Cardiology in the Young (2013), **23**, 89–98
doi:10.1017/S1047951112000431

Original Article

Risk of congenital heart defects is influenced by genetic variation in folate metabolism

Karen E. Christensen,[1] Yassamin Feroz Zada,[2] Charles V. Rohlicek,[3] Gregor U. Andelfinger,[4,5] Jacques L. Michaud,[6] Jean-Luc Bigras,[5] Andrea Richter,[6] Marie-Pierre Dubé,[2] Rima Rozen[1]

[1]*Departments of Pediatrics and Human Genetics, McGill University-Montreal Children's Hospital Research Institute;* [2]*Institut de Cardiologie de Montréal/StatGen;* [3]*Division of Cardiology, Montreal Children's Hospital, McGill University Health Centre;* [4]*Department of Pediatrics, Cardiovascular Genetics;* [5]*Service of Pediatric Cardiology;* [6]*Division of Medical Genetics, Research Center of CHU Sainte Justine, University of Montréal, Montreal, Quebec, Canada*

Abstract Genetic disturbances in folate metabolism may increase risk for congenital heart defects. We examined the association of heart defects with four polymorphisms in folate-related genes (methylenetetrahydrofolate reductase (*MTHFR*) c.677C > T, *MTHFR* c.1298A > C, methionine synthase reductase (*MTRR)* c.66A > G, and reduced folate carrier (*SLC19A1*) c.80A > G) in a case–control study of children (156 patients, 69 controls) and mothers of children with heart defects (181 patients, 65 controls), born before folic acid fortification. *MTRR* c.66A > G in children modified odds ratios for overall heart defects, specifically ventricular septal defect and aortic valve stenosis (p-value below 0.05). The 66GG and AG genotypes were associated with decreased odds ratios for heart defects (0.42, 95% confidence interval (0.18–0.97) and 0.39 (0.18–0.84), respectively). This overall association was driven by decreased risk for ventricular septal defect for 66GG and AG (odds ratio 0.32 (0.11–0.91) and 0.25 (0.09–0.65)) and decreased odds ratio for aortic valve stenosis for 66AG (0.27 (0.09–0.79)). The association of ventricular septal defect and 66AG remained significant after correction for multiple testing ($p = 0.0044$, multiple testing threshold $p = 0.0125$). Maternal *MTHFR* 1298AC genotype was associated with increased odds ratio for aortic valve stenosis (2.90 (1.22–6.86), $p = 0.0157$), but this association did not meet the higher multiple testing threshold. No association between *MTHFR* c.677C > T or *SLC19A1* c.80A > G and heart defect risk was found. The influence of folate-related polymorphisms may be specific to certain types of heart defects; larger cohorts of mothers and children with distinct sub-classes are required to adequately address risk.

Keywords: Ventricular septal defect; genetic polymorphism; one-carbon folate metabolism; methylenetetrahydrofolate reductase; methionine synthase reductase

Received: 4 November 2011; Accepted: 6 March 2012; First published online: 5 April 2012

CONGENITAL HEART DEFECTS OCCUR IN APPROXIMATELY 1/100 live births[1] and represent the major cause of infant death due to birth defects.[2] Early studies in rats indicated that heart defects are among the folate-sensitive birth defects, which include neural tube defects and cleft lip and palate.[3] More recent human studies suggest that periconceptional folate supplementation reduces the incidence of heart defects, in particular conotruncal defects and ventricular septal defect.[4,5] In Canada, fortification of grain products with folic acid has been linked to a significant decrease in heart defects, particularly conotruncal defects.[6] Increased folate intake may prevent heart defects by

Correspondence to: Dr Rima Rozen, PhD, FRSC, FCAHS, FCCMG, McGill University-Montreal Children's Hospital Research Institute, 4060 Ste Catherine West, Room 200, Montreal, Quebec, Canada H3Z 2Z3. Tel: +514 412 4358; Fax: +514 412 4331; E-mail: rima.rozen@mcgill.ca

lowering maternal homocysteine levels, as maternal hyperhomocysteinaemia is associated with a greater than fourfold increase in risk for heart defects.[7] However, it is possible that homocysteine as such does not cause heart defects, but that it acts as a biomarker of disturbed folate metabolism or cellular methylation reactions that may disrupt embryonic development.

The link between low folate/high homocysteine and heart defect incidence implies that single-nucleotide polymorphisms in the folate pathways may be genetic risk factors for these disorders. A number of polymorphisms in folate pathway genes have been identified that appear to affect protein function and/or folate metabolism and thus may affect the risk for heart defects (for a review, see Christensen and Rozen[8]). On this basis, we selected four variants to examine in a cohort of congenital heart defect patients and their mothers: methylenetetrahydrofolate reductase (*MTHFR*) c.677C > T and c.1298A > C, methionine synthase reductase (*MTRR*) c.66A > G and reduced folate carrier (*SLC19A1*) c.80A > G. These polymorphisms were selected on the basis of their reported associations with risk for neural tube defects, and observations of cardiac defects in mouse models.

MTHFR catalyses the reduction of methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is required for the remethylation of homocysteine to methionine. The *MTHFR* c.677C > T variant (p.Ala222Val, dbSNP ID: rs1801133) results in a thermolabile protein associated with reduced enzyme activity in vivo.[9,10] This variant has been found to increase plasma homocysteine, particularly in combination with low folate levels,[11] and is a risk factor for neural tube defects.[12] The *MTHFR* c.1298A > C variant (p.Glu429Ala, rs1801131) has been reported to modestly reduce MTHFR activity in vivo,[10] although it may not influence homocysteine levels[13,14] or neural tube defect risk.[15] The c.1298A > C variant is in linkage disequilibrium with the c.677C > T variant (the 677TT/1298CC genotype is rarely observed), which complicates the analysis of its effects.[16] In mice, MTHFR-deficient females have greater numbers of offspring with heart defects than wild-type mice; the majority of observed defects were ventricular septal defects.[17]

