Research Article

Gene-Gene Interactions in the Folate Metabolic Pathway and the Risk of Conotruncal Heart Defects

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Conotruncal and related heart defects (CTRD) are common, complex malformations. Although there are few established risk factors, there is evidence that genetic variation in the folate metabolic pathway influences CTRD risk. This study was undertaken to assess the association between inherited (i.e., case) and maternal gene-gene interactions in this pathway and the risk of CTRD. Case-parent triads (n = 727), ascertained from the Children’s Hospital of Philadelphia, were genotyped for ten functional variants of nine folate metabolic genes. Analyses of inherited genotypes were consistent with the previously reported association between MTHFR A1298C and CTRD (adjusted $P = .02$), but provided no evidence that CTRD was associated with inherited gene-gene interactions. Analyses of the maternal genotypes provided evidence of a MTHFR C677T/CBS 844ins68 interaction and CTRD risk (unadjusted $P = .02$). This association is consistent with the effects of this genotype combination on folate-homocysteine biochemistry but remains to be confirmed in independent study populations.

1. Introduction

Congenital heart defects (CHDs) are a heterogeneous group of malformations with a birth prevalence that approaches 1 per 100 [1]. In addition to being the most common type of structural birth defect, CHDs have a major impact on pediatric morbidity and mortality [2]. Although CHDs can occur in association with several known teratogenic (e.g., anticonvulsants and maternal pregestational diabetes) and genetic (e.g., 22q11 deletion and Alagille) syndromes [3, 4], most CHDs (approximately 80%) appear to be nonsyndromic [5] and are thought to have a complex etiology involving interactions between several factors. However, relatively little is known about the specific risk factors for non-syndromic CHDs, and there are currently no strategies for reducing the public health impact of these conditions [3].

The identification of CHD risk factors is complicated by several issues. For example, since CHDs develop during gestation, studies aimed at identifying genetic risk factors should consider the effects of both the maternal genotype and the inherited genotype (i.e., the genotype inherited by the case) [6]. Further, as CHDs are complex conditions, the identification of risk factors may require the simultaneous assessment of multiple factors (e.g., gene-gene interactions). In addition, since CHDs are a heterogeneous group of conditions, analyses may need to be restricted to subgroups of phenotypes that are likely to be relatively homogeneous [7].

The present study was undertaken to extend our studies of the relationship between conotruncal and related heart defects (CTRD), which are thought to comprise a relatively homogeneous subset of CHDs, and variation within genes in the folate metabolic pathway. This pathway was selected for analysis given evidence that, similar to neural tube defects, the risk of CHDs in general, and of CTRD in particular, is influenced by maternal folate status (reviewed in [3]), as well as variation within genes that comprise the folate metabolic pathway [8–11]. As there are few other clues regarding the causes of non-syndromic CTRD, additional studies of the association between genetic variation within the folate...
metabolic pathway and the risk of CTRD are strongly warranted. Moreover, confirmation of an association between CTRD and the folate metabolic pathway would suggest potential, targeted risk reduction strategies (e.g., women with high-risk genotypes could be targeted for interventions aimed at increasing folic acid supplementation). We have previously analyzed these data using a sequential (i.e., SNP-by-SNP) approach to assess associations between CTRD and both the maternal genotype and the inherited genotype. Here, we summarize additional analyses that consider potential maternal and inherited gene-gene interaction effects, as well as heterogeneity in the effect of the inherited genotype across the CTRD component phenotypes.

2. Materials and Methods

2.1. Study Subjects. Details of this family-based, case-parent triad study have been provided previously [8]. Briefly, individuals with a CTRD were ascertained through the clinical practices of the Children’s Hospital of Philadelphia between 1997 and 2007. Patients of either sex and of any race/ethnicity, with a diagnosis of tetralogy of Fallot (TOF), D-transposition of the great arteries (D-TGA), double outlet right ventricle (DORV), truncus arteriosus (TA), interrupted aortic arch (IAA), conoventricular or posterior malalignment type ventricular septal defect (VSD), or an isolated aortic arch anomaly (AAA) were eligible to be cases in this study. Potential subjects who had a recognized syndrome, including the 22q11 deletion syndrome, were excluded so as to reduce etiologic heterogeneity among the cases.

Blood samples were collected from cases prior to a blood transfusion at the time of surgical or other interventions. Blood, buccal, or saliva samples were collected from each participating parent, however, participation of both parents was not required [12].

