MTHFR Mutations and Conotruncal Defects

Prevalence in Affected Children and Their Mothers

A Thesis

by

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Précis

I have always been interested in clinical research, primarily in medical genetics, human development, and epidemiology. To that end, I enrolled in the combined MD/MPH program at OHSU in 1997. The following summer, I signed up for a course in nutritional epidemiology. I'm sorry to say that this choice was motivated more by a need to fulfill credits than by an inherent thirst for knowledge about the subject. In retrospect, however, this serendipitous selection affected my subsequent career path more significantly than any other decision since choosing to go into medicine.

In addition to taking classes that summer, I was charged with finding a clinical research internship. Fortunately, the course's professor needed a research assistant to work on a prospective study of dietary folic acid in pregnancy and conotruncal heart defects. As I had some work experience in genetics, Cynthia Morris agreed to take me on to track down the results of genetic tests ordered on participants in the study.

Reviewing the literature regarding genetic influences on folate metabolism for this internship, I came across reports linking MTHFR mutations with neural tube defects, much as dietary folate had previously been linked with these defects. Since no reports of MTHFR mutations and congenital heart defects had been published, this was an ideal topic for a thesis project in genetic epidemiology.

The process of completing this project taught me more about epidemiology and running a clinical research project than I ever thought I would know at this stage of my professional development. In addition, this research strongly influenced my decision to become a pediatrician. I look forward to continued investigation in this and related areas.

Acknowledgments

A grateful mind
By owing owes not, but still pays, at once
Indebted and discharg'd.

John Milton
Paradise Lost

I have been fortunate enough to receive such tremendous guidance and support that this potentially overwhelming project was able to come to fruition with relative ease. A page or two of acknowledgments in no way approaches of my debt of gratitude.

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Alix Seif
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I can no other answer make but thanks,
And thanks.

William Shakespeare
Twelfth Night

Abstract

Introduction: This study investigated the prevalence of the thermolabile form of methylenetetrahydrofolate reductase (MTHFR) in children born with conotruncal heart defects and their mothers. A homozygous point mutation at position 677 causes this enzyme to lose function at physiologic temperatures, resulting in reduced serum folate and increased homocysteine. A second mutation at position 1298 causes a similar reduction in enzyme function in the compound heterozygous state with the 677 mutation. This study investigated the interaction between MTHFR mutations in mothers of affected infants and nutritional and environmental factors in pregnancy.

Method: Subjects were recruited from the Oregon Congenital Heart Defect Registry (ORCHD), which is a population-based registry of children who underwent surgery for congenital heart defects. Beginning in 1958, all children with one of 14 types of heart defects requiring surgical repair who are residents of Oregon at the time of their surgery have been entered into the registry and followed every two to three years. Information collected in this database includes demographics, perioperative clinical state, details of surgery, and post-operative follow-up for major morbidity and mortality.

Cases were defined as children with conotruncal defects born after 1988 and their mothers and were recruited from ORCHD (n=110). Affected controls were children with patent ductus arteriosus (PDA) and their mothers, also recruited from the registry from the same secular cohort (n=55). Mothers were interviewed about periconceptional vitamin use, dietary and lifestyle habits. Buccal swabs were collected from mothers and children for PCR analysis of the MTHFR 677 C→T and 1298 A→C mutations.

Results: MTHFR mutations alone were not significant predictors of conotruncal defects in mothers or children. However, among women who used prenatal vitamins three or fewer days per week in the first 12 weeks of pregnancy, high-risk genotypes (677TT/any 1298, 677CT/1298AC, 677CT/1298CC) increased the risk of having a child with a conotruncal defect over women with low-risk genotypes [odds ratio (OR) 3.05, 95% confidence interval (CI) 0.89-10.45]. Conversely, taking a prenatal vitamin more than three days per week was protective for women with high-risk genotypes (OR 0.27, 95% CI 0.06-1.24). Furthermore, consumption of three or more servings of legumes per week is significantly protective against conotruncal defects (OR 0.23, 95% CI 0.09-0.59); legumes are one of the top three sources of folate in the American diet. Finally, postconceptional tobacco use was significantly negatively associated with conotruncal defects in this study (OR 0.35, 95% CI 0.12-0.99).

Conclusions: MTHFR mutations interact with low consumption of prenatal vitamin use to increase risk of conotruncal heart defects. This increased risk may be overcome by using prenatal vitamins more than three times per week in the first trimester of pregnancy. In addition, a diet high in legumes is significantly protective against conotruncal defects. Use of an affected control group in this study may have revealed an association between smoking and patent ductus arteriosus, rather than a protective effect of tobacco against conotruncal defects. A second phase of this study including a population-based cohort of unaffected controls recruited from birth certificate data and frequency-matched by year of birth is currently underway.

Introduction

The question of how low maternal serum folate levels might affect fetal growth and development has been a popular topic of research for the past two decades. The focus of much of the seminal work on folic acid and birth defects has been on dietary intake of naturally occurring and especially supplemental folate before and during pregnancy. With the recent discovery of point mutations in methylenetetrahydrofolate reductase (MTHFR) which diminish the enzyme's function, a new area of study has arisen, namely that of a genetic etiology of low maternal serum folate leading to increased incidences of congenital malformations and fetal loss.

Thus far, evidence linking dietary folate and neural tube defects (NTDs) has been more robust than evidence linking dietary folate and other defects, including conotruncal heart defects (CTDs). However, a sophisticated new avenue of investigation has been in the gene-nutrient interaction for folate and NTDs. The mutations studied are not always associated with the defects alone, but rather in the setting of low dietary folate. Given that the birth prevalence of CTDs is much lower than that of NTDs, a subtle dietary effect may be difficult to establish. On the other hand, an interaction between a genetic risk factor and a dietary one may be easier to demonstrate if the association is strengthened by an interaction effect.

An examination of the prevalence of the mutations in children with conotruncal defects and their mothers would have considerable epidemiological value, since low serum folate and the MTHFR mutations are both highly prevalent. Furthermore, dietary intervention for low folate in the setting of a MTHFR mutation is effective in correcting

the deficiency, and such correction might be protective against both neural tube and congenital heart defects (CHD) and potentially against various other congenital defects.

This study has the following objectives:

1) To test the hypothesis that mothers of children with conotruncal heart defects will have a higher prevalence of 677TT or 677CT/1298AC than mothers of unaffected children. This will be specifically determined by comparing prevalence of the mutation among mothers of children with conotruncal defects versus mothers of children without such defects.

2) To test the hypothesis that the mother's genotype is more important than the child's in determining outcome. This will be specifically determined by comparing genotype risk among mothers versus among children both alone and in interactions with established risk factors.

3) To test the hypothesis that mothers of children with CTD have lower intakes of folic acid than mothers of controls. This will be specifically examined by comparing the folic acid intake of case mothers with that of control mothers.

4) To test the hypothesis that high folic acid intake is associated with reduced risk of CTD in the presence of MTHFR mutations. This will be specifically determined by comparing odds ratios for folic acid in cases and controls in the presence of MTHFR mutations and in the absence of MTHFR mutations.

If confirmed, a significant difference between case and control groups would enable physicians to screen women with previous children with conotruncal defects for the MTHFR mutations, in order to estimate risk better for future pregnancies. As people

with the MTHFR mutation tend to have much higher homocysteine levels than expected at low levels of folate intake, this would add support to animal studies showing a potentially teratogenic effect of homocysteine. Finally, if additional folate intake is necessary to overcome the faulty enzyme, knowing a patient's MTHFR status would enable the physician to counsel the patient about how much folate she needs to consume in order to attain healthy serum levels of folate and homocysteine.

Much work has been done relating low serum folic acid to a variety of birth defects from neural tube defects to conotruncal defects. The epidemiology of low serum folate is linked both to dietary and supplemental issues, as well as to defects in the molecular biology of the folic acid pathway.

Conotruncal defects

Congenital heart defects affect an estimated 10 out of every 1000 (1%) live births, with reports ranging from 3-5 per 1000 to 12 per 1000. [1, 2] However, prenatal detection has the potential to increase the overall incidence of these defects, as many pregnancies that would not have an outcome of a live-born affected child would be detected. One strictly prenatal study in which fetuses were diagnosed at 18 weeks gestation showed a prevalence of 11-12 per 1000, at the upper end of the overall prevalence. [3] As a subset, conotruncal anomalies make up an estimated 0.732 per 1000 live births, or about 0.1% of all live births (10% of all cardiac anomalies). [4] Affected infants are typically cyanotic because of mixing of the pulmonary and systemic circulations. Almost all of these lesions are either fatal without correction or result in severely compromised quality of life.

Conotruncal defects arise early in pregnancy, typically in the first 6-8 weeks of development. A complex interaction of neural crest cells most likely from branchial arches III and IV contributes to the formation of the conotruncal cushions which spiral up to form the cardiac outflow tracts. [5] Defects arise from abnormal septation of the conotruncus, resulting in transposition of the great arteries (TGA), tetralogy of Fallot (ToF), pulmonary atresia with ventricular septal defect (PA+VSD),[a] and truncus arteriosus (TruncA). [6] Related defects include aorticopulmonary window (APW), interrupted aortic arch (IAA), double-outlet right ventricle (DORV), subarterial (type 1) VSDs [7] and double-outlet left ventricle (DOLV). [8]

Folic acid metabolism and homocysteine

Folic acid is a water-soluble B-group vitamin found in leafy green vegetables, legumes and citrus fruits. Its primary function relates to donation of methyl groups to various compounds in DNA synthesis and cell growth. In addition to providing key reagents for nucleotide synthesis, it is responsible for regeneration of the "universal" methyl donor, S-adenosylmethionine (SAM) from homocysteine. Of note, folic acid is the fully oxidized, synthetic form of the compound and is not found in nature; however, forms of this pterin occur naturally in various stages of methylation. [9] The tetrahydrofolates are the active forms of the compound; fully reduced forms accept methyl, formyl and methylene groups. The rate-limiting step of thymidylate synthesis, essential for DNA production, requires 5,10-methylene tetrahydrofolate as a reagent. Formation of this key coenzyme is dependent on adequate stores of vitamin B_{12}

[a] These defects are referred to as "tetralogy of Fallot with pulmonary atresia" (TetPA) in the present study.

(cobalamin), as well as folate. [10] The methylene tetrahydrofolate reductase (MTHFR) enzyme is the catalyst for the transformation from 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, which then donates a methyl group to homocysteine to form SAM with the methionine synthase enzyme. Tetrahydrofolate is then recycled into its 5,10-methylenated form to start the cycle again. [11]

Homocysteine is a byproduct of metabolism of the non-essential amino acid methionine. Homocysteine may be remethylated into SAM, as above, or converted into cystathionine by cystathionine synthase with vitamin B_6 as a cofactor. Cystathionine is a precursor to cysteine, which eventually forms glutathione, which is essential to normal red blood cell membrane synthesis. [9]

Folate and neural tube defects

Neural tube defects result from a failure of complete midline closure of the embryonic neural tube in the first 10-12 weeks of development. Defects include the spina bifida and anencephaly variants. In the mid-1990s, birth prevalence in the United States was about 4 per 1,000 live births.[12]

In the earliest investigations, recurrence risk of neural tube defects was notably attenuated in women who took a multivitamin regularly.[13] A subsequent case-control study in the United States found a significantly reduced risk of NTD among women taking multivitamins periconceptionally [odds ratio (OR) 0.40; 95% confidence interval (CI) 0.25-0.63]. [14] Folate was identified as the likely protective agent when a large randomized controlled trial found a relative risk (RR) of 1.95 (CI 1.23-3.09) for NTD in women who took trace elements in multivitamins for the month prior and two months

post conception versus women who took 0.8 mg folic acid (FA). [15] A second large trial showed a protective effect for supplemental folic acid in women at high risk of having NTD-affected children (RR 0.28; CI 0.12-0.71); other vitamins showed no protective effect. [16] Most recently, a population-based randomized clinical trial in China demonstrated a large risk reduction in a high-risk population (birth prevalence 4.8 per 1,000 live births - risk reduction 85%, 95% CI 62-94%). This group found a smaller risk reduction in a lower-risk population (birth prevalence 1.0 per 1,000 live births – risk reduction 41%, 95% CI 3-64%) with supplementation of 400 mg of folic acid daily from the premarital examination through the end of the first trimester of pregnancy. [17]

An Irish case-control study shows a dose-effect relationship between red cell folate (RCF), which is an indicator of stored or long-term folate intake, and risk of NTD. Respectively, the relative risk (RR) per 1,000 births falls from 6.6 to 3.2, 2.3, 1.6, and 0.8 as RCF levels increase from 0-399 to 340-352, 453-679, 680-905, and ≥906 nmol/L. [18] A case-control study of families with a history of spina bifida shows a significant elevation the plasma homocysteine (pHcy) levels of affected families, as well as a significantly decreased mean plasma folate (pFA) level. [19] Animal models also support a relationship between folate and neural tube formation: mouse models of dietary deficiency, folate-antagonist teratogen administration, and knockout genes for folate-sensitive NTDs are all associated with neural tube defects. [20]

A recent review of the literature asserts that obstetricians should advise their patients to take 0.4 mg supplements of folic acid daily and 4 mg if there is a previous history of NTD-affected offspring. [21] As many as 50% of all neural tube defects may be preventable by increasing folic acid intake. [22] A systematic review of periconceptional

folate supplementation from the Cochrane Database showed a substantial risk reduction (RR 0.28; 95% CI 0.13, 0.58) with a number needed to treat of 847 to prevent a case of NTD. Of note, multivitamin supplementation was not found to reduce the risk of NTD significantly (RR 0.61, 95% CI 0.26, 1.46). [23]

One proposed mechanism for the effect of folic acid is its reduction of plasma homocysteine, which is found to be elevated in mothers of infants with NTDs. In one study, blood samples from pregnant women were analyzed for Hcy, methylmalonic acid (MMA), pFA, RCF, and B_{12}. Mothers with affected infants had significantly higher pHcy levels (μ=8.62 [SD 2.8] μmol/L) when compared to women with unaffected children matched for B_{12} (μ =7.96 [2.5] μmol/L; p=0.03). The authors suggest that a defect in Hcy metabolism is likely related to increased NTD incidence. [24] A study of non-pregnant women with NTD-affected children found similarly elevated Hcy in the NTD group. The authors propose a defect in remethylation of Hcy to methionine, secondary to either an acquired (dietary) or inherited disorder of folate metabolism. [25] Exogenous administration of homocysteine to avian embryos has been shown to induce neural tube, craniofacial, and multiple defects by inhibiting the function of N-methyl-D-aspartate receptors in the neural epithelium. As a teratogen, homocysteine works in apparent synergy with ethanol and NMDA-specific calcium channel blockers.[26]

Other proposed mechanisms revolve around folate receptors in the neural crest cells. In addition to defects in folate metabolism resulting in low levels of usable folate, other folate-sensitive gene candidates have been identified, including folate-receptor alpha, which is responsible for folate transport into cells. [27] Since neural crest cells have

such high mitotic indices, requiring relatively high levels of folate, defects in these receptors may have effects on neural tube formation. [28]

Folate and conotruncal defects

Given that neural tube defects are midline defects, considerable interest has arisen in looking at a relationship between folate and homocysteine status and other midline defects, notably conotruncal heart defects. As with neural tube defects, early studies focused on dietary intake of folic acid, with studies of folate metabolism emerging later.

