Published in final edited form as:

Mol Genet Metab. 2007 May ; 91(1): 104–110.

Polymorphisms in Methionine Synthase Reductase and Betaine-Homocysteine S-Methyltransferase Genes: Risk of Placental Abruption

Cande V. Ananth, PhD, MPH¹, Denise Elsassner, MPH¹, Wendy L. Kinzler, MD², Morgan R. Peltier, PhD², Darios Getahun, MD, MPH¹, Daniel Leclerc, PhD³, Rima R. Rozen, PhD³, and
For the New Jersey-Placental Abruption Study Investigators

*1*Division of Epidemiology and Biostatistics, Department of Obstetrics, Gynecology, and Reproductive Sciences, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA

*2*Division of Maternal-Fetal Medicine, Department of Obstetrics, Gynecology, and Reproductive Sciences, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA

*3*Departments of Human Genetics, Pediatrics and Biology, McGill University, Montreal, Quebec, Canada

Abstract

Objective—Methionine Synthase Reductase (MTRR) and Betaine-Homocysteine S-Methyltransferase (BHMT) are 2 enzymes that regulate homocysteine metabolism. Elevated homocysteine (hyperhomocysteinemia) is associated with adverse pregnancy outcomes and vascular disease. We assessed whether polymorphisms in MTRR (66A→G; I22M) and BHMT (742G→A; R239Q) were associated with abruption. We further evaluated whether homocysteine levels differed between cases and controls for MTRR and BHMT genotypes.

Methods—Data were derived from the New Jersey Placental Abruption Study (NJ-PAS)—an ongoing, multicenter, case-control study since August 2002. Women with a clinical diagnosis of abruption were recruited as incident cases (n=196), and controls (n=191) were matched to cases based on maternal race/ethnicity and parity. Total plasma homocysteine concentrations were evaluated in a subset of 136 cases and 136 controls. DNA was genotyped for the MTRR and BHMT polymorphisms.

Results—Frequencies of the minor allele of MTRR were 40.8% and 42.2% in cases and controls, respectively (adjusted OR 0.79, 95% CI 0.45, 1.40). The corresponding rates for BHMT were 33.9% and 31.7%, respectively (adjusted OR 1.93, 95% CI 0.99, 4.09). Distributions for the homozygous mutant form of MTRR were similar between cases and controls (OR 1.18, 95% CI 0.62, 2.24). The rate of homozygous mutant BHMT genotype was 2.8-fold (OR 2.82, 95% CI 1.84, 4.97) higher in cases than controls. Stratification of analyses based on maternal race did not reveal any patterns in association.

Conclusions—In this population, there was an association between the homozygous mutant form of BHMT (742G→A) polymorphism and increased risk for placental abruption.

Correspondence and reprint requests Cande V. Ananth, PhD, MPH, Division of Epidemiology and Biostatistics, Department of Obstetrics, Gynecology, and Reproductive Sciences, UMDNJ-Robert Wood Johnson Medical School, 125 Paterson Street, New Brunswick, NJ 08901-1977, Tel: (732) 235-7940/Fax: (732) 235-6627, Email: cande.ananth@umdnj.edu

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Keywords

Placental abruption; gene-gene interaction; MTRR; BHMT; homocysteine; folate deficiency

Placental abruption, an obstetric complication in which the implanted placenta prematurely shears off from the endometrium, complicates 1 in 100 pregnancies [1;2]. It is a serious complication resulting in painful vaginal bleeding and portends high risks of an array of adverse perinatal and reproductive outcomes [3;4;5]. The etiology of abruption remains speculative [6], but women of advanced maternal age, multiparity, smokers, cocaine users, folate deficiency, hypertensive disorders, prolonged rupture of membranes, previous cesarean delivery, and those with intra-amniotic infections are at increased risk [1;5;7;8;9;10;11;12;13;14;15;16]. Women with a previous abruption are at greatest risk (over 10-fold) risk of recurrent abruption, suggesting a strong genetic etiologic mechanism [17;18].