2.2. Laboratory Methods. DNA, regardless of sample type, was extracted using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s protocol. Ten polymorphisms from nine genes in the folate metabolic pathway, including eight single nucleotide polymorphisms: BHMT G742A (rs3733890), MCP1 A251G (rs1024611), MTHFR C677T (rs1801133), MTHFR A1298C (rs1801131), MTR A2756G (rs1805087), MTRR A66G (rs18013940), SHMT C1420T (rs1979277), TCN2 C777G (rs1801198) and two insertion/deletion alleles: CBS 844ins68 and DHFR intron 1 19-base pair deletion, were genotyped as previously described [8]. Each of these variants has been shown to influence the function of the resulting gene product [13–22]. Genotyping of single nucleotide polymorphisms was conducted in the High-Throughput Genotyping Core Laboratory at the Molecular Diagnosis and Genotyping Facility at the University of Pennsylvania, using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, California). The insertion/deletion polymorphisms were genotyped using published polymerase chain reaction-based assays [8, 23] and visualized on agarose gels.

2.3. Statistical Methods. All statistical analyses included a single case per family (e.g., in a family with two affected offspring, one child was randomly selected to serve as the case and the other child was not included in the study). None of the cases were known to be related. Case and parental characteristics were summarized using counts and proportions. For each analyzed genetic variant, the proportion of samples for which a genotype could not be assigned, the proportion of samples that yielded discrepant results on repeated genotypes, and the proportion of triads that had genotype combinations that were incompatible with Mendelian inheritance were determined. For each sample, the number of genotyping failures (i.e., genotypes that could not be assigned or were discrepant across repeated genotypes) was determined. These analyses were performed using SAS version 9.1 (SAS Institute Inc).

Multifactor dimensionality reduction-phenomics (MDR-P) [24] was used to assess the association between inherited gene-gene interactions and CTRD and to determine whether the association was influenced by heterogeneity across the seven specific CTRD component phenotypes included in the case definition [24], MDR-P uses a permutation method to estimate P-values that are adjusted for multiple testing. All one-, two-, three-, and four-locus models derived from the ten genotyped folate metabolic genetic variants were assessed. These analyses were implemented with the computer program MDR-Phenomics [24], using data from triads with complete genotype data for all variants included in a given model.

Given that MDR-P uses information on the transmission of alleles from parents to an affected offspring to assess associations, this method cannot be used to assess maternal gene-gene interactions in case-parent triad data. Consequently, a case-only approach was used to assess maternal gene-gene interactions [25]. Specifically, for case mothers, all pairwise gene-gene combinations were assessed (e.g., BHMT G742A/MCP1 A251G, BHMT G742A/MTHFR C677T). Chi-square tests were used to obtain unadjusted P-values. All case-only analyses were restricted to data from white mothers due to the potential for population stratification bias [26]. These analyses were performed using SAS version 9.1 (SAS Institute Inc).

Maternal gene-gene combinations that were associated with CTRD in the case-only analyses (i.e., unadjusted P < .05) were investigated using log-linear models for joint effects in order to obtain effect estimates [27]. To test the no-interaction null hypothesis, we calculated a 2-degree-of-freedom likelihood ratio test (LRT) statistic as twice the difference of the log likelihoods for the log-linear model that included two parameters indexing the inherited genotype for SNP1, two parameters indexing the maternal genotype for SNP1, and two interaction terms representing the product of maternal SNP1-SNP2 pairwise genotypes and a reduced model that excluded the interaction terms. These analyses were run using LEM [28], a program for log-linear
Table 1: Characteristics of study cases and parents, Children's Hospital of Philadelphia, 1997–2007.

| Characteristic                     | Total (%) (n = 727) |
|------------------------------------|---------------------|
| Offspring sex                      |                     |
| Male                               | 430 (59.2)          |
| Female                             | 297 (40.8)          |
| Race/ethnicity (parental mating pairs) |                 |
| White                              | 525 (72.2)          |
| Black                              | 74 (10.2)           |
| Hispanic                           | 22 (3.0)            |
| Asian                              | 20 (2.8)            |
| Mixed                              | 86 (11.8)           |
| Primary diagnosis                  |                     |
| Tetralogy of Fallot (TOF)          | 279 (38.4)          |
| D-transposition of the great artery (D-TGA) | 152 (20.9) |
| Ventricular septal defect (VSD)*    | 146 (20.1)          |
| Double outlet right ventricle (DORV)| 72 (9.9)           |
| Isolated aortic arch anomaly (AAA) | 38 (5.2)            |
| Truncus arteriosus (TA)            | 21 (2.9)            |
| Interrupted aortic arch (IAA)      | 19 (2.6)            |

