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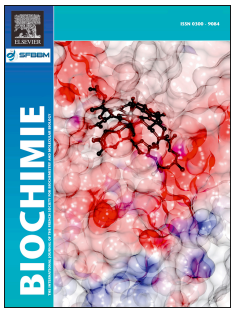

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Impact of the *MTHFR* C677T polymorphism on one-carbon metabolites: evidence from a randomised trial of riboflavin supplementation

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Highlights

- The *MTHFR* 677TT genotype is associated with a 24-87% increased risk of hypertension
- Riboflavin (the precursor for the MTHFR cofactor, FAD), lowers BP in TT adults
- Perturbed one-carbon metabolism may influence the BP phenotype linked with TT genotype
- SAM concentrations and SAM:SAH ratio were lower in individuals with the TT genotype
- In the TT genotype group, SAM and cystathionine increased in response to riboflavin

Key words

MTHFR, riboflavin, S-adenosylmethionine, one-carbon metabolism, hypertension.

Abbreviations

5-MTHF, 5-methyltetrahydrofolate; ANOVA, analysis of variance; BHMT, betaine-homocysteine methyltransferase; BP, blood pressure; CßS, cystathionine ß-synthase; cv, coefficient of variation; CVD, cardiovascular disease; EGRac, estimated glutathione reductase activation coefficient; FAD, flavin adenine dinucleotide; GWAS, genome wide association study; HPLC-ESI-MS/MS, high-performance liquid chromatography; electrospray positive ionization tandem mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; MTHFR, methylenetetrahydrofolate reductase; NICHE, Nutrition Innovation Centre for Food and Health; NORCCAP; Norwegian Colorectal Cancer Prevention; PLP, pyridoxal-5′-phosphate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SD, standard deviation.

Abstract

Homozygosity for the C677T polymorphism in *MTHFR* (TT genotype) is associated with a 24-87% increased risk of hypertension. Blood pressure (BP) lowering was previously reported in adults with the TT genotype, in response to supplementation with the MTHFR cofactor, riboflavin. Whether the BP phenotype associated with the polymorphism is related to perturbed one-carbon metabolism is unknown. This study investigated one carbon metabolites and their responsiveness to riboflavin in adults with the TT genotype. Plasma samples from adults (*n* 115) screened for the *MTHFR* genotype, who previously participated in RCTs to lower BP, were analysed for methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), betaine, choline and cystathionine by liquid chromatography tandem mass spectrometry (LC-MS/MS). The one-carbon metabolite response to riboflavin (1.6 mg/d; *n* 24) or placebo (*n* 23) for 16 weeks in adults with the TT genotype was also investigated. Plasma SAM (74.7 ± 21.0 vs 85.2 ± 22.6 nmol/L, P=0.013) and SAM:SAH ratio (1.66 ± 0.55 vs 1.85 ± 0.51, P=0.043) were lower and plasma homocysteine was higher (P=0.043) in TT, compared to CC individuals. In response to riboflavin, SAM (P=0.008) and cystathionine (P=0.045) concentrations increased, with no responses in other one-carbon metabolites. These findings confirm perturbed one-carbon metabolism in individuals with the *MTHFR* 677TT genotype, and for the first time demonstrate that SAM, and cystathionine, increase in response to riboflavin supplementation in this genotype group. The genotype-specific, one-carbon metabolite responses to riboflavin intervention observed could offer some insight into the role of this gene-nutrient interaction in blood pressure.

1.0 Introduction

Hypertension is a major modifiable risk factor for stroke and cardiovascular disease (CVD), and a leading cause of premature mortality worldwide, responsible for over 10 million deaths annually [1]. The pathophysiology of hypertension is complex, involving the interaction of genetics, environmental factors and physiological mechanisms [2]. Genome wide association studies (GWAS) have linked a number of genetic loci with hypertension [3,4], including a region near the gene encoding the folate metabolising enzyme, methylenetetrahydrofolate reductase (MTHFR). The common *MTHFR* C677T polymorphism produces an enzyme with reduced activity [5] owing to lowered affinity for its riboflavin cofactor, (flavin adenine dinucleotide, FAD) [6]. The homozygous *MTHFR* 677TT genotype affects 2-32% of populations worldwide [7] and meta-analyses have estimated that the variant TT genotype is associated with 24-87% increased risk of hypertension and increased risk for CVD by up to 40% [8]. Previous studies conducted at this Centre have demonstrated that BP is highly responsive to riboflavin supplementation, with evidence that systolic BP can be lowered by between 6 to 14 mmHg in individuals with the TT genotype [9–11]. This gene-nutrient interaction thus offers a novel, nutritional approach for BP management among adults with the C677T variant in *MTHFR*, although the underlying mechanism remains unexplained. It is possible the phenotype of elevated BP and its response to riboflavin may be owing to perturbations in one-carbon metabolism in affected individuals; however, this mechanism has not been previously investigated.

In one-carbon metabolism, FAD-dependent MTHFR generates 5-methyltetrahydrofolate (5-MTHF), which is involved in the remethylation of homocysteine to methionine, the pre-cursor to S-adenosylmethionine (SAM; **Figure 1**). As the principal methyl donor, SAM transfers methyl groups to over 100 methyltransferases involved in numerous biochemical pathways including DNA methylation, histone modification and neurotransmitters [12]. This

transfer, in turn, leads to the formation of S-adenosylhomocysteine (SAH), which is subsequently metabolised to homocysteine. DNA methylation, an epigenetic process involved in gene transcription and expression, has been implicated in a number of disease states across the life-cycle, including CVD [13]. The ratio of SAM:SAH has been sometimes used as a marker of methylation potential, although the validity of this indicator requires confirmation [14]. Choline and betaine can also serve as alternative methyl donors in homocysteine remethylation as part of the betaine-homocysteine methyltransferase (BHMT) pathway [15]. Homocysteine can be removed through irreversible condensation with serine to cystathionine via the action of cystathionine ß-synthase (CßS), in the pyridoxal-5′-phosphate (PLP)-dependent transsulfuration pathway. Regulation of the methylation cycle is essential to ensure sufficient supply of SAM to methyltransferase reactions. This is achieved through the action of SAM as an allosteric inhibitor of MTHFR and an allosteric activator of CßS, thus controlling one-carbon flux and homocysteine levels [16].

