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Association between Selected Folate Pathway Polymorphisms and Nonsyndromic Limb Reduction Defects: A Case-Parental Analysis

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SUMMARY

Inadequate folate status due to either genetic variation or nutritional deficiencies has been associated with an increased risk of congenital malformations including orofacial clefting, limb, cardiac and neural tube defects. Few epidemiologic studies have examined the association between limb reduction defects (LRDs) and folate-related genetic polymorphisms other than *MTHFR* 677C→T. We conducted a case–parental analysis of 148 families who participated in the National Birth Defects Prevention Study (NBDPS) to examine the association between nonsyndromic transverse and longitudinal LRDs with five single nucleotide polymorphisms (SNPs) in genes encoding for enzymes in folate and methionine pathways. Log-linear Poisson regression, adapted for analysis of case–parental data, assuming an additive genetic model was used to estimate genetic relative risks and 95% confidence intervals for the association between LRDs and each SNP. Among women who did not take multivitamin supplements, the *MTHFR* 677T variant acts via the offspring's genome to increase the risk of LRDs. No association between LRDs and any fetal SNP was found among women who used multivitamin supplements. These results suggest the possibility that initiating folic acid supplementation prior to pregnancy may reduce the risk of having a LRD-affected pregnancy, especially in women whose offspring inherit one or two copies of the *MTHFR* 677T variant.

Keywords

Folic acid; homocysteine; *MTHFR*; polymorphisms; case-parental analysis

INTRODUCTION

Limb reduction defects (LRDs) affect approximately five to seven infants in every 10,000 live births.^{1–4} The cause of most limb defects is thought to be multi-factorial, involving both environmental exposures and genetic factors. However, despite the extensive knowledge gained from linkage and animal studies that have identified over 50 genes involved in limb development,^{5–7} we know very little about the genetic etiology of nonsyndromic congenital limb deficiencies.

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Epidemiologic studies have provided evidence of a decreased risk of LRDs associated with pre-pregnancy or early pregnancy supplementation with multivitamin containing folic acid.^{8–10} Folic acid is essential for normal DNA synthesis and for normal cellular methylation reactions. Inadequate folate status due to either genetic variations in folate-related genes or nutritional deficiencies compromises the folate-B12 dependent methionine synthase activity, increasing plasma homocysteine levels and decreasing methionine levels.^{11–13} This imbalance has been implicated in increased risk of congenital malformations including cardiac, neural tube defects, orofacial clefts and limb reduction defects.^{14–18}

Studies have shown that the effect of folic acid intake on pregnancy outcomes is modified by variants in both maternal and fetal genes that code for critical enzymes in the folate and methionine pathways. Although most of the evidence supports a strong association between folate pathway gene polymorphisms with neural tube defects (NTDs), associations with other malformations have also been reported.^{19–21} Similar associations are also suspected with limb reduction defects. Few studies have examined the association between genetic polymorphisms with LRDs.^{17, 18, 22–24}

However, with the exception of *MTHFR* 677C→T polymorphism,^{18, 23} no study has reported examining polymorphisms in folate and methionine pathway genes. Carmichael et al. genotyped 96 cases with longitudinal, transverse or amniotic band limb deficiency defects and 437 non-malformed controls from California (1987–1988 birth cohort). They did not find a significant association between the *MTHFR* 677C>T polymorphism and limb defects (Heterozygote (CT) OR=1.0, 95% CI=0.6, 1.5; Homozygote(TT) OR=0.9, 95% CI=0.5, 1.9). Shashi et al.'s study was a case report of a mother homozygous for T allele that delivered a child with a transverse terminal limb defect.

We conducted a case–parental analysis of children with LRDs and their parents, participants in the National Birth Defects Prevention Study (NBDPS), to examine the association between nonsyndromic limb reduction defects and five functional SNPs within four folate and methionine pathway genes: the methylenetetrahydrofolate reductase (*MTHFR*) gene, the transcobalamin II (*TCN2*) gene, the methionine synthase reductase (*MTRR*) gene and the betaine-homocysteine methyltransferase (*BHMT*) gene (Table I).