*Conoventricular or posterior malalignment type ventricular septal defect; coarctation of the aorta was present in 16 of the case individuals with a VSD.

analysis with missing data that allows information from triads that have not been completely genotyped (e.g., father not available) to be included in the analysis for any given variant [12]. To reduce concerns regarding possible mating stratification bias [26, 29], only data from triads in which both parents were reported to be white were used in these analyses. Given the exploratory nature of these analyses, both unadjusted and adjusted (i.e., Bonferroni corrected) P-values are presented.

3. Results

Details of the study sample have been provided elsewhere [8]. Briefly, there were 727 case-parent triads in which the case individual had a CTRD (Table 1). The most common diagnoses among the cases were tetralogy of Fallot (38.4 percent), D-transposition of the great arteries (20.9 percent), and ventricular septal defect (20.1 percent). There was a predominance of males among the cases (59.2 percent), and the majority of parents were reported to be white (72.2 percent).

DNA samples were available for 1991 members of the study triads (i.e., 537 complete triads and 190 case-parent pairs). Genotype call rates for these 1991 samples ranged from 96% to 98% for each variant; the proportion of samples that provided discrepant results on repeat genotypes ranged from 0% to 0.8%; and the proportion of triads with genotype combinations that were incompatible with Mendelian inheritance ranged from 0.7% to 2.5% (n = 5–19 families) per variant. On the basis of these results, all of the genotypes were considered to be of sufficiently high quality to include in the subsequent statistical analyses.

However, all genotype data from families that included at least one genotype combination that was incompatible with Mendelian inheritance were omitted from all analyses (n = 225 samples from 75 triads with a Mendelian inconsistency for one or more variant). In addition, all genotype data from individual samples that failed (i.e., no genotype called) or provided discrepant results on repeat genotyping for four or more of the genotyped variants were omitted from all analyses (n = 55 samples). After the above-mentioned exclusions, 652 families (90%) were available for analysis, and the number of useable genotypes for each of the variants ranged from 1685 to 1715.

In our previous SNP-by-SNP analyses of these data [8], three of the ten folate metabolic variants were found to be associated with CTRD at P (unadjusted) < .05: MTR A2756G (maternal effect, P = .04), CBS 844ins68 (inherited effect, P = .05), and MTHFR A1298C (inherited effect, P = .002). However, only the association with the inherited MTHFR A1298C genotype remained significant when the false discovery rate was controlled at 0.05 (unadjusted P = .0021 < .0025).

MDR-P was used to evaluate inherited gene-gene interactions and heterogeneity across the seven CTRD component phenotypes. Using this approach, the only model achieving significance was the one-locus model for the MTHFR A1298C variant (adjusted P = .02). There was no evidence of heterogeneity in the association of this variant across the seven CTRD component phenotypes, and no other one-, two-, three-, or four-locus model had an adjusted P value < .05 (Table 2).

The case-only approach was used to assess the association of CTRD with maternal gene-gene interactions (Table 3). Using this approach, two maternal gene-gene combinations were associated with CTRD with unadjusted P values less than .05: MTHFR C677T/CBS 844ins68 (unadjusted P = .01) and MTHFR A1298C/CBS 844ins68 (unadjusted P = .02). Based on the data presented in Table 4, the MTHFR 677 TT genotype is over-represented and the MTHFR 1298 CC genotype is under-represented among case-mothers with the CBS NN genotype. As these two MTHFR variants are in strong linkage disequilibrium [8, 30, 31], and given prior evidence that homocysteine levels are influenced by a MTHFR C677T/CBS 844ins68 interaction [32], only the MTHFR C677T/CBS interaction was evaluated using log-linear analyses.