Several studies have demonstrated an inverse relationship between maternal folic acid intake and incidence of conotruncal defects. In 1995, Shaw, et al., found a reduced risk of all conotruncal defects among multivitamin users [odds ratio (OR) 0.53, 95%CI 0.34-0.85]. [29] A similar overall reduction in all conotruncal defects was found in a second case-control study for women who used a multivitamin periconceptionally versus women who did not use a supplement (OR 0.57; 95% CI 0.33-1.00). This study found the greatest risk reduction for isolated conotruncal defects (OR 0.41, 0.2-0.84), whereas neither noncardiac (OR 0.91, CI 0.33-2.52) nor syndromic defects showed a significant reduction in risk (OR 1.82, CI 0.31-10.67). The most greatly affected anatomic subgroup of cardiac anomalies was transposition of the great arteries, with an OR of 0.36 (0.15-0.89). [30]

A large but non-population-based case-control study found no significant effect of maternal folate intake on transposition of the great vessels (OR 1.04, 0.5-2.2), [31] and a second found no effect for any conotruncal defects. [32] In addition, the Cochrane review found no significant risk reduction by folate supplementation for conotruncal defects (RR

0.74, 95% CI 0.14, 3.32), limb reduction defects (RR 0.59, 95% CI 0.04, 8.34), or orofacial clefts (RR 0.76, 95% CI 0.24, 2.37). When all defects including NTDs are combined, folate is not found to reduce risk significantly with a relative risk of 0.76 (95% CI 0.38, 1.51). [23] On the other hand, a prospective population-based study in Denmark found a significant difference in all malformations that are thought to develop before 7 weeks gestation, provided they began folate supplementation before 7 weeks. [33]

Both animal and human studies show evidence of an effect of elevated homocysteine on conotruncal development. Avian studies show a teratogenic effect of homocysteine when given during the period of conotruncal septation, with significant risk reduction with concomitant folate administration. [34] In humans, Kapusta, et al. found elevated homocysteine levels in mothers of children with congenital heart defects versus mothers of unaffected infants; folate levels were not significantly different between groups, suggesting a defect in homocysteine catabolism. [35]

A recent study investigating folate antagonists further supports the hypothesis that low maternal folate may increase the risk of having a child with a congenital heart defect. This case-control study showed an association between this class of drug and congenital heart defects (OR 2.1; 95% CI 1.5-3.0). The authors looked at two classes of folate antagonists: dihydrofolate reductase inhibitors, including trimethoprim and sulfasalazine, and "other" folate antagonists, including anticonvulsants, such as phenytoin, carbamazepine, and valproate. Dihydrofolate reductase inhibitors were associated with an increased risk of congenital heart defect in the absence of folate supplementation (OR 7.7; 95% CI 2.28-21.7) that returned to baseline risk with supplement use (OR 1.5; 95% CI 0.6-3.8). The anticonvulsants, however, produced a smaller risk in the absence of

folate supplementation (OR 2.0; 95% CI 1.1-3.7), which did not improve with folate supplementation (OR 2.3; 95% CI 1.1-4.7). [36]

MTHFR and folate

Altered activity of the enzyme methylenetetrahydrofolate reductase (MTHFR) results in elevations in pHcy and decreased pFA. The enzyme is responsible for conversion of methylenetetrahydrofolate to 5-methyltetrahydrofolate. Only the 5-methylated form can serve as the methyl donor to homocysteine, forming tetrahydrofolate and methionine. Importantly, the MTHFR product, 5-methyltetrahydrofolate, is the predominant form found in plasma, whereas the enzyme substrate, methylenetetrahydrofolate, is the stored form found in erythrocytes. Hence, mutant MTHFR produces elevated RCF and decreased pFA. [37]

Point mutations in the gene encoding this enzyme occur frequently enough to be classified as polymorphisms. A substitution of the nucleotide cytosine with a thymine at position 677 results in the more commonly studied mutation, MTHFR 677 C→T. This substitution results in thermolability of this protein at physiologic temperatures. Homozygotes for this mutation are estimated to have approximately 50% of the normal enzyme function *in vitro*. A second point mutation occurs at position 1298 with an adenosine to cytosine mutation (1298 A→C). This mutation is less often included in studies but is associated with a similar reduction in enzyme function in the heterozygous state when combined with a heterozygous 677 mutation to the enzyme function seen with homozygous 677 mutations *in vivo*, although this finding was not replicated *in vitro*. [38]

Most gene frequency studies find a baseline frequency for the homozygous MTHFR 677 C→T mutation to be approximately 10%. [39, 40] In certain populations, however, the prevalence is much higher; for example, in healthy Italian subjects the baseline prevalence for the homozygous mutation is as high as 16% (versus a reported value of 11.5% in the US and Australia). [41] However, homocysteine levels in this population were comparable to those of other European studies. This last observation points to the interaction between dietary intake of folic acid and this malfunctioning enzyme, as cultural factors may influence intake. In fact, the mutation may only be problematic in the setting of dietary deficiency in folate. [42] For example, dietary patterns may explain in part why Ashkenazi Jews have a low NTD prevalence despite increased frequency of 26.4% for the homozygous mutation. [43]

A recent pooled estimate of homozygote prevalence shows a range from 7.5% - 13% for most of Europe, North America and Japan, while data from Italy and from Hispanic Americans show higher prevalences (over 15%), and Sub-Saharan Africans and African-Americans have less than 2% prevalences. Homozygous infants have a pooled odds ratio of 1.73 (1.39-2.16) for NTD, and homozygous mutant mothers have a pooled OR of 2.05 (1.47-2.86). Neither heterozygous mothers nor children show a significantly elevated risk. Of note, fathers' MTHFR status is unrelated to infant outcome (OR 1.18; 0.65-2.12). [44]

In particular, patients with elevated pHcy are more likely to carry the thermolabile enzyme than controls. One study shows patients with coronary vascular disease have a trend towards increased risk of being homozygous (OR 3.1, 95% CI 1.0-9.2), [45] while in another study 73% of subjects with elevated pHcy are homozygous for the mutation

versus 10% of normal controls ($p < 0.001$). [46] Importantly, this study also demonstrates that most of these patients reach normal values of serum homocysteine and of serum folate after several weeks of daily supplementation with only 0.2 mg folic acid, and the rest normalize with a much higher dose of five mg per day. Other studies cite improvement with values ranging from 0.5-1 mg per day. [47] The RDA currently recommends 400 μg per day of supplemental folic acid. [48]

MTHFR and Neural Tube Defects

A gene frequency analysis of families with histories of spina bifida shows that mothers of children with spina bifida are significantly more likely than mothers of controls to be homozygous for the mutant MTHFR 677C→T gene (OR 3.7, 95% CI 1.5-9.1). [37] Affected children are also more likely to be homozygous for the mutant (OR 2.9, 95% CI 1.0-7.9). Another case control study reports an odds ratio of 7.2 (95% CI 1.8-30.3) for affected infants to be homozygous for the mutation, as well as an OR of 2.4 for babies with one or two mutant alleles (95% CI 1.1-5.2). [49] Hyperhomocysteinemia has also been linked to repeated fetal loss, although mutation prevalence was not found to be significantly different among cases and matched controls. [50] One case-control demonstrated significant risks of NTD in children (OR 2.56; 95% CI 1.28-5.13) and mothers (OR 3.05; 95% CI 1.54-6.03) with low red blood cell folate, which are intensified in conjunction with homozygous MTHFR 677 mutations (OR 13.43; 95% CI 2.49-72.33 for children; OR 3.28; 95% CI 0.84-12.85 for mothers), suggesting a gene-nutrient interaction. [51]

A second mutation site (1298A→C) in the gene has also been identified and linked to reduced MTHFR activity. One study found a 10% homozygous prevalence, which conferred 60% enzyme activity, as well as an overall compound heterozygosity (one each of the 1298A→C and 677C→T mutations) prevalence of 15% with a more severe reduction in enzyme function. [52] Another study found a compound heterozygosity prevalence to be 21-22% for two US populations. [43] While neither homozygotes nor heterozygotes for the 1298 mutation had increased pHcy or decreased pFA, compound heterozygotes did have significantly affected values. Furthermore, NTD patients show a trend towards increased prevalence of compound heterozygosity (OR 2.04; CI 0.9-4.7). [53] A recent case-control study of NTD-affected children (N=148) also found a trend towards a compound heterozygous effect; cases had a 24% prevalence compared with only 16% in controls ($p = 0.07$). [54]

None of the above studies observed any patients homozygous for both mutations. Furthermore, a study of fetal viability looking at neonatal cord blood and spontaneously aborted fetal tissue found no instances of double homozygous mutants in the cord blood samples; whereas, the fetal samples yielded all possible genotypes, implying sufficient enzyme impairment as to be incompatible with life. [55] Subsequently, investigations of compound genotypes and spina bifida revealed a single example of 677TT/1298CC out of 808 Hispanic participants of Mexican descent, in a mother of an affected child; the same group of investigators found no double mutants in a U.S. sample of 482 subjects of European descent. [56] Of note, this publication does not mention how the samples were obtained nor does it address the possibility that this finding may represent a mosaic carrier of the double mutant genotype. In any case, if a double germline mutation is

compatible with life, it remains exceedingly rare and as yet unreported in an individual of European descent.

MTHFR and Conotruncal Defects

Two studies investigating the MTHFR mutation and congenital heart defects have been published since the present study began. Both studies have focused on the child's genotype rather than the mother's. Neither of these studies looked at the combined risk of heterozygous 677 and 1298 mutations.

Junker, et al. looked at the 677 mutation in a wide variety of non-syndromic structural heart defects, only one of which was strictly conotruncal (D-TGA). This study found an overall OR 2.2 (95% CI 1.2-4.3). While D-TGA was not significantly associated with an increased mutation rate, this study included only nine such patients. [57] Although the authors report significant associations for individual defects, their cell sizes are notably small (e.g. pulmonic stenosis is listed as the strongest association, however, two out of a total of three patients are MTHFR-positive). The overall result remains compelling, however.

In a study looking at amniocyte genotype and amniotic fluid homocysteine levels, Wenstrom, et al. demonstrate both elevated amniotic fluid homocysteine levels in pregnancies with isolated congenital heart defects compared with normal pregnancies and elevated risk of MTHFR 677 mutation. Amniotic fluid homocysteine levels greater than the 90th percentile had a significantly increased risk of congenital heart defect (OR 3.5, 95% CI 1.2-10.2). Similarly, affected pregnancies were also more likely to have heterozygous or homozygous MTHFR mutations (OR 3.6, 95% CI 1.3-9.8). Finally,

pregnancies meeting both criteria were at markedly increased risk for congenital heart defects versus pregnancies not meeting both criteria (OR 34.7, 95% CI 1.7-694.3). [58] This study would have been further strengthened by assessment of mother's serum homocysteine and genotype.

Since maternal and fetal genotypes are not independent variables, one would expect that if there is an association with the mutation and the defect, the only way to know whether the mother's or the child's genotype is truly responsible for the outcome is to investigate both and examine discordant mother-child pairs to assess risk. From a mechanistic standpoint, this finding might elucidate whether the defect is a result of overproduction of homocysteine in the very early embryo or whether the mother's deranged homocysteine catabolism bathes the developing embryo in a teratogen.

Folate Intake in the US

Data from several population-based studies indicate that folate intake for women of childbearing age is consistently below what is currently recommended. Analysis of data collected by the United States Department of Agriculture in the Continuing Survey of Food Intakes of Individuals (CSFII) shows that only 30% of lower income women (income < 131% of the poverty level) and 50% of higher income women (>300% of poverty) achieved the Recommended Dietary Allowance (RDA) of 180 μg folic acid. [59] Data from phase I of the Third National Health and Nutrition Examination Survey (NHANES III) show mean values which surpass the RDAs for folate at the time, but which fall far short of the current recommendation of 400 μg of folate daily. [60]

As a measure of public health education outcomes, a cross-sectional telephone survey was conducted which showed that 44.3% of American women of childbearing age use a supplement containing folic acid (32.2% daily and 12.1% less than daily). Of note, fewer women aged 18-24 took a folic acid supplement (daily use 23% versus less than daily use 10%) than women aged 25-34 (daily 36% versus less than daily 14%) or aged 35-45 years (daily 35% versus less than daily use 12%). Only 45% of women who reported hearing about the Public Health Service's recommendation to take a folate supplement actually took one on a daily basis. [61] A Georgia study showed that 71% of all respondents (women aged 15-44) report never having heard a recommendation to take folic acid. [62] Young women are also largely unaware of the need to increase folate consumption. Among 16-19 year-olds, only 14% knew of the recommendation, while 41% of a population of undergraduates were familiar with the suggestion. [63]

The most exciting feature of this study is the potential for successful intervention. In 1996, the United States Food and Drug Administration implemented a policy requiring folate fortification of most flour, rice, noodles, and grain products in order to help women approach the recommended folate intake of 400 μg per day. [64] A recent study notes that this fortification of 140 μg folic acid per 100 g of grain showed significant increases in mean plasma folate, as well as a reduction in prevalence of low folate concentrations (<3 ng/ml or 7 nmol/l). Similarly, fortification resulted in significant decreases in mean plasma pHcy and in the prevalence of pHcy >13mmol/l. [65]

A 12-week interventional study showed that non-consumers of folate-fortified products (less than once per week) consumed 78 mg per day of folic acid less than regular consumers. This resulted in a drop of 11 nmol/L in red cell folate over a 12-week

period. [66] Evidence suggests that consistent, low-level folate intake is slightly more effective at lowering plasma homocysteine than less frequent but higher doses. [67] Finally, the most compelling evidence that the intervention has been successful is in the drop in birth prevalence of NTDs since fortification went into effect (prevalence ratio 0.81; 95% CI 0.75-0.87). [12]

Methods

This is a case-control study of the prevalence of the MTHFR 677C→T and 1298A→C mutations in children with conotruncal heart defects and their mothers versus children with non-conotruncal heart defects and their mothers. Subjects recruited from the Oregon Registry of Congenital Heart Defects (ORCHD) were interviewed and tested for the mutations.

Oregon Registry of Congenital Heart Defects

The Oregon Registry of Congenital Heart Defects (ORCHD) is a population-based registry of children with congenital heart defects. Beginning in 1958, children with heart defects requiring surgical repair who are residents of Oregon[b] have been entered into the registry and followed every two to three years. Information collected in this database includes demographics, perioperative clinical state, details of surgery, and post-operative follow-up for major morbidity and mortality.

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[b] The catchment area includes a part of southwest Washington considered to be a part of the Greater Portland community.

There are 14 heart defects included in the registry; Table 1 shows the defects and their total numbers since 1958, and Table 2 shows the number of each type of heart defect entered in the registry since 1991 by year. Three conotruncal defects are not included in this database: L-transposition of the great arteries (L-TGA), interrupted aortic arch (IAA), and double outlet right ventricle (DORV).

Table 1: Total Defects in ORCHD since 1958[c]	**Number**
Tetralogy of Fallot (ToF)	*525*
Ventriculoseptal defect (VSD)	617
Atrial septal defect (ASD)	678
Coarctation of the aorta (CoA)	600
Aortic stenosis (AS)	252
Pulmonary stenosis (PS)	279
D-Transposition of the Great Arteries (D-TGA)	*233*
Patent ductus arteriosus (PDA)	*653*
Partial atrioventriculosetpal defect (pAVSD)*	125
Complete atrioventriculosetpal defect (cAVSD)*	186
Pulmonary atresia (PA)**	35
*Pulmonary atresia with ventriculoseptal defect (PA+VSD)***	*53*
Total anomalous pulmonary venous return (TAPVR)	83
Truncus arteriosus (TruncA)	*35*
Total	**4354**

*AVSD is also known as atrioventricular canal (AVC)
**PA+VSD is also known as Tetralogy-type pulmonary atresia (TetPA)
Study defects in italics

Table 2: ORCHD Defects since 1991	**1991**	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**	***Total***
ToF	14	10	12	16	9	13	10	4	0	***88***
D-TGA	10	9	8	6	9	10	4	7	0	***63***
PA+VSD/TetPA	2	5	4	3	5	6	0	1	0	***26***
TruncA	1	2	0	5	1	0	1	1	0	***11***
Total CTD	**28**	**26**	**25**	**33**	**26**	**29**	**16**	**13**	**0**	***196***
PDA	23	16	18	16	20	17	12	5	1	*128*

[c] Totals are updated through 1999.

Benefits of using this population include its statewide catchment area and its comprehensive nature. Demographic information is readily available, and frequent follow-ups ensured reasonably up-to-date addresses and phone numbers for most subjects. Both cases and controls were recruited through the same database, since all PDAs requiring surgery are also included in this registry. Importantly, the database provides a sufficient number of potential case recruits for this scarce population.

This population is geographically widespread throughout the state, so practical aspects of sample collection and interviewing were more difficult than if the population were drawn only from the Portland metropolitan region. Furthermore, as entry into the database is automatic upon surgical repair, some of these subjects would not have volunteered participation in ORCHD and were reluctant to participate in the present study. Women who terminate affected pregnancies or whose babies are stillborn are not included. Only surgical patients are entered into the database, and then largely only patients with relatively straightforward abnormalities; therefore, patients whose defects did not require surgery were missed, as were patients with overly complex defects. As mentioned earlier, there are three specific types of CTD (IAA, DORV, and L-TGA) that are not included in the database.

Children with syndromes were excluded from the study, as many CTDs are associated with known and unknown genetic syndromes. Participants were specifically

asked about a history of microdeletion of chromosome 22q.[d] Mothers who were not English speaking were also excluded from the study, due to difficulties in obtaining truly informed consent and interviews. Deceased children or mothers were excluded, as were index children who were adopted or the product of donor eggs.

Study Design

Patients and their mothers were recruited from ORCHD. Cases were CTD-affected children and their mothers. Cases included children with tetralogy of Fallot with and without pulmonary atresia, truncus arteriosus and transposition of the great arteries.