Higher concentrations of SAM and SAH have been reported in TT relative to CC adults in some [17,18] but not all [19,20] studies. In observational analysis of 10,601 Norwegian adults elevated homocysteine and decreased betaine were reported in TT compared to CC genotype groups, with no influence of genotype on other one-carbon metabolites [21–23]. Sub-optimal status of the B vitamins, folate, riboflavin, PLP and cobalamin, which act as nutritional cofactors for the key enzymes in the one-carbon pathway (**Figure 2**), have been previously shown to result in elevated homocysteine in adults generally and particularly by *MTHFR* genotype [24,25]. The effect of intervention with one or a combination of these B vitamins has been shown to modulate homocysteine concentrations [26–28]; however, the effect on other one-carbon metabolites has not been widely investigated, and few studies have considered the effect of the *MTHFR* 677TT genotype.

Therefore, the aim of this study is to investigate the impact of the *MTHFR* C677T polymorphism on one-carbon metabolite status and the responsiveness of one-carbon metabolites to riboflavin supplementation (1.6mg/day) in adults with the *MTHFR* 677TT genotype. The findings of this study could contribute to our understanding of the mechanism underpinning the BP phenotype related to this gene-nutrient interaction.

2.0 Materials and Methods

2.1 Subjects and samples

Plasma samples from participants who had previously participated in studies at the Nutrition Innovation Centre for Food and Health (NICHE), Ulster University, and had been screened for the *MTHFR* 677TT genotype were accessed for the current study. In all cases, participants provided informed, written consent and agreed for samples to be used in subsequent studies. Samples were accessed from the GENOVIT study (ORECNI ref 08/NIR03/40) [9], the GENOVIT follow-up study (ORECNI ref 08/NIR03/40) [10] and the RIBOGENE study (ORECNI/12/0338). Ethical approval for the analysis reported in the current study was granted by Ulster University Research Ethics Committee (FCBMS-18-040). All three studies had identical inclusion (pre-screened for *MTHFR* C677T polymorphism) and exclusion (history of gastrointestinal, hepatic or renal disease, consumers of B vitamin supplements, use of medication known to interfere with B vitamin metabolism) criteria. Clinic BP was measured in accordance with guidelines from the National Institute of Care and Excellence [29]. In brief, after ten minutes at rest, BP was measured in the reference arm, i.e. the arm with the highest BP, with the participant in the seated position. Mean BP was calculated as the average of two BP readings within 5mmHg, with a maximum of six readings obtained. Anthropometry, health and lifestyle information and blood samples were collected according to appropriate standardised operating procedures as part of each study, described in detail elsewhere [9,10].

The analysis for the current study consisted of both an observational and an intervention phase. In the observational phase, participants with the TT genotype were age-matched with a similar number of individuals with the CC genotype and compared for general characteristics and one-carbon metabolite biomarker status. In the intervention phase, biomarker status of methionine, SAM, SAH, SAM:SAH ratio, betaine, choline and cystathionine in response to intervention with riboflavin (*n* 24) and placebo (*n* 23) were investigated (**Figure 2**).

2.2. Blood sampling

Venipuncture of a vein in the antecubital fossa was conducted by a trained phlebotomist with the participant in a non-fasting state. A 25ml blood sample was obtained into two EDTA vacutainers (9ml and 4ml) and two serum vacutainers (8ml and 4ml). All tubes, apart from the 4ml EDTA tube, were placed immediately on ice and centrifuged at 3000 rpm for 15 minutes at 4º Celsius, within 30 minutes of the venipuncture. Plasma, serum and buffy coat were removed at this stage. The erythrocytes in the 9ml EDTA tube were thrice washed with phosphate buffered saline and these washed red cells were used for erythrocyte glutathione reductase activation coefficient (EGRac) analysis. The 4ml EDTA tube was rolled for 30 minutes, and 50µl was added to 450µl of 1% ascorbic acid solution (1 in 10 dilution), from which red blood cell folate was determined. All fractions were labelled and stored at -80º Celsius in alarm-controlled freezers with batch analysis of biomarkers conducted at the end of the study. The samples did not undergo any freeze-thaw cycles between initial storage and analysis.

2.3 B vitamin biomarker analysis

Riboflavin status was determined at Ulster University using the erythrocyte glutathione reductase activation coefficient (EGRac) assay, which measures the enzyme activity of glutathione reductase before and after in vitro reactivation with its prosthetic group FAD, as described elsewhere [10]. EGRac is calculated as the ratio of FAD-stimulated to unstimulated

enzyme activity, with values <1.3 indicating optimal riboflavin status, 1.3-1.4 suboptimal status and >1.4 signifying deficiency. Red blood cell folate concentrations, a long-term biomarker of folate status was measured by microbiological assay using *Lactobacillus casei*, as described by Molloy & Scott [30]. Plasma PLP, as a marker of vitamin B6 status, was analysed by HPLC [31]. Plasma homocysteine was analysed by fluorescence polarisation immunoassay for plasma homocysteine [32].

2.4 Metabolite analysis

One-carbon metabolites, apart from homocysteine, were analysed at the Center of Metabolomics, Baylor Scott & White Research Institute (Dallas, Texas 75226). Determination of methionine, SAM, SAH, betaine, choline and cystathionine in plasma was performed by high performance liquid chromatography coupled with electrospray positive ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using a method previously described with some minor modification [33]. In brief, 20µl of plasma was added to 180µl of isotope internal standards and loaded into a microtiter plate before being centrifuged for 60 minutes prior to analysis. The calibration curve for SAM and SAH was 25-400-nmol/L, for methionine, betaine and choline; 3.1-50 nmol/L and for cystathionine: 125-2000 nmol/L. Two levels of quality control samples were used to monitor within and between day precision of the method. In all cases, the coefficient of variation (cv) was less than 15% for all metabolites.