The *MTHFR* gene encodes for the enzyme that catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is essential for re-methylation of homocysteine to methionine. Two single nucleotide polymorphisms (SNPs) in *MTHFR*, 677C→T (rs1801133) and 1298A→C (rs1801131), are associated with decreased enzyme activity.²⁵ In addition to 5-methyltetrahydrofolate, vitamin B12, as methylated cobalamin, is also essential for re-methylation of homocysteine. Cobalamin is only able to enter cells or pass the placenta boundary when bound to transcobalamin. The 776 C→G (rs1801198) polymorphism in the cobalamin transporter *TCN2* gene has been suggested to affect transcobalamin activity and consequently cobalamin status.^{26, 27} The *MTRR* gene product is required to maintain methionine synthase in an active state via reduction of vitamin B12. The polymorphism, 66A→G (rs1801394), was reported to increase NTD risk when B12 status is low and/or when *MTHFR* 677C→T is present.²⁸ The *BHMT* gene converts homocysteine to methionine using betaine. A common polymorphism, 742G→A (rs3733890), is believed to modify susceptibility to spina bifida^{29–31} and possibly other birth defects.

Several studies have also identified behavioral factors in addition to vitamin supplementation that modify LRD risk. Some evidence suggests that maternal obesity, alcohol consumption or smoking early in pregnancy may disturb the folate-pathway

increasing the risk of malformations including LRDs.^{32–35} Consequently, we also examined the interaction between these lifestyle factors and the five candidate SNPs.

METHODS

Case selection

Cases were ascertained through the National Birth Defects Prevention Study (NBDPS). Details regarding the NBDPS methodology have previously been published.^{36, 37} Briefly, the NBDPS is an ongoing, IRB-approved case-control/case-parental study intended to identify the etiology of more than 30 nonsyndromic structural birth defects, including limb reduction defects. Case infants were identified from among prevalent cases through population-based birth defect surveillance systems in participating sites. All surveillance systems are within a state department of health or a *bona fide* agent of the state and as such mandated to identify cases of birth defects using multiple sources including in-patient and out-patient discharge records, electronic medical records and labor and delivery logs. An expert panel of pediatric clinical dysmorphologists developed uniform diagnostic criteria for each phenotype. Most cases were diagnosed and ascertained prior to their first birthday. NBDPS-eligible case infants were those who had no known single-gene disorder or chromosomal abnormality and were diagnosed with at least one of the eligible nonsyndromic structural birth defects. Infants who were adopted or in foster care were ineligible to participate in the NBDPS study. For the current study we included cases and their parents, if the case had an upper or lower limb transverse or longitudinal limb reduction defect. Transverse LRD cases were those with absent distal segments and intact proximal structure, whereas longitudinal LRD cases were those with preaxial or postaxial ray deficiencies. Six families with infants with intercalary LRD were excluded from this study. We also excluded five families where the mother reported having pre-gestational diabetes.

Maternal interview data

Mothers of cases were interviewed using a structured Computer Assisted Telephone Interview (CATI) that was specifically designed for the NBDPS. In this questionnaire, the respondent was asked whether they took multivitamins, smoked or drank alcoholic beverages before and/or after conception, and if so, during which months. Participants were also asked their pre-pregnancy weight and height, educational level and household income.

Periconceptional intake of vitamin supplements

The protective effect of folic acid is greatest when supplementation occurs prenatally and continues through early limb development, but has little or no effect after the first two months of pregnancy.⁸ Therefore, periconceptional intake of vitamin supplements was examined for two time periods. The first was defined as reporting intake of any multivitamin, prenatal vitamin or single vitamin labeled as folic acid any time from one month before the estimated date of conception through the first month of pregnancy, where the second was defined as reporting intake of any multivitamin, prenatal vitamin or single vitamin labeled as folic acid any time from one month before the estimated date of conception through the second month of pregnancy.

Genotyping

DNA samples were extracted from buccal cell samples obtained from cases and parents. Extracted DNA was stored at and retrieved from the central repository (CASPIR) at CDC. Available Arkansas DNA samples were identified locally and retrieved from our genomic laboratory freezer using our electronic inventory system. Each sample was quantified using

quantitative real-time polymerase chain reaction (RT-PCR) to determine the concentration of amplifiable DNA (TaqMan® RNase P Detection Reagents, Applied Biosystems Incorporated). Only samples with sufficient genomic DNA (0.1 ng/μl) were analyzed. Ten to twenty nanograms of each DNA sample were then amplified using whole genome amplification (WGA) techniques in a total volume of 50 μl. PCR reactions using 1 μl of the WGA product were then used for SNP genotyping using Applied Biosystems Incorporated (ABI) TaqMan® SNP assays. All assays were conducted in the Hobbs Birth Defects Genomics Laboratory.