Controls were patent ductus arteriosus (PDA)-affected children and their mothers from the registry. Patent ductus arteriosus is a persistence past the age of 3 months of the fetal circulatory architecture, which shunts blood from the right ventricle away from the deoxygenated lungs and directly into the aorta and back to the oxygenated placental circulation. Normally, the ductus arteriosus closes in the first 48-72 hours of life; however, the fetal circulation may persist in cases of neonatal illness, prematurity, and duct-dependent cardiac lesions. There may also be an idiopathic persistent ductus. Many

[d] The 22q11 microdeletion is associated with the DiGeorge/Velocardiofacial family of cardiac and facial anomalous syndromes (DG/VCFS). The mutation is thought to impair proper migration of the branchial arches, affecting palato-facial and cardiac development, especially that of the conotruncus. One study estimates the UK prevalence at 13 per 100,000 live births. [68] In conotruncal defects, deletions were found in 50% of patients with interrupted aortic arch, 34.5% of patients with truncus arteriosus, and 15.9% with tetralogy of Fallot. Two out of six patients with posterior malalignment ventricular septal defect and one of 20 patients with double outlet right ventricle had the deletion, and no patient with transposition of the great vessels had a deletion. Deletions were also found to be more prevalent in patients with any anomalies of the aortic arch or pulmonary vessels than in patients with normal aortic arch and any other abnormality. [69] Of note, many patients with these deletions have very subtle phenotypic manifestations that go undetected by clinicians. Although chromosomal analysis for this deletion is emerging as the standard of care for children with cardiac defects, many practitioners still elect not to perform this relatively costly study on everyone. Without testing the children ourselves, we cannot be certain that children who have never been tested are truly negative for DG/VCFS.

PDAs close with medical intervention or with time; others must be closed by placement of a clip via thoracotomy. Newer techniques include thoracoscopic placement of a clip or arterial placement of coils. Only children with PDAs lasting past 3 months of age are included in ORCHD, and of these 100% have had surgical closure by one of these two methods. Prevalence is approximately 0.5 per 1,000. [70]

The advantage of using PDA patients and their mothers as controls was first that they were from the same catchment area and time period as the cases, and PDAs are not conotruncal defects. Furthermore, PDAs are not associated with FA level. Prematurity, which is correlated with PDA, [71] is not affected by multivitamin use. [72] Using mothers of children with malformations as controls reduces the risk of recall bias, since mothers of other affected children are more likely to have similar patterns of self-scrutiny in reporting their historical information. [73] Importantly, however, the surgical or angiographic repairs of PDAs are minimal in severity compared with the often multiple open-heart procedures required for repair of most conotruncal defects.

Subjects were initially contacted by mail about obtaining mucosal samples and participating in an interview; the recruitment letter was followed by a phone call in which recruitment was finalized and arrangements to obtain the samples were made. A mailed follow-up letter and consent form[e] followed, to be returned with the used buccal swab kit. If the mother was willing to participate in the interview at the time of recruitment, consent to participate was implied at that time, and she was subsequently mailed a consent form, swab kit and instruction sheet. No interviews or DNA results were used

[e] The consent form was accompanied by a glossary of terms, including definitions of "congenital", "genetic", and "risk factor gene" versus "disease gene".

unless a signed consent form was on file. All recruitment, data collection and storage, and sample analysis protocols were approved by the Oregon Health and Science University Institutional Review Board (OHSU IRB #5720).

Mothers were interviewed about demographic and ethnic information, obstetric history, family and personal medical history, periconceptional lifestyle habits, supplement use and diet. Interview data were recorded on standardized forms maintained in locked drawers. The results were coded and entered into a flat data file maintained on a password-protected drive.

Patients and their mothers were asked for a buccal swab for MTHFR analysis. This non-invasive method of obtaining tissue samples likely increased participation, especially for the children. In one study, 97.4% of mailed buccal samples yielded at least one genotype; [74] subjects in this study were asked for multiple swabs in order to maintain backup samples in case of testing failure.

Each consenting subject was sent two kits for obtaining the mucosal samples in a standardized, sterile manner, as specified by the testing laboratory's protocol. Each kit included four individually wrapped sterile cotton swabs, which were clearly labeled for mother and for child. Samples were mailed to the participants only with coded identifiers in postage-paid envelopes. Once returned, they were stored in a refrigerator in one of the investigator's locked office.

After samples were collected and encoded, they were sent in batches to Thetagen Laboratory in Seattle, Washington for PCR analysis of the gene with the MTHFR 677 and 1298 probes. The automated system employed is 99-100% accurate for gene amplification. [75, 76] As the results were performed using laboratory research protocols,

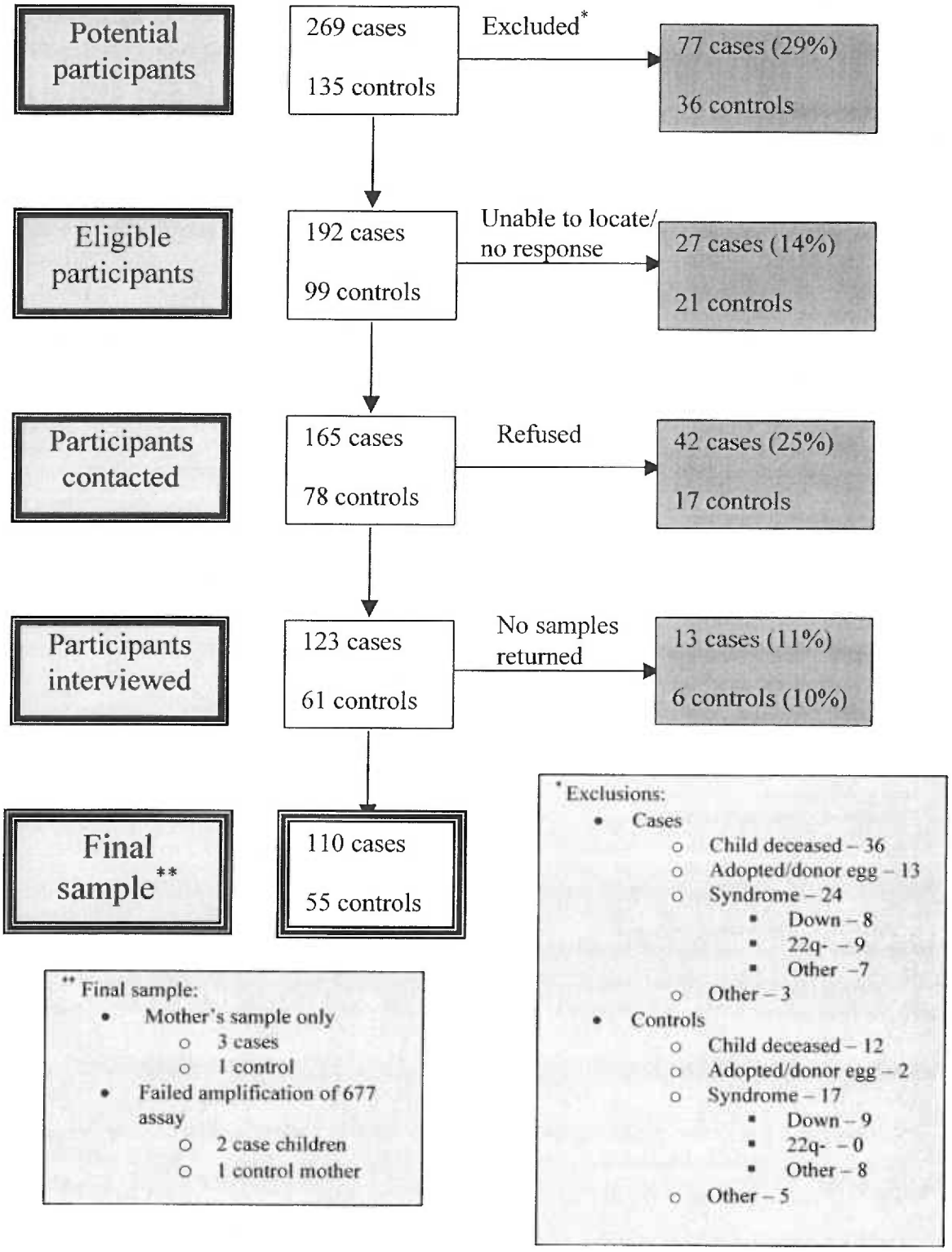
rather than clinical protocols, the results were not released to the participants. Unfortunately, Thetagen discontinued this assay in January 2001. The remaining samples were sent to Pro-ADN Laboratories in Montréal, Canada. Approximately 50 backup samples on previously analyzed subjects were sent as quality control; the results showed 100% concordance between labs. The results of the MTHFR mutation analysis were linked with the interview data set by the coded unique identifiers.

Encoded data were imported into the SPSS statistical software package (version 10.1.3, SPSS Inc., Chicago, IL). Univariate analyses were computed using Crosstabs with χ^2 statistics and Mantel-Haenszel odds ratios for 2x2 tables. Binomial logistic regression models were created for continuous and dichotomized variables of empirical significance (tobacco use, alcohol use, supplement use and diet) using case and control status as the outcome. In addition, logistic regressions were repeated including interaction terms computing these *a priori* lifestyle variables and maternal and child genotypes.

Results

Of the initial 404 potential participants (269 cases and 135 controls) from the ORCHD database (Figure 1), 192 cases and 99 controls were eligible for inclusion. Of these, 123 cases and 61 control mother-child pairs were enrolled in the study. Swab samples were obtained for 110/123 case mothers who were interviewed and 107 case children. Of controls, swabs were obtained for 55/61 mothers and 54 children. Two case children and one control mother had samples for which the 677 assay failed amplification.

Figure 1: Participation and Recruitment



Demographics

Case and control mothers were similar in mean age, education, employment at the time of conception, and insurance status during pregnancy. The groups did not differ significantly by race, although control mothers were more likely to be of Hispanic origin than case mothers (10.9% vs. 1.8%, p=0.06). Part of the interview involved ascertaining ethnicity, and participants reported up to two ethnicities for themselves and for the index children's fathers. Cases and controls showed no significant differences in ethnicity, although Mexican heritage approached significance (p=0.08). Paternal age, race, Hispanic origin and ethnicity were not significant, with the exception of Pacific Islanders, which comprised 3.6% of the controls and none of the cases (p=0.04).

Mean child age at the time of interview differed significantly between cases and controls, with cases being approximately 1.5 years younger than controls (mean 6.6 vs. 8.2, p<0.01). Median date of birth also differed significantly (cases: November 19, 1993; controls: November 3, 1991; p<0.01).

Table 3: Demographic variables		Case (n=110)		Control (n=55)		p
		N	%	N	%	
Mother	Mean age @ conception (years)	27.6±5.9		27.2±6.1		0.68*
	High school diploma or less	48	43.6	23	41.8	0.82**
	Employment @ conception					
	Working for wages	70	63.6	33	60.0	
	Self-employed	6	5.5	4	7.3	
	Unemployed	4	3.6	3	5.4	0.39**
	Homemaker	18	16.4	14	25.5	
	Student	10	9.1	1	1.8	
	Unable to work/other	2	1.8	0	0	
	Insurance during pregnancy					
	None/self-pay	7	6.4	3	5.5	0.96**
	Private	74	67.3	38	69.1	
	Public	29	26.4	14	25.5	

	Race					
	Caucasian	106	96.4	48	87.3	
	Native American	1	0.9	4	7.3	0.11**
	African American	0	0	0	0	
	Asian American or Pacific Islander	1	0.9	1	1.8	
	Unspecified	2	1.8	2	3.6	
	Hispanic	*2*	*1.8*	*6*	*10.9*	*0.06***
	Ethnicity†					
	Southern European	8	7.3	4	7.3	1.00**
	Northern European	93	84.5	41	74.5	0.12**
	Asian	0	0	1	1.8	0.16**
	Pacific Islander	1	0.9	0	0	0.48**
	Native American	9	8.2	9	16.4	0.11**
	Sub-Saharan African	0	0	0	0	
	Ashkenazi Jew	0	0	0	0	
	French Canadian	1	0.9	1	1.8	0.62**
	Mexican	2	*1.8*	4	*7.3*	*0.08***
	Unknown	11	10.0	5	9.1	0.85**
	Other	0	0	1	0.6	0.16**
Father	Mean age @ conception (years)	30.3±6.7		29.5±6.7		0.44*
	Race					
	Caucasian	104	94.5	49	89.1	
	Native American	0	0	0	0	0.30**
	African American	0	0	1	1.8	
	Asian American or Pacific Islander	1	0.9	2	3.6	
	Unspecified	5	4.5	3	5.5	
	Hispanic	12	10.9	5	9.1	0.40**
	Ethnicity†					
	Southern European	16	14.5	4	7.3	0.18**
	Northern European	71	64.5	35	63.6	0.91**
	Asian	2	1.8	1	1.8	1.00**
	Pacific Islander	***0***	***0***	***2***	***3.6***	***0.04*****
	Native American	*10*	*9.1*	*1*	*1.8*	*0.08***
	Sub-Saharan African	0	0	1	1.8	0.16**
	Ashkenazi Jew	1	0.9	2	3.6	0.22**
	French Canadian	2	1.8	1	1.8	1.00**
	Mexican	7	6.4	4	7.3	0.83**
	Unknown	19	17.3	8	14.5	0.66**
	Other	0	0	0	0	
Child	*Mean age @ interview*	***6.6±3.1***		***8.2±3.0***		***<0.01****
	Median date of birth	***11/19/93***		***11/03/91***		***<0.01****

*T-test for independent samples

** Pearson χ^2

† Participants were allowed to report up to two ethnicities; totals ≠ 100%

Medical history

Case and control mothers had similar overall pregnancy histories, with no significant differences between mean gravidity, live births, stillbirths, spontaneous

abortions, therapeutic abortions, molar pregnancies/blighted ova, or ectopic pregnancies. Neither group was significantly more likely to have had a planned index pregnancy or a twin or multiple index pregnancy, and both case and control mothers first discovered or suspected their respective pregnancies at similar gestations (mean 4.0 weeks for cases and 4.5 weeks for controls, $p=0.35$). Birth order for the index child was also non-significant between the groups, with index pregnancies as the first, second, or third or later pregnancies occurring at similar frequencies between the groups. Case children were more likely to have other defects than their heart defect (24.5%) than controls (12.7%; $p=0.08$).

The affected controls were more likely to have a positive family history of heart defects than cases. A sibling with a heart defect occurred in 9.1% of controls and only 0.9% of cases ($p<0.01$). Controls were also more likely to have any family history of heart defect (29.1%) than cases (15.5%, $p=0.04$). Maternal and paternal heart defects were equally prevalent among cases and controls.

Specific aspects of mother's periconceptional medical history did not predict risk of conotruncal defects. Similar proportions of case and control mothers had diabetes mellitus diagnosed before or during pregnancy, and while four case mothers used periconceptional insulin (3.6%) and no control mothers did, this difference was not significant. Control mothers were slightly more likely than cases to have had a history of seizure disorder. None of the women in the study used any folate-antagonizing anticonvulsants in the periconceptional period. One control mother reported using lithium in the periconceptional period; this did not constitute a significant association.

Table 4: Pregnancy History	Case (n=110)		Control (n=55)		p
	N	%	N	%	
Total pregnancy history (mean)					
Gravidity	3.7±2.0		3.7±2.3		0.96*
Live births	2.7±1.5		2.8±1.9		0.48*
Still births	0.0±0.1		0		0.48*
Spontaneous abortions	0.7±1.2		0.7±1.3		0.82*
Therapeutic abortions	0.4±0.6		0.2±0.5		0.14*
Molar pregnancies/blighted ova	0.0±0.1		0.0±0.2		0.22*
Ectopic pregnancies	0.0±0.2		0		0.15*
Index pregnancy					
Planned	55	50.0	29	52.7	0.74**
Twin or multiple gestation	5	4.5	1	1.8	0.38**
Index child with other defects	*27*	*24.5*	*7*	*12.7*	*0.08***
Birth order					
First pregnancy	26	23.6	14	25.5	
Second pregnancy	30	27.3	21	38.2	0.25 **
Third or later pregnancy	54	49.1	20	36.4	
Mean gestation when suspected or discovered (weeks)	4.0±2.9		4.5±3.3		0.35*
Family history					
Sibling with heart defect	***1***	***0.9***	***5***	***9.1***	***<0.01****
Sibling with other defect	22	20.0	7	12.7	0.25**
Maternal heart defect	6	5.5	1	1.8	0.28**
Paternal heart defect	1	0.9	2	3.6	0.37**
Any family history of heart defect	***17***	***15.5***	***16***	***29.1***	***0.04****
Other maternal medical illness					
Diabetes mellitus diagnosed before or during pregnancy	13	11.8	5	9.1	0.60**
Periconceptional insulin use	4	3.6	0	0	0.15**
History of seizure disorder †	*2*	*1.8*	*4*	*7.3*	*0.08***
Periconceptional lithium use	0	0	1	1.8	0.16**

*T-test for independent samples

**Pearson χ^2

†No patients reported periconceptional use of valproate, carbamazepine or phenytoin

Lifestyle factors

Postconceptional tobacco use was more likely to be associated with control status than case status. On average, controls smoked 1.6 cigarettes per day more than cases in this period, which approached statistical significance (mean case consumption 2.5±6.0 cigarettes per day, mean control 4.4±8.5; p=0.10). Furthermore, postconceptional consumption of more than 10 cigarettes (½ pack) per day was associated with a significantly decreased risk of case status [odds ratio (OR) 0.35, 95% confidence interval

(CI) 0.12-0.99]. Pre- and periconceptional use of more than 10 cigarette per day were not significant, but did have odds ratios of similar magnitude to the postconceptional effect. Smoking 100 or more cigarettes in the participant's lifetime was not a significant risk factor for conotruncal defects.