2.5 Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS; version 25.0; SPSS UK Ltd, Chertsey, UK). Normality tests were carried out on the data and data not normally distributed were log transformed before analysis was conducted. Differences in general characteristics and one-carbon metabolite status between genotype groups (observational cohort) were determined using independent samples t-test. Chi square test was

used for comparison between categorical variables. To determine the response to intervention, within-between repeated-measures ANCOVA was used, controlling for baseline EGRac. The between-participant factor was the intervention group (placebo compared with riboflavin), and the within-participant factor was time (before compared with after intervention). Results are presented as mean (SD), unless otherwise stated. P<0.05 was considered significant in all analysis carried out. Network analysis was performed with visualisation of the networks in a circular layout in corrplot and qgraph packages from R (version 3.3.0; R Core Team 2016, Vienna, Austria; www.R-project.org).

3.0 Results

Available plasma samples and data from adults (*n* 115) screened for the *MTHFR* genotype, and who previously participated in trials to lower BP were accessed. In the observational cohort, there were no significant differences in general characteristics between *MTHFR* genotype groups (**Table 1)**. EGRac, the functional indicator of riboflavin status, was similar across the groups. PLP, serum and red blood cell folate concentrations were significantly lower in those with the TT compared to CC genotype. As previously reported, both systolic and diastolic BP were significantly elevated in the TT relative to CC genotype groups (mean difference 16.6 ± 3.4 mmHg, P<0.001; 9.0 ± 13.5 mmHg, P<0.001, respectively), and those with the TT genotype were more likely to be classed as hypertensive according to current NICE guidelines [29]. There was no difference in use of anti-hypertensive medications between groups (75% of CC and 83% of TT genotype, P=0.308).

In relation to one-carbon metabolites, elevated homocysteine (10.4 ± 3.0 vs 9.3 ± 2.5 µmol/L P=0.043), lower SAM concentrations (74.7 ± 21.0 vs 85.2 ± 22.6 nmol/L P=0.013) and lower SAM:SAH ratio (1.66 ± 0.55 vs 1.85 ± 0.51, P=0.043) was observed in the TT compared to the CC genotype (**Table 2)**. No differences were observed for methionine, SAH, betaine, choline or

cystathionine by genotype group. Network analysis showed that the nature and strength of interrelationships of metabolites and B vitamins within one-carbon metabolism were influenced by *MTHFR* genotype (**Figure 3**).

Table 1
Characteristics of participants by *MTHFR* genotype (observational cohort *n* 115)

	***MTHFR* 677CC (*n* 68)**	***MTHFR* 677TT (*n* 47)**	***P* value[1]**
Age (years)	54.7 (6.0)	54.3 (6.0)	0.807
Male sex *n* (%)	58 (85)	37 (79)	0.361
BMI (kg/m^2)	29.1 (4.9)	29.1 (4.6)	0.956
Diabetes mellitus *n* (%)	8 (12)	5 (11)	0.851
Smoker *n* (%)	16 (24)	17 (36)	0.141
Family history CVD *n* (%)	31 (46)	34 (72)	0.229
B vitamin biomarkers			
Red blood cell folate (nmol/L)	1055 (557)	809 (385)	0.045
Serum folate (nmol/L)	12.2 (8.0)	6.7 (4.0)	<0.001
PLP (nmol/L)	72.0 (38.3)	47.5 (22.2)	<0.001
EGRac (riboflavin status)	1.37 (0.18)	1.36 (0.14)	0.788
Blood Pressure			
Systolic BP (mmHg)	128.0 (16.6)	144.7 (19.2)	<0.001
Diastolic BP (mmHg)	78.8 (11.9)	87.1 (12.4)	<0.001
Pulse pressure (mmHg)	49.3 (12.4)	57.3 (16.6)	0.004
Hypertensive[2] *n* (%)	17 (25)	29 (62)	<0.001
BP medications *n* (%)	51 (75)	39 (83)	0.308

Values are mean (SD). [1] P values refer to differences between genotype groups compared using independent samples t-test. Chi square test used for comparison between categorical variables. P<0.05 considered significant. [2]Hypertension defined as a BP reading of ≥140mmHg systolic and/or ≥90mmHg diastolic BP [31]. BP, blood pressure; CVD, cardiovascular disease; EGRac, erythrocyte glutathione reductase activation coefficient (a marker of riboflavin status where lower EGRac values indicate better riboflavin status); PLP, plasma-5'-pyridoxal phosphate.

Table 2
One-carbon metabolites by *MTHFR* genotype (observational cohort *n* 115)

	***MTHFR* 677CC (*n* 68)**	***MTHFR* 677TT (*n* 47)**	***P* value[1]**
Homocysteine (µmol/L)	9.3 (2.5)	10.4 (3.0)	0.043
Methionine (µmol/L)	29.5 (7.2)	30.3 (6.7)	0.450
SAM (nmol/L)	85.2 (22.6)	74.7 (21.0)	0.013
SAH (nmol/L)	45.0 (10.9)	46.8 (9.8)	0.320
SAM:SAH ratio	1.85 (0.51)	1.66 (0.55)	0.043
Betaine (µmol/L)	53.1 (13.7)	50.5 (15.8)	0.194
Choline (µmol/L)	9.7 (2.1)	9.8 (2.7)	0.869
Cystathionine (nmol/L)	243 (96)	248 (118)	0.965

Values are mean (SD). [1]Differences between genotype groups compared using independent t-tests. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. P<0.05 considered significant.