Studies have shown that folate deficiency is associated with increased homocysteine levels [19]. In a normally functioning metabolic state, methionine produces homocysteine as an intermediate step before either transsulfuration via cystathionine into cysteine or remethylation to methionine [20]. This remethylation may be folate-dependent, or may use betaine, a metabolite of choline [21]. Methionine synthase, a vitamin B₁₂-dependent enzyme, utilizes 5-methyltetrahydrofolate as the carbon donor for folate-dependent homocysteine remethylation; methionine synthase requires activation by methionine synthase reductase (MTRR). Betaine-homocysteine methyltransferase (BHMT) utilizes betaine as the carbon donor. Therefore, the improper function of remethylation enzymes, due to mutation or to insufficient intake of relevant nutrients, may result in elevated homocysteine levels and contribute to thrombophilias, and possibly, placental abruption.

This study focuses on variants in two of the enzymes responsible for the remethylation of homocysteine, MTRR 66A→G and BHMT 742G→A [21]. While BHMT, unlike MTRR, is not directly involved in the folate-dependent pathway, experiments have shown that homocysteine flux through the BHMT pathway is enhanced when folate-dependent remethylation is disrupted [22]. Betaine can effectively lower plasma homocysteine levels and there is a significant negative correlation between plasma homocysteine and plasma betaine, as shown in men with coronary artery disease [22]. Thus, BHMT may play a critical role in the remethylation of homocysteine when the folate-dependent pathway is compromised by either genetic or dietary factors.

We hypothesized that the risk of placental abruption would be higher among women carrying the mutant allele of the MTRR and BHMT polymorphisms. We tested this hypothesis in a case-control study to examine the associations between MTRR and BHMT polymorphisms and placental abruption. We also examined if plasma homocysteine, folate and vitamin B₁₂ levels differed between cases and controls.

Material and Methods

The New Jersey—Placental Abruption Study

The New Jersey—Placental Abruption Study (NJ—PAS) is an ongoing, multicenter, case-control study of placental abruption, with subjects recruited from Saint Peter's University Hospital (since August 2002) and Robert Wood Johnson University Hospital (since July 2003) in New Brunswick, NJ. Both hospitals are tertiary, level III referral centers located within a mile of each other. Ethics approval from the institutional review boards of both hospitals, as well as that of UMDNJ-Robert Wood Johnson Medical School, NJ, was received.

Placental abruption cases and controls

Cases that were eligible for inclusion were women with a clinical diagnosis of placental abruption (at ≥ 20 weeks) before or during delivery, by the attending obstetrician. The definition of placental abruption included painful vaginal bleeding or hemorrhage accompanied by documented fetal distress, uterine pain or tenderness, or uterine hypertonicity. In the absence of any of these clinical hallmarks for abruption, if the delivered placenta showed signs of retroplacental bleeding or retroplacental hematoma/clots on the placental surface or disc, then these patients were also eligible for inclusion as potential cases. In addition, if there was an antepartum sonographic diagnosis of placental abruption, then these cases were also considered. Cases were identified by reviewing daily hospital delivery logs at both hospitals, and/or by referral by the physician, nurse, or obstetrics and gynecology residents.

Controls were identified from viewing daily delivery logs in both hospitals and were matched to cases on parity (nulliparous, primiparous, or parity ≥ 2), and maternal race/ethnicity (non-Hispanic White, non-Hispanic Black, Hispanic, or other race/ethnicity). Women with a diagnosis of placenta previa in the index pregnancy or with abruption in any of their previous pregnancies were excluded from the eligible pool of controls.

All cases and controls were recruited immediately following delivery. Upon obtaining consent, patients were approached during their postpartum period, and a structured in-person interview questionnaire was administered (lasting approximately 20 minutes) to collect details regarding maternal and paternal demographic, lifestyle, behavioral and general health conditions. Upon completing the interviews, a blood draw was completed. In addition, consent was also obtained to abstract the medical and prenatal care records from the index and all previous pregnancies and outcomes. A majority of patient contact and recruitment were completed within 24 hours, and all within 72 hours following delivery.

Polymorphisms in the MTRR and BHMT genes

Maternal peripheral blood samples were collected in 7 Vacutainer tubes (BD Vacutainer, Preanalytical Solutions, Franklin Lakes NJ), and were immediately transported to the laboratory on ice. Three tubes of blood samples were collected in Buffered Citrate solution 0.105 M, three contained potassium EDTA 7.5 mg, and one contained no additives. Plasma and serum were harvested by centrifugation of blood (at 1,500 g for 20 minutes at 4°C) within one hour after collection. Once the specimens were centrifuged, they were divided into aliquots, and frozen immediately at -70°C in preparation for subsequent analysis.