Alcohol is not significantly associated with conotruncal heart defects in this study. Mean use, any use, and more than three drinks per week were not significant in preconceptional, postconceptional or periconceptional periods. Binge drinking was also not significantly associated with conotruncal defects.

Table 5a: Lifestyle factors Comparison of means		**Case (n=110)**		**Control (n=55)**		**p***
		N	**%**	**N**	**%**	
Tobacco	Mean use (cigarettes per day)					
	Preconceptional	3.5±7.2		4.7±8.7		0.36
	Postconceptional	*2.5±6.0*		*4.4±8.5*		*0.10*
	Periconceptional	3.0±6.5		4.6±8.5		0.20
	Median use (cigarettes per day)					
	Preconceptional	0		0		
	Postconceptional	0		0		
	Periconceptional	0		0		
Alcohol	Mean use (drinks per week)					
	Preconceptional	3.4±18.8		1.3±3.0		0.38
	Postconceptional	1.0±3.4		0.4±1.8		0.28
	Periconceptional	2.2±9.9		0.9±2.3		0.33
	Median use (drinks per week)					
	Preconceptional	0		0		
	Postconceptional	0		0		
	Periconceptional	0		0		
	Mean episodes of binge drinking	2.3±13.4		0.5±1.5		0.33
	Median episodes of binge drinking	0		0		

*T-test for independent samples

Table 5b: Lifestyle factors Binary logistic regression		**Case (n=110)**		**Control (n=55)**		**OR**	**95% CI**	
		N	**%**	**N**	**%**			
Tobacco	Mean use (cigarettes per day)							
	Preconceptional	Continuous variable				0.98	0.94	1.02
	Postconceptional					*0.96*	*0.92*	*1.01*
	Periconceptional					0.97	0.93	1.02
	>100 cigarettes in lifetime	48	43.6	25	45.5	0.93	0.49	1.78

	Any use							
	Preconceptional	30	27.3	17	30.9	0.84	0.41	1.70
	Postconceptional	27	24.5	16	29.1	0.79	0.38	1.64
	Periconceptional	30	27.3	17	30.9	0.84	0.41	1.70
	10 or more cigarettes per day							
	Preconceptional	14	12.7	9	16.4	0.75	0.30	1.84
	Postconceptional	7	6.4	9	16.4	***0.35***	***0.12***	***0.99***
	Periconceptional	13	11.8	10	18.2	0.60	0.25	1.48
Alcohol	Mean use (drinks per week)							
	Preconceptional	Continuous variable				1.04	0.94	1.14
	Postconceptional					1.09	0.93	1.28
	Periconceptional					1.06	0.93	1.20
	Mean episodes of binge drinking	Continuous variable				1.06	0.92	1.21
	Any binge drinking	17	15.5	10	18.2	0.82	0.35	1.94
	Any use							
	Preconceptional	58	52.7	23	41.8	0.84	0.41	1.70
	Postconceptional	33	30.0	10	18.2	0.79	0.38	1.64
	Periconceptional	61	55.5	23	41.8	0.84	0.41	1.70
	More than 3 drinks per week							
	Preconceptional	15	13.6	6	10.9	1.29	0.47	3.53
	Postconceptional	8	7.3	2	3.6	2.08	0.43	10.14
	Periconceptional	12	10.9	3	5.5	2.12	0.57	7.86

Diet and supplementation

Vitamin use was not generally found to have significant associations with conotruncal defects. The exception is the association between periconceptional use of prenatal vitamins three or more days per week and increased risk of case status (OR 3.62, 95% CI 1.02-12.80). Prenatal vitamin use in the preconceptional or postconceptional period and multivitamin use in any period were not significantly associated with increased risk of conotruncal defects. Conversely, any use of prenatal vitamins in the post- or periconceptional periods was associated with a non-significant but much reduced risk of conotruncal defect (OR 0.59, 95% CI 0.20-1.70 in both). Furthermore, daily use of either a multivitamin or a prenatal vitamin was not associated with significant risk increase or reduction.

Table 6a: Supplement use Comparison of means		Case (n=110)		Control (n=55)		p*
		N	%	N	%	
Multivitamin	Mean use (days per week)					
	Preconceptional	1.2±2.3		1.3±2.6		0.75
	Postconceptional	0.5±1.5		0. 7±1.6		0.56
	Periconceptional	0.9±1.7		1.0±2.0		0.64
	Median use (days per week)					
	Preconceptional	0		0		
	Postconceptional	0		0		
	Periconceptional	0		0		
Prenatal vitamin	Mean use (days per week)					
	Preconceptional	0.6±1.8		0.2±0.9		0.13
	Postconceptional	3.8±2.3		3.7±2.0		0.75
	Periconceptional	2.2±1.7		2.0±1.1		0.32
	Median use (days per week)					
	Preconceptional	0		0		
	Postconceptional	4		4		
	Periconceptional	2		2		
Multi- or Prenatal vitamin	Mean use (days per week)					
	Preconceptional	1.8±2.9		1.6±2.6		0.57
	Postconceptional	4.3±2.6		4.4±2.6		0.95
	Periconceptional	3.1±2.4		3.0±2.2		0.76
	Median use (days per week)					
	Preconceptional	0		0		
	Postconceptional	5		5		
	Periconceptional	2.5		2.5		

* T-test for independent samples

Table 6b: Supplement use Binary logistic regression		Case (n=110)		Control (n=55)		OR	95% CI	
		N	%	N	%			
Multivitamin	Mean use (days per week)	Continuous variable						
	Preconceptional					0.98	0.86	1.12
	Postconceptional					0.94	0.76	1.16
	Periconceptional					0.96	0.80	1.15
	Any use							
	Preconceptional	27	24.5	13	23.6	1.05	0.49	2.24
	Postconceptional	17	15.5	11	20.0	0.73	0.32	1.69
	Periconceptional	28	25.5	13	23.6	1.10	0.52	2.35
	3 or more days per week							
	Preconceptional	20	18.2	11	20.0	0.89	0.39	2.02
	Postconceptional	6	5.5	7	12.7	0.40	0.13	1.24
	Periconceptional	15	13.6	10	18.2	0.71	0.30	1.71

Prenatal vitamin	Mean use (days per week)							
	Preconceptional	Continuous variable				1.22	0.93	1.61
	Postconceptional					1.03	0.88	1.19
	Periconceptional					1.12	0.90	1.39
	Any use							
	Preconceptional	13	11.8	3	5.5	2.32	0.63	8.52
	Postconceptional	94	85.5	50	90.9	0.59	0.20	1.70
	Periconceptional	94	85.5	50	90.9	0.59	0.20	1.70
	3 or more days per week							
	Preconceptional	11	10.0	2	3.6	2.94	0.63	13.77
	Postconceptional	64	58.2	31	56.4	1.08	0.56	2.07
	Periconceptional	19	17.3	3	5.5	***3.62***	***1.02***	***12.80***
Multi- or Prenatal vitamin	Mean use (days per week)							
	Preconceptional	Continuous variable				1.04	0.92	1.16
	Postconceptional					1.00	0.88	1.13
	Periconceptional					1.02	0.89	1.18
	Any use							
	Preconceptional	36	32.7	16	29.1	0.84	0.42	1.71
	Postconceptional	97	88.2	51	92.7	0.59	0.18	1.89
	Periconceptional	97	88.2	51	92.7	0.59	0.18	1.89
	3 or more days per week							
	Preconceptional	30	27.3	13	23.6	1.21	0.57	2.57
	Postconceptional	72	65.5	36	65.5	1.00	0.51	1.98
	Periconceptional	36	32.7	13	23.6	1.57	0.75	3.29

In addition to their vitamin use, participants were also questioned about their periconceptional intake of the top three sources of folate in the American diet, orange juice, ready-to-eat cereal, and legumes, defined as peas, lentils, pinto, navy, kidney or other dried beans. Importantly, these are not the foods highest in folate content, but rather the vehicle for the bulk of folate consumption in the American diet.

Neither orange juice nor ready-to-eat cereal was significantly associated with conotruncal defects in any period assessed. However, controls consumed approximately one more serving than cases per week in all three periods ($p \leq 0.01$ for each comparison), with an estimated risk reduction of 16-19% per serving per week. Mothers who consumed more than three servings per week had a 72-77% risk reduction.

Total consumption of these three sources of folate was significantly different between cases and controls with 3-4% risk reduction per serving per week in the post- and periconceptional periods. While consumption of nine or more total servings of either

orange juice, ready-to-eat cereal or legumes per week was not significantly associated with conotruncal defects in the time periods studied, the odds ratios were consistently approximately 0.5 with upper confidence intervals approaching one.

	Table 7a: Diet Comparison of means	**Case (n=110)**		**Control (n=55)**		**p***
		N	**%**	**N**	**%**	
Orange juice	Mean consumption (servings per week)					
	Preconceptional	2.8±6.2		3.2±6.5		0.69
	Postconceptional	2.7±4.5		3.6±6.7		0.30
	Periconceptional	2.8±5.2		3.4±6.6		0.48
	Median consumption (servings per week)					
	Preconceptional	1		2		
	Postconceptional	1		2		
	Periconceptional	1.5		2		
Cereal	Mean consumption (servings per week)					
	Preconceptional	2.8±2.4		3.0±2.7		0.54
	Postconceptional	2.8±2.6		3.0±2.9		0.74
	Periconceptional	2.8±2.4		3.0±2.7		0.63
	Median consumption (servings per week)					
	Preconceptional	2		3		
	Postconceptional	2		3		
	Periconceptional	2		3		
Legumes	***Mean consumption (servings per week)***					
	Preconceptional	***1.3±1.5***		***2.4±3.9***		***0.01***
	Postconceptional	***1.3±1.5***		***2.6±3.9***		***<0.01***
	Periconceptional	***1.3±1. 5***		***2.5±3.9***		***<0.01***
	Median consumption (servings per week)					
	Preconceptional	1		1		
	Postconceptional	1		1		
	Periconceptional	1		1		
Total food	Mean consumption (servings per week)					
	Preconceptional	6.9±7.2		8.6±8.0		0.17
	Postconceptional	***6.9±5.9***		***9.2±8.8***		***0.05***
	Periconceptional	*6.9±6.4*		*8.9±8.3*		*0.09*
	Median consumption (servings per week)					
	Preconceptional	6		7		
	Postconceptional	6		7		
	Periconceptional	6		7		

*T-test for independent samples

Table 7b: Diet Binary logistic regression		Case (n=110)		Control (n=55)		OR	95% CI	
		N	%	N	%			
Orange juice	Mean consumption (servings per week)							
	Preconceptional	Continuous variable				0.99	0.94	1.04
	Postconceptional					0.97	0.92	1.03
	Periconceptional					0.98	0.93	1.04
	3 or more servings per week							
	Preconceptional	21	19.1	11	20.0	0.94	0.42	2.13
	Postconceptional	27	22.7	16	29.1	0.79	0.38	1.64
	Periconceptional	25	32.7	14	25.5	0.86	0.41	1.83
Cereal	Mean consumption (servings per week)							
	Preconceptional	Continuous variable				0.96	0.85	1.09
	Postconceptional					0.98	0.87	1.11
	Periconceptional					0.97	0.85	1.10
	3 or more servings per week							
	Preconceptional	25	22.7	14	25.5	0.92	0.47	1.83
	Postconceptional	36	32.7	19	34.5	1.09	0.55	2.16
	Periconceptional	38	34.5	18	32.7	0.92	0.47	1.82
Legumes	***Mean consumption (servings per week)***							
	Preconceptional	Continuous variable				***0.84***	***0.72***	***0.98***
	Postconceptional					***0.81***	***0.69***	***0.95***
	Periconceptional					***0.82***	***0.69***	***0.97***
	3 or more servings per week							
	Preconceptional	7	6.4	11	20.0	***0.27***	***0.10***	***0.75***
	Postconceptional	8	7.3	14	25.5	***0.23***	***0.09***	***0.59***
	Periconceptional	8	7.3	13	23.6	***0.25***	***0.10***	***0.66***
	3 or more servings per week, non-Hispanic							
	Preconceptional	6	5.6	5	10.2	0.52	0.15	1.79
	Postconceptional	7	6.5	8	16.3	*0.36*	*0.12*	*1.04*
	Periconceptional	7	6.5	7	14.3	*0.42*	*0.14*	*1.26*
Total food	Mean consumption (servings per week)							
	Preconceptional	Continuous variable				0.97	0.93	1.01
	Postconceptional					***0.96***	***0.91***	***1.00***
	Periconceptional					*0.96*	*0.92*	*1.01*
	9 or more servings per week							
	Preconceptional	20	18.2	16	29.1	*0.54*	*0.25*	*1.16*
	Postconceptional	21	19.1	16	29.1	*0.58*	*0.27*	*1.22*
	Periconceptional	20	18.2	16	29.1	*0.54*	*0.25*	*1.16*

Maternal genotype

Mother's genotype alone was not significantly associated with increased risk of conotruncal heart defects. Specifically, neither the homozygous nor the heterozygous 677 C→T mutation was a significant predictor of case status, nor was the homozygous 1298 A→C mutation. The heterozygous 1298 A→C mutation was nearly significantly almost

twice the risk of conotruncal defect over the wild type for this allele (OR 1.92, 95% CI 0.96-3.83). The combined genotypes individually conferred no significant excess risk, and neither did a comparison of empirically grouped high (677 CT/1298 AC, 677 CT/1298 CC, 677 TT/any) and low risk genotypes (677 CC/1298 AA, 677 CC/1298 AC, 677 CC/1298 CC, 677 CT/1298 AA). Consistent with other reports, no mothers with double homozygous mutations were observed.

Table 8: Maternal genotype		Cases (N=110)		Controls (N=55)		OR	95% CI*	
		N	%	N	%			
677	CC-Wild	48	43.6	24	44.4	1.00		
	CT-Heterozygote	49	44.5	22	40.7	1.11	0.55	2.25
	TT-Mutant	13	11.8	8	14.8	0.81	0.30	2.23
1298	AA-Wild	43	39.1	30	54.5	1.00		
	AC-Heterozygote	55	50.0	20	36.4	*1.92*	*0.96*	*3.83*
	CC-Mutant	12	10.9	5	9.1	1.67	0.53	5.25
677/1298	CC-Wild/AA-Wild	8	7.3	7	13.0	1.00		
	CC-Wild /AC-Heterozygote	29	26.4	12	22.2	2.12	0.63	7.14
	CC-Wild /CC-Mutant	11	10.0	5	9.3	1.93	0.45	8.33
	CT-Heterozygote/AA-Wild	22	20.0	14	25.9	1.38	0.41	4.64
	CT-Heterozygote/AC-Heterozygote	26	23.6	8	14.8	2.84	0.79	10.30
	CT-Heterozygote/CC-Mutant	1	0.9	0	0	–		
	TT-Mutant/CC-Wild	13	11.8	8	14.8	1.42	0.37	5.45
	TT-Mutant/AC-Heterozygote	0	0	0	0	–		
	TT-Mutant/CC-Mutant	0	0	0	0	–		
	Low risk genotype (CC/AA, CC/AC, CC/CC, CT/AA)	70	63.6	38	70.4	1.00		
	High risk genotype (CT/AC, CT/CC, TT/any)	40	36.4	16	29.6	1.36	0.67	2.74

*Binary logistic regression

Maternal genotype and smoking showed no significant interaction, and postconceptional tobacco use was negatively associated with conotruncal heart defects in this study. Controlling for maternal genotype slightly decreased the risk associated with tobacco use (OR 0.20, 95% CI 0.05-0.82) with no significantly greater effect among the high-risk genotype group. Similarly, controlling for maternal genotype made no difference in findings for alcohol use as a risk factor for conotruncal heart defects.