MTRR catalyses the regeneration of the cobalamin cofactor of methionine synthase and may also help stabilise and activate methionine synthase.[18] Methionine synthase uses the 5-methyltetrahydrofolate generated by MTHFR to remethylate homocysteine, producing methionine. If MTRR activity is disrupted, it results in a functional deficiency of methionine synthase.[19] The *MTRR* c.66A > G variant (p.Ile22Met, rs1801394) has been reported to have different biochemical properties from the wild-type residue in vitro;[20] however, the effect of the mutation in vivo is not clear. Although this variant protein does not appear to independently affect homocysteine levels,[13,14] the 66GG genotype has been reported to significantly decrease homocysteine levels in *MTHFR* 677TT individuals.[14] This variant has also been reported to influence risk for neural tube defects, although results of these studies have been mixed.[15,21–23] In mouse models, MTRR deficiency causes increased plasma homocysteine[24] and has been found to increase the incidence of ventricular septal defect.[25]

Reduced folate carrier (gene name: *SLC19A1*) is a bidirectional transporter that carries reduced folates such as methyltetrahydrofolate. The effect of the *SLC19A1* c.80A > G variant (p.His27Arg, rs1051266) on the protein is not clear; one study found no effect,[26] whereas another found that it decreased transport of the folate analog methotrexate.[27] This variant does not appear to affect plasma homocysteine or folate levels.[13] The *SLC19A1* c.80A > G variant may be a folate-responsive risk factor for neural tube defects in some populations, although reports are inconsistent.[28,29] In mice, reduced folate carrier-deficient embryos die before implantation in the absence of maternal folate supplementation; heart defects such as conotruncal defects and ventricular septal defect have been reported in folate-supplemented reduced folate carrier-deficient embryos at later stages of development.[30]

The aim of this study is to assess the impact of these polymorphisms on heart defect risk in a Canadian cohort born before mandatory folate fortification. This is the first investigation of the impact of the *MTHFR* c.677C > T, *MTHFR* c.1298A > C, *MTRR* c.66A > G, and *SLC19A1* c.80A > G variants in a Canadian cohort.

Materials and methods

Human subjects

The subjects in this study are a subset of a previously described cohort.[31] DNA from patients and control subjects was obtained from blood spots and stored as reported.[31] Informed consent was obtained from all study participants before sample collection. The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki with approval from the Institutional Review Boards at the Montreal Children's Hospital and CHU Sainte-Justine. The subjects were from the province of Quebec, Canada, of Northern European background, born before December 31, 1996, before the introduction of mandatory folic acid fortification in Canada in 1998. Demographic information and use of folic acid

https://doi.org/10.1017/S1047951112000431 Published online by Cambridge University Press

Table 1. Congenital heart defect diagnoses in patients and mothers.

	n (%)		
Type of congenital heart defect	**Children (n = 156)**	**Mothers (n = 181)**	**Mother–child pairs (n = 153)**
Ventricular septal defect	44 (28.2)	51 (28.2)	44 (28.8)
Tetralogy of Fallot	34 (21.8)	42 (23.2)	34 (22.2)
Aortic valve stenosis	32 (20.5)	38 (21.0)	30 (19.6)
Transposition of the great arteries	20 (12.8)	21 (11.6)	19 (12.4)
Atrial septal defect	11 (7.1)	12 (6.6)	11 (7.2)
Atrioventricular septal defect	9 (5.8)	10 (5.5)	9 (5.9)
Truncus arteriosus	2 (1.3)	2 (1.1)	2 (1.3)
Double outlet right ventricle	1 (0.6)	1 (0.6)	1 (0.7)
Pulmonary stenosis	2 (1.3)	2 (1.1)	2 (1.3)
Coarctation of the aorta	1 (0.6)	2 (1.1)	1 (0.7)

supplements during pregnancy was obtained using a questionnaire administered at sample collection. As in other studies,[32–34] mothers were considered unsupplemented if use of supplements began only after the pregnancy was known. Congenital heart defects were diagnosed by echocardiography, as described.[31] Only patients and mothers of children with non-syndromic heart defects were included in this study. Control samples were collected from mothers and children unaffected by heart defects who presented at the Montreal Children's Hospital for outpatient blood sampling.

The subjects include 156 children with congenital heart defects, 181 mothers of children with congenital heart defects, 69 control children, and 65 control mothers (Table 1). There were 216 mother–child pairs within the cohort, that is, 153 patients and 63 controls. Congenital heart defects in the patients were aortic valve stenosis, atrial septal defect, atrioventricular septal defect, coarctation of the aorta, double-outlet right ventricle, pulmonary stenosis, transposition of the great arteries, tetralogy of Fallot, truncus arteriosus, and ventricular septal defect (Table 1).

Genotyping

Genotypes were determined by restriction fragment length polymorphism analysis at the Research Institute of the Montreal Children's Hospital. Genotyping of *MTHFR* c.677C > T was carried out using the sense primer 5′-TGAAGGA-GAAGGTGTCTGCGGGA-3′ and the antisense primer 5′-GATGCCCATGTCGGTTCATGCCTT-3′. Following 35 cycles of polymerase chain reaction amplification (94°C, 1 minute; 68°C, 1 minute; 72°C, 2 minutes), the 104 base-pair amplicon was digested with Hinf1 to generate the 104 base-pair 677C or 79 base-pair 677T fragments. Genotyping for *MTHFR* c.1298A > C, *MTRR* c.66A > G, and *SLC19A1* c.80A > G was carried out as described, except that the restriction enzyme Hinp1I was used for *SLC19A1*.[35–37] One case mother could not be genotyped for *MTRR* c.66A > G or *SLC19A1* c.80A > G, and thus was excluded from the analysis of those polymorphisms.