In the log-linear analyses, the maternal CBS 844ins68 IN and II genotypes were combined, due to the small number of II genotypes (n = 4), and the two resulting categories (NN versus IN + II) were used to stratify the data. An unrestricted model, which provides effect estimates for heterozygotes and for rare homozygotes, was fitted to the maternal MTHFR C677T genotype data. For these analyses, data from mothers who were genotyped for the MTHFR C677T but not the CBS 844ins68 variant were excluded (n = 2). Mothers who were CBS NN and MTHFR 677 TT had a 1.85-fold (95 percent confidence interval: 1.13, 3.02) higher risk of having a child with a CTRD as compared to those who were CBS NN and MTHFR 677 CC. This association was not seen in mothers with the CBS IN or II genotypes (Table 5). The unadjusted
Table 2: MDR-P results (adjusted \( P \) values) for all 2-locus models of the inherited genotype and CTRD, Children’s Hospital of Philadelphia, 1997–2007.

| BHMT G742A 844ins68 | CBS 844ins68 | DHFR A251G | MCP1 A1298C | MTHFR C677T | MTHFR A1298C | MTR A2756G | MTRR A66G | SHMT C1420T | TCN2 C777G |
|----------------------|-------------|------------|-------------|-------------|-------------|------------|----------|-----------|-----------|
| BHMT G742A           | 0.75        | 0.27       | 0.69        | 0.43        | 0.18        | 0.50       | 0.68     | 0.92      | 0.57      |
| CBS 844ins68         | 0.40        | 0.38       | 0.59        | 0.09        | 0.48        | 0.48       | 0.45     | 0.45      | 0.63      |
| DHFR 19-bp del       | 0.92        | 0.60       | 0.72        | 0.82        | 0.28        | 0.26       | 0.28     | 0.26      | 0.86      |
| MCP1 A251G           | 0.58        | 0.83       | 0.60        | 0.53        | 0.47        | 0.47       | 0.47     | 0.47      | 0.67      |
| MTHFR C677T          | N/A*        | 0.30       | 0.60        | 0.43        | 0.10        |            |          |           |           |
| MTHFR A1298C         | 0.13        | 0.23       | 0.13        | 0.32        | 0.57        | 0.13       | 0.74     | 0.13      | 0.32      |
| MTR A2756G           | 0.57        | 0.13       | 0.32        | 0.14        | 0.17        |            |          |           |           |
| MTRR A66G            | 0.88        | 0.18       | 0.93        | 0.43        | 0.10        |            |          |           |           |

* This interaction was not assessed as the two MTHFR variants are in strong linkage disequilibrium.

Table 3: Case-only results (unadjusted \( P \) values) for all pairwise combinations of maternal genotypes and CTRD, Children’s Hospital of Philadelphia, 1997–2007.

| BHMT G742A 844ins68 | CBS 844ins68 | DHFR A251G | MCP1 A1298C | MTHFR C677T | MTHFR A1298C | MTR A2756G | MTRR A66G | SHMT C1420T | TCN2 C777G |
|----------------------|-------------|------------|-------------|-------------|-------------|------------|----------|-----------|-----------|
| BHMT G742A           | 0.95        | 0.64       | 0.30        | 1.15        | 0.50        | 0.43       | 0.38     | 0.26      | 0.92      |
| CBS 844ins68         | 0.28        | 0.98       | 0.84        | 0.48        | 0.92        | 0.48       | 0.27     | 0.26      | 0.36      |
| DHFR 19-bp del       | 0.95        | 0.98       | 0.59        | 0.14        | 0.81        | 0.48       | 0.14     | 0.14      | 0.17      |
| MCP1 A251G           | 0.71        | 0.65       | 0.64        | 0.57        | 0.83        | 0.48       | 0.47     | 0.47      | 0.97      |
| MTHFR C677T          | N/A*        | 0.97       | 0.41        | 0.57        | 0.57        | 0.48       | 0.47     | 0.47      | 0.58      |
| MTHFR A1298C         | 0.57        | 0.14       | 0.14        | 0.14        | 0.14        | 0.14       | 0.14     | 0.14      | 0.74      |
| MTR A2756G           | 0.42        | 0.57       | 0.97        | 0.48        | 0.57        | 0.48       | 0.47     | 0.47      | 0.57      |
| MTRR A66G            | 0.92        | 0.28       | 0.43        | 0.43        | 0.43        | 0.43       | 0.43     | 0.43      | 0.43      |

* This interaction was not assessed as the two MTHFR variants are in strong linkage disequilibrium.