Table 9: Maternal genotype + lifestyle factors			Cases (N=110)	Controls (N=55)	OR	95% CI*	
Tobacco	Preconceptional	Low risk + 10 or fewer cigarettes per day	63	30	1.00		
		High risk + 10 or fewer cigarettes per day	33	15	1.05	0.50	2.22
		Low risk + more than 10 cigarettes per day	7	8	0.42	0.14	1.26
		High risk + more than 10 cigarettes per day	7	1	7.63	0.66	87.90
		Low risk + no use	52	25	1.00		
		High risk + no use	28	12	1.12	0.49	2.57
		Low risk + any use	18	13	0.67	0.28	1.57
		High risk + any use	12	4	1.93	0.40	9.31
	Postconceptional	Low risk + 10 or fewer cigarettes per day	67	31	1.00		
		High risk + 10 or fewer cigarettes per day	36	14	1.19	0.56	2.52
		Low risk + more than 10 cigarettes per day	3	7	***0.20***	***0.05***	***0.82***
		High risk + more than 10 cigarettes per day	4	2	2.16	0.40	38.98
		Low risk + no use	54	26	1.00		
		High risk + no use	29	12	1.16	0.51	2.64
		Low risk + any use	16	16	0.64	0.27	1.55
		High risk + any use	11	4	1.77	0.36	8.73
	Periconceptional	Low risk + 10 or fewer cigarettes per day	63	30	1.00		
		High risk + 10 or fewer cigarettes per day	34	14	1.16	0.54	2.47
		Low risk + more than 10 cigarettes per day	7	8	0.42	0.14	1.26
		High risk + more than 10 cigarettes per day	6	2	2.97	0.39	22.83
		Low risk + no use	52	25	1.00		
		High risk + no use	28	12	1.12	0.49	2.57
		Low risk + any use	18	13	0.67	0.28	1.57
		High risk + any use	12	4	1.93	0.40	9.31
Alcohol	Preconceptional	Low risk + 3 or fewer drinks per week	61	33	1.00		
		High risk + 3 or fewer drinks per week	34	15	1.23	0.59	2.57
		Low risk + more than 3 drinks per week	9	5	0.97	0.30	3.15
		High risk + more than 3 drinks per week	6	1	2.72	0.22	32.92
		Low risk + no use	36	20	1.00		
		High risk + no use	16	11	0.81	0.32	2.07
		Low risk + any use	34	18	1.05	0.78	2.31
		High risk + any use	24	5	3.14	0.73	13.59
	Postconceptional	Low risk + 3 or fewer drinks per week	66	37	1.00		
		High risk + 3 or fewer drinks per week	36	15	1.35	0.65	2.78
		Low risk + more than 3 drinks per week	4	1	2.24	0.24	20.81
		High risk + more than 3 drinks per week	4	1	0.74	0.03	17.92
		Low risk + no use	51	31	1.00		
		High risk + no use	26	13	1.22	0.55	2.71
		Low risk + any use	19	7	1.65	0.62	4.37
		High risk + any use	14	3	1.41	0.25	7.87
	Periconceptional	Low risk + 3 or fewer drinks per week	63	36	1.00		
		High risk + 3 or fewer drinks per week	35	15	1.33	0.64	2.77
		Low risk + more than 3 drinks per week	7	2	2.00	0.39	10.15
		High risk + more than 3 drinks per week	5	1	1.07	0.07	16.91
		Low risk + no use	33	20	1.00		
		High risk + no use	16	11	0.88	0.34	2.27
		Low risk + any use	37	18	1.25	0.57	2.75
		High risk + any use	24	5	2.65	0.61	11.45

* Binary logistic regression

Multivitamin use alone was not significantly associated with conotruncal defects in any period. Postconceptional prenatal vitamin use approaches significance among high-risk genotype mothers. Compared with mothers with low-risk genotypes and low prenatal vitamin consumption (3 or fewer days per week), high-risk mothers are three times as likely to have a child with conotruncal defects (OR 3.05, 95% CI 0.89-10.45). Conversely, mothers with the high-risk genotypes and more than 3 days per week of prenatal vitamin use had a reduced risk of conotruncal defects (OR 0.27, 95% CI 0.06-1.24). Use of either multivitamins or prenatal vitamins conveyed similar risk estimates (OR 4.66, 95% CI 0.93-23.32 for high risk + 3 or fewer days per week, and OR 0.19, 95% CI 0.03-1.16 for high risk + more than 3 days per week).

Table 10: Maternal genotype + supplement use			**Cases (n=110)**	**Controls (n=55)**	**OR**	**95% CI***	
Multivitamin	**Pre**	Low risk + 3 or fewer days per week	55	31	1.00		
		High risk + 3 or fewer days per week	35	12	1.64	0.75	3.62
		Low risk + more than 3 days per week	15	7	1.21	0.45	3.28
		High risk + more than 3 days per week	5	4	0.36	0.06	2.10
	Post	Low risk + 3 or fewer days per week	66	34	1.00		
		High risk + 3 or fewer days per week	38	13	1.52	0.71	3.28
		Low risk + more than 3 days per week	4	4	0.92	0.33	2.56
		High risk + more than 3 days per week	2	3	0.38	0.05	2.85
	Peri	Low risk + 3 or fewer days per week	58	31	1.00		
		High risk + 3 or fewer days per week	37	13	1.52	0.71	3.28
		Low risk + more than 3 days per week	12	7	0.92	0.33	2.56
		High risk + more than 3 days per week	3	3	0.38	0.05	2.85
		Low risk + no use	51	30	1.00		
		High risk + no use	31	11	1.66	0.73	3.77
		Low risk + any use	19	8	1.40	0.55	3.58
		High risk + any use	9	5	0.46	0.09	2.26
Prenatal vitamin	**Pre**	Low risk + 3 or fewer days per week	63	37	1.00		
		High risk + 3 or fewer days per week	36	15	1.41	0.68	2.91
		Low risk + more than 3 days per week	7	1	4.11	0.49	35.66
		High risk + more than 3 days per week	4	1	0.41	0.02	9.17
	Post	Low risk + 3 or fewer days per week	28	19	1.00		
		High risk + 3 or fewer days per week	18	4	*3.05*	*0.89*	*10.45*
		Low risk + more than 3 days per week	42	19	1.50	0.68	3.32
		High risk + more than 3 days per week	22	12	*0.27*	*0.06*	*1.24*

	Peri	Low risk + 3 or fewer days per week	58	37	1.00		
		High risk + 3 or fewer days per week	33	14	1.50	0.71	3.18
		Low risk + more than 3 days per week	12	1	7.65	0.96	61.34
		High risk + more than 3 days per week	7	2	0.19	0.01	2.83
		Low risk + no use	9	5	1.00		
		High risk + no use	7	0	–		
		Low risk + any use	61	33	1.03	0.32	3.32
		High risk + any use	33	16	–		
Multi- or prenatal vitamin	Pre	Low risk + 3 or fewer days per week	49	30	1.00		
		High risk + 3 or fewer days per week	31	11	1.73	0.76	3.94
		Low risk + more than 3 days per week	21	8	1.61	0.63	4.08
		High risk + more than 3 days per week	9	5	0.40	0.08	1.96
	Post	Low risk + 3 or fewer days per week	24	16	1.00		
		High risk + 3 or fewer days per week	14	2	*4.66*	*0.93*	*23.32*
		Low risk + more than 3 days per week	46	22	1.40	0.62	3.14
		High risk + more than 3 days per week	26	14	*0.19*	*0.03*	*1.16*
	Peri	Low risk + 3 or fewer days per week	46	30	1.00		
		High risk + 3 or fewer days per week	28	11	1.67	0.72	3.83
		Low risk + more than 3 days per week	24	8	1.96	0.78	4.92
		High risk + more than 3 days per week	12	5	0.48	0.10	2.29
		Low risk + no use	7	4	1.00		
		High risk + no use	6	0	–		
		Low risk + any use	63	34	1.06	0.29	3.88
		High risk + any use	34	16	–		

*Binary logistic regression

As with the unadjusted dietary comparisons, orange juice failed to show any significant effects in the combined regression models. Ready-to-eat cereal, however, was a significant risk factor in the adjusted model. Specifically, high-risk mothers who consumed more than three servings per week of cereal in the preconceptional period had a significantly increased risk of conotruncal defects compared to low-risk women who consumed three or fewer servings per week (OR 5.78, 95% CI 1.13-146.96). Periconceptional use was also associated with increased risk (OR 4.46, 95% CI 0.96-20.68).

Legumes did not show a significant interaction effect with genotype but retained their independent association (OR 0.25, 95% CI 0.08-0.81 in the postconceptional period and OR 0.29, 95% CI 0.09-0.96 in the periconceptional period). These last data may account for the near-significance of the total servings variable, which also lacks an

interaction effect. Low-risk mothers who consumed more than nine total servings of these folate-containing foods had a reduced risk of conotruncal defects (OR 0.45, 95% CI 0.18-1.13 for both post- and periconceptional consumption).

Table 11: Maternal genotype + diet			Cases (n=110)	Controls (n=55)	OR	95% CI*	
Orange juice	Pre	Low risk + 3 or fewer servings per week	57	30	1.00		
		High risk + 3 or fewer servings per week	32	13	1.30	0.59	2.83
		Low risk + more than 3 servings per week	13	8	0.86	0.32	2.29
		High risk + more than 3 servings per week	8	3	1.27	0.22	7.47
	Post	Low risk + 3 or fewer servings per week	54	28	1.00		
		High risk + 3 or fewer servings per week	29	11	1.37	0.60	3.14
		Low risk + more than 3 servings per week	16	10	0.83	0.33	2.07
		High risk + more than 3 servings per week	11	5	1.01	0.21	4.78
	Peri	Low risk + 3 or fewer servings per week	55	30	1.00		
		High risk + 3 or fewer servings per week	30	11	1.49	0.65	3.83
		Low risk + more than 3 servings per week	15	8	1.02	0.39	2.69
		High risk + more than 3 servings per week	10	5	0.72	0.15	3.56
Cereal	Pre	Low risk + 3 or fewer servings per week	49	24	1.00		
		High risk + 3 or fewer servings per week	25	11	1.11	0.47	2.63
		Low risk + more than 3 servings per week	21	14	0.74	0.32	1.69
		High risk + more than 3 servings per week	15	5	1.80	0.41	7.98
	Post	Low risk + 3 or fewer servings per week	50	23	1.00		
		High risk + 3 or fewer servings per week	22	13	0.78	0.33	1.81
		Low risk + more than 3 servings per week	20	15	0.61	0.27	1.41
		High risk + more than 3 servings per week	18	3	***5.78***	***1.13***	***146.96***
	Peri	Low risk + 3 or fewer servings per week	50	22	1.00		
		High risk + 3 or fewer servings per week	22	12	0.81	0.34	1.91
		Low risk + more than 3 servings per week	20	16	0.55	0.24	1.26
		High risk + more than 3 servings per week	18	4	*4.46*	*0.96*	*20.68*
Legumes	Pre	Low risk + 3 or fewer servings per week	65	32	1.00		
		High risk + 3 or fewer servings per week	38	12	1.56	0.72	3.38
		Low risk + more than 3 servings per week	5	6	0.41	0.12	1.45
		High risk + more than 3 servings per week	2	4	0.39	0.04	3.51
	Post	Low risk + 3 or fewer servings per week	65	29	1.00		
		High risk + 3 or fewer servings per week	37	12	1.38	0.63	3.02
		Low risk + more than 3 servings per week	5	9	***0.25***	***0.08***	***0.81***
		High risk + more than 3 servings per week	3	4	0.98	0.13	7.35
	Peri	Low risk + 3 or fewer servings per week	65	30	1.00		
		High risk + 3 or fewer servings per week	37	12	1.42	0.65	3.11
		Low risk + more than 3 servings per week	5	8	***0.29***	***0.09***	***0.96***
		High risk + more than 3 servings per week	3	4	0.84	0.11	6.39
Total food	Pre	Low risk + 9 or fewer servings per week	59	27	1.00		
		High risk + 9 or fewer servings per week	31	12	1.18	0.53	2.65
		Low risk + more than 9 servings per week	11	11	*0.46*	*0.18*	*1.19*
		High risk + more than 9 servings per week	9	4	1.90	0.36	9.95
	Post	Low risk + 9 or fewer servings per week	58	26	1.00		
		High risk + 9 or fewer servings per week	31	13	1.07	0.48	2.37
		Low risk + more than 9 servings per week	12	12	*0.45*	*0.18*	*1.13*
		High risk + more than 9 servings per week	9	3	2.81	0.50	15.78

	Peri	Low risk + 9 or fewer servings per week	58	26	1.00		
		High risk + 9 or fewer servings per week	32	13	1.10	0.50	2.44
		Low risk + more than 9 servings per week	12	12	*0.45*	*0.18*	*1.13*
		High risk + more than 9 servings per week	8	3	2.42	0.42	13.78

*Binary logistic regression

Child's genotype

As with the mothers, the child's genotype alone was not a significant predictor of conotruncal defects, although the 1298 CC (homozygous mutant) genotype neared significance for predicting case status (OR 2.63, 95% CI 1.00-6.94). The combined genotypes were not individually associated with conotruncal defects nor were the grouped genotypes. One child had a double-mutant genotype.

Table 12: Child's genotype		Cases (n=110)		Controls (n=55)		OR	95% CI*	
		N	%	N	%			
677	CC-Wild	52	49.5	22	40.7	1.00		
	CT-Heterozygote	44	41.9	25	46.3	0.75	0.37	1.50
	TT-Mutant	9	8.6	7	13.0	0.54	0.18	1.65
1298	AA-Wild	38	35.5	28	51.9	1.00		
	AC-Heterozygote	44	41.1	19	35.2	1.71	0.83	3.53
	CC-Mutant	25	23.4	7	13.0	*2.63*	*1.00*	*6.94*
677/1298	CC-Wild/AA-Wild	11	10.5	7	13.0	1.00		
	CC-Wild /AC-Heterozygote	19	18.1	9	16.7	1.34	0.39	4.62
	CC-Wild /CC-Mutant	22	21.0	6	11.1	2.33	0.63	8.64
	CT-Heterozygote/AA-Wild	17	16.2	15	27.8	0.72	0.22	2.34
	CT-Heterozygote/AC-Heterozygote	25	23.8	9	16.7	1.77	0.52	5.96
	CT-Heterozygote/CC-Mutant	2	1.9	1	1.9	1.27	0.10	16.81
	TT-Mutant/CC-Wild	8	7.6	6	11.1	0.85	0.21	3.51
	TT-Mutant/AC-Heterozygote	0	0	1	1.9	–		
	TT-Mutant/CC-Mutant	1	1.0	0	0	–		
	Low risk genotype (CC/AA, CC/AC, CC/CC, CT/AA)	69	65.7	37	68.5	1.00		
	High risk genotype (CT/AC, CT/CC, TT/any)	36	34.3	17	31.5	1.14	0.56	2.29

*Binary logistic regression

In general, interaction variables for the children paralleled those of the mothers although with attenuated effects. Postconceptional tobacco had a significantly reduced risk in the low-risk genotype group (OR 0.24, 95% CI 0.08-0.79). Alcohol was not associated with outcome.