Statistical methods

Case–control tests of single-nucleotide polymorphism association without controlling for covariates were first used to compare the frequency of polymorphism alleles in two well-defined cohorts of patients diagnosed with the heart defects under study and unaffected controls. Case–control tests were performed for mother and child cohorts separately. Polymorphism impact on heart defect risk was analysed by diagnosis; owing to small numbers, coarctation of the aorta, double-outlet right ventricle, pulmonary stenosis, and truncus arteriosus were not analysed separately. Single-nucleotide polymorphism association with heart defect risk overall was analysed by grouping all diagnoses together.

Case–control association tests included the chi-square test on genotype, the chi-square test on alleles and the Cochran–Armitage trend test on genotypes. A total of 100,000 permutations were used to obtain the exact p-values by the Monte Carlo method.[38] Single-nucleotide polymorphisms were tested for Hardy–Weinberg equilibrium using an exact test[39] based on the conditional probability of genotype counts given allelic counts and the hypothesis of allelic independence using 100,000 permutations to get the exact p-values by Monte Carlo permutation.

A logistic regression model was used to evaluate the genotypic effect for the homozygous, heterozygous, dominant, and recessive models for each polymorphism. *MTHFR* 677T, *MTHFR* 1298C, *MTRR* 66G,

https://doi.org/10.1017/S1047951112000431 Published online by Cambridge University Press

and *SLC19A1* 80G were set as the risk alleles. All statistical analyses were conducted with SAS 9.1 including SAS genetics. Results with p-values less than or equal to 0.05 were considered to be of interest; to correct for multiple testing, the significance threshold was adjusted to less than or equal to 0.0125 using the Bonferroni correction.

Results

Descriptive data

Cohort Demographics. Demographic information for the cohort – maternal age at birth of index child, child's sex, multivitamin use – is shown in Table 2. Maternal age in the control group was significantly higher than that in the case groups, p is equal to 0.0121 for heart defects overall. Although significant, this approximately 1-year increase in maternal age would not be expected to affect heart defect risk. The proportion of female children is also higher in the control groups than that in the case groups.

In the mothers' cohort, supplemental vitamin use questions were completed by 57% of patients' mothers and 72% of control mothers (Table 2). Of those, only 11% of patients' mothers and 4% of control mothers reported using multivitamins containing folic acid before conception. Owing to these small numbers, a separate analysis of supplemented and unsupplemented mothers was not performed. The majority of mothers in this study did not use folic acid supplements during the periconceptional period. Of those that responded, 50% of patients' mothers and 68% of control mothers reported using multivitamins only after the pregnancy was discovered; as in other studies, we consider these mothers to be unsupplemented.[32–34] Materna was the most commonly named supplement in all groups. As the majority of participants were mother–child pairs, reported use of multivitamins containing folic acid was not meaningfully different in the children's cohort. Given that all of the participants in this study were born before folic acid supplementation in Canada, and the majority of mothers used folic acid supplements only after the periconceptional period, this study is more likely to detect folate-responsive single-nucleotide polymorphism associations than post-fortification cohorts.

Hardy–Weinberg equilibrium and linkage

In the mothers' cohort, no significant deviations from the Hardy–Weinberg equilibrium were observed; frequency of polymorphisms and Hardy–Weinberg equilibrium results are reported in Supplementary Table S1. In the children, there were no deviations from the Hardy–Weinberg equilibrium for any polymorphism or diagnosis except for *MTHFR* c.677C > T in children with ventricular septal defect and tetralogy of Fallot, which results in Hardy–Weinberg equilibrium deviation for heart defects overall for this polymorphism – frequency of single-nucleotide polymorphisms and Hardy–Weinberg equilibrium results in Supplementary Table S2. These deviations could be due to genetic associations with heart defects; however, they were not significant after correction for multiple testing.

As expected, the *MTHFR* c.677C > T and c.1298A > C variants were in linkage disequilibrium with a D′ value of 1. The 677TT/1298CC genotype was not observed in this cohort.

Association of folate-related single-nucleotide polymorphisms with risk for congenital heart defects

The associations between heart defect risk and the variant allele, homozygous variant genotype and heterozygous variant genotype were evaluated for each of the four polymorphisms. The associations of

Table 2. Demographic description of the cohort.

	Patients		Controls	
	Children	Mothers	Children	Mothers
Child's sex				
Male	64	75	21	18
Female	64	70	48	45
Unknown	28	36	0	2
Maternal age				
Mean ± SEM (n[1])	27.9 ± 0.4 (111)	28.0 ± 0.4 (121)	29.9 ± 0.6 (59)	29.8 ± 0.6 (57)
Periconceptional				
Number responded (%)	84 (54)	103 (57)	46 (67)	47 (72)
Supplement use				
Yes (%)	7/84 (8)	11/103 (11)	2/46 (4)	2/47 (4)
No (%)	77/84 (92)	92/103 (89)	44/46 (96)	45/47 (96)

[1]Number of mothers that provided maternal age at birth of index child

https://doi.org/10.1017/S1047951112000431 Published online by Cambridge University Press

heart defect risk using recessive and dominant effect models were also assessed by logistic regression. No significant associations were observed for atrial septal defect, atrioventricular septal defect, transposition of the great arteries, and tetralogy of Fallot in either mothers or children. Complete results including calculated odds ratios, upper and lower 95% confidence limits, and p-values for the four polymorphisms in the mothers are reported in Supplementary Tables S3 and S4; complete results for the children in Supplementary Tables S5 and S6. Tables 3–6 show the results for the diagnosis groups for which there were findings of interest (overall heart defect risk, aortic valve stenosis, and ventricular septal defect).

The maternal *MTHFR* 1298AC genotype was associated with increased odds ratios for aortic valve stenosis (odds ratio (AC versus CC): 2.90 (1.22–6.86), p = 0.0157; odds ratio (AC and CC versus AA): 2.39 (1.04–5.47), p = 0.0398) (Tables 3 and 4). However, these associations did not meet the higher threshold for significance (p = 0.0125) after correction for multiple testing. No maternal effect on heart defect risk was observed for *MTHFR* c.677C > T, *MTRR* c.66A > G, and *SLC19A1* c.80A > G.