4. Discussion

We have previously reported that the inherited MTHFR A1298C genotype is associated with the risk of CTRD, and others have observed a similar association in CHD samples including, but not limited to CTRD [8, 9]. Our results using MDR-P are consistent with these previous findings and provide evidence that this association is similar...
across the seven component CTRD phenotypes (TOF, DTGA, VSD, DORV, AAA, TA, and IAA). However, based on these analyses, there is no evidence that the inherited MTHFR A1298C genotype influences the risk of CTRD via interactions with the other measured genotypes, or that other measured folate metabolic genotype combinations influence CTRD risk.

The SNP-by-SNP analyses of these data provided little evidence that the risk of CTRD is influenced by maternal genotype for any of the measured variants [8]. However, analyses of pairwise maternal gene-gene combinations suggested that a maternal MTHFR C677T/CBS 844ins68 interaction is associated with the risk of CTRD in offspring. Specifically, based on our analyses of these data, women with the MTHFR TT genotype appear to be at increased risk of having a child with CTRD, relative to women with CC genotype, only if they also carry the CBS NN genotype. Although this association did not achieve statistical significance after correction for multiple testing, it is in line with studies showing that the high homocysteine, low folate phenotype commonly observed in individuals with the MTHFR 677 TT genotype is present only among individuals with the CBS 844ins68 NN genotype [32–34]. Further, there is some evidence that interactions between these two variants may influence the risk of neural tube defects [35–38].

This study had several strengths, including a large sample size and consideration of the joint effects of genetic variants within the folate metabolic pathway. In addition, the effects of both the maternal and inherited genotypes were considered, as was heterogeneity of the effects of the inherited genotype across the component CTRD phenotypes. It is of note that in this large sample, the association between CTRD risk and the inherited MTHFR A1289C genotype did not appear to differ across the various CTRD phenotypes, which provides support for the inclusion of the full range of CTRD phenotypes in studies aimed at identifying CTRD risk factors.

As with all studies, this study also had limitations. Although this is the largest and most comprehensive study of CTRD and genes in the folate metabolic pathway conducted to date, it included only ten variants in nine folate metabolic genes. Further, heterogeneity within the case group may have influenced the observed associations, although results from MDR-P provide some evidence that the component CTRD phenotypes can be combined for analysis. In addition, the power to detect weak to moderate gene-gene interaction effects was low, and this limitation was further compounded by the relatively large number of interactions that were evaluated. Hence, although the observed association between CTRD and the maternal MTHFR C677T/CBS 844ins68 interaction is consistent with the biochemical consequences of this gene-gene combination, it is based on small numbers and may represent a false-positive finding.

5. Conclusions

The results of our study are consistent with the previous studies in this and other populations that indicate an association between the inherited MTHFR A1298C genotype and CHDs. In addition, our results suggest that this association is similar for each of the CTRD component phenotypes and, therefore, provides some support for pooling data from the component phenotypes in analyses aimed at identifying CTRD risk factors. The results of these analyses also indicate that a maternal MTHFR C677T/CBS 844ins68 interaction may be associated with the risk of CTRD in offspring. However, this finding requires confirmation in independent study samples. Hence, larger studies, which include additional folate metabolic pathway genes and a more extensive set of SNPs, are needed to more fully elucidate the role of folate in CTRD.

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References

[1] L. D. Botto, A. Correa, and J. D. Erickson, “Racial and temporal variations in the prevalence of heart defects,” Pediatrics, vol. 107, no. 3, p. E32, 2001.
[2] A. Christianson, C. P. Howson, and B. Modell, Global Report on Birth Defects, March of Dimes, White Plains, NY, USA, 2006.
[3] K. J. Jenkins, A. Correa, J. A. Feinstein, et al., “Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on cardiovascular disease in the young: endorsed by the American Academy of Pediatrics,” Circulation, vol. 115, no. 23, pp. 2995–3014, 2007.
[4] M. E. Pierpont, C. T. Basson, D. W. Benson Jr., et al., “Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on cardiovascular disease in the young: endorsed by the American Academy of Pediatrics,” Circulation, vol. 115, no. 23, pp. 3015–3038, 2007.
[5] P. S. Harper, Practical Genetic Counselling, Hodder Arnold, New York, NY, USA, 2004.
[6] L. E. Mitchell and C. R. Weinstein, “Evaluation of offspring and maternal genetic effects on disease risk using a family-based approach: the ‘pent’ design,” American Journal of Epidemiology, vol. 162, no. 7, pp. 676–685, 2005.
[7] L. D. Botto, A. E. Lin, T. Riehle-Colarusso, S. Malik, and A. Correa, “Seeking causes: classifying and evaluating congenital heart defects in etiologic studies,” Birth Defects Research Part A, vol. 79, no. 10, pp. 714–727, 2007.
[8] E. Goldmuntz, S. Woyciechowski, D. Renstrom, et al., “Variants of folate metabolism genes and the risk of conotruncal cardiac defects,” Circulation, vol. 1, pp. 126–132, 2008.
[9] C. A. Hobbs, S. J. James, A. Parsian, et al., “Congenital heart defects and genetic variants in the methylenetetrahydrofolate
reductase gene,” *Journal of Medical Genetics*, vol. 43, no. 2, pp. 162–166, 2006.