Table 13: Child's genotype + lifestyle factors			Cases (n=110)	Controls (n=55)	OR	95% CI*	
Tobacco	Preconceptional	Low risk + 10 or fewer cigarettes per day	60	28	1.00		
		High risk + 10 or fewer cigarettes per day	31	17	0.85	0.41	1.79
		Low risk + more than 10 cigarettes per day	9	9	0.47	0.17	1.30
		High risk + more than 10 cigarettes per day	5	0	–		
		Low risk + no use	46	22	1.00		
		High risk + no use	29	15	0.93	0.41	2.07
		Low risk + any use	23	15	0.73	0.32	1.67
		High risk + any use	7	2	2.47	0.38	16.20
	Postconceptional	Low risk + 10 or fewer day per day	64	28	1.00		
		High risk + 10 or fewer cigarettes per day	34	17	0.88	0.42	1.82
		Low risk + more than 10 cigarettes per day	5	9	***0.24***	***0.08***	***0.79***
		High risk + more than 10 cigarettes per day	2	0	–		
		Low risk + no use	48	23	1.00		
		High risk + no use	30	15	0.96	0.43	2.12
		Low risk + any use	21	14	0.72	0.31	1.66
		High risk + any use	6	2	2.09	0.31	14.10
	Periconceptional	Low risk + 10 or fewer cigarettes per day	60	27	1.00		
		High risk + 10 or fewer cigarettes per day	32	17	0.85	0.40	1.78
		Low risk + more than 10 cigarettes per day	9	10	0.41	0.15	1.11
		High risk + more than 10 cigarettes per day	4	0	–		
		Low risk + no use	46	22	1.00		
		High risk + no use	29	15	0.93	0.41	2.07
		Low risk + any use	23	15	0.73	0.32	1.67
		High risk + any use	7	2	2.47	0.38	16.20
Alcohol	Preconceptional	Low risk + 3 or fewer drinks per week	60	32	1.00		
		High risk + 3 or fewer drinks per week	30	16	1.00	0.48	2.10
		Low risk + more than 3 drinks per week	9	5	0.96	0.30	3.11
		High risk + more than 3 drinks per week	6	1	3.33	0.28	40.40
		Low risk + no use	36	20	1.00		
		High risk + no use	14	11	0.71	0.27	7.85
		Low risk + any use	33	17	1.08	0.47	2.40
		High risk + any use	22	6	2.67	0.63	11.30
	Postconceptional	Low risk + 3 or fewer drinks per week	62	35	1.00		
		High risk + 3 or fewer drinks per week	35	17	1.16	0.57	2.37
		Low risk + more than 3 drinks per week	7	2	1.98	0.39	10.04
		High risk + more than 3 drinks per week	1	0	–		
		Low risk + no use	45	29	1.00		
		High risk + no use	28	15	1.20	0.55	2.63
		Low risk + any use	24	8	1.93	0.77	4.88
		High risk + any use	8	2	1.11	0.16	7.49
	Periconceptional	Low risk + 3 or fewer drinks per week	62	34	1.00		
		High risk + 3 or fewer drinks per week	31	17	1.00	0.49	2.06
		Low risk + more than 3 drinks per week	7	3	1.28	0.31	5.27
		High risk + more than 3 drinks per week	5	0	–		
		Low risk + no use	33	20	1.00		
		High risk + no use	14	11	0.77	0.29	2.03
		Low risk + any use	36	17	1.28	0.58	2.86
		High risk + any use	22	6	2.25	0.53	9.49

* Binary logistic regression

Postconceptional maternal multivitamin use was associated with reduced risk of conotruncal defects in children with low-risk genotypes only (OR 0.24, 95% CI 0.06-1.00). The inverse relationship among high-risk/low-consumption and high-risk/high-consumption is evident and approaches significance (OR 2.59, 95% CI 0.81-8.28 and OR 0.25, 95% CI 0.06-1.09, respectively).

Table 14: Child's genotype + supplement use			Cases (n=110)	Controls (n=55)	OR	95% CI*	
Multivitamin	Pre	Low risk + 3 or fewer days per week	55	28	1.00		
		High risk + 3 or fewer days per week	30	15	1.02	0.47	2.20
		Low risk + more than 3 days per week	14	9	0.79	0.31	2.05
		High risk + more than 3 days per week	6	2	1.89	0.27	13.49
	Post	Low risk + 3 or fewer days per week	66	31	1.00		
		High risk + 3 or fewer days per week	33	16	0.97	0.47	2.02
		Low risk + more than 3 days per week	3	6	*0.24*	*0.06*	*1.00*
		High risk + more than 3 days per week	1	1	6.19	0.40	97.22
	Peri	Low risk + 3 or fewer days per week	59	29	1.00		
		High risk + 3 or fewer days per week	31	15	1.02	0.48	2.17
		Low risk + more than 3 days per week	10	8	0.61	0.22	1.72
		High risk + more than 3 days per week	5	2	1.97	0.26	15.03
		Low risk + no use	52	26	1.00		
		High risk + no use	25	15	0.83	0.38	1.85
		Low risk + any use	17	11	0.77	0.32	1.89
		High risk + any use	11	2	2.00	0.66	27.55
Prenatal vitamin	Pre	Low risk + 3 or fewer days per week	63	36	1.00		
		High risk + 3 or fewer days per week	32	16	1.14	0.55	2.36
		Low risk + more than 3 days per week	6	1	3.43	0.40	29.59
		High risk + more than 3 days per week	4	1	0.58	0.03	13.38
	Post	Low risk + 3 or fewer days per week	25	18	1.00		
		High risk + 3 or fewer days per week	18	5	2.59	0.81	8.28
		Low risk + more than 3 days per week	44	19	1.67	0.74	3.75
		High risk + more than 3 days per week	18	12	*0.25*	*0.06*	*1.09*
	Peri	Low risk + 3 or fewer days per week	57	36	1.00		
		High risk + 3 or fewer days per week	30	15	1.26	0.60	2.67
		Low risk + more than 3 days per week	12	1	7.58	0.95	60.79
		High risk + more than 3 days per week	6	2	0.20	0.01	2.94
		Low risk + no use	11	5	1.00		
		High risk + no use	5	0	–		
		Low risk + any use	58	32	0.82	0.26	2.58
		High risk + any use	31	17	–		

Multi- or prenatal vitamin	Pre	Low risk + 3 or fewer days per week	49	27	1.00		
		High risk + 3 or fewer days per week	27	14	1.06	0.48	2.36
		Low risk + more than 3 days per week	20	10	1.10	0.45	2.69
		High risk + more than 3 days per week	9	3	1.41	0.26	7.80
	Post	Low risk + 3 or fewer days per week	21	31	1.00		
		High risk + 3 or fewer days per week	14	5	1.73	0.51	5.95
		Low risk + more than 3 days per week	48	24	1.24	0.53	2.89
		High risk + more than 3 days per week	22	12	0.53	0.12	2.38
	Peri	Low risk + 3 or fewer days per week	47	27	1.00		
		High risk + 3 or fewer days per week	23	14	0.94	0.42	2.13
		Low risk + more than 3 days per week	22	10	1.26	0.52	3.06
		High risk + more than 3 days per week	13	3	2.09	0.39	11.12
		Low risk + no use	9	4	1.00		
		High risk + no use	4	0	–		
		Low risk + any use	60	33	0.81	0.23	2.83
		High risk + any use	32	17	–		

*Binary logistic regression

Maternal diet was only significant for legume consumption, independent of child's genotype in adjusted models (preconceptional OR 0.19, 95% CI 0.05-0.96; postconceptional OR 0.15, 95% CI 0.63-0.97; periconceptional OR 0.17, 95% CI 0.05-0.58). Periconceptional consumption of more than nine total servings of high-folate foods was negatively associated with conotruncal heart defects (OR 0.44, 95% CI 0.17-1.11).

Table 15: Child's genotype + diet			Cases (n=110)	Controls (n=55)	OR	95% CI*	
Orange juice	Pre	Low risk + 3 or fewer servings per week	57	28	1.00		
		High risk + 3 or fewer servings per week	30	15	0.98	0.46	2.12
		Low risk + more than 3 servings per week	12	9	0.66	0.25	1.74
		High risk + more than 3 servings per week	6	2	2.29	0.32	16.49
	Post	Low risk + 3 or fewer servings per week	51	27	1.00		
		High risk + 3 or fewer servings per week	29	12	1.28	0.56	2.90
		Low risk + more than 3 servings per week	18	10	0.95	0.39	2.35
		High risk + more than 3 servings per week	7	5	1.89	0.12	3.03
	Peri	Low risk + 3 or fewer servings per week	54	28	1.00		
		High risk + 3 or fewer servings per week	28	12	1.21	0.54	2.74
		Low risk + more than 3 servings per week	15	9	0.86	0.34	2.22
		High risk + more than 3 servings per week	8	5	0.79	0.16	3.98
Cereal	Pre	Low risk + 3 or fewer servings per week	49	24	1300		
		High risk + 3 or fewer servings per week	22	11	0.98	0.41	2.35
		Low risk + more than 3 servings per week	20	13	0.75	0.32	1.77
		High risk + more than 3 servings per week	14	6	1.55	0.36	6.74
	Post	Low risk + 3 or fewer servings per week	46	24	1.00		
		High risk + 3 or fewer servings per week	23	12	1.00	0.43	2.35
		Low risk + more than 3 servings per week	23	13	0.92	0.40	2.14
		High risk + more than 3 servings per week	13	5	1.47	0.33	6.60

	Peri	Low risk + 3 or fewer servings per week	47	24	1.00		
		High risk + 3 or fewer servings per week	22	10	1.12	0.46	2.75
		Low risk + more than 3 servings per week	22	13	0.86	0.37	2.01
		High risk + more than 3 servings per week	14	7	1.05	0.25	4.47
Legumes	Pre	Low risk + 3 or fewer servings per week	65	28	1.00		
		High risk + 3 or fewer servings per week	33	15	0.95	0.45	2.02
		Low risk + more than 3 servings per week	4	9	***0.19***	***0.05***	***0.96***
		High risk + more than 3 servings per week	3	2	3.56	0.37	34.50
	Post	Low risk + 3 or fewer servings per week	65	26	1.00		
		High risk + 3 or fewer servings per week	32	14	0.91	0.35	2.33
		Low risk + more than 3 servings per week	4	11	***0.15***	***0.63***	***0.97***
		High risk + more than 3 servings per week	4	3	4.01	0.76	1.64
	Peri	Low risk + 3 or fewer servings per week	65	27	1.00		
		High risk + 3 or fewer servings per week	32	14	0.95	0.44	2.05
		Low risk + more than 3 servings per week	4	10	***0.17***	***0.05***	***0.58***
		High risk + more than 3 servings per week	4	3	3.51	0.45	27.13
Total food	Pre	Low risk + 9 or fewer servings per week	58	27	1.00		
		High risk + 9 or fewer servings per week	28	11	1.19	0.52	2.73
		Low risk + more than 9 servings per week	11	10	0.51	0.19	1.35
		High risk + more than 9 servings per week	8	6	1.02	0.21	5.05
	Post	Low risk + 9 or fewer servings per week	56	25	1.00		
		High risk + 9 or fewer servings per week	29	13	1.00	0.45	2.23
		Low risk + more than 9 servings per week	13	12	0.48	0.19	1.21
		High risk + more than 9 servings per week	7	4	1.62	0.31	8.58
	Peri	Low risk + 9 or fewer servings per week	57	25	1.00		
		High risk + 9 or fewer servings per week	29	13	0.98	0.44	2.19
		Low risk + more than 9 servings per week	12	12	*0.44*	*0.17*	*1.11*
		High risk + more than 9 servings per week	7	4	1.79	0.34	9.53

*Binary logistic regression

Discussion

In statistical models including both maternal genotype and folate-containing supplement use, this study found increased risk of having a child with a conotruncal heart defect among mothers with MTHFR mutations and low consumption of supplements. Compared with the low-risk/low-supplement use group, mothers with high-risk genotypes with the most supplement use showed a significantly decreased risk, suggesting that the increased risk of the genotype alone is obviated by supplement use.

While children with high-risk genotypes whose mothers used supplements regularly showed a decreased risk of conotruncal heart defects, the risk reduction was attenuated compared with mothers with high-risk genotypes. Furthermore, the high-risk

genotype alone in the child did not confer an increased risk of these defects among women who used vitamins three or fewer days per week. These observations together entail the mother's genotype having more influence on conotruncal development than the child's does.

An unexpected finding was the strongly negative association between tobacco use and conotruncal defects. Given the use of affected controls in this study, this may represents a positive association between tobacco and patent ductus arteriosus; this would be best determined by an investigation of this factor in unaffected controls.

In addition, this study may have identified the second reported case of a living individual with double homozygous mutations in the MTHFR gene. Notably, this child has an MTHFR defect. This child's genotype must be confirmed with repeat testing, as must his mother's, as their 1298 genotypes are incompatible. Should the genotypes be confirmed, the next step will be to establish whether the child has a germline versus a somatic (i.e. mosaic) mutation.

Demographics

Markers of socioeconomic status – maternal age, education, employment, insurance status, and race – showed no significant differences between cases and controls. Control mothers were slightly more likely to be of Hispanic origin than case mothers. This difference in cases and controls may be the result of confounding by an independent association between Hispanic ethnicity and control status. As women of Hispanic origin are more likely to have a homozygous MTHFR 677 mutation than the North American population overall, the effect that this difference should have is a

diminution of power to detect an effect of MTHFR 677 mutations. Furthermore, considering the use of affected controls, this finding may represent an association between Hispanic ethnicity and patent ductus arteriosus that has not been described previously.

Paternal markers of socioeconomic status were also not significantly different. In fathers, Hispanic ethnicity was not significantly related to case or control status, as it was for mothers. Case fathers were more likely to be Native American and less likely to be Pacific Islanders than control fathers; however, cell sizes were small enough that these associations were most likely spurious.

Mean age of case and control children differed by approximately 2 years ($p<0.01$). Possible explanations for this include increased mortality among cases and a secular effect of improved treatments among the control. As participants and non-participants among the controls did not differ significantly in age, the former hypothesis is more likely.

Obstetric History

Case and control mothers showed no significant difference in total number of pregnancies, live births, stillbirths, spontaneous and therapeutic abortions, molar pregnancies or ectopic pregnancies. Neither cases nor controls were more likely to have had unplanned pregnancies or to have had multiple index gestations. Birth order was also not a significant predictor of conotruncal defects. Gestational age when pregnancy was first discovered or suspected was also not a significant predictor of case status, which is important as an indicator of periconceptional self-care habits and recall.

Of note, index children were about twice as likely to have had other malformations than control children (p=0.08). This may be the result of the associations between folate and MTHFR and midline defects, in general, or it may represent some degree of misclassification of children with chromosome 22q microdeletions as cases.

Family and Maternal Medical History

Affected controls were nearly ten times as likely as cases to have a sibling with a heart defect and approximately twice as likely to have any family history of congenital heart defect. Patent ductus arteriosus does have an established familial inheritance, although this is generally as part of a syndrome, such as Char syndrome or other heart-hand syndromes. [77-80] However, given that the phenotypes of these syndromes may be quite subtle, some of these familial defects may in fact be associated with autosomal dominant inheritance of a syndrome with only mild facial differences, which went undetected.

The lack of association of parental heart defects with conotruncal defects, on the other hand, is counterintuitive, given the premise of the paper. This lack of effect may represent a survival phenomenon, in that potential parents with conotruncal defects may be less likely to survive to reproductive age or may be more reluctant to bear children either for personal health reasons or out of fear of having affected children. Notably, survival to reproductive age is a relatively recent phenomenon in this population.

History of seizure disorder was negatively associated with conotruncal heart defects in this study. This finding is most likely spurious, as this finding represents four controls and two cases. Furthermore, none of the participants in this study used any of the

anticonvulsant medications associated with congenital heart defects in the periconceptional period.

Lifestyle Factors

The most striking finding of the lifestyle variables is the strongly negative association between tobacco use in the first trimester of pregnancy and conotruncal defects in this study. Cases were about one third as likely as controls to have smoked 10 or more cigarettes per day in the first twelve weeks of gestation. While this observation may truly represent a protective effect of tobacco use against conotruncal heart defects, this may be an example of a "bonus" finding brought out by the use of affected controls. An association between tobacco use and PDA has been neither reported nor refuted in the literature, and evaluation of this risk factor in unaffected controls would best clarify whether tobacco is truly protective against conotruncal heart defect or whether tobacco use early in pregnancy is in fact a risk factor for patent ductus arteriosus.

Alcohol use, on the other hand, was not significantly associated with conotruncal heart defects, as might be expected given its association with low folate levels. Of note, heavy alcohol use (more than three drinks per week) does show a consistently increased risk that does not reach statistical significance in this study. This lack of association may reflect a small sample size and ensuing insufficient power to detect an effect of such a prevalent risk factor. Furthermore, many heavy drinkers were likely excluded from the study, as children with fetal alcohol syndrome and fetal alcohol effects were excluded from this study. Binge drinking was also not a predictor of conotruncal defects, as expected given the proposed mechanism of alcohol's effect on folate, which is primarily

through chronic use as a caloric replacement with only minor additional impairment of folate metabolism.

Supplement Use

Unexpectedly, prenatal vitamin use conferred an increased risk of conotruncal defects. This effect is only true for women taking a prenatal vitamin three or more days per week in the periconceptional period as a whole, rather than for either pre- or postconceptional prenatal vitamin use. This observed effect may represent some reporting bias in frequency of vitamin use among mothers of children with severe heart defects versus control mothers whose lives were likely much less significantly influenced by their children's usually substantially less severe heart defects.

Multivitamin use was consistently protective against conotruncal heart defects, although not statistically significantly so. Of note, postconceptional multivitamin use approaches statistical significance with a 60% reduction in risk. Combining the protective multivitamin variable with the higher-risk prenatal vitamin variable yields no effect for use of either type of vitamin three or more days per week in any period.

Diet

Dietary folate consumption was approximated by reported weekly servings of the top three sources of folate in the American diet. This crude assessment was chosen in order to estimate the gross influence of dietary folate in three brief questions.

Weekly consumption of neither orange juice nor cereal predicted conotruncal defects; however, legumes were strongly protective in all three periods. The study

population had more Hispanic mothers in the control group than the case group, and an independent association between ethnicity and diet may account in part for this effect. However, analysis of legume consumption excluding Hispanic participants yields a similar risk reduction, albeit with wider confidence intervals.