As previously reported [9–11], significant decreases were observed in both systolic (-14.0 ± 15.3 mmHg, P=0.030) and diastolic BP (-8.2 ± 11.1 mmHg, P=0.013) in response to riboflavin supplementation which resulted in a significant decrease in EGRac (-0.15 ± 0.16, P<0.001), indicating improved riboflavin status, in those with the *MTHFR* 677TT genotype (**Figure 4**). No change in red blood cell folate was observed (data not shown).

Response of one-carbon metabolites to riboflavin intervention among individuals with the TT genotype in *MTHFR* is presented in **Table 3**. Plasma homocysteine decreased by 0.5 ± 1.7 µmol/L in the riboflavin group, albeit an effect that was non-significant compared to the placebo group. Mean plasma SAM concentration increased significantly in response to riboflavin supplementation by 19.5 ± 20.6 (P=0.021), where the nature of this effect was only strengthened when adjusted for baseline riboflavin status (P=0.008). Plasma cystathionine

concentrations increased by 50.7 ± 92.5 nmol/L (P=0.021), in response to riboflavin supplementation. No other metabolites were affected by riboflavin intervention.

Table 3
One-carbon metabolite response to riboflavin intervention in adults with the *MTHFR* 677TT genotype **(***n* 47**)**

	Placebo (n 23)		Riboflavin (n 24)		*P* Value[1]	
	Pre	**Post**	**Pre**	**Post**	**Model 1**	**Model 2**
Homocysteine (μmol/L)	10.2 (3.4)	9.9 (3.4)	10.0 (2.4)	9.5 (2.0)	0.860	0.548
Methionine (μmol/L)	29.4 (5.7)	29.5 (6.6)	30.5 (7.0)	33.8 (10.0)	0.213	0.310
SAM (nmol/L)	74.4 (23.9)	74.3 (18.6)	72.3 (20.1)	91.8 (27.3)	0.021	0.008
SAH (nmol/L)	42.9 (8.9)	36.9 (11.5)	48.1 (9.0)	43.3 (8.8)	0.287	0.295
SAM:SAH ratio	1.73 (0.56)	1.96 (0.60)	1.58 (0.57)	2.14 (0.78)	0.192	0.182
Betaine (μmol/L)	46.7 (16.1)	48.2 (15.9)	51.6 (15.5)	53.9 (18.0)	0.854	0.777
Choline (μmol/L)	9.3 (2.7)	9.3 (2.5)	10.0 (2.61)	10.0 (2.9)	0.642	0.816
Cystathionine (nmol/L)	206 (61)	196 (73)	215 (78)	266 (114)	0.021	0.045

Values presented as mean (SD). [1] *P* value refers to time*treatment interaction (repeated measures ANOVA, comparing the effect of treatment vs placebo over time). Model 1: unadjusted, Model 2: adjusted for baseline EGRac. P<0.05 considered significant. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

4.0 Discussion

The findings of the current study report for the first time that that plasma concentrations of the one-carbon metabolites, SAM and cystathionine, increase significantly in response to riboflavin supplementation in individuals with the *MTHFR* C677T polymorphism. Coincident with this finding, we also observed lower concentrations of plasma SAM in TT compared to CC genotype adults. Indeed, after intervention with riboflavin in adults with the TT genotype, SAM concentrations increased to levels similar to those observed in adults with the CC genotype at baseline. The changes in plasma SAM and cystathionine concentrations in response to riboflavin intervention are consistent with the genotype specific BP response previously reported in response to supplementation with riboflavin, raising the possibility that the effect of this gene-nutrient interaction on BP may be influenced by the cofactor requirements.

To our knowledge, this is the first study to investigate the effect of intervention with riboflavin on SAM concentrations in adults with the *MTHFR* 677TT genotype. Previous investigations have, however, considered the effect of folic acid supplementation on one-carbon metabolites. In a small sub-group of *MTHFR* 677TT patients from the Verona Heart Study Project, 5mg/d folic acid resulted in significant increases in SAM by 13nmol/L and SAM:SAH ratio by 3.3, in addition to the expected reductions in homocysteine following 8 weeks of treatment [35]. The extent of the response in SAM of almost 20 nmol/L observed in the current study in response to riboflavin is even greater than these previous observations [35]. As the principal methyl donor, SAM-dependent methylation regulates fundamental biological processes including nuclear transcription, cell signalling, mRNA translation and DNA synthesis [12] and altered DNA methylation has previously been observed in TT relative to CC adults [36]. Supplementing with B vitamins to regulate concentrations of SAM in adults with perturbed one-carbon metabolism owing to genetic variants, could thus

potentially have important implications for CVD health outcomes. Previous studies have linked methylation with hypertension; however, no one has considered the C677T polymorphism in *MTHFR* or its relationship with SAM or BP. This is the first study to show that riboflavin supplementation in those with the mutant genotype affects concentrations of SAM and thus, possibly methylation potential. A recent meta-analysis reported lower global methylation levels with higher systolic BP, diastolic BP and hypertension [37]. The same meta-analysis also reported lower methylation levels of a number of candidate genes with increased BP; however, *MTHFR* has not yet been considered to any great extent. While hypertension was not considered in a meta-analysis by Amenyah et al., lower global methylation was reported in those with the TT genotype in combination with low folate status [38].

Choline and 5-MTHF are considered fungible methyl group sources in one-carbon metabolism, and methyl groups from choline can also facilitate homocysteine remethylation via the BHMT pathway [15]. In a study of folate-deficient males with the TT genotype, intervention with 2,200 mg/day choline over 12 weeks was found to significantly increase plasma SAM concentrations compared to lower choline doses of 300-500 mg which were associated with a decreased SAM concentration [18]. Whilst these studies investigated one-carbon nutrients, BP was not considered. To date, research examining the effect of supplementation with B vitamins on one-carbon metabolites in adults with the *MTHFR* 677TT genotype has predominantly focused on the established phenotype of elevated homocysteine. Numerous meta-analyses demonstrating the responsiveness of homocysteine to supplementation with a combination of B vitamins have been published [24,25,39]; however, other one-carbon metabolites apart from homocysteine have received little attention in studies of this nature. Studies at our Centre have previously reported that riboflavin

supplementation lowers homocysteine in TT, but not CC, individuals, although, the response of other one-carbon metabolies were not considered [9,26].