[10] G. M. Shaw, D. M. Iovannisci, W. Yang, et al., “Risks of human conotruncal heart defects associated with 32 single nucleotide polymorphisms of selected cardiovascular disease-related genes,” *American Journal of Medical Genetics*, vol. 138, no. 1, pp. 21–26, 2005.

[11] G. M. Shaw, W. Lu, H. Zhu, et al., “118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects,” *BMC Medical Genetics*, vol. 10, article 49, 2009.

[12] C. R. Weinberg, “Allowing for missing parents in genetic studies of case-parent triads,” *American Journal of Human Genetics*, vol. 64, no. 4, pp. 1186–1193, 1999.

[13] K.-A. da Costa, O. G. Kozyreva, J. Song, J. A. Galanko, L. M. Fischer, and S. H. Zeisel, “Common genetic polymorphisms affect the human requirement for the nutrient choline,” *FASEB Journal*, vol. 20, no. 9, pp. 1336–1344, 2006.

[14] B. H. Rovin, L. Lu, and R. Saxena, “A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression,” *Biochemical and Biophysical Research Communications*, vol. 259, no. 2, pp. 344–348, 1999.

[15] P. Frost, H. J. Blom, R. Milos, et al., “A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase,” *Nature Genetics*, vol. 10, no. 1, pp. 111–113, 1995.

[16] L. Kluitjman, P. Tran, B. Christensen, S. Sibani, and R. Rozen, “A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity,” *Molecular Genetics and Metabolism*, vol. 64, no. 3, pp. 169–172, 1998.

[17] D. L. Harmon, J. V. Woodside, J. W. G. Yarnell, et al., “The common ‘thermolabile’ variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinemia,” *QJM*, vol. 89, no. 8, pp. 571–577, 1996.

[18] D. J. Gaughan, L. A. J. Kluitjman, S. Barbaux, et al., “The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations,” *Atherosclerosis*, vol. 157, no. 2, pp. 451–456, 2001.

[19] S. G. Heil, N. M. J. van der Put, E. T. Waas, M. Den Heijer, F. J. M. Trijbels, and H. J. Blom, “Is mutated serine hydroxymethyltransferase (SHMT) involved in the etiology of neural tube defects?” *Molecular Genetics and Metabolism*, vol. 73, no. 2, pp. 164–172, 2001.

[20] J. A. Lievers, L. A. Afman, L. A. J. Kluitjman, et al., “Polymerizations in the transcobalamin gene: association with plasma homocysteine in healthy individuals and vascular disease patients,” *Clinical Chemistry*, vol. 48, no. 9, pp. 1383–1389, 2002.

[21] L. A. J. Kluitjman, G. H. J. Boers, F. J. M. Trijbels, H. M. A. van Lith-Zanders, L. P. W. J. van den Heuvel, and H. J. Blom, “A common 8441NS68 insertion variant in the cystathionine β-synthase gene,” *Biochemical and Molecular Medicine*, vol. 62, no. 1, pp. 23–25, 1997.

[22] R. D. Kalmbach, S. F. Choumenkovitch, A. P. Troen, P. F. Jacques, R. D’Agostino, and J. Selhub, “A 19-base pair deletion polymorphism in dihydrofolate reductase is associated with increased unmetabolized folic acid in plasma and decreased red blood cell folate,” *Journal of Nutrition*, vol. 138, no. 12, pp. 2322–2327, 2008.