While consumption of three or more servings of legumes per week may itself be protective against conotruncal defects, this variable may also serve as a marker for a healthy diet. Legume consumption is likely largely responsible for the protective effect of consumption of nine or more total servings of these foods per week.

Maternal Genotype

Maternal mutations at the 677 position of the MTHFR gene are not associated with conotruncal defects in this study. On the other hand, mutations at the 1298 locus confer increased risk, as does the compound heterozygous genotype. Given the Hispanic skew of the control population and the fact that Hispanic individuals are nearly twice as likely as the U.S. population as a while to have the MTHFR 677 mutation, this lack of significance may be due to effect modification. When grouped as a single variable, all high-risk genotypes combined show an increased risk over the low-risk group, but this odds ratio is not statistically significant.

Maternal Genotype and Lifestyle

Periconceptional tobacco and alcohol use show no significant interaction effects with maternal genotype. Postconceptional tobacco use of ½ pack per day or more is strongly negatively associated with conotruncal defects in the low-risk genotype group,

as is consistent with the unadjusted finding. Notably, the risk of conotruncal defects among high-risk genotype smokers is twice that of low-risk non-smokers, although not at a significant level. While tobacco use may be associated with patent ductus arteriosus, it may also be associated with conotruncal defects in high-risk populations. Further study with unaffected controls and larger sample sizes may elucidate these questions.

Maternal Genotype and Supplement Use

While none of the findings in these adjusted models reaches statistical significance, periconceptional vitamin use is protective against conotruncal heart defects in mothers with high-risk MTHFR genotypes. Furthermore, the MTHFR genotype alone is a risk factor for conotruncal defects among mothers who take vitamins fewer than three days per week.

High-risk mothers who take multivitamins three or fewer days per week in the postconceptional period are 50% more likely to have children with conotruncal heart defects than low-risk mothers. Women with high-risk MTHFR mutations who take prenatal vitamins three or fewer days per week are three times as likely as low-risk women to have an affected child. The genotype confers a nearly five-fold risk on women who use either a multi- or a prenatal vitamin three or fewer days per week in the postconceptional period. A high-risk genotype confers a markedly increased risk when adjusted for supplement use.

Conversely, high-risk mothers who take a multivitamin more than three days per week have a risk reduction of about 60% versus low-risk mothers who take them three or fewer days per week in any period. Prenatal vitamins confer about 70% risk reduction;

this is in marked contrast to the unadjusted finding of increased association of prenatal vitamin use with conotruncal defects. Use of either type of vitamin three or more days a week reduces risk by nearly 80%. This evidence suggests that the increased risk of the genotype can be overcome by supplementation. Although these odds ratios show relatively strong associations, none is statistically significant. Overall, these data suggest that this study may lack adequate power to detect the full effect of this interaction, given the small sample size.

Maternal Genotype and Diet

In the adjusted model, the strong negative association between legume consumption and conotruncal heart defects persists between the low-risk/high consumption versus low-risk/low-consumption groups. All of the odds ratios for the high risk/low consumption groups were above one for both orange juice and legumes, although none reached statistical significance, suggesting that a diet low in orange juice and legumes confers increased risk of having a child with a conotruncal defect among women with MTHFR mutations.

For individuals with high levels of cereal consumption, however, the odds ratios were significantly increased among women with high-risk genotypes. Typically, many ready-to-eat breakfast cereals were fortified to some extent, even before all grains were fortified in the U.S.; in other words, most cereals have some folic acid, and several brands now contain 100% of the recommended daily allowance. Given that folate and MTHFR status are associated with conotruncal heart defects, as demonstrated by the interaction between vitamin use and genotype, two possible explanations for this

unexpected finding for cereal arise. First, there may be a teratogenic effect of one of the nutrients in cereal or some feature that lowers serum folate; however, if this were the case, one might expect to see a risk increase among low-risk/high consumption groups. On the other hand, cereal may actually be a confounder as a marker of poor overall diet and may represent a diet high in "convenience" foods and low in folate-rich vegetables. Additionally, cereal may be a marker of higher degrees of pregnancy-associated nausea, in which case mothers are losing nutrients by eating fewer total calories and/or by frequent vomiting.

Child's Genotype

Child's MTHFR 677 genotype showed no association with conotruncal heart defects. The 1298 heterozygous and homozygous mutations were associated with an increased risk of conotruncal defects. When analyzed together, 677 and 1298 genotypes showed no consistently increased risk by individual compound genotypes or when grouped by high- and low-risk categories.

Of note, there was one child with a double homozygous mutant genotype. This child had tetralogy of Fallot with pulmonary atresia and was the only affected child of triplets resulting from *in vitro* fertilization. He had no other congenital defects. The child's mother had a history of eight prior spontaneous abortions. She was of strictly Northern European ancestry with no Hispanic heritage. The child's father was of Southern European but non-Hispanic ethnicity and also was of Native American origin. The mother's genotype was 677TT/1298AA.

If these are the correct genotypes, at least one of the child's 1298 alleles must be the result of a *de novo* mutation. The first step in confirming whether this is a real result is to repeat the assays of both mother and child, given the discrepant 1298 findings of the mother and child. If the repeated tests confirm the initial finding, the rarity of this genotype might warrant further contact with this family in order to assess whether the child has a germline versus a mosaic double mutant genotype, as well as an assessment of the father's and siblings' genotypes.

Child's Genotype and Maternal Supplement Use

The interactions between child's genotype and maternal vitamin use range from no interaction effect to effects that parallel the interaction between maternal genotype and supplement use. Specifically, postconceptional use of prenatal vitamin use more than three days per week confers a 75% risk reduction when the child has a high-risk genotype. Conversely, the high-risk genotype is associated with increased risk of conotruncal defects when mothers took prenatal vitamins three days per week or less, although the relationship is weaker than with maternal genotype. This suggests that maternal genotype has more influence on outcome than the child's genotype. Maternal diet and lifestyle factors show no interaction with child's genotype, although the risks among low-risk genotype groups remain consistent, as expected.

Study Limitations

The most influential limitation of this study is the small sample size, especially in the control group. Many of the observed effects were of strong in magnitude but with

wide confidence intervals including one. While this may truly represent a series of purely chance findings, the consistency of the observations both within the study and with the background evidence leading to the proposed hypotheses suggests that the sample population may have demonstrated insufficient power to detect the full effects of the risk factors.

Affected controls were selected in order to minimize recall bias and to utilize the same recruitment population base as cases. Unfortunately, the use of affected controls may have limited the study in several ways. First, the number of available affected controls was fewer than the number of cases in the same time period. This was due in part to differences in prevalence of PDA versus conotruncal defects, but was also due to differential participation in ORCHD. Furthermore, in order to reduce recall bias, cases and affected controls should have a similar degree of severity; this is clearly not the case among PDA-affected families when compared to conotruncal defect-affected families.

On the other hand, finding an affected control group that would be truly comparable to these cases in every way except defect type without significant confounding would be quite difficult. Neural tube and other midline defects are not eligible due to likely associations with folate. Using children with chromosomal abnormalities would introduce age and possibly other lifestyle biases. Furthermore, the logistics of locating population-based affected controls would likely have been prohibitive, introducing additional biases.

In addition to the differences in severity of case and control malformations, another potential source of recall bias is the difference in mean child age at interview. This is most likely the result of increased mortality among the cases; however, as the

control children are older, reporting inaccuracy may be greater in the control mothers, as their pregnancies occurred further back in time than the case mothers' pregnancies. On the other hand, control mothers were less likely to have consumed grains fortified with folate, since they were more likely to have had their children before 1996. This may have resulted in effect modification; case mothers may have been consuming higher quantities of protective folate than control mothers. Frequency matching by year of birth would decrease the likelihood of this difference in mean age.

The dietary variables assessed in the interview were crude, largely in order to minimize the time commitment of the participants. Ideally, diet would be assessed using a validated dietary questionnaire assessing food frequency and caloric intake, such as the Block Food Frequency Questionnaire. However, an assessment of dietary intake as far as 12 years prior to the interview would be of dubious accuracy. The ideal assessment of diet would be prospective.

Finally, although the ORCHD database is an excellent source of medical history for many of the participants, the health information gathered in the present study was largely reported by the participating mothers. Ideally, medical histories would have been confirmed by maternal and child medical records. Of specific concern is diagnosis of chromosome 22 microdeletion. While many mothers would know whether their child had been tested, some mothers are undoubtedly and understandably overwhelmed by the immediate needs of their acutely ill child and may not remember whether the test had been performed or what it showed. Furthermore, although many practitioners, especially in the Portland area, test all children with conotruncal defects for the 22q deletion, others do not. This is most likely to be true with older children but remains the case even now.

As a result, some cases in the study may have undetected velocardiofacial syndrome as the cause of their conotruncal defects.

Future Research

In order to improve sample size and to provide a comparison group for both cases and affected controls, a second phase of this project has been implemented. A population-based group of unaffected controls and their mothers is currently being recruited using Oregon birth certificate data. Controls are frequency-matched by year of birth and excluded in case of heart defect or any of the exclusion criteria applied to cases and affected controls. This phase of the study has been undertaken in conjunction with the Oregon Health Division and has received approval from its IRB, as well as the OHSU IRB. The target recruitment is 150 healthy controls, which should result in sufficient power to detect the effects observed in the initial phase. In addition, this population-based cohort will provide a potential comparison group for future investigations of other defects in the state of Oregon. Specifically, this group should provide an adequate comparison for the potential effect of tobacco use on patent ductus arteriosus.

While a case-control study design is well suited to a preliminary study of a rare outcome, it is particularly susceptible to bias, especially recall bias. In general the best way to minimize bias is to conduct a randomized control trial; however, since the relationship between neural tube defects and folic acid has been established, use of a placebo would be unethical. The next best study design would be a prospective study following diet and supplement use among pregnant women with and without MTHFR mutations with follow-up of outcome and subsequent MTHFR testing of the fetus or

neonate. This design would also enable study of other defects that might be associated with a gene-nutrient interaction between folate and MTHFR mutations.

An additional avenue for establishing a relationship between folate, MTHFR mutations and conotruncal heart defects is a recently completed prospective study of pregnant women and dietary folic acid. This National Institutes of Health-funded study resulted in stored blood samples of approximately 500 women. Data will be available on the red cell and plasma folate, homocysteine, and vitamin B_{12} levels of the mothers during their pregnancies. In addition, this study collected detailed supplement-use histories and food frequency questionnaires on participants. A nested case-control study, in which the stored blood of mothers of affected and unaffected infants were tested for MTHFR mutations, would be extremely informative, despite that the study is not population-based and that the child's genotype would not be available.

These data represent the first evidence of a gene-nutrient interaction among folate and MTHFR mutations and conotruncal heart defects. The potential impact of these findings is that women may significantly reduce their risk of having a child with a tragic heart defect by as simple, safe, and inexpensive an intervention as a folic acid supplement. While this evidence would not likely change current recommendations for pregnant women, mothers of affected children might be able to decrease their risk of having additional children with conotruncal defects. Moreover, this finding would further justify the controversial decision to fortify the nation's grain supply with folic acid. Recent technological advances have significantly improved survival and quality of life for children with conotruncal heart defects, but no intervention is comparable to prevention.

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Appendix 1: Recruitment letter

Date

FIELD(4) FIELD(3)
FIELD(5)
FIELD(6), FIELD(7) FIELD(8)

Dear Ms. FIELD(3);

I am a researcher at Oregon Health Sciences University (OHSU) studying how diet affects the way certain genes function. I am now working with Drs. Cynthia Morris and Mark Reller on a project to find out if a gene called MTHFR is related to congenital heart defects. Your name was chosen because you have a child born with a heart defect who has been followed in the long-term follow-up study of heart defects after surgery. You and your child are invited to take part in this study of the MTHFR gene.

The study would involve about an hour of your time, plus about 10 minutes for your child. Your participation involves three steps:

1) Read, sign and return the white consent form in the prepaid envelope.
2) Answer questions about your medical and dietary history in a telephone interview. This will take about 20 minutes.
3) Rub two cotton swabs on the inside of your mouth to collect cells for analysis of the gene. This will take about 10 minutes total, including reading the instructions and packaging and mailing both sets of swabs.

If you allow your child to participate, you will need to rub swabs in his or her mouth. You will also need to sign and return the child's white consent form.

This study involves *NO* invasive procedures – only swabbing the inside of your mouth. All information we get from you, including the answers to your interview questions and your DNA analysis, will be kept completely confidential. You will be notified if we discover a result that might have a negative impact on your health or your child's health. You may also decide whether you would like for us to inform your health care provider of such a result. Please see the consent forms for more details about the study.

We believe that this study will provide important information about the causes of congenital heart disease. If you join this study, you will make a valuable contribution to this area of research. You may help scientists learn how to prevent babies in the future from being born with certain heart defects.

Thank you for thinking about helping us with this study. Please feel free to call me at (503) 494-1314 if you have any questions. Either I or a research assistant will call you in the next few weeks to find out if you are willing to join our study.

Best wishes,

Piper Hackett, BS
Research Assistant

Alix Seif, MA
Project Director

Does the MTHFR gene cause congenital heart defects?
Mother's Consent Form

Oregon Health Sciences University
3181 S.W. Sam Jackson Park Rd.
Mail Code: BICC
Portland, OR 97201

Investigators:

Alix Seif	(503) 494-0567
Dr. Cynthia Morris, Principal Investigator	(503) 494-3262
Dr. Mark Reller	(503) 494-3187

Purpose:
You have been invited to participate in this study because your child was diagnosed with a heart defect and has been followed up in a long-term study of heart defects after surgery or because you participated in a prenatal study of heart defects at OHSU. The purpose of the study is to determine whether the MTHFR gene affects the risk of having a baby with a certain type of heart defect known as a conotruncal defect. If your child does not have a conotruncal type of defect, information we get from you will be used to compare with mothers of children with conotruncal defects.

Procedures:

1) You will be contacted by telephone and asked about your personal and family medical history, including some questions about your pregnancy with this child. This should take approximately 20 minutes.

2) You will be mailed a kit for doing a sterile mouth swab. This will require you to rub a swab on the inside of your cheek. We will send you instructions on how to use this kit and a postage-paid envelope for you to return the swab. This should take approximately 10 minutes.

3) Your sample will be sent to a laboratory for DNA analysis of the MTHFR gene.

Results:
You will not be contacted unless your DNA test result is clinically important. This result will be reported both to you and your primary care provider, if you agree to release the information. In case of a clinically important result, you or your provider may wish to have the test repeated in a clinical laboratory. Please note that this gene is a risk factor gene (not a disease gene).

Risks and discomforts:
There are no known risks associated with the mouth swab method of sample collection. However, a breach of confidentiality could affect your insurability or employability.

Benefits:
You may or may not personally benefit from being in this study. However, if you choose to join this study, you may contribute valuable new information which may benefit patients in the future. The MTHFR gene is known to affect the risk of other birth defects and adult heart disease. If you have a clinically important result, you may benefit in the future from early treatment.

Alternatives:
You may choose not to participate in this study. There will be no effect on your standard medical care.

Confidentiality:
The answers you give us and your test results will be kept confidential. A code number will be assigned to you, your cells and your DNA. Only the investigators named on this consent form will be authorized to link the code number to you. Laboratory personnel will be given only the code number which will not identify you. All samples will be destroyed at the end of the study. Neither your name nor any identifiers will be used for publication or publicity. According to Oregon law, suspected child abuse or elder abuse must be reported to appropriate authorities.

Costs:
There are no costs for participation in this study. If a clinically important result is found, you may choose to undergo additional clinical tests, physicians visits, treatments, or genetic counseling. You will be responsible for any such costs.

Liability:
It is not the policy of the funding agency for this research project in which you are participating to compensate or provide medical treatment for human subjects in the event the research results in physical injury.

The Oregon Health Sciences University is subject to the Oregon Tort Claims Act (ORS 30.260 through 30.300). If you suffers any injury and damage from this research project through the fault of the University, its officers or employees, you have the right to bring legal action against the University to recover the damage done to you subject to the limitations and conditions of the Oregon Tort Claims Act. You have not waived your legal rights by signing this form. If you have further questions, please call the OHSU Legal Department at (503) 494-5222.

If you have any questions about this study, please call Alix Seif at (503) 494-0567or Cynthia Morris, PhD at (503) 494-3262. If you have any questions about your rights as a research subject, you may contact the Oregon Health Sciences University Institutional Review Board at (503) 494-7887.

Participation:
Your participation is completely voluntary. You may refuse to participate, or you may withdraw from the study at any time without affecting your relationship with or treatment at the Oregon Health Sciences University or other care providers. If in the future you decide you no longer want to participate in this research, we will destroy all identifying information and will not use your DNA in future studies.

You have either read this consent form or had it read to you. You will receive a copy of this form. Your signature below indicates that you have read this document and agree to participate in this study.