Genotypes were determined by PCR amplification and restriction digestion. Restriction sites were created through the use of an oligonucleotide in which one nucleotide was altered to generate a restriction site when found in combination with one of the tested alleles. The presence of another restriction site in the amplicon served as a positive control for digestion. Amplifications were performed in a 50 μ l reaction with 0.2 to 2 μ l of genomic DNA template, 250 ng of each specific primer, in the presence of 1.5 mM MgCl₂, 0.2 mM dNTPs and 1.25 U (0.25 μ l) Platinum Taq DNA Polymerase (Invitrogen), in the 1X Buffer recommended by the manufacturer.

For the analysis of the MTRR 66A→G (I22M) polymorphism, PCR-based amplification of a 105 bp DNA fragment was performed in the presence of the sense primer 5'-AGCAGGGACATGCAAAGGCCATCGCAGAAGACAT-3' and the antisense primer 5'-CTCTAACCTTATCGGATACACTAATA-3'. Amplification was performed by denaturing the DNA for 2 min at 94°C, followed by 35 cycles of heating at 94°C for 1 min., 58°C for 1 min., and 72°C for 2 minutes. Digestion of the PCR products with *NspI* generates a 97 bp

fragment for the A allele (a fragment of 8 bp is also generated but not visible) and a fragment of 74 bp is observed following digestion of the G allele (fragments of 23 bp and 8 bp are not visible).

For analysis of the BHMT 742G→A (R239Q) polymorphism, a 171 bp gene fragment was produced by PCR using the sense primer 5'-TGCTGGTTTCTGGTGCATCCCTAA-3' and the antisense primer 5'-AAGGGCTGACTCATCAGGTGAGCTTTGAGT-3'. Amplification involved an initial denaturation for 2 min. at 94°C, followed by 35 cycles of 1 min. 94°C, 1 min. 64°C and 2 min. 72°C. After digestion of the products with *Hinf*I, a band of 141 bp is observed on polyacrylamide gels for products containing the G allele sequences (segments of 19 bp and 11 bp are also generated but are usually not visible) and a segment of 160 bp is observed for the A allele (a fragment of 11 bp is also generated but not visible).

Biochemical analysis

Plasma was shipped in dry ice to the laboratory for a total homocysteine, folate and vitamin B₁₂ assays. Approximately 1.0 ml blood was drawn and aliquoted in EDTA tubes. The plasma was then separated and stored in Eppendorf tubes and stored at -70°C. These specimens were processed for homocysteine metabolism using the Abbott IMX technology, which is based on a fluorescence polarizing immunoassay technique [23]. Plasma folate and vitamin B₁₂ were determined using the Abbott Diagnostic IMX based on a microparticle enzyme immunoassay, as directed by the manufacturer's protocols [23]. The coefficients of variation for folate and vitamin B₁₂ were both <4%.

Statistical analysis

We examined the distributions of maternal age (<19, 19-24, 25-34, and ≥35 years), maternal education (less than high school or high school or more of completed schooling), marital status (single or married), lack of prenatal care, prepregnancy body-mass index, smoking, alcohol and cocaine use before and during pregnancy (yes/no) as well as the matching factors parity (nulliparous, primiparous, and parity ≥2), and maternal race/ethnicity (Caucasian, African-American, Hispanic, or other race/ethnicity) in relation to abortion cases and controls. Body-mass index was calculated as the ratio of weight (in kilograms) over squared-height (in meters).

Test for departure from the Hardy-Weinberg equilibrium for the MTRR and BHMT alleles was assessed by the Chi-square analysis [24]. Allele and genotype frequencies of the MTRR and BHMT polymorphisms, with bootstrap-generated 95% confidence intervals were calculated, and compared between cases and controls. The bootstrap samples were based on 10,000 replications to ensure stability in parameter estimates. We derived the unadjusted odds ratio with 95% confidence interval as a measure of effect. Given that this was a matched case-control study by design, all preliminary analyses were based on a matched analysis. However, since the results of these matched analyses did not differ from those based on an unmatched analysis (not shown), we report the results of the unmatched analysis.