In children, the *MTRR* c.66A > G variant was associated with decreased risk for heart defects. The G allele, the GG and the AG genotypes decreased odds ratios for heart defects overall (odds ratio (G versus A): 0.66 (0.44–0.98), p = 0.0532; odds ratio (GG versus AA): 0.42 (0.18–0.97), p = 0.0423; odds ratio (AG versus AA): 0.39 (0.18–0.84), p = 0.0168) (Table 5). When genotypes were combined using the dominant model (AG and GG versus AA), the odds ratio was 0.40 (0.19–0.83), p = 0.0140 (Table 6). None of these models met the higher threshold for significance after correction for multiple testing. The relationship of *MTRR* c.66A > G and heart defect risk appears to be driven by associations with risk for aortic valve stenosis and ventricular septal defect. Decreased odds ratios for aortic valve stenosis were found for both the AG genotype (odds ratio (AG versus AA): 0.27 (0.09–0.79), p = 0.0162) (Table 5) and the dominant model (odds ratio (AG and GG versus AA): 0.32 (0.12–0.83), p = 0.0191) (Table 6); these associations did not meet the threshold of p = 0.0125 for significance after correction for multiple testing. The c.66A > G variant appears to be linked with ventricular septal defect risk at both the allele and genotype levels: odds ratio (G versus A): 0.54 (0.32–0.93), p = 0.0295; odds ratio (GG versus AA): 0.32 (0.11–0.91), p = 0.0329; odds ratio (AG versus AA): 0.25 (0.09–0.65), p = 0.0044) (Table 5). Consistent with these observations, the dominant model was highly significant for ventricular septal

Table 3. Association of maternal folate-related single-nucleotide polymorphisms with risk of congenital heart defects in children.

		Allelic				Homozygous				Heterozygous			
Defect	SNP	OR	Lower CL	Upper CL	p-exact	OR	Lower CL	Upper CL	p-value	OR	Lower CL	Upper CL	p-value
All CHD													
	MTHFR c.677C > T	1.11	0.73	1.69	0.6716	1.16	0.48	2.81	0.7349	1.22	0.66	2.26	0.5200
	MTHFR c.1298A > C	0.96	0.61	1.50	0.9069	0.69	0.25	1.86	0.4636	1.20	0.65	2.21	0.5636
	MTRR c.66A > G	0.73	0.49	1.10	0.1517	0.53	0.23	1.23	0.1418	0.73	0.34	1.58	0.4212
	SLC19A1 c.80A > G	0.83	0.56	1.25	0.4153	0.68	0.30	1.56	0.3616	0.86	0.42	1.76	0.6776
AS													
	MTHFR c.677C > T	1.15	0.64	2.06	0.6569	1.15	0.32	4.14	0.8263	1.43	0.60	3.43	0.4199
	MTHFR c.1298A > C	1.44	0.78	2.64	0.2752	0.79	0.15	4.31	0.7865	2.90	1.22	6.86	0.0157
	MTRR c.66A > G	0.98	0.55	1.73	1.0000	0.87	0.28	2.71	0.8169	0.69	0.23	2.05	0.5007
	SLC19A1 c.80A > G	0.82	0.47	1.45	0.5633	0.64	0.19	2.16	0.4724	1.01	0.37	2.72	0.9898
VSD													
	MTHFR c.677C > T	1.57	0.93	2.66	0.1076	2.31	0.75	7.05	0.1425	2.01	0.86	4.65	0.1050
	MTHFR c.1298A > C	0.60	0.32	1.12	0.1238	0.44	0.11	1.84	0.2619	0.61	0.27	1.39	0.2389
	MTRR c.66A > G	0.68	0.40	1.15	0.1825	0.46	0.16	1.34	0.1557	0.66	0.25	1.72	0.3973
	SLC19A1 c.80A > G	0.78	0.46	1.31	0.3554	0.60	0.21	1.73	0.3472	0.66	0.27	1.61	0.3615

AS = aortic valve stenosis; CHD = congenital heart defects; CL = 95% confidence limit; *MTHFR* = methylenetetrahydrofolate reductase; *MTRR* = methionine synthase reductase; OR = odds ratio; *SLC19A1* = reduced folate carrier; SNP = single-nucleotide polymorphism; VSD = ventricular septal defect

https://doi.org/10.1017/S1047951112000431 Published online by Cambridge University Press

Table 4. Association of maternal folate-related single-nucleotide polymorphisms with risk of congenital heart defects in children, recessive and dominant effects.

		Recessive				Dominant			
Defect	SNP	OR	Lower CL	Upper CL	p-value	OR	Lower CL	Upper CL	p-value
All CHD									
	MTHFR c.677C > T	1.04	0.46	2.36	0.9188	1.21	0.68	2.16	0.5201
	MTHFR c.1298A > C	0.64	0.24	1.68	0.3670	1.08	0.61	1.90	0.8035
	MTRR c.66A > G	0.67	0.36	1.24	0.2022	0.65	0.31	1.35	0.2481
	SLC19A1 c.80A > G	0.76	0.39	1.46	0.4044	0.80	0.41	1.58	0.5191
AS									
	MTHFR c.677C > T	0.94	0.29	3.05	0.9221	1.37	0.59	3.14	0.4622
	MTHFR c.1298A > C	0.46	0.09	2.34	0.3496	2.39	1.04	5.47	0.0398
	MTRR c.66A > G	1.14	0.49	2.63	0.7584	0.76	0.28	2.11	0.6027
	SLC19A1 c.80A > G	0.64	0.24	1.71	0.3726	0.88	0.34	2.29	0.8009
VSD									
	MTHFR c.677C > T	1.52	0.57	4.07	0.4073	2.08	0.93	4.62	0.0734
	MTHFR c.1298A > C	0.52	0.13	2.11	0.3590	0.57	0.26	1.22	0.1480
	MTRR c.66A > G	0.62	0.27	1.41	0.2532	0.58	0.23	1.43	0.2380
	SLC19A1 c.80A > G	0.80	0.33	1.90	0.6073	0.64	0.27	1.49	0.3020

AS = aortic valve stenosis; CHD = congenital heart defects; CL = 95% confidence limit; *MTHFR* = methylenetetrahydrofolate reductase; *MTRR* = methionine synthase reductase; OR = odds ratio; *SLC19A1* = reduced folate carrier; SNP = single-nucleotide polymorphism; VSD = ventricular septal defect

defect (odds ratio (AG and GG versus AA): 0.27 (0.11–0.66), p = 0.0040) (Table 6). The association of ventricular septal defect risk with the AG genotype and the dominant model were significant after correction for multiple testing. The *MTHFR* c.677C > T, *MTHFR* c.1298A > C, and *SLC19A1* c.80A > G polymorphisms were not associated with heart defect risk in the children.

