.04(0.73)	0.815
Female	46.71(1.98)	47.08(2.22)	45.71(1.24)	45.38(1.40)	0.995
GNA12					
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
Male	0.69(0.05)	0.71(0.04)	0.77(0.11)	0.58(0.03)	0.129
Female	0.69(0.06)	0.81(0.07)	0.60(0.04)	0.71(0.15)	0.791

IGF2					
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**
Male	45.63(0.87)	43.81(1.08)	44.86(1.23)	46.30(0.96)	**0.017**
Female	45.36(1.36)	45.18(1.63)	44.94(1.10)	45.44(1.00)	0.629
MMP9					
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
Male	4.15(0.32)	3.58(0.24)	4.07(0.38)	3.40(0.18)	0.852
Female	3.70(0.32)	3.93(0.30)	4.31(0.18)	3.88(0.25)	0.061
NOS3					
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
Male	7.81(0.54)	8.77(0.69)	8.03(0.53)	7.56(0.54)	0.116
Female	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; *GNA12*, Guanine nucleotide-binding protein, alpha-12; *IGF2*, Insulin-like growth factor II; *MMP9*, Matrix metalloproteinase 9; *NOS3*, Nitric oxide synthase 3.