Distributions of total plasma concentrations of total homocysteine, folate and vitamin B₁₂ were compared between cases and controls. To meet the assumptions of normality and equal variances in the distributions of these analyses between cases and controls, the Box-Cox transformation was applied by allowing λ , the transformation parameter, to range between -4 and 4 in 0.05 increments [25]. The optimally transformed variables were then fitted to a general linear model (dependent variable). Differences in the (transformed) analytes were compared between cases and controls, as well as within allele and genotype combinations of MTRR and BHMT.

To address bias due to confounding, we adjusted the associations between the MTRR and BHMT polymorphisms and abruption for several potential confounders through a multivariable logistic regression analysis. These confounders included, in addition to parity and maternal race/ethnicity (matching factors), maternal age, marital status, lack of prenatal care, smoking during pregnancy, and prepregnancy body mass index. In addition, we also examined gene-gene interactions in the MTRR and BHMT polymorphisms and risk of placental abruption. For analysis pertaining to plasma homocysteine, folate and vitamin B₁₂, we fitted multivariable linear regression models to assess differences in these analytes between cases and controls after adjusting for confounders (listed above). Finally, the entire analysis was replicated after stratification by maternal race/ethnicity.

Results

Of a total recruitment of 202 cases and 197 controls, 196 cases and 191 controls had complete information on the 2 polymorphisms, and of these cases and controls, homocysteine, folate and vitamin B₁₂ assays were completed in 137 cases and 134 controls. The distribution of maternal age, parity, race/ethnicity, marital status, and pre-pregnancy maternal body-mass index were similar between cases and controls (Table 1). Cases were more likely to smoke than controls. Total plasma homocysteine, folate and vitamin B₁₂ levels were similar between cases and controls.

The minor allele (G) frequencies of MTRR (66A→G) in abruption cases and controls were 40.8% and 42.2%, respectively (Table 2). In comparison to the A/A genotype of MTRR, the frequency of the mutant form (G/G) was 21.4% and 20.4% in cases and controls, respectively. Adjustment for confounders did not reveal any association of MTRR polymorphism and abruption risk. Frequencies of the minor allele (A) of BHMT were 33.9% and 31.7% in cases and controls, respectively. In comparison to women carrying the G/G genotype of BHMT, the mutant form (A/A) was strongly associated with increased risk of abruption (OR 2.82, 95% CI 1.84, 4.97).

We examined the joint association between MTRR and BHMT polymorphisms and risk of abruption (Table 3). Homozygotes for the BHMT mutant allele (A/A) were associated with increased risk for abruption with the wild-type (A/A) and heterozygous (A/G) forms of the MTRR polymorphism. There did not appear to be any increased risk for abruption when mutant genotypes for both MTRR and BHMT were present in combination, although the numbers in this group were limited. Women carrying the wild-type form of MTRR (A/A) but with the mutant form of BHMT (A/A) were at significantly increased risk for abruption (OR 4.80, 95% CI 1.19, 19.41). Similarly, women carrying the heterozygous mutant form of MTRR (A/G) and the homozygous form of BHMT (A/A) were also at increased risk for abruption (OR 2.44, 95% CI 1.03, 8.40).

Differences in total homocysteine, folate and vitamin B₁₂ concentrations between cases and controls were examined by genotypes of MTRR and BHMT mutations (Table 4). Among women carrying the wild-type form of MTRR (A/A), homocysteine concentrations were significantly lower in cases than controls. Women carrying the wild-type and heterozygous mutant form of BHMT (G/G and G/A) had higher levels of homocysteine in cases than controls.

Comment

These data suggest that the homozygous mutant form of the BHMT polymorphism (742G→A) was associated with increased risk of abruption. While the distribution of the MTRR (66A→G) genotypes was similar between abruption cases and controls, women carrying the homozygous

mutant genotype of BHMT (A/A) and either the wild-type (A/A) or heterozygous form (A/G) of MTRR carried increased risk of abruption. Our study is the first to report the association between the BHMT mutation and abruption risk.