To assure genotyping proficiency, high quality and high concordance rates among NBDPS laboratories, annual quality assessment and laboratory proficiency evaluations are conducted by the CDC as described in Supplement 1 (Documentation, External Quality Assurance Protocol). The Hobbs Birth Defects Genomics Laboratory has had consistent superior performance in these evaluations.

Statistical data analysis

Prior to statistical analysis, genotypic data were checked for Mendelian inheritance errors. Genotypes for five families with unresolved transmission errors were set to missing. Raw genotyping data are summarized in the supplemental tables. Genotyping call rates vary by family member. Call rates were higher among mothers (90%), followed by fathers (88%) and infants (86%). To make use of genotyping data from all available families, including incomplete triads, a log-linear model (Poisson) in conjunction with an expectation-maximization (EM) algorithm developed by Weinberg et al. for the analysis of case-parent data was used.^{38–40} Similar to the transmission disequilibrium test (TDT), this log-linear approach regards the association only within families, and consequently, it is robust to confounding by population stratification when testing the offspring's genotype. An advantage of this model is that it allows the estimation of the genetic relative risks and corresponding 95% confidence intervals for the association between LRDs and both maternal and offspring genotype independently.⁴¹ To assess the significance of the maternal and offspring genotypes on LRD risk, likelihood-ratio tests (LRT) were computed by comparing a full model that included maternal and offspring genotypes to reduced models that included either the maternal or offspring genotypes only. These models were estimated assuming additive genetic risks (log-additive). Similar log-linear models were also used to estimate genetic risk for transverse and longitudinal LRDs separately.

An extension of a log-linear model approach for assessing gene-environment interactions was used to test for interactions between selected maternal characteristics (obesity, folate supplementation, smoking and alcohol use) and fetal and maternal genotypes.⁴² This interaction analysis was performed using all families and also complete trios and dyads only. Significant interactions were further examined through stratification. The log-linear analyses were carried out using the LEM program without assuming Hardy-Weinberg equilibrium.⁴³

RESULTS

A total of 148 families with an infant with either transverse or longitudinal LRD met study criteria. Of these, 52 (35.1%) were complete triads, 30 (20.3%) parent-child dyads, 28 (18.9%) mother and father dyads and the remaining 38 (25.7%) were individuals, mainly mothers. Characteristics of mothers from these families are summarized in Table II. Over 65% of mothers were Caucasian, 25.7% were Hispanic and only 3.4% were African-American. The average maternal age at delivery was 27 years. Over 54% of women reported taking supplements at any time from one month prior to conception until the end of the first month of pregnancy, whereas almost 80% of women reported taking supplements by the end of the second month of pregnancy. Twenty-two percent of women reported smoking and

31.8% reported consuming alcohol at any time one month before pregnancy through the end of the first trimester. Twenty-eight (18.9%) mothers were obese (BMI $\geq$ 30) at the estimated time of conception.

The estimated genetic relative risks (RR) and 95% confidence intervals (CI) for the association between limb reduction defects and maternal and fetal genotypes, as well as the results from the likelihood-ratio tests are summarized in Table III. For some of the polymorphism, the risk of LRD changes as the number of variant alleles increases. However, none of the SNPs were found to be significantly associated with LRDs at the 5% level.

When testing for interaction between the five SNPs and obesity, maternal folic acid supplementation, smoking or alcohol use, a significant interaction was only found between the offspring's *MTHFR* 677 C $\rightarrow$ T polymorphism and maternal multivitamin supplementation early in pregnancy when only triads and dyads were analyzed (interaction $p=0.038$), but not when singletons were included ($p=0.2525$). This interaction was further examined by stratifying by maternal supplementation using all families (Figure 1). Among women who did not take supplements periconceptionally, one month before conception through the first month of pregnancy, fetuses who carried one or two copies of the 677 T allele of the *MTHFR* gene were more likely to have a transverse or longitudinal limb reduction defect than fetuses who did not carry any copies of the T allele (RR: 3.43; 95% CI: 1.26, 9.37 and RR: 11.79; 95% CI: 1.58, 87.86 respectively). The likelihood ratio test provided further evidence that the minor allele of the 677 C $\rightarrow$ T polymorphism increases the risk of LRDs via the offspring's genotype (LRT $p=0.0054$), but not the maternal genotype (LRT $p=0.6683$), among women who did not use supplements periconceptionally.