Discussion

Studies of animal models suggest that heart defects associated with low folate/high homocysteine may result from abnormal differentiation, migration, and apoptosis in neural crest cells affecting primarily the interventricular septum and the conotruncal region.[40,41] Several studies of the effects of folic acid/multivitamin supplementation also suggest an association between folate deficiency and conotruncal defects/ventricular septal defect.[4,42] In this cohort, we found potential associations of single-nucleotide polymorphisms in enzymes of folate metabolism with ventricular septal defect and aortic valve stenosis. Aortic valve stenosis has not been commonly reported to be a folate-responsive heart defect; however, patients with aortic valve stenosis have not been included in many of these studies.

In this study, a significant association between the *MTRR* c.66A > G variant in children and risk for certain types of heart defects was observed. After correction for multiple testing, the *MTRR* 66AG genotype and the dominant model (AG and GG versus AA) were found to significantly decrease risk for ventricular septal defect. Maternal c.66A > G genotype had no effect on heart defect risk in this population. The results of published studies of this variant and heart defect risk have been mixed. This variant, in either the mother or child, was found to have no impact on heart defect risk in mixed pre- and post-fortification American cohorts – grouped conotruncal and left-sided cardiac defects, respectively[43,44] – or in children with conotruncal defects in a pre-fortification American cohort.[45] In contrast to our findings, in the Dutch population, which is not folic acid fortified, maternal *MTRR* 66GG was found to increase risk for non-conotruncal heart defects – including aortic valve stenosis, pulmonary stenosis, coarctation of the aorta, atrial septal defect and others – when combined with vitamin B_{12} deficiency.[46] However, in a second Dutch cohort, consisting primarily of conotruncal and septal defects, there was no association between either maternal or inherited 66GG genotype and heart defects, regardless of B_{12} status.[47] Similarly conflicting results have been obtained from investigations of this variant and neural tube defect risk.[15,21–23] These contrasting results suggest that the effect of the *MTRR* variant may depend on other factors – for example, genetic, nutritional, environmental – within the population, or that this variant may only affect risk for specific subtypes of heart defects. In mice, both maternal and embryonic MTRR deficiency were found to increase ventricular septal defect incidence in

https://doi.org/10.1017/S1047951112000431 Published online by Cambridge University Press

Table 5. Association of folate-related single-nucleotide polymorphisms in children with risk of congenital heart defects.

Defect	SNP	Allelic				Homozygous				Heterozygous			
		OR	Lower CL	Upper CL	p-exact	OR	Lower CL	Upper CL	p-value	OR	Lower CL	Upper CL	p-value
All CHD													
	MTHFR c.677C > T	1.33	0.87	2.05	0.2039	1.74	0.71	4.22	0.2232	1.21	0.65	2.23	0.5471
	MTHFR c.1298A > C	1.17	0.74	1.83	0.5695	1.18	0.39	3.61	0.7657	1.27	0.70	2.31	0.4298
	MTRR c.66A > G	0.66	0.44	0.98	0.0532	0.42	0.18	0.97	0.0423	0.39	0.18	0.84	0.0168
	SLC19A1 c.80A > G	1.07	0.72	1.60	0.7596	1.17	0.52	2.62	0.7068	1.24	0.60	2.57	0.5532
AS													
	MTHFR c.677C > T	1.28	0.69	2.40	0.5167	0.80	0.15	4.32	0.7909	2.33	0.95	5.72	0.0659
	MTHFR c.1298A > C	1.38	0.73	2.64	0.4033	1.17	0.20	6.77	0.8615	1.91	0.79	4.60	0.1481
	MTRR c.66A > G	0.62	0.34	1.12	0.1303	0.39	0.13	1.22	0.1055	0.27	0.09	0.79	0.0162
	SLC19A1 c.80A > G	0.92	0.51	1.66	0.8796	0.84	0.26	2.75	0.7761	0.94	0.33	2.65	0.9087
VSD													
	MTHFR c.677C > T	1.58	0.91	2.77	0.1145	2.53	0.87	7.37	0.0882	0.99	0.42	2.34	0.9852
	MTHFR c.1298A > C	1.19	0.66	2.15	0.6469	1.38	0.34	5.69	0.6544	1.20	0.54	2.66	0.6605
	MTRR c.66A > G	0.54	0.32	0.93	0.0295	0.32	0.11	0.91	0.0329	0.25	0.09	0.65	0.0044
	SLC19A1 c.80A > G	1.05	0.61	1.80	0.8918	1.12	0.38	3.34	0.8351	1.20	0.45	3.18	0.7102

AS = aortic valve stenosis; CHD = congenital heart defects; CL = 95% confidence limit; *MTHFR* = methylenetetrahydrofolate reductase; *MTRR* = methionine synthase reductase; OR = odds ratio; *SLC19A1* = reduced folate carrier; SNP = single-nucleotide polymorphism; VSD = ventricular septal defect

offspring.[25] However, it is not clear that the 66G variant causes in vivo MTRR deficiency.[20,48]