The BHMT mutation is found in exon 6 of the BHMT gene at nucleotide position 742. This mutation leads to the substitution of an arginine to a glutamine residue (742G→A) at amino acid position 239.[27;28] It has been suggested that altered functioning of BHMT may result in elevated homocysteine levels. Given the underlying thrombotic phenomenon of abruption, we hypothesized that the MTRR and BHMT mutations might influence this phenotypic disease state through an elevation in homocysteine. Homocysteine, a natural byproduct of normal metabolism, is associated with inflammatory responses. Hyperhomocysteinemia has been shown to be associated with vascular thrombotic disease states [29;30;31]. Cases with the wild-type and heterozygous BHMT genotypes (G/G and G/A) showed higher homocysteine levels than the controls with the same genotypes (Table 4). This was not observed in the comparison of cases and controls with the homozygous mutant BHMT genotype (A/A), although the number of subjects with the A/A genotype is low. It is not surprising that cases have higher homocysteine levels than controls, although it remains puzzling as to why this pattern was evident only with certain subgroups in our study. Despite these modest differences in plasma homocysteine, it is important to note that the overall homocysteine levels in this study are low, and the folate levels high, due to folate fortification of food in the United States. Nonetheless, since the bloods were procured in the non-fasted state, at various times of the day, the biochemical measurements must be interpreted with caution.

We also examined the interaction of the MTRR and BHMT polymorphisms in relation to placental abruption. The risk of abruption was most highly associated with the homozygous mutant BHMT genotype, in women with the wild-type and heterozygous forms of the MTRR genotypes. The number of women with homozygous mutant genotypes for both MTRR and BHMT was low; there was no association with abruption in this group. Whether the observed association with BHMT is causative or if it merely serves as a marker of impending risk for thrombotic events, remains unclear.

Limitations and strengths

The findings of our study must be interpreted with some caution owing to certain limitations. Despite being statistically significant, the fairly wide confidence intervals of effect measures warrant some caution. Although our study had sufficient statistical power for overall analysis, the stratified analysis by maternal race (not shown) may have been underpowered. This study was designed to account for population stratification by matching all abruption cases and controls by maternal race/ethnicity. Moreover, the distribution of race categories in this study favors more of the high-risk patients (higher proportions of African-American and Hispanic patients) insofar as abruption risk is concerned [1]. All laboratory personnel were blinded to case-control status, so the potential for a diagnostic bias is unlikely. These analyses also incorporate careful adjustments for several confounders through a multivariable logistic regression.

Conclusions

In summary, this study demonstrates an association between the mutant BHMT enzyme and increased risk for abruption. Future studies may benefit from evaluating these associations from fetal DNA, as well an examination of an interaction between maternal and fetal genotypes in the BHMT mutation, on the risk of abruption. An examination of the interaction of the BHMT polymorphism with other genetic variants and with other environmental modifiers would be useful in understanding the heterogeneous etiology of placental abruption.

ACKNOWLEDGMENTS

This research was funded by the National Institutes of Health (HD038902) awarded to Dr. Ananth. Dr. Peltier was supported through the NIH-Loan Repayment Program and through a Foundation of UMDNJ research support. The authors thank Susan Fosbre for help during the preparation of the manuscript.

APPENDIX

Investigators currently participating or who have been involved in the New Jersey— Placental Abruptio Study include Cande V. Ananth, PhD, MPH (Principal investigator), Darios Getahun, MD, MPH, Neela Srinivas, MD, MPH, Celeste DeMarco, RN, BSN, Denise Elsasser, MPH, Yu-Ling Lai, RN and Shelby Pitts, RN (Division of Epidemiology and Biostatistics), John C. Smulian, MD, MPH, Wendy L. Kinzler, MD, Morgan R. Peltier, PhD, and Marian Lake, RN, MPH (Division of Maternal-Fetal Medicine), Department of Obstetrics, Gynecology, and Reproductive Sciences; Claire Philipp, MD (Department of Medicine), all at UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ; and George G. Rhoads, MD, MPH (Department of Epidemiology) and Dirk F. Moore, PhD (Department of Biostatistics), UMDNJ-School of Public Health, Piscataway, NJ.