Because a large proportion of families include only a single member, we conducted a sensitive analysis to examine the impact of the missing data on our results. We replicated the previous analysis using only complete trios and dyads. A similar pattern of results were obtained using this reduced sample. When all LRD defects were examined, the offspring's LRT p -value for the 677 C $\rightarrow$ T polymorphism was 0.0029 for families whose mothers did not use multivitamin supplements and $p=0.9755$ for families whose mothers use multivitamin supplements.

When the second periconceptional exposure window was examined, one month before conception through the second month of pregnancy, a significant interaction was not detected between the offspring's *MTHFR* 677 C $\rightarrow$ T variant and maternal multivitamin supplementation. However, women in 80% of families reported taking supplements by the end of the second month of pregnancy, mainly prenatal vitamins, thus making it difficult to assess the impact of supplement use beyond the first month of pregnancy.

Of the 148 families, 92 had a child with a transverse limb reduction defect, 53 families had a child with longitudinal limb reduction defects and three families had a child with both transverse and longitudinal limb reduction defects. The estimated genetic RR and 95% CI for the association between transverse limb reduction defects and maternal and fetal genotypes are summarized in Table IV. None of the SNPs were found to be significantly associated with transverse LRDs at the 5% level. Table IV also summarizes the estimated genetic RR and 95% CI for the association between longitudinal limb reduction defects and maternal and fetal genotypes. No significant association was detected between longitudinal limb reduction defects and any of the five SNPs.

A suggestive significant interaction was found between multivitamin use and the *MTHFR* 677C $\rightarrow$ T variant when analyzing all the 95 families with transverse LRDs (interaction $p=0.0603$) and a significant interaction was found when the sample was restricted to only

triads and dyads (interaction $p=0.0182$). This interaction was also examined by stratifying by maternal supplementation (Figure 2). Among women who did not take supplements periconceptionally, one month before conception through the first month of pregnancy, fetuses who carried one or two copies of the 677 T allele of the *MTHFR* gene were more likely to have a transverse limb reduction defect than fetuses who did not carry any copies of the T allele (RR: 3.41; 95% CI: 1.22, 9.49 and RR: 11.60; 95% CI: 1.49, 89.98 respectively). The likelihood ratio test also provided evidence that the minor allele of the 677 C→T polymorphism increases the risk of LRDs via the offspring's genotype (LRT $p=0.0074$), but not the maternal genotype (LRT $p=0.9825$), among women who did not use supplements periconceptionally. When only complete trios and dyads with transverse LRDs were examined, the offspring's LRT p -value for the 677 C→T polymorphism was 0.0059 for families whose mothers did not use multivitamin supplements and $p=0.9575$ for families whose mothers use multivitamin supplements.

Of the 56 families that had a child with a longitudinal limb reduction defect, only women in 27 families reported not taking multivitamin supplements one month before conception through the first month of pregnancy. Because of the small sample size, a relative risk could not be reliably estimated for families whose mother did not take multivitamin supplements during the periconceptional period.

DISCUSSION

Ample nucleic acid synthesis and proper methylation reactions during fetal development are essential for cell replication, migration and differentiation, as well as for maintaining chromosome integrity.⁴⁴ Central to this process is the folate metabolic pathway in which folate-derived compounds serve as the donor of methyl groups for methylation and nucleotide synthesis. Reactions within the folate pathway are complex, involving multiple genes, enzymes and cofactors. Consequently, the availability of sufficient methyl groups depends on a complex and not well understood interplay of environmental, lifestyle and genetic factors.

Folate deficiency and polymorphisms in folate pathway genes may modulate risk of congenital malformations by affecting the bioavailability of methyl groups for DNA methylation reactions or nucleotide synthesis.⁴⁵ Folate deficiency and variants in folate pathway genes have been associated with increased risk of various nonsyndromic congenital malformations including neural tube defects,^{46, 47} oral facial clefts, limb reduction defects and selected heart defects.^{17, 48–50} In 2006 using California population-based case and control samples, Carmichael et al. did not find a significant association between multivitamin supplementation and LRD (RR: 0.6; 95% CI: 0.4, 1.2).¹⁷ In a more recent case-control study using NDBPS data, Robitaille et al. failed to show an association between the use of a supplement containing folic acid and either transverse (RR: 1.10; 95% CI: 0.82–1.47) or longitudinal LRDs (RR: 0.87; 95% CI: 0.59–1.28).⁵¹ However, neither of these studies examine genetic polymorphisms in conjunction with maternal intake of folic acid supplementation.