We did not find an association between heart defect risk and *MTHFR* c.677C > T genotype in this pre-fortification Canadian cohort. The results of studies of this variant and heart defects in other non-fortified populations have been mixed. Some groups have reported increased risk for certain subtypes of heart defects associated with maternal[32] or inherited[49,50] TT genotype, whereas others have not observed these effects.[45,51–53] In the Dutch population, the risk for conotruncal defects associated with maternal *MTHFR* 677TT genotype was increased in those who did not consume folic acid supplements during the periconceptional period.[32] Consistent with those findings, studies of *MTHFR* c.677C > T in folic acid fortified populations have found no link with heart defect risk,[43,44,54,55] except in combination with maternal obesity.[34]

In this cohort, maternal *MTHFR* 1298AC genotype was associated with a possible increase in risk for aortic valve stenosis. This contrasts with the findings from a pre-fortification American cohort that did not observe any association between this variant and aortic valve stenosis risk.[55] In other non-fortified populations, this variant was not linked to risk for conotruncal defects[45,52] or a mixed group of heart defects.[53] In a Dutch study, non-fortified population, the 1298AC and CC genotypes in children were reported to decrease risk for a mixed group of heart defects, compared with those with the 1298AA genotype, unless their mothers consumed folic acid supplements;[33] maternal genotype had no effect on risk in unsupplemented mothers, but the 1298AC and CC genotypes increased heart defect risk relative to the 1298AA genotype in mothers that were folic acid supplemented. However, the 1298AC and CC genotypes in children were also associated with a decreased risk for heart defects in a folic acid fortified cohort,[54] and the 1298CC genotype in children was found to protect against conotruncal defects in a mixed pre- and post-fortification American cohort.[43] No association between this variant and risk for left-sided cardiac defects was found in a second mixed pre- and post-fortification American cohort.[44] Clearly, the impact of this variant on heart defect risk, particularly in combination with folic acid intake, requires further investigation.

In this study, the *SLC19A1* c.80A > G variant was also not associated with heart defects. In contrast, the 80GG and AG genotypes showed an increase in risk for conotruncal defects in a Californian population[37] and heart defects in general – predominantly ventricular septal defect – in a Chinese population.[56] In those studies, low folate levels increased the risk attributed to the

https://doi.org/10.1017/S1047951112000431 Published online by Cambridge University Press

Table 6. Association of folate-related single-nucleotide polymorphisms in children with the risk of congenital heart defects, recessive and dominant effects.

		Recessive				Dominant			
Defect	SNP	OR	Lower CL	Upper CL	p-value	OR	Lower CL	Upper CL	p-value
All CHD									
	MTHFR c.677C > T	1.60	0.68	3.72	0.2789	1.33	0.75	2.35	0.3225
	MTHFR c.1298A > C	1.07	0.36	3.15	0.9071	1.26	0.71	2.22	0.4296
	MTRR c.66A > G	0.79	0.42	1.47	0.4562	0.40	0.19	0.83	0.0140
	SLC19A1 c.80A > G	1.00	0.53	1.89	0.9966	1.22	0.61	2.41	0.5729
AS									
	MTHFR c.677C > T	0.51	0.10	2.54	0.4101	1.96	0.82	4.68	0.1276
	MTHFR c.1298A > C	0.85	0.16	4.65	0.8546	1.79	0.77	4.19	0.1789
	MTRR c.66A > G	0.89	0.35	2.26	0.8132	0.32	0.12	0.83	0.0191
	SLC19A1 c.80A > G	0.88	0.34	2.29	0.7888	0.91	0.34	2.40	0.8423
VSD									
	MTHFR c.677C > T	2.54	0.93	6.94	0.0687	1.35	0.63	2.90	0.4343
	MTHFR c.1298A > C	1.28	0.32	5.05	0.7245	1.23	0.57	2.62	0.5985
	MTRR c.66A > G	0.76	0.32	1.79	0.5324	0.27	0.11	0.66	0.0040
	SLC19A1 c.80A > G	0.99	0.42	2.30	0.9756	1.17	0.47	2.95	0.7330

AS = aortic valve stenosis; CHD = congenital heart defects; CL = 95% confidence limit; *MTHFR* = methylenetetrahydrofolate reductase; *MTRR* = methionine synthase reductase; OR = odds ratio; *SLC19A1* = reduced folate carrier; SNP = single-nucleotide polymorphism; VSD = ventricular septal defect

variant. However, this association was not observed in a second study of the Californian population.[45]

The associations with heart defect risk for the *MTHFR* c.1298A > C variant in mothers and *MTRR* c.66A > G variants in children may reflect the metabolic role of these enzymes. MTHFR produces the major circulating form of folate supplied to embryos during development;[57] therefore, maternal MTHFR deficiency may cause foetal folate deficiency resulting in abnormalities, whereas MTHFR deficiency in the child may be compensated for by the supply of methyltetrahydrofolate from the mother. MTRR supports the folate-dependent remethylation of homocysteine within cells and tissues. Therefore, MTRR could have a greater role in one-carbon metabolism within developing embryonic tissues, leading to metabolic changes that affect heart defect incidence.

The largely unsupplemented nature of this cohort provides the opportunity to identify folate-responsive relationships between these polymorphisms and heart defect risk that may not be evident in more recent North American cohorts. Clearly, gene–nutrient interactions may play an important role in the impact of variants of folate metabolism on heart defect risk, and should be considered in future studies. In this study, the number of patients was small when subdivided by diagnosis; despite this limitation, we observed some associations. Nonetheless, our findings should be considered preliminary and further investigation of these gene variants should be performed in larger cohorts.

In conclusion, we identified possible associations between certain types of congenital heart defects and single-nucleotide polymorphisms in genes of folate metabolism. The *MTRR* c.66A > G variant in children may protect against specific heart defects, such as ventricular septal defect. Additional studies of folate-related variants and heart defect risk in larger cohorts should be focused on specific classes of defects, with particular emphasis on the potential for genetic interactions between mother and child.

Acknowledgements

We thank Qing Wu, Liyuan Deng, and Roxanne Gendron for technical assistance. This work was supported by grants from the Canadian Institutes of Health Research, grant number GMHD-79045 (GUA, AR, RR) and a Fellowship from the Montreal Children's Hospital Research Institute (KEC).

