Lack of Maternal Folic Acid Supplementation Is Associated with Heart Defects in Down Syndrome: A Report from the National Down Syndrome Project

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BACKGROUND: Maternal folic acid supplementation has been associated with a reduced risk for neural tube defects and may be associated with a reduced risk for congenital heart defects and other birth defects. Individuals with Down syndrome are at high risk for congenital heart defects and have been shown to have abnormal folate metabolism. **METHODS:** As part of the population-based case-control National Down Syndrome Project, 1011 mothers of infants with Down syndrome reported their use of supplements containing folic acid. These data were used to determine whether a lack of periconceptional maternal folic acid supplementation is associated with congenital heart defects in Down syndrome. We used logistic regression to test the relationship between maternal folic acid supplementation and the frequency of specific heart defects correcting for maternal race or ethnicity, proband sex, maternal use of alcohol and cigarettes, and maternal age at conception. **RESULTS:** Lack of maternal folic acid supplementation was more frequent among infants with Down syndrome and atrioventricular septal defects (odds ratio [OR], 1.69; 95% confidence interval [CI], 1.08–2.63; $p = 0.011$) or atrial septal defects (OR, 1.69; 95% CI, 1.11–2.58; $p = 0.007$) than among infants with Down syndrome and no heart defect. Preliminary evidence suggests that the patterns of association differ by race or ethnicity and sex of the proband. There was no statistically significant association with ventricular septal defects (OR, 1.26; 95% CI, 0.85–1.87; $p = 0.124$). **CONCLUSIONS:** Our results suggest that lack of maternal folic acid supplementation is associated with septal defects in infants with Down syndrome. *Birth Defects Research (Part A) 91:885–893, 2011.* © 2011 Wiley-Liss, Inc.

Key words: atrial septal defect; atrioventricular septal defect; congenital heart defect; Down syndrome; folic acid

INTRODUCTION

Individuals with Down syndrome (DS), the clinical consequence of trisomy 21, exhibit a wide range of phenotypes. Congenital heart defects (CHDs) occur in approximately 40% of DS cases and range from small atrial septal defects (ASD) or ventricular septal defects (VSD) to complete atrioventricular septal defects (AVSD) and other serious heart defects, such as tetralogy of Fallot (TOF; Freeman et al., 1998, 2008). We recently reported that the frequencies of AVSD and secundum ASD (ASD

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II), but not VSD, in the DS population vary by sex and race or ethnicity of the proband, suggesting underlying genetic or racially or ethnically specific environmental risk factors (Freeman et al., 2008). Compared to non-Hispanic white infants with DS, non-Hispanic black infants have double the risk and Hispanic infants have half the risk for an AVSD. In addition, there is a nearly twofold excess of female probands among DS cases with an AVSD, despite a male-to-female ratio of 1.15 among all infants with DS (Kallen et al., 1996; Freeman et al., 2008). An excess of female nonsyndromic AVSD cases has also been noted (Ferencz et al., 1997).

Folate, a vital nutrient, donates methyl groups for purine and pyrimidine synthesis, methylation of DNA and proteins, and conversion of homocysteine to methionine. DNA methylation is used for critical cellular functions such as imprinting, X-chromosome inactivation, and long-term gene silencing (Bernstein et al., 2007). The importance of the folate pathway in development is clear from the association between maternal folic acid intake and neural tube defects (NTDs; Wald and Sneddon, 1991; Czeizel and Dudas, 1992). In 1992, the U.S. Public Health Service issued a recommendation that all fertile women consume 0.4 mg of folic acid per day to reduce the risk of NTDs in offspring (Centers for Disease Control and Prevention, 1992). Studies of maternal folic acid supplementation in the etiology of CHD (Shaw et al., 1995; Botto et al., 1996; Scanlon et al., 1998; Botto et al., 2004; Bailey and Berry, 2005; van Beynum et al., 2010) and other birth defects (Bailey and Berry, 2005; Botto et al., 2004) suggest a role for the folate pathway, although significant associations have not been seen in all studies.

The many functions of the folate pathway are mediated by enzymatic processes. Several studies have found that polymorphisms in folate pathway genes such as 5,10-methylenetetrahydrofolate reductase (*MTHFR*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), and 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) reduce the enzyme activity of their gene products (Frosst et al., 1995; van der Put et al., 1997; Weisberg et al., 1998; Harmon et al., 1999). Polymorphisms in these pathway genes and others, such as the chromosome 21-linked reduced folate carrier gene (*SLC19A1*), have been associated with CHD in some, but not all studies (Shaw et al., 2003; McBride et al., 2004; Shaw et al., 2005; Hobbs et al., 2006; Pei et al., 2006; van Beynum et al., 2006; Goldmuntz et al., 2008; Locke et al., 2010; Mitchell et al., 2010).