The results from our study provide additional evidence of the importance of multivitamin supplementation and genetic polymorphisms in the etiology of nonsyndromic transverse and longitudinal limb reduction defects. Our case-parental triad analysis found a significant interaction between the *MTHFR* 677C→T genotype and the use of supplements. Among women who did not use folic acid supplements, carrying one or two copies of the fetal *MTHFR* 677 T allele increased the risk of LRDs relative to those who did not carry any copy of the T allele. However, among women who reported taking folic acid supplements, the *MTHFR* 677 T allele did not confer a greater risk than the *MTHFR* 677 C allele. Women

were at significantly higher risk of having a child with either a transverse or a longitudinal limb reduction defect if they did not initiate multivitamin supplementation before the end of the first month of pregnancy and their offspring inherited one or two copies on the *MTHFR* 677T allele. Our results suggest the possibility that failing to initiate multivitamin supplementation, including folic acid, prior to pregnancy may significantly increase the risk of having a LRD-affected pregnancy, especially in families where the child may inherit either one or two copies of *MTHFR* 677T allele. This finding is particularly interesting in the context that the vast majority of conceptions included in this study occurred after mandatory folic acid fortification. A much larger assessment of SNPs and genes in these pathways is needed to more fully evaluate the relationship between folate and LRDs.

MTHFR catalyzes the irreversible reduction of 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate, the product needed for the conversion of homocysteine to methionine. The substitution of cytosine (C) by thymine (T) at position 677 within the *MTHFR* gene causes an amino acid change (alanine to valine) in the *MTHFR* protein that reduces enzyme activity. It has been estimated that enzyme activity of heterozygous CT may be reduced by approximately 35% and by as much as 70% in homozygous TT, compared to the wild homozygous CC.⁵² However, the phenotypic expression of the *MTHFR* 677 C→T mutation will vary depending on the folate status of the individual. A high dietary intake of folate may effectively neutralize the negative impact of the mutation and restore normal activity,⁵³ whereas insufficient intake of folate in the presence of the mutation leads to reduced synthesis of methionine and an increase in intracellular homocysteine, compromising essential methylation reactions.

Several methodological limitations of our study should be considered. First, the folate and methionine metabolic pathways involve multiple genes, enzymes and many other micronutrients and metabolites. Our analysis was limited to a few SNPs in four genes. Other genetic variants in these or other folate and methionine pathway genes may contribute or modify the risk of having a pregnancy affected by LRD. Second, we did not have sufficient statistical power to examine interactions between SNPs because of sample size. It is possible that the effect of alleles in two or more of these SNPs could modify the risk of LRDs. Third, because of the limited number of families in the study with longitudinal LRDs we were unable to examine the association between SNPs and specific LRD phenotypes. Fourth, the use of supplements beyond the first month of pregnancy could not be assessed because of the large number of women reporting supplementation starting during the second month of pregnancy. Fifth, information regarding periconceptional folic acid supplementation, smoking and alcohol use relies on maternal recall of both the exposure event and the timing. Errors in reporting can lead to misclassification of exposure status. Sixth, the small number of families, especially complete trios, in the study is a major limitation for the gene-environment interactions and the analysis of main effects and could be a possible explanation for the absence of significant associations with other SNPs. Lastly, Mendelian inheritance errors were checked by examining the compatibility between parental and offspring genotypes when possible.

It is possible that the observed interaction between the *MTHFR* 677C→T polymorphism and multivitamin supplementation is spurious due to multiple testing. However, this result relates most directly to the hypothesis of interest and thus has the potential of being real. A more meticulous evaluation of the relationship between genetic variants in the folate and methionine pathway genes with LRDs will require the examination of a more comprehensive list of SNPs and genes and a large sample of cases so that a more thorough analysis of gene-gene and gene-environment interactions can be conducted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Estimated relative risks of limb reduction defects associated with the child or mother carrying zero, one, or two copies of the *MTHFR* 677 T variant allele stratified by supplemental vitamin use one month before conception through the first month of pregnancy (results from log-linear model using all available families). National Birth Defects Prevention Study.

Figure 2.