Plasma cystathionine significantly increased in response to riboflavin supplementation in the current analysis. It is possible that increased availability of SAM, an allosteric regulator of cystathionine ß-synthase (CßS), in response to riboflavin may potentially have activated CßS, thereby increasing homocysteine elimination from the one-carbon pathway and generating cystathionine [16]. In addition, riboflavin administered at the same dose as the current study (1.6 mg/day) has previously been found to improve PLP status in older adults [40] and may thus augment the activity of PLP-dependent CßS. Consistent with earlier findings reported by Midttun and colleagues [23] lower PLP concentrations were observed in the current study in participants with the TT genotype. Those with the *MTHFR* 677TT genotype have reduced affinity for their riboflavin cofactor, FAD [6], thus are likely to have an increased requirement for riboflavin. Considering that cells appear to have a tendency to spare FAD at the expense of FMN [41] it is possible that FMN-dependent pathways (such as the pathway required to convert vitamin B6 into active PLP) may be compromised in those with the mutant genotype, leading to reduced vitamin B6 metabolism and thus lower PLP concentrations.

A paucity of evidence exists with respect to investigating the impact of *MTHFR* genotype on SAM and SAH concentrations. In a cohort of Mexican-American males, Shin *et al.*, reported increased concentrations of SAH and decreased SAM:SAH ratio in those with the TT compared to the CC genotype [18]. Davis *et al.*, observed elevated SAM in young females with the TT relative to CC genotype; however this was not significant [17]. This is in contrast with the findings of the current analysis, where decreased SAM was observed in the TT compared to CC genotype group. Increased transmethylation reaction flux (i.e. conversion of SAM to SAH) has been found in females with the TT compared to the CC genotype [42]. A

number of studies have found that the TT genotype is not a determinant of SAM or SAH [17,43] but folate status appears to be an important modulator of this effect [20]. Pertubations in one-carbon metabolism can impair the synthesis of SAM, and potentially lead to epigenetic alerations (specifically aberrant DNA methylation); correspondingly global DNA hypomethylation has been previously reported in individuals with the TT compared to CC genotype [44,45]. The ratio of SAM:SAH has been proposed by some as an indicator of methylation potential, although confirmation of its validity remains to be established. Methylation regulation enzymes are differentially expressed in human tissues, leading to tissue-specific SAM and SAH regulation and therefore methylation capacity. Thus systemic SAM:SAH ratio is not necessarily a meaningful indicator of methylation potential in all tissues [14]. In the current analysis, lower SAM:SAH ratio was observed in the TT compared to CC genotype group, driven by the reduced SAM concentrations. However, these results are at odds with another study that reported the *MTHFR* genotype did not influence the ratio of SAM:SAH [43].

The observational results of the current analysis are broadly in agreement with those of the Norwegian Colorectal Cancer Prevention (NORCCAP) study, where differences in one-carbon metabolite status in individuals with the TT relative to the CC genotype were reported in 10,601 adults aged 50–64 years [21–23]. In agreement with our baseline analysis, these studies also reported the expected phenotype of elevated homocysteine, lower folate and lower PLP concentrations in the TT compared to the CC genotype. No *MTHFR* genotype effect was noted in relation to methionine, choline and cystathionine. One notable difference in the observed associations reported in the Norwegian cohort compared to the current cohort is betaine, where concentrations among Norwegians were significantly lower in those with the TT genotype compared to non-TT genotypes [22]. Betaine has been suggested as a

preferential methyl donor in TT males relative to CC males [46]; however, in our analysis no genotype effect was noted with respect to betaine.

4.1 Strengths and limitations

This is the first study to consider the effect of the MTHFR cofactor, riboflavin, on one-carbon metabolites in adults stratified by *MTHFR* genotype. Samples from a number of carefully conducted randomised controlled trials utilising identical dose, duration and study protocols were accessed. The one-carbon metabolite analysis, which is known to pose analytical challenges, was conducted at a Centre with considerable expertise in laboratory analysis of one-carbon metabolite biomarkers. Furthermore, EGRac is considered the gold standard method for measurement of long-term riboflavin status and this measure was available for all participants. One limitation of the current study is the relatively small sample size which may have limited the ability to detect small differences in certain metabolites either between genotypes or in response to riboflavin. Additional biomarker information, in particular 5-MTHF, which is generated by the MTHFR enzyme, might further add to our understanding of the role of this gene-nutrient interaction in BP regulation. The intervention could also be extended to those with *MTHFR* 677CC genotype.

5.0 Conclusion

In conclusion, this study shows evidence of perturbed one-carbon metabolism in individuals with the *MTHFR* C677T polymorphism, in particular reduced concentrations of the principal methyl donor, SAM. This study provides the first evidence that altered one-carbon flux may be alleviated through riboflavin supplementation in individuals with the C677T variant in *MTHFR*. The findings of this study may shed some light on the mechanism underpinning the elevated BP phenotype related to this gene-nutrient interaction, which, in turn could influence health outcomes in adult cohorts. Future studies investigating the effect of riboflavin and

other B vitamins on one-carbon metabolite concentrations, are needed to further explore the potential mechanisms underlying the effect of this gene-nutrient interaction on BP among individuals with the *MTHFR* 677TT genotype.

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Contributors

MR, TB and BWP conducted the analysis. GH and AMcM collected the original samples under the supervision of CH, MW, HMcN and JJS. MR analysed the data. AMcC conducted network analysis. MR wrote the initial draft of the manuscript with critical input from MW and HMcN. MW and HMcN had primary responsibility for the final content and all authors provided important revisions. All authors read and approved the final manuscript.