Other investigators that were involved with the study included Rima R. Rozen, PhD and Jacques Genest, MD (McGill University, Montreal, Canada); Susan Shen-Schwarz, MD (Department of Pathology, Saint Peter's University Hospital, New Brunswick, NJ); and Vinay Prasad, MD (Department of Pediatric Pathology, Arkansas Children's Hospital, University of Arkansas Medical Sciences, Little Rock, AR)

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Table 1
Distribution of placental abruption cases and controls according to selected maternal characteristics: The New Jersey Placental Abruption Study

Maternal characteristics	Abruptio cases		Controls		P-value
	n	%	n	%	
Study site					
Saint Peter's UH	87	44.4	56	29.3	0.002
Robert Wood Johnson UH [†]	109	55.6	135	70.7	
Maternal age (years) [‡]					0.605
<19	5	2.6	4	2.1	
19-24	32	16.3	28	14.6	
25-34	98	50.0	97	50.8	
≥35	61	31.1	62	32.5	0.868
Parity					
Nulliparous	71	36.2	69	36.1	
Primiparous	70	35.7	66	34.6	
Parity ≥2	55	28.1	56	29.3	0.760
Maternal race/ethnicity					
Caucasian	65	33.2	74	38.7	
African-American	35	17.9	30	15.7	
Hispanic	64	32.7	59	30.9	0.034
Other race/ethnicity	32	16.3	28	14.7	
No prenatal care	11	5.6	3	1.6	
Education below high school	83	42.3	62	32.5	
Single marital status	64	32.7	51	26.7	0.045
Smoking during pregnancy	21	10.7	7	3.7	
Multiple births (twin and triplet)					
Pre-pregnancy BMI [‡]					
Pre-pregnancy BMI [‡]		25.0 ± 5.8		24.7 ± 5.2	0.627
Gestational age (weeks) [‡]		32.8 ± 5.3		37.7 ± 2.7	
Plasma homocysteine (μmol/L) [‡]		5.7 ± 2.3		5.5 ± 2.1	
Plasma folate (nmol/L) [‡]		41.5 ± 17.1		43.7 ± 16.8	
Plasma vitamin B ₁₂ (pmol/L) [‡]		267 ± 118		247 ± 99	0.158

UH, University hospital; BMI, body-mass index Analyses pertaining to homocysteine, folate and vitamin B12 assays were based on 136 abruption cases and 136 controls

[†] Recruitment of patients at this center was initiated in 2003

[‡] Data are expressed as mean (standard deviation)

Table 2
Allele and genotype frequencies of MTRR and BHMT mutations and associations with placental abruption: The New Jersey Placental Abruption Study

	Abruption cases (n=196)		Controls (n=191)		Odds ratio (95% confidence interval)	
	n	%	n	%	Unadjusted	Adjusted†
MTRR (66A→G)						
Allele frequency						
A	116	59.2	100	57.8	1.00 (Reference)	1.00 (Reference)
G	80	40.8	81	42.2	0.94 (0.58, 1.54)	0.79 (0.45, 1.40)
Genotype frequency						
A/A	78	39.8	69	36.1	1.00 (Reference)	1.00 (Reference)
A/G	76	38.8	83	43.5	0.81 (0.52, 1.27)	0.89 (0.53, 1.47)
G/G	42	21.4	39	20.4	1.04 (0.61, 1.81)	1.18 (0.62, 2.24)
BHMT (742G→A)						
Allele frequency						
G	130	66.1	130	68.3	1.00 (Reference)	1.00 (Reference)
A	66	33.9	61	31.7	1.50 (0.78, 2.87)	1.93 (0.99, 4.09)
Genotype frequency						
G/G	88	44.9	87	45.5	1.00 (Reference)	1.00 (Reference)
G/A	83	42.4	87	45.6	0.94 (0.62, 1.44)	0.90 (0.56, 1.45)
A/A	25	12.8	17	8.9	1.46 (0.74, 2.88)	2.82 (1.84, 4.97)

P-values for the Hardy-Weinberg equilibrium test for cases and controls were 0.008 and 0.139, respectively, for the MTRR and 0.432 and 0.614, respectively, for the BHMT polymorphisms. Odds ratios were adjusted for study site, maternal race/ethnicity, parity, maternal age, education, marital status, pre-pregnancy body-mass index and smoking during pregnancy through multivariable logistic regression.