My DNA results may be released to my health care provider.

Agree [ ] Don't agree [ ]

(Check one)

Provider's name & phone number: _____

_____ _____
Signature ***Date***

_____ _____
Signature of parent or guardian, if subject is a minor *Date*

_____ _____
Signature of investigator ***Date***

Glossary of terms:

congenital - means "born with". Congenital problems can have many causes. Some are genetic, some are environmental, and some are a combination of both. Many congenital problems have causes that scientists and health care providers do not understand yet.

genetic - means "pertaining to a gene". Everyone inherits 2 copies of all possible genes -- one from each parent. Some genes are "disease genes", meaning that a mutation in these genes will almost definitely cause a certain disease process in the individual.

Other genes are "risk factor genes". A person with a mutation in this kind of gene is more likely to get the disease than people without the mutation. However, not everyone with the mutation gets the disease. MTHFR is this type of gene.

mutation - a change in the DNA code in your body. Some mutations cause no change in the way a person's body functions. Other mutations are capable of causing a disease or increasing a person's risk of getting a disease.

enzyme - a protein that causes chemical reactions needed to keep your body functioning.

MTHFR gene - full name: methylenetetrahydrofolate reductase gene. This gene carries the code for an enzyme that helps your body use folic acid from the food you eat. Mutations in this gene, combined with certain dietary habits, can sometimes cause increased blood levels of a chemical called homocysteine. This chemical is thought to increase the risk of getting heart disease as an adult and may play a role in causing certain birth defects.

conotruncal heart defects - These heart defects happen when a certain part of the heart (the "conotruncus") does not grow properly during fetal development. There are several different types of heart defect in this category. The cause of this type of defect is unknown.

Child's Assent Form

Prevalence of Homozygous MTHFR 677C→Mutation in Children with Conotruncal Heart Defects and their Mothers

Oregon Health and Science University

For children ages 10 and over:

I understand what this research study is about. I know it may or may not help me. I also understand that this research may help doctors learn more about heart defects. I have thought about being in this research study. I have asked and received answers to my questions.

I agree to be in this research study. I know that I don't have to agree. Even though I agree now, I know I may feel differently later on and may choose to change my mind. I know that I may talk to my parents and the researchers about not being in this study at any time.

_____ _____

Child's Name/Signature Date

MOTHER'S
ID Number: _____

Collecting Mouth Cells

Your pack contains: 1 storage bag with ID label
2 sterile swabs

How to Use the Cotton Swabs

The goal is to trap some of the skin cells from inside your mouth on the cotton swab.

1) - Open the first swab packet, and remove the swab from its wrapping.
Keep the wrapper in order to store the swab later.

2) - Rub or scrape the end of the swab with some pressure along the **inside** of the **right side** of your mouth, including the inside of your cheek and lips.
- Do this for about 20 seconds.
- Repeat on the **left side** of your mouth.
This should NOT hurt at all.

3) - After using the swab, place it back in the wrapper it came in.
- Place the swab in the plastic bag that is labeled with your ID number, and seal the bag.
Be sure that the number written on the bag matches the number on the top of this page.

4) - Repeat steps 1-3 with the second packet **at least 3 hours** but **not more than 24 hours** later.

5) - Once you have used both swabs, please put the sealed bags and a signed copy of the consent form into the envelope provided.
- Mail the envelope **within 24 hours of using the swabs.**
If the swabs are not mailed right away, they may get contaminated.

Important points

- It is very important that **there are no bits of food** in your mouth.
- Please **DO NOT use the swabs within an hour of brushing your teeth**.
Brushing may remove some of the cells that you need to collect.
- If you have not already returned it, be sure to include your **SIGNED consent form** in the envelope.

If you have any questions about this procedure, please call us at (503) 494-0567.
We really appreciate your time and effort for this important part of our study.
Thanks for all of your help!

Appendix 5: Study Questionnaire

MTHFR-CTD Study data form

Thank you for agreeing to participate in this study. Information we get from you today may help us learn more about what causes certain heart defects with the goal of helping future babies to be born healthy. This interview should take about 15 or 20 minutes. Your answers will be kept confidential. You may refuse to answer any question.

During this interview, we will be focusing on you and your pregnancy with your child with a heart defect (state child's name if known/living). At any time during the interview, if you are unsure about which pregnancy we are referring to, or if you do not understand a question, please feel free to ask me to stop and clarify.

Mother's last name

Mother's first name

Mother's Code #:

Mother's birth date ___ / ___ / ___

Child's last name

Child's first name

Child's Code #:

Child's birth date ___ / ___ / ___

The following questions are mainly for demographic purposes, such as what you would be asked in a census. Since some genes occur more frequently in certain ethnic groups than in others, I will also ask some questions about your ethnic heritage and that of the child's natural father.

1. What race do you consider yourself to be?

☐ Caucasian American

☐ African American

☐ Native American

☐ Asian American or Pacific Islander

2. Are you of Hispanic origin? If yes, from which region?

☐ No ☐ Cuban

☐ Mexican American ☐ Dominican

☐ Central American ☐ Other: _____ Please Specify

☐ South American ☐ Unsure

☐ Puerto Rican

3. What ethnicity predominates in your family background? Please limit to the top two.

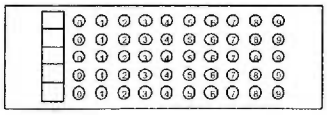
☐ Southern European ☐ Native American

☐ Northern European ☐ Sub-Saharan African

☐ Asian ☐ North African

☐ Pacific Islands ☐ Ashkenazi Jew

☐ Mid East ☐ Other _____ Please Specify



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Mother's Code #: | | | | | | | | |

4. What level of education had you completed at the time you became pregnant with this child?

☐ some high school

☐ high school diploma

☐ some college

☐ college graduate

☐ some graduate school

☐ graduate degree

5. What was your employment status at the time you became pregnant?

☐ working for wages

☐ self-employed

☐ unemployed more than one year

☐ unemployed less than or equal to one year

☐ homemaker

☐ student

☐ unable to work

☐ other

6. Did you have health insurance during pregnancy? If yes, what type?

☐ none/self-pay

☐ private

☐ public (includes Oregon Health Plan, CareOregon)

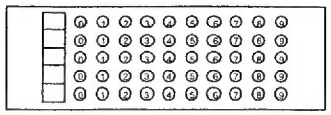
☐ I don't know

7. What was your marital status at the time you became pregnant?

☐ Married living w/partner ☐ Single living alone

☐ Married living alone ☐ Divorced

☐ Single living w/partner ☐ Widowed



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8. How old was the father of this baby at the time you became pregnant?

9. What is his race?

☐ Caucasian American

☐ African American

☐ Native American

☐ Asian American or Pacific Islander

☐ Unknown/Unsure

10. Is he of Hispanic origin? If yes, from which region?

☐ No ☐ Cuban

☐ Mexican American ☐ Dominican

☐ Central American ☐ Other:_____
Please Specify

☐ South American ☐ unsure

☐ Puerto Rican

11. What ethnicity predominates in his family background? Please limit to the top two.

☐ Southern European ☐ Native American

☐ Northern European ☐ Sub-Saharan African

☐ Asian ☐ North African

☐ Pacific Islands ☐ Ashkenazi Jew

☐ Mid East ☐ Othe _____
Please Specify

Mother's Code #: ☐☐☐☐☐☐☐☐

Now I'm going to ask you about your reproductive history.

12. How many times have you been pregnant, including pregnancies ending in live birth, stillbirth, miscarriage,molar or ectopic pregnancy or abortion? ☐☐

Starting with the first time you were pregnant, what was the outcome of that pregnancy?
Was this pregnancy with the same father as (index child's)?
Was this baby born with a heart defect?
Any other defects?

Index Child?		LB	SB >20 wks GA	SAB ≤ 20 wks GA	TAB	M	E	Same FOB Yes	Same FOB No	CHD Yes	CHD No	Other Dfx Yes	Other Dfx No
☐	Pregnancy # ☐☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
☐	Pregnancy # ☐☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
☐	Pregnancy # ☐☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
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☐	Pregnancy # ☐☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Pregnancy #	Name:	DOB:	Type of defect:
☐☐			
☐☐			
☐☐			
☐☐			
☐☐			
☐☐			
☐☐			

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Referring to your pregnancy with (index child). Mother's Code #: | | | | | | | | |

14. Was this pregnancy planned? ☐ Yes ☐ No

15. At any time during the three months before you became pregnant, did you use birth control? What kind?

☐ Hormonal ☐ Barrier ☐ IUD ☐ Other ☐ None

16. Do you or your child have any chromosomal probles that you're aware of? One example might be Down's Syndrome.

☐ MOB ☐ Child ☐ Both ☐ None

Details: _____
Type of Defect

17. Is there any history of chromosome 22 problems, like DiGeorge's syndrome, Velocardiofacial or VCF syndrome, Shprintzen's syndrome or CATCH-22, in your family?

☐ Yes ☐ No

Details: _____
Name, relationship, description of syndrome

The next set of questions is about you and your family.

18. Were you born with a heart defect? ☐ Yes ☐ No

Do you know what type? Details: _____

Did this require surgery? ☐ Yes ☐ No
Details: _____

19. Was either of your parents born with a heart defect? ☐ Mother ☐ Father ☐ Both ☐ Neither

Do you know what type? Details: _____

Did this require surgery? Mother: ☐ Yes ☐ No
Details: _____
Father: ☐ Yes ☐ No
Details: _____

20. How many brothers do you have by both of your parents? | |

21. Do you have any half-brothers? ☐ Yes ☐ No

How many on your mother's side? | |

How many on your father's side? | |

22. How many sisters do you have by both of your parents? | |

23. Do you have any half-sisters? ☐ Yes ☐ No

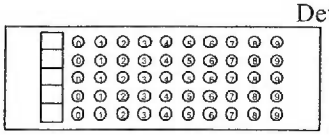
How many on your mother's side? | |

How many on your father's side? | |

24. Were any of your brothers or sisters born with a heart defect? ☐ Yes ☐ No Sib 1 | | Sib 2 | | SiblingCode: BB, SB, BM, BF, SM, SF

Do you know what type? Details: _____

Did this require surgery? Sib 1: ☐ Yes ☐ No
Details: _____
Sib 2: ☐ Yes ☐ No
Details: _____



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Mother's Code #: | | | | | | | | |

The next set of questions is about the father and his family.

25. Was the father born with a heart defect? ☐ Yes ☐ No

Do you know what type? Details:_____

Did this require surgery? ☐ Yes ☐ No

Details:_____

26. Was either of the father's parents born with a heart defect? ☐ Mother ☐ Father ☐ Both ☐ Neither

Do you know what type? Details:_____

Did this require surgery? Mother: ☐ Yes ☐ No

Details:_____

Father: ☐ Yes ☐ No

Details:_____

27. How many brothers does the father have by both of his parents? | |

28. Does the father have any half-brothers? ☐ Yes ☐ No

How many on his mother's side? | |

How many on his father's side? | |

29. How many sisters does he have by both of his parents? | |

30. Does he have any half-sisters? ☐ Yes ☐ No

How many on his mother's side? | |

How many on his father's side? | |

31. Were any of his brothers or sisters born with a heart defect? ☐ Yes ☐ No Sib 1 | | Sib 2 | | SiblingCode: BB, SB, BM, BF, SM, SF

Do you know what type? Details:_____

Did this require surgery? Sib 1: ☐ Yes ☐ No

Details:_____

Sib 2: ☐ Yes ☐ No

Details:_____

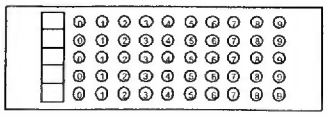
32. Does the father have any children not belonging to you? ☐ Yes ☐ No

How many? | |

. Were any of these children born with a heart defect? ☐ Yes ☐ No

Do you know what type? Details:_____

Did this require surgery? ☐ Yes ☐ No



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Mother's Code #: | | | | | | | | |

The next set of questions is about your medical history around the time you became pregnant. We will be focussing on the period beginning three months before you became pregnant through the first three months of your pregnancy, in other words, the first trimester or first twelve weeks of your pregnancy. Please remember that this includes the period of time when you might not have known you were pregnant yet.

33. As a reference, how far along were you (in weeks) when you suspected or discovered you were pregnant? | |

34. Have you ever been told by a healthcare provider that you have diabetes? ☐ Yes ☐ No

Was this diagnosed before or during your pregnancy? ☐ Yes ☐ No

At any time during the three months before the pregnancy began through the first three months of the pregnancy, did you take insulin? ☐ Yes ☐ No

35. Have you ever been told by a health care provider that you have epilepsy or seizures? ☐ Yes ☐ No

At any time during the three months before the pregnancy began did you take any of the following medications:

-phenytoin/Dilantin ☐ Yes ☐ No

-trimethadione/Tridione ☐ Yes ☐ No

-valproic acid/Depakene ☐ Yes ☐ No

At any time during the first three months of your pregnancy did you take any of the following medications:

-phenytoin/Dilantin ☐ Yes ☐ No

-trimethadione/Tridione ☐ Yes ☐ No

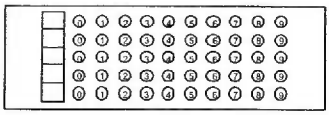
-valproic acid/Depakene ☐ Yes ☐ No

36. Have you ever been told by a health care provider that you have a disease like lupus, rheumatoid arthritis, scleroderma, or amyloidosis? ☐ Yes ☐ No

☐ lupus ☐ rheumatoid arthritis ☐ scleroderma ☐ amyloidosis ☐ other _____ Please Specify

Was that diagnosed either before or during your pregnancy? ☐ Yes ☐ No

37. At any time during the three months before the pregnancy began through three months into your pregnancy, did you take lithium? ☐ Yes ☐ No



Mother's Code #: | | | | | | | | |

Now I'm going to ask about your lifestyle habits, like smoking and vitamin use. Please remember that your answers are confidential and that you may refuse to answer any question.

38. Have you smoked more than 100 cigarettes in your lifetime? ☐ Yes ☐ No

At any time during the three months before the pregnancy began did you smoke cigarettes? ☐ Yes ☐ No

On average, how many cigarettes did you smoke per day? | |

At any time during the first three months of the pregnancy did you smoke cigarettes, even before you knew you were preganant? ☐ Yes ☐ No

On average, how many cigarettes did you smoke per day? | |

39. At any time during the three months before the pregnancy began did you drink any alcoholic beverages such as beer, wine, wine cooler, or liquor? ☐ Yes ☐ No

On average, how many drinks did you have per week? | |

At any time during the first three months of the pregnancy did you drink any alcoholic beverages, even before you knew you were pregnant? ☐ Yes ☐ No

On average, how many drinks did you have per week? | |

How many times did you have 5 or more drinks on one occasion during both of these periods? | |

40. At any time during the three months before the pregnancy began did you take any multivitamins regularly excluding prenatal vitamins? ☐ Yes ☐ No

On average, how many days per week did you take them? | |

At any time during the first three months of the pregnancy did you take any multivitamins regularly? ☐ Yes ☐ No

On average, how many days per week did you take them? | |

41. At any time during the three months before the pregnancy began did you take any prenatal vitamins regularly? ☐ Yes ☐ No

On average, how many days per week did you take them? | |

At any time during the first three months of the pregnancy did you take any prenatal vitamins regularly? ☐ Yes ☐ No

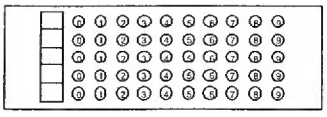
On average, how many days per week did you take them? | |

42. At any time during the three months before the pregnancy began did you take a Vitamin A supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

At any time during the first three months of the pregnancy did you take a Vitamin A supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |



43. At any time during the three months before the pregnancy began did you take a Vitamin B or B-complex supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

At any time during the first three months of the pregnancy did you take a Vitamin B or B-complex supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

44. At any time during the three months before the pregnancy began did you take a folic acid supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

At any time during the first three months of the pregnancy did you take a folic acid supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

45. At any time during the three months before the pregnancy began did you take a Vitamin C supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

At any time during the first three months ofthe pregnancy did you take a Vitamin C supplement regularly? ☐ Yes ☐ No

On average, how many days per week did you take it? | |

During the three months before the pregnancy began through the first three months of the pregnancy, how many servings a week on average did you consume of the following:

	PRE Number per week:	POST Number per week:
46. Orange or grapefruit juice (1 serving = 1 cup)?		
47. Ready-to-eat breakfast cereal (1 cup)?		
48. Legumes, like peas, pinto beans, navy beans, kidney beans, lentils, or other kinds of dried beans (1/2 cup)?		

This is the end of the interview. Do you have any questions or comments?

_____

_____

_____

Thank you very much for helping us with our study today.