Estimated relative risks of transverse limb reduction defects associated with the child or mother carrying zero, one, or two copies of the *MTHFR* 677 T variant allele stratified by supplemental vitamin use one month before conception through the first month of pregnancy (results from log-linear model using all available families). National Birth Defects Prevention Study.

Genes and SNPs included in the current analysis testing the association between genetic polymorphisms in the folate and methionine pathway with limb reduction defects

Table 1

Gene Name	Gene symbol	Location	SNP	dbSNP ID	NM Number	Amino acid change
Betaine-homocysteine methyltransferase	BHMT	5q13.1-q15	742G→A	rs3733890	NM_001713	Arg239Gln
Methylenetetrahydrofolate reductase	MTHFR	1p36.3	677C→T	rs1801133	NM_005957	Ala222Val
		1p36.3	1298A→C	rs1801131	NM_005957	Glu429Ala
Methionine synthase reductase	MTRR	5p15.3-15.2	66A→G	rs1801394	NM_002454	Ile22Met
Transcobalamin II	TCN2	22q12.2	776C→G	rs1801198	NM_000355	Pro259Arg

Characteristics of women with offspring affected by nonsyndromic transverse and longitudinal limb reduction defects: National Birth Defects Prevention Study

Table II

Characteristic	All cases		Triads/dyads (N=82)		Mother & Father only or singletons only (N=66)		P-value
	N	%	N	%	N	%	
Mother's race							
Caucasian	97	65.5	53	64.6	44	66.7	
African American	5	3.4	4	4.9	1	1.5	
Hispanic	38	25.7	20	24.4	18	27.3	
Other	8	5.4	5	6.1	3	4.6	0.6757
Mother's age at delivery (years)							
<20	16	10.8	5	6.1	11	16.7	
20-24	38	25.7	23	28.1	15	22.7	
25-29	35	23.6	21	25.6	14	21.2	
30-34	43	29.1	23	28.1	20	30.3	
≥35	16	10.8	10	12.2	6	9.1	0.3008
Household income							
<\$10,000	30	20.3	17	20.7	13	19.7	
\$10,000-\$30,000	36	24.3	20	24.4	16	24.2	
\$30,000-\$50,000	33	22.3	16	19.5	17	25.8	
≥\$50,000	41	27.7	25	30.5	16	24.2	
Missing	8	5.4	4	4.9	4	6.1	0.7601
Mother's education							
<12 years	23	15.5	11	13.4	12	18.2	
HS/GRE	39	26.4	22	26.8	17	25.8	
Some college	46	31.1	26	31.7	20	30.3	
4 or more years	39	26.4	22	26.8	17	25.8	
Missing	1	0.7	1	1.2	0	0.0	0.9002
Multivitamin supplementation: one month before conception through the first month of pregnancy							
No	68	45.9	36	43.9	32	48.5	
Yes	80	54.1	46	56.1	34	51.5	0.5782

Characteristic	All cases		Triads/dyads (N=82)		Mother & Father only or singletons only (N=66)		P-value
	N	%	N	%	N	%	
Multivitamin supplementation: one month before conception through the second month of pregnancy							
No	30	20.3	15	18.3	15	22.7	0.5047
Yes	118	79.7	67	81.7	51	77.3	
Smoker: one month before conception through the first trimester of pregnancy							
No	114	77.0	64	78.1	50	75.8	0.6381
Yes	33	22.3	17	20.7	16	24.2	
Missing	1	0.7	1	1.2	0	0.0	
Alcohol use: one month before conception through the first trimester of pregnancy							
No	98	66.2	52	63.4	46	69.7	0.4604
Yes	47	31.8	28	34.2	19	28.8	
Missing	3	2.0	2	2.4	1	1.5	
BMI (kg/m ²)							
Normal (18.5 to 24.9)	76	51.4	43	52.4	33	50.0	0.4508
Underweight (<18.5)	8	5.4	6	7.3	2	3.0	
Overweight (25.0 to 29.9)	27	18.2	12	14.6	15	22.7	
Obese (≥30)	28	18.9	17	20.7	11	16.7	
Missing	9	6.1	4	4.9	5	7.6	

Estimated genetic relative risks (RR) and 95% confidence intervals (CI) of nonsyndromic transverse and longitudinal limb reduction defects in 148 families from the National Birth Defects Prevention Study