Supplementary materials

For supplementary material referred to in this article, please visit http://dx.doi.org/doi:10.1017/S1047951112000431

References

1. Hoffman JI, Kaplan S. The incidence of congenital heart disease. J Am Coll Cardiol 2002; 39: 1890–1900.
2. Boneva RS, Botto LD, Moore CA, Yang Q, Correa A, Erickson JD. Mortality associated with congenital heart defects in the United States: trends and racial disparities, 1979–1997. Circulation 2001; 103: 2376–2381.

3. Baird CD, Nelson MM, Monie IW, Evans HM. Congenital cardiovascular anomalies induced by pteroylglutamic acid deficiency during gestation in the rat. Circ Res 1954; 2: 544–554.
4. Bailey LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. Am J Clin Nutr 2005; 81(5): 1213S–1217S.
5. van Beynum IM, Kapusta L, Bakker MK, den Heijer M, Blom HJ, de Walle HE. Protective effect of periconceptional folic acid supplements on the risk of congenital heart defects: a registry-based case-control study in the northern Netherlands. Eur Heart J 2010; 31: 464–471.
6. Ionescu-Ittu R, Marelli AJ, Mackie AS, Pilote L. Prevalence of severe congenital heart disease after folic acid fortification of grain products: time trend analysis in Quebec, Canada. BMJ 2009; 338: b1673.
7. Verkleij-Hagoort A, Bliek J, Sayed-Tabatabaei F, Ursem N, Steegers E, Steegers-Theunissen R. Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. Am J Med Genet A 2007; 143A: 952–960.
8. Christensen KE, Rozen R. Genetic variation: effect on folate metabolism and health. In: Bailey LB (ed.). Folate in Health and Disease, second edition. CRC Press, Boca Raton, FL, 2010, pp 75–110.
9. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995; 10: 111–113.
10. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998; 64: 169–172.
11. Jacques PF, Bostom AG, Williams RR, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 1996; 93: 7–9.
12. van der Put NM, Steegers-Theunissen RP, Frosst P, et al. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 1995; 346: 1070–1071.
13. Fredriksen A, Meyer K, Ueland PM, Vollset SE, Grotmol T, Schneede J. Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. Hum Mutat 2007; 28: 856–865.
14. Yang QH, Botto LD, Gallagher M, et al. Prevalence and effects of gene–gene and gene–nutrient interactions on serum folate and serum total homocysteine concentrations in the United States: findings from the third National Health and Nutrition Examination Survey DNA Bank. Am J Clin Nutr 2008; 88: 232–246.
15. Relton CL, Wilding CS, Pearce MS, et al. Gene–gene interaction in folate-related genes and risk of neural tube defects in a UK population. J Med Genet 2004; 41: 256–260.
16. Brown NM, Pratt VM, Buller A, et al. Detection of 677CT/1298AC "double variant" chromosomes: implications for interpretation of MTHFR genotyping results. Genet Med 2005; 7: 278–282.
17. Li D, Pickell L, Liu Y, Wu Q, Cohn JS, Rozen R. Maternal methylenetetrahydrofolate reductase deficiency and low dietary folate lead to adverse reproductive outcomes and congenital heart defects in mice. Am J Clin Nutr 2005; 82: 188–195.
18. Yamada K, Gravel RA, Toraya T, Matthews RG. Human methionine synthase reductase is a molecular chaperone for human methionine synthase. Proc Natl Acad Sci U S A 2006; 103: 9476–9481.
19. Gulati S, Chen Z, Brody LC, Rosenblatt DS, Banerjee R. Defects in auxiliary redox proteins lead to functional methionine synthase deficiency. J Biol Chem 1997; 272: 19171–19175.
20. Olteanu H, Munson T, Banerjee R. Differences in the efficiency of reductive activation of methionine synthase and exogenous electron acceptors between the common polymorphic variants of human methionine synthase reductase. Biochemistry 2002; 41: 13378–13385.
21. Wilson A, Platt R, Wu Q, et al. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab 1999; 67: 317–323.
22. van der Linden IJ, den Heijer M, Afman LA, et al. The methionine synthase reductase 66A > G polymorphism is a maternal risk factor for spina bifida. J Mol Med 2006; 84: 1047–1054.
23. O'Leary VB, Mills JL, Pangilinan F, et al. Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Mol Genet Metab 2005; 85: 220–227.
24. Elmore CL, Wu X, Leclerc D, et al. Metabolic derangement of methionine and folate metabolism in mice deficient in methionine synthase reductase. Mol Genet Metab 2007; 91: 85–97.
25. Deng L, Elmore CL, Lawrance AK, Matthews RG, Rozen R. Methionine synthase reductase deficiency results in adverse reproductive outcomes and congenital heart defects in mice. Mol Genet Metab 2008; 94: 336–342.
26. Whetstine JR, Gifford AJ, Witt T, et al. Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His27 and Arg27 Carriers. Clin Cancer Res 2001; 7: 3416–3422.
27. Baslund B, Gregers J, Nielsen CH. Reduced folate carrier polymorphism determines methotrexate uptake by B cells and CD4+ T cells. Rheumatology (Oxford) 2008; 47: 451–453.
28. Pei L, Zhu H, Ren A, et al. Reduced folate carrier gene is a risk factor for neural tube defects in a Chinese population. Birth Defects Res A Clin Mol Teratol 2005; 73: 430–433.
29. Shaw GM, Lammer EJ, Zhu H, Baker MW, Neri E, Finnell RH. Maternal periconceptional vitamin use, genetic variation of infant reduced folate carrier (A80G), and risk of spina bifida. Am J Med Genet 2002; 108: 1–6.
30. Taparia S, Gelineau-van Waes J, Rosenquist TH, Finnell RH. Importance of folate–homocysteine homeostasis during early embryonic development. Clin Chem Lab Med 2007; 45: 1717–1727.
31. Christensen KE, Rohlicek CV, Andelfinger GU, et al. The MTHFD1 p.Arg653Gln variant alters enzyme function and increases risk for congenital heart defects. Hum Mutat 2009; 30: 212–220.
32. van Beynum IM, Kapusta L, den Heijer M, et al. Maternal MTHFR 677C > T is a risk factor for congenital heart defects: effect modification by periconceptional folate supplementation. Eur Heart J 2006; 27: 981–987.
33. van Driel LM, Verkleij-Hagoort AC, de Jonge R, et al. Two MTHFR polymorphisms, maternal B-vitamin intake, and CHDs. Birth Defects Res A Clin Mol Teratol 2008; 82: 474–481.
34. Hobbs CA, Cleves MA, Karim MA, Zhao W, MacLeod SL. Maternal folate-related gene environment interactions and congenital heart defects. Obstet Gynecol 2010; 116: 316–322.
35. Leclerc D, Sibani S, Rozen R. Molecular Biology of methylenetetrahydrofolate reductase (MTHFR) and overview of mutation/polymorphisms. In: Ueland PM and Rozen R (eds). MTHFR Polymorphisms and Disease. Landes Bioscience, Georgetown, Texas, 2005, pp 1–20.
36. Ananth CV, Elsasser DA, Kinzler WL, et al. Polymorphisms in methionine synthase reductase and betaine-homocysteine S-methyltransferase genes: risk of placental abruption. Mol Genet Metab 2007; 91: 104–110.
37. Shaw GM, Zhu H, Lammer EJ, Yang W, Finnell RH. Genetic variation of infant reduced folate carrier (A80G) and risk of