In addition to studies of folate pathway gene polymorphisms in nonsyndromic CHD, the biochemical consequences of trisomy 21 led us to consider the folate pathway to explain the increased risk for CHD among infants with DS. Enzymatic and biochemical evidence suggests that individuals with DS have abnormal folate and homocysteine metabolism. Overexpression of the cystathionine beta synthase gene (*CBS*), located on chromosome 21, creates a functional folate deficiency in tissues with trisomy 21 (Chadefaux et al., 1985). Folate pathway components such as homocysteine, methionine, S-adenosyl methionine, and S-adenosylhomocysteine are reduced in individuals with DS (Pogribna et al., 2001). In support of this hypothesis, we recently showed an associated between polymorphisms in *SLC19A1* and AVSD in DS (Locke et al., 2010). We uses the large population-based epidemiologic dataset collected through the National Down Syndrome Project (NDSP) to test the hypothesis that maternal folic acid supplementation prior to fetal heart development is associated with CHD in DS.

MATERIALS AND METHODS

Population Ascertainment

Based at Emory University in Atlanta, Georgia, the NDSP enrolled families of infants with DS born from 2001 through 2004 at six sites across the country. Each site was linked to a birth defects surveillance system. We previously reported the details of ascertainment and recruitment (Freeman et al., 2007). All NDSP sites obtained institutional review board approvals and informed consent from participants.

The NDSP included live-born infants with standard trisomy 21 or mosaic trisomy 21 born during the study period to English- or Spanish-speaking mothers. Infants with DS resulting from a translocation were excluded, as were families whose infants died after birth and before study enrollment. Those excluded because the infant died before enrollment represented <5% of identified cases and did not differ proportionally in maternal race or ethnicity or proband sex from those who were live born. For this study, we have further excluded infants with mosaic trisomy 21 and those with both trisomy 21 and another clinically relevant chromosome abnormality.

Race and ethnicity of the mother was determined by the self-reporting. Methods for the collection and abstraction of medical records documenting CHD and other birth defects were described previously (Freeman et al., 2008). Each recruitment site abstracted infant medical records and entered the information onto a structured clinical form, which was reviewed by a single clinically trained individual. A pediatric cardiologist was consulted as necessary. Each occurrence of a specific type of CHD was counted. For example, infants with more than one heart defect were included as cases in each relevant group. Complex heart defects (e.g., complete AVSD, TOF) were counted as single defects. Only those clearly described as ASD II were counted as ASD II. Control infants were those with a structurally normal heart, patent foramen ovale, or patent ductus arteriosus. The use of echocardiography to document normal heart status at five of six recruitment sites was more than 90% and at the sixth site (selected geographic area in California) was more than 70% among probands with DS. In the remaining cases, physical examination was used (Freeman et al., 2008).

Determination of Maternal Behavior and Exposures

Participating mothers completed questionnaires administered by trained study personnel at the time of enrollment in the NDSP (Freeman et al., 2007). Using data from these questionnaires, we determined maternal use of supplements containing folic acid, use of alcohol and cigarettes, and education. Mothers were asked about prenatal vitamin, vitamin, and supplement intake for three periods: before pregnancy, the first 3 months of pregnancy, and after the first 3 months of pregnancy. We assigned mothers to *supplemented*, *nonsupplemented*, *uncertain*, or *missing* folic acid use groups. Human heart development occurs between the fourth and eighth weeks

Table 1
National Down Syndrome Project: A comparison of the frequency of heart defects, sex ratios, and maternal race or ethnicity between all eligible and enrolled infants with DS

	All eligible cases (Freeman et al., 2008)		All eligible cases with maternal questionnaire (current study)		
Proband characteristic	n	%	n	%	*p* value
Heart Defect					
Atrioventricular septal defect (AVSD)	252	17.2	178	16.5	0.66
Atrial septal defect (ASD II)[a]	273	18.6	203	18.8	0.88
Ventricular septal defect (VSD)[b]	282	19.2	213	19.7	0.73
Tetralogy of Fallot (TOF)	39	2.7	26	2.4	0.70
Other	19[c]	1.3	17[d]	1.6	0.55
Heart defect summary					0.77
Cases with more than one heart defect	649	44.2	483	44.8	
Cases with no heart defect	820	55.8	596	55.2	
Total with heart defect information	1469		1079		
Sex of proband					0.49
Female	682	46.4	516	48	
Male	787	53.6	563	52	
Total	1469		1079		
Race or Ethnicity of Mother					0.06
White	624	43.4	510	48.2	
Black	183	12.7	111	10.5	
Hispanic	569	39.5	399[e]	38.0	
Asian	63	4.4	37	3.5	
Total	1439		1057		

[a]Secundum ASD II. Excludes patent foramen ovale (PFO) and PFO versus ASD II.
[b]Excludes VSD that is part of an AVSD or TOF.
[c]Includes double-outlet right ventricle (n = 6), coarctation of the aorta (n = 6), dextrocardia (n = 2), and right aortic arch (n = 5).
[d]Includes double-outlet right ventricle (n = 6), coarctation of the aorta (n = 6), dextrocardia (n = 2), right aortic arch (n = 3).
[e]Includes 390 white Hispanics and 9 black Hispanics.