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Figure legends

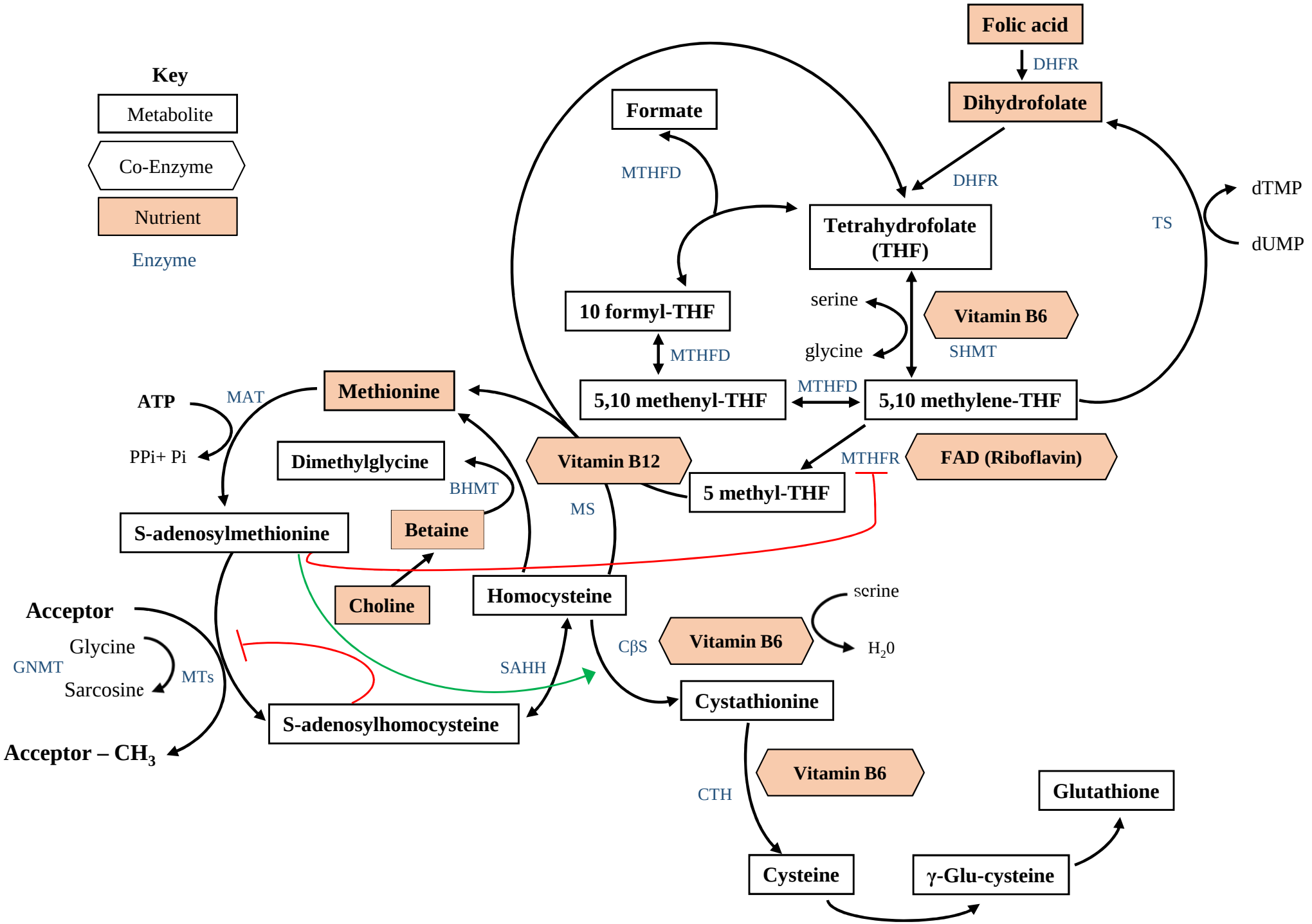
Figure 1. Overview of one-carbon metabolism. **Abbreviations:** BHMT, betaine-homocysteine methyltransferase; CβS, cystathionine-β-synthase; CTH, cystathionine γ-lyase; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FAD, flavin adenine dinucleotide; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MT, methyltransferases; MTHFR, methylenetetrahydrofolate reductase; MTHFD, methylenetetrahydrofolate dehydrogenase; SAHH, S-adenosyl homocysteine hydrolase; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase. Adapted from James *et al.* [47].

Figure 2. Flow diagram of study population. [1]CC (wild type) and TT (homozygous) genotypes for the *MTHFR* C677T polymorphism.

Figure 3. Network analysis to show interrelationships within one-carbon metabolism by *MTHFR* genotype group: CC, panel a; TT, panel b. Positive and inverse associations

indicated by green and red edges, respectively. Strength of association indicated by edge thickness. **Abbreviations:** Smk, smoking; SBP, systolic blood pressure; BMI, body mass index; Met, methionine; HCY, homocysteine; Cys, cystathionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SSr, SAM:SAH ratio; Cho, choline; Bet, betaine; B2, riboflavin; PLP, Pyridoxal-5'-phosphate; RCF, red blood cell folate.

Figure 4. Change in riboflavin biomarker (panel a), systolic BP (panel b), and diastolic BP (panel c) in response to supplementation with placebo or riboflavin (1.6mg/d) for 16 weeks. For riboflavin biomarker, a decrease in EGRac indicates an improvement in riboflavin status.