orofacial and conotruncal heart defects. Am J Epidemiol 2003; 158: 747–752.
38. Westfall PH, Young SS. Resampling-based multiple testing. John Wiley & Sons, Inc., New York, 1993.
39. Guo SW, Thompson EA. Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 1992; 48: 361–372.
40. Boot MJ, Steegers-Theunissen RP, Poelmann RE, Van Iperen L, Lindemans J, Gittenberger-de Groot AC. Folic acid and homocysteine affect neural crest and neuroepithelial cell outgrowth and differentiation in vitro. Dev Dyn 2003; 227: 301–308.
41. Tang LS, Wlodarczyk BJ, Santillano DR, Miranda RC, Finnell RH. Developmental consequences of abnormal folate transport during murine heart morphogenesis. Birth Defects Res A Clin Mol Teratol 2004; 70: 449–458.
42. Czeizel AE. Reduction of urinary tract and cardiovascular defects by periconceptional multivitamin supplementation. Am J Med Genet 1996; 62: 179–183.
43. Goldmuntz E, Woyciechowski S, Renstrom D, Lupo PJ, Mitchell LE. Variants of folate metabolism genes and the risk of conotruncal cardiac defects. Circ Cardiovasc Genet 2008; 1: 126–132.
44. Mitchell LE, Long J, Garbarini J, Paluru P, Goldmuntz E. Variants of folate metabolism genes and risk of left-sided cardiac defects. Birth Defects Res A Clin Mol Teratol 2010; 88: 48–53.
45. Shaw GM, Lu W, Zhu H, et al. 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. BMC Med Genet 2009; 10: 49.
46. van Beynum IM, Kouwenberg M, Kapusta L, et al. MTRR 66A > G polymorphism in relation to congenital heart defects. Clin Chem Lab Med 2006; 44: 1317–1323.
47. Verkleij-Hagoort AC, van Driel LM, Lindemans J, et al. Genetic and lifestyle factors related to the periconception vitamin B12 status and congenital heart defects: a Dutch case–control study. Mol Genet Metab 2008; 94: 112–119.
48. Olteanu H, Wolthers KR, Munro AW, Scrutton NS, Banerjee R. Kinetic and thermodynamic characterization of the common polymorphic variants of human methionine synthase reductase. Biochemistry 2004; 43: 1988–1997.
49. Junker R, Kotthoff S, Vielhaber H, et al. Infant methylenetetrahydrofolate reductase 677TT genotype is a risk factor for congenital heart disease. Cardiovasc Res 2001; 51: 251–254.
50. Lee CN, Su YN, Cheng WF, et al. Association of the C677T methylenetetrahydrofolate reductase mutation with congenital heart diseases. Acta Obstet Gynecol Scand 2005; 84: 1134–1140.
51. Shaw GM, Iovannisci DM, Yang W, et al. Risks of human conotruncal heart defects associated with 32 single nucleotide polymorphisms of selected cardiovascular disease-related genes. Am J Med Genet A 2005; 138: 21–26.
52. Storti S, Vittorini S, Lascone MR, et al. Association between 5,10-methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and conotruncal heart defects. Clin Chem Lab Med 2003; 41: 276–280.
53. Xu J, Xu X, Xue L, et al. MTHFR c.1793G > A polymorphism is associated with congenital cardiac disease in a Chinese population. Cardiol Young 2010; 20: 318–326.
54. Hobbs CA, James SJ, Parsian A, et al. Congenital heart defects and genetic variants in the methylenetetrahydroflate reductase gene. J Med Genet 2006; 43: 162–166.
55. McBride KL, Fernbach S, Menesses A, et al. A family-based association study of congenital left-sided heart malformations and 5,10 methylenetetrahydrofolate reductase. Birth Defects Res A Clin Mol Teratol 2004; 70: 825–830.
56. Pei L, Zhu H, Zhu J, Ren A, Finnell RH, Li Z. Genetic variation of infant reduced folate carrier (A80G) and risk of orofacial defects and congenital heart defects in China. Ann Epidemiol 2006; 16: 352–356.
57. Henderson GI, Perez T, Schenker S, Mackins J, Antony AC. Maternal-to-fetal transfer of 5-methyltetrahydrofolate by the perfused human placental cotyledon: evidence for a concentrative role by placental folate receptors in fetal folate delivery. J Lab Clin Med 1995; 126: 184–203.