Gene-gene interactions between MTRR and BHMT mutations and risk of placental abruption: The New Jersey Placental Abruption Study

Table 3

Genotypes		Placental abruption: (%)		Odds ratio (95% confidence interval)	
MTRR (66A→G)	BHMT (742G→A)	Cases (n=196)	Controls (n=191)	Unadjusted	Adjusted [†]
A/A	G/G	16.3	18.9	1.00 (Reference)	1.00 (Reference)
A/A	G/A	18.4	15.2	1.40 (0.71, 2.76)	1.62 (0.76, 3.49)
A/A	A/A	5.1	2.1	2.81 (0.80, 9.84)	4.80 (1.19, 19.41)
A/G	G/G	18.4	18.3	1.16 (0.60, 2.25)	1.48 (0.69, 3.15)
A/G	G/A	15.3	20.9	0.84 (0.43, 1.65)	0.91 (0.42, 1.96)
A/G	A/A	5.1	4.2	1.41 (0.50, 4.00)	2.44 (1.03, 8.40)
G/G	G/G	10.2	18.4	1.41 (0.62, 3.17)	2.14 (0.84, 5.46)
G/G	G/A	18.6	9.4	1.06 (0.47, 2.40)	1.38 (0.53, 3.59)
G/G	A/A	2.6	2.6	1.13 (0.30, 4.25)	1.23 (0.28, 5.41)

[†] Odds ratios were adjusted for study site, maternal race/ethnicity, parity, maternal age, education, marital status, pre-pregnancy body-mass index and smoking during pregnancy through multivariable logistic regression

Table 4
Distribution (mean $\pm$ standard deviation) of total plasma homocysteine, folate, and vitamin B₁₂ among abruption cases and controls by genotypes of the MTRR and BHMT polymorphisms

	Plasma homocysteine (μ mol/L)			Plasma folate (nmol/L)			Plasma vitamin B ₁₂ (pmol/L)		
	Cases (n=136)	Controls (n=136)	P-value [‡]	Cases (n=136)	Controls (n=136)	P-value [‡]	Cases (n=136)	Controls (n=136)	P-value [‡]
MTRR (66A→G)									
A/A	5.9 $\pm$ 2.1	6.1 $\pm$ 2.3	0.011	42.9 $\pm$ 16.5	43.7 $\pm$ 15.7	0.300	303 $\pm$ 138	247 $\pm$ 94	<0.001
A/G	5.6 $\pm$ 2.0	5.7 $\pm$ 1.5	0.389	42.5 $\pm$ 16.7	45.4 $\pm$ 14.8	0.117	264 $\pm$ 132	267 $\pm$ 108	0.664
G/G	6.2 $\pm$ 2.7	5.5 $\pm$ 2.8	0.471	42.0 $\pm$ 15.3	41.2 $\pm$ 16.1	0.163	245 $\pm$ 122	220 $\pm$ 76	0.342
P-value [‡]	0.587	0.077		0.930	0.470		0.043	0.553	
BHMT (742G→A)									
G/G	6.0 $\pm$ 2.1	5.6 $\pm$ 2.4	0.031	41.5 $\pm$ 17.9	42.3 $\pm$ 15.1	0.002	260 $\pm$ 145	238 $\pm$ 96	0.921
G/A	5.9 $\pm$ 2.5	5.5 $\pm$ 1.7	<0.001	43.1 $\pm$ 14.6	44.8 $\pm$ 15.3	0.715	306 $\pm$ 135	271 $\pm$ 106	<0.001
A/A	4.9 $\pm$ 1.5	5.7 $\pm$ 2.1	0.089	44.2 $\pm$ 16.0	51.1 $\pm$ 16.6	0.006	245 $\pm$ 60	219 $\pm$ 43	0.441
P-value [‡]	0.051	0.736		0.404	0.123		0.844	0.333	

Differences in homocysteine, folate and vitamin B₁₂ values between cases and controls were adjusted for study site, year recruited to study, maternal race/ethnicity, parity, maternal age, education, marital status, pre-pregnancy body-mass index and smoking during pregnancy through multivariable linear regression models

[‡]Tests of significance were performed after Box-Cox transformations were applied to homocysteine, folate, and vitamin B₁₂ concentrations

[‡]P-values correspond to a test of linear trend in analyses across genotype