of pregnancy (calculated from the last menstrual period; Sadler, 2005). Mothers who were taking a vitamin or supplement containing folic acid before becoming pregnant and those who began taking such a supplement within the first 4 weeks of pregnancy were assigned to the supplemented group. Those who began taking a supplement during or after the eighth week of pregnancy and those who took no supplement were assigned to the nonsupplemented group. Although formation of the cardiac septa begins during the sixth week of pregnancy, we conservatively excluded those with uncertain supplementation (those whose folic acid supplementation started between the fourth and eighth weeks of pregnancy). Those with missing data were excluded from the analysis. Maternal education was determined to be either less than or at least a high school education. Mothers were asked about alcohol and cigarette use during two time periods: the first month and the second through third months of pregnancy. We used information about use during the first month instead of the second through third months of pregnancy, because the exposure was higher. For alcohol use, those who reported consuming at least one alcoholic drink per week during the time period were considered to have used alcohol. For cigarette use, those who smoked at least one cigarette per week during the time period were considered to have smoked.

Statistical Analysis

We used chi-square analysis in comparisons of frequency distributions between case groups. For each CHD, we used logistic regression analysis adjusting for maternal race or ethnicity and proband sex to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association of CHD with folic acid supplementation, using infants with DS without CHD as controls. Maternal age at birth of the infant, maternal education, alcohol use, and smoking did not contribute significantly in the AVSD or VSD model (i.e., step-wise removal of each did not change the OR by >10%) and were removed. Maternal smoking (at least one cigarette per day) in the first month of pregnancy contributed significantly to the ASD II model and was included in all ASD II models, except Hispanics alone because of the small number of Hispanic mothers who smoked (n = 7/390). In addition, none of the interaction terms with folic acid use and the primary covariates were significant, and none contributed significantly to the model as determined by the log-likelihood method. Regardless of statistical significance of the interaction terms, we stratified each analysis by factors previously known to be associated with AVSD—namely proband sex and race/ethnicity. Statistical analysis was performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC).

Our primary hypothesis that lack of folic acid supplementation increases the risk for CHD is unidirectional. Therefore, we provide *p* values for CIs around the ORs for lack of folic acid supplementation reflecting a one-sided test at a significance level of 0.05. Applying a Bonferroni correction for the three hypotheses originally tested (the association of lack of maternal folic acid use and AVSD, ASD II, or VSD in the proband) adjusts the

Table 2
The frequency of heart defects in probands with DS stratified by maternal demographics and exposures and proband sex

Exposure	No CHD n (%)	AVSD n (%)	ASD II n (%)	VSD n (%)
Race/Ethnicity				
White	282 (51)	96 (56)	79 (42)	92 (45)
Black	49 (9)	34 (20)	30 (16)	24 (12)
Hispanic	221 (40)	41 (24)	81 (43)	89 (43)
Proband Sex				
Female	253 (46)	109 (64)	102 (54)	93 (45)
Male	299 (54)	62 (36)	88 (46)	112 (55)
Supplement Use				
Missing	9 (2)	4 (2)	1 (1)	3 (1)
Uncertain	100 (18)	42 (25)	37 (19)	36 (18)
Yes	239 (43)	60 (35)	64 (34)	80 (40)
No	204 (37)	65 (38)	88 (46)	86 (22)
Maternal Age				
<35 years	282 (51)	97 (58)	97 (51)	117 (57)
≥35 years	270 (49)	74 (43)	93 (49)	88 (43)
Maternal education				
<High school	113 (20)	21 (12)	47 (25)	52 (25)
≥High school	439 (80)	150 (88)	143 (75)	153 (75)
Alcohol (1st month)				
Missing	7 (1)	0 (0)	3 (1)	2 (1)
≥1 per wk	43 (8)	13 (8)	7 (4)	15 (7)
<1 per wk	502 (91)	158 (92)	180 (95)	188 (92)
Alcohol (2nd/3rd month)				
Missing	2 (<1)	0 (0)	1 (<1)	2 (1)
≥1 per wk	7 (1)	5 (3)	4 (2)	4 (2)
<1 per wk	543 (98)	166 (97)	185 (97)	199 (97)
Smoking (1st month)				
Missing	4 (<1)	0 (0)	2 (1)	2 (1)
Yes	60 (11)	19 (11)	9 (5)	15 (7)
No	488 (88)	152 (89)	179 (94)	188 (92)
Smoking (2nd /3rd month)				
Missing	6 (1)	0 (0)	1 (1)	2 (1)
Yes	42 (8)	15 (9)	7 (4)	14 (7)
No	504 (91)	156 (91)	182 (95)	189 (92)
Total	552	171	190	205

significance level to $p = 0.017$. No correction was applied to the post hoc stratified analyses.

RESULTS

Study Population

The NDSP identified 1469 infants with DS and, of those families, 1079 (73.5%) participated by completing a maternal questionnaire and providing access to medical records. There was no difference in the frequency of CHD, maternal race or ethnicity, or proband sex ratio between eligible infants and enrolled infants (Table 1).

The study population consisted of 510 non-Hispanic white mothers (referred to as *white*), 111 non-Hispanic black mothers (referred to as *black*), and 390 white Hispanic mothers for a total of 1