[23] H. Gellekink, H. J. Blom, I. M. van der Linden, and M. den Heijer, “Molecular genetic analysis of the human dihydrofolate reductase gene: relation with plasma total homocysteine, serum and red blood cell folate levels,” *European Journal of Human Genetics*, vol. 15, no. 1, pp. 103–109, 2007.

[24] H. Mei, M. L. Cuccaro, and E. R. Martin, “Multifactor dimensionality reduction-phenomics: a novel method to capture genetic heterogeneity with use of phenotypic variables,” *American Journal of Human Genetics*, vol. 81, no. 6, pp. 1251–1261, 1997.

[25] Q. Yang, M. J. Khoury, F. Sun, and W. D. Flanders, “Case-only design to measure gene-gene interaction,” *Epidemiology*, vol. 10, no. 2, pp. 167–170, 1999.

[26] L.-Y. Wang and W.-C. Lee, “Population stratification bias in the case-only study for gene-environment interactions,” *American Journal of Epidemiology*, vol. 168, no. 2, pp. 197–201, 2008.

[27] D. M. Umbach and C. R. Weinberg, “The use of case-parent triads to study joint effects of genotype and exposure,” *American Journal of Human Genetics*, vol. 66, no. 1, pp. 251–261, 2000.

[28] J. K. Vermunt, LEM: A General Program for the Analysis of Categorical Data, Tilberg University, Tilberg, The Netherlands, 1997.

[29] C. R. Weinberg, A. J. Wilcox, and R. T. Lie, “A log-linear approach to case-parent-triad data: assessing effects of disease genes that act either directly or through maternal effects and that may be subject to parental imprinting,” *American Journal of Human Genetics*, vol. 62, no. 4, pp. 969–978, 1998.

[30] V. M. Guillem, M. Collado, M. J. Terol, et al., “Role of MTHFR (677, 1298) haplotype in the risk of developing secondary leukemia after treatment of breast cancer and hematological malignancies,” *Leukemia*, vol. 21, no. 7, pp. 1413–1422, 2007.

[31] S. G. Reeves, C. Meldrum, C. Groombridge, et al., “MTHFR 677 C > T and 1298 C > T polymorphisms and the age of onset of colorectal cancer in hereditary nonpolyposis colorectal cancer,” *European Journal of Human Genetics*, vol. 17, no. 5, pp. 629–635, 2009.

[32] C. M. Summers, A. L. Hammons, L. E. Mitchell, et al., “Influence of the cystathionine β-synthase 844ins68 and methylenetetrahydrofolate reductase 677 C > T polymorphisms on folate and homocysteine concentrations,” *European Journal of Human Genetics*, vol. 16, no. 8, pp. 1010–1013, 2008.

[33] V. de Stefano, V. Dekou, V. Nicaud, et al., “Linkage disequilibrium at the cystathionine β synthase (CBS) locus and the association between genetic variation at the CBS locus and plasma levels of homocysteine,” *Annals of Human Genetics*, vol. 62, no. 6, pp. 481–490, 1998.

[34] V. Dekou, V. Gudnason, E. Hawe, G. J. Miller, D. Stansbie, and G. J. Miller, “Influence of the cystathionine β-synthase 844ins68 and methylenetetrahydrofolate reductase 677 C > T polymorphisms on folate and homocysteine concentrations,” *European Journal of Human Genetics*, vol. 16, no. 8, pp. 1010–1013, 2008.

[35] L. D. Botto and P. Mastroiacovo, “Exploring gene-gene interactions in the etiology of neural tube defects,” *Clinical Genetics*, vol. 53, no. 6, pp. 456–459, 1998.

[36] R. Ropers, R. C. E. Melvin, D. Siegel, et al., “Updated investigations of the role of methylenetetrahydrofolate reductase in human neural tube defects,” *Clinical Genetics*, vol. 63, no. 3, pp. 210–214, 2003.

[37] B. Richter, K. Stengmann, B. Roper, et al., “Interaction of folate and homocysteine pathway genotypes evaluated in susceptibility to neural tube defects (NTD) in a German...
population,” *Journal of Human Genetics*, vol. 46, no. 3, pp. 105–109, 2001.

[38] M. C. Speer, J. Nye, D. McLone, et al., “Possible interaction of genotypes at cystathionine $\beta$-synthase and methylenetetrahydrofolate reductase (MTHFR) in neural tube defects,” *Clinical Genetics*, vol. 56, no. 2, pp. 142–144, 1999.