Key
Metabolite
Co-Enzyme
Nutrient
Enzyme
Folic acid
DHFR
Dihydrofolate
Formate
MTHFD
DHFR
Tetrahydrofolate (THF)
TS
dTMP
dUMP
10 formyl-THF
MTHFD
serine
glycine
Vitamin B6
SHMT
5,10 methenyl-THF
MTHFD
5,10 methylene-THF
ATP
MAT
Methionine
PPi+ Pi
Dimethylglycine
BHMT
Vitamin B12
MS
5 methyl-THF
MTHFR
FAD (Riboflavin)
S-adenosylmethionine
Betaine
Choline
Homocysteine
Acceptor
Glycine
GNMT
Sarcosine
MTs
Acceptor – CH3
SAHH
S-adenosylhomocysteine
serine
CβS
Vitamin B6
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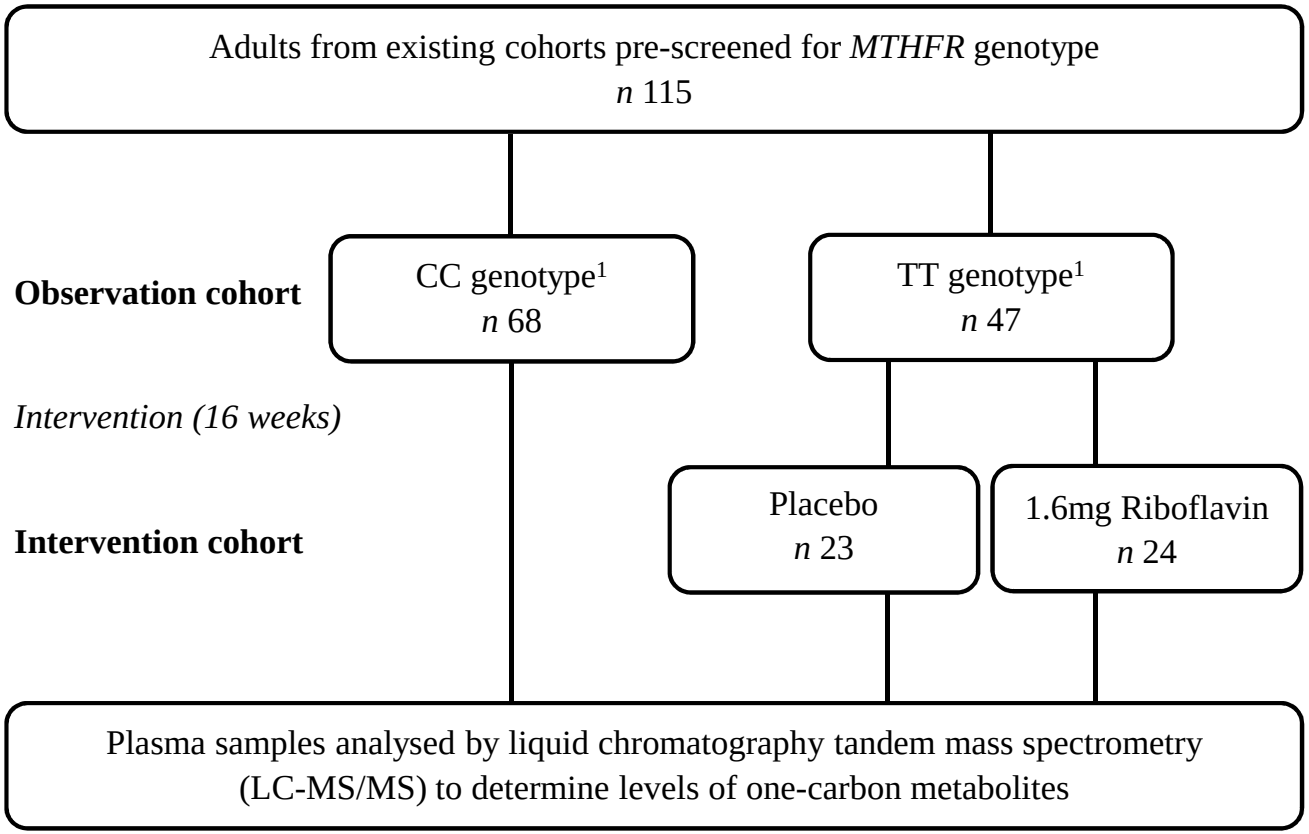
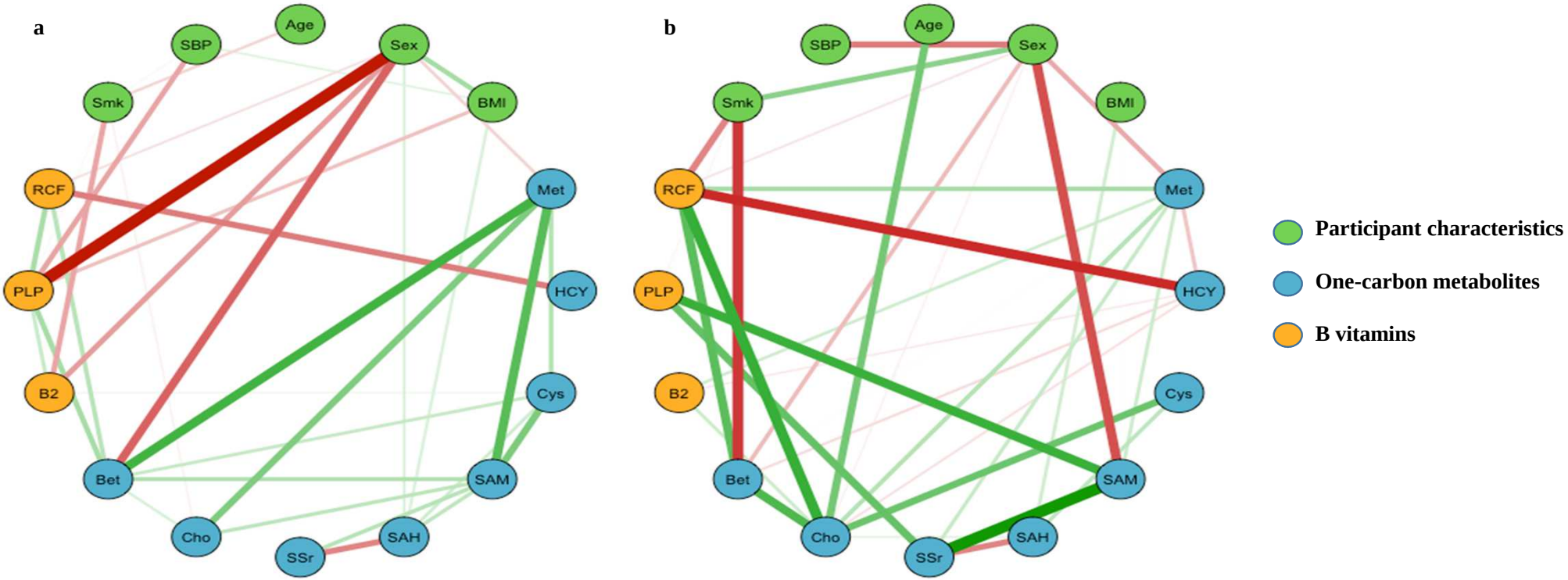
CTH
Vitamin B6
Glutathione
Cysteine
γ-Glu-cysteine