Table III

	<i>MTHFR</i> 677C→T	<i>MTHFR</i> 1298A→C	<i>TCN2</i> 776C→G	<i>MTRR</i> 66A→G	<i>BHMT</i> 742G→A
	RR [95% CI]	RR [95% CI]	RR [95% CI]	RR [95% CI]	RR [95% CI]
Offspring					
One copy	1.31 [0.77, 2.21]	1.00 [0.56, 1.77]	1.06 [0.64, 1.77]	0.92 [0.50, 1.69]	1.10 [0.66, 1.85]
Two copies	1.71 [0.60, 4.87]	1.00 [0.32, 3.15]	1.13 [0.41, 3.14]	0.84 [0.25, 2.85]	1.22 [0.43, 3.42]
LR (p value)	0.3119	1.0000	0.8119	0.7792	0.7085
Mother					
One copy	0.76 [0.50, 1.15]	1.05 [0.60, 1.82]	1.07 [0.71, 1.60]	0.94 [0.66, 1.35]	0.83 [0.53, 1.31]
Two copies	0.58 [0.25, 1.32]	1.10 [0.37, 3.31]	1.14 [0.50, 2.57]	0.89 [0.43, 1.83]	0.69 [0.28, 1.71]
LR (p value)	0.1854	0.8652	0.7607	0.7514	0.4192

LR, likelihood ratio test; *MTHFR*, methylenetetrahydrofolate reductase; *TCN2*, transcobalamin II; *MTRR*, methionine synthase reductase; *BHMT*, betaine-homocysteine methyltransferase.

Estimated genetic relative risks (RR) and 95% confidence intervals (CI) of nonsyndromic transverse limb reduction defects and of nonsyndromic longitudinal limb reduction defects in families from the National Birth Defects Prevention Study

Table IV

	<i>MTHFR</i> 677C→T	<i>MTHFR</i> 1298A→C	<i>TCN2</i> 776C→G	<i>MTRR</i> 66A→G	<i>BHMT</i> 742G→A
	RR [95% CI]	RR [95% CI]	RR [95% CI]	RR [95% CI]	RR [95% CI]
Transverse limb reduction defects (n=95 families [†])					
Offspring					
One copy	1.49 [0.81, 2.73]	1.13 [0.60, 2.13]	1.09 [0.59, 2.03]	1.01 [0.50, 2.04]	0.86 [0.46, 1.61]
Two copies	2.21 [0.66, 7.43]	1.29 [0.36, 4.55]	1.20 [0.35, 4.11]	1.01 [0.25, 4.17]	0.75 [0.22, 2.59]
LR (p value)	0.1934	0.6947	0.7731	0.9862	0.6454
Mother					
One copy	0.77 [0.44, 1.32]	1.30 [0.63, 2.68]	1.44 [0.84, 2.47]	0.92 [0.56, 1.51]	0.98 [0.56, 1.71]
Two copies	0.59 [0.20, 1.74]	1.68 [0.39, 7.19]	2.07 [0.70, 6.11]	0.85 [0.32, 2.29]	0.95 [0.31, 2.94]
LR (p value)	0.3252	0.4823	0.1760	0.7518	0.9347
Longitudinal limb reduction defects (n=56 families [†])					
Offspring					
One copy	1.10 [0.41, 2.98]	0.84 [0.26, 2.67]	1.00 [0.42, 2.39]	0.70 [0.21, 2.27]	1.75 [0.69, 4.44]
Two copies	1.22 [0.17, 8.85]	0.70 [0.07, 7.15]	1.00 [0.17, 5.71]	0.48 [0.05, 5.16]	3.05 [0.47, 19.73]
LR (p value)	0.8432	0.7640	0.9950	0.5444	0.2317
Mother					
One copy	0.74 [0.40, 1.37]	0.70 [0.30, 1.63]	0.68 [0.35, 1.33]	1.02 [0.62, 1.68]	0.52 [0.23, 1.18]
Two copies	0.54 [0.16, 1.89]	0.49 [0.09, 2.67]	0.47 [0.12, 1.78]	1.03 [0.38, 2.81]	0.27 [0.05, 1.39]
LR (p value)	0.3337	0.4060	0.2547	0.9510	0.0998

[†]Three families had children affected by both transverse and longitudinal limb reduction defects.

NE, not estimable; *MTHFR*, methyltetrahydrofolate reductase; *TCN2*, transcobalamin II; *MTRR*, methionine synthase reductase; *BHMT*, betaine-homocysteine methyltransferase.