Adults from existing cohorts pre-screened for *MTHFR* genotype
n 115
Observation cohort
CC genotype[1]
n 68
TT genotype[1]
n 47
Intervention (16 weeks)
Intervention cohort
Placebo
n 23
1.6mg Riboflavin
n 24
Plasma samples analysed by liquid chromatography tandem mass spectrometry
(LC-MS/MS) to determine levels of one-carbon metabolites


a
Age
SBP
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b
Age
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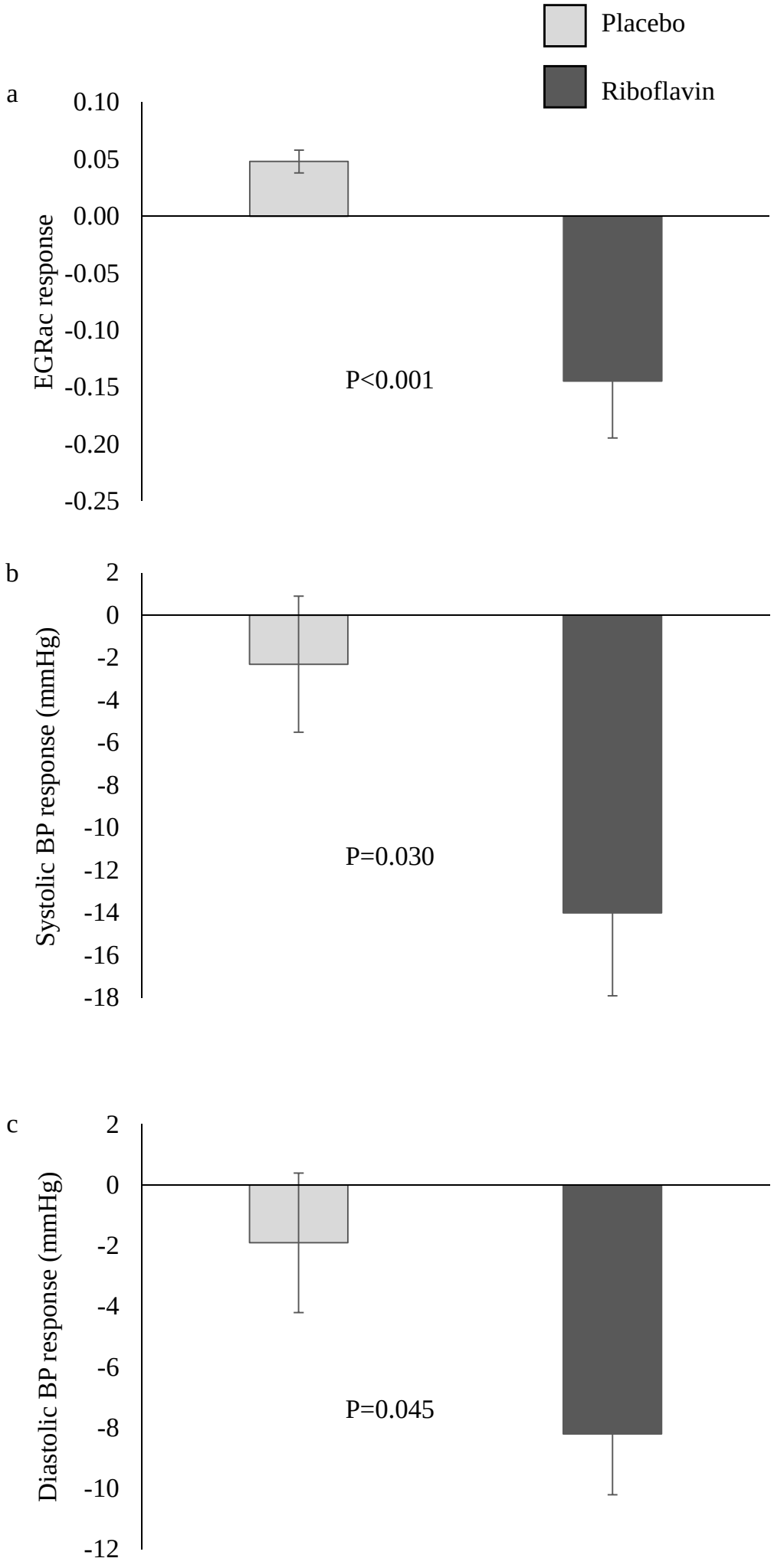
SAH
SSr
Participant characteristics
One-carbon metabolites
B vitamins


Placebo
Riboflavin
a
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
EGRac response
P<0.001
b
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
Systolic BP response (mmHg)
P=0.030
c
2
0
-2
-4
-6
-8
-10
-12
Diastolic BP response (mmHg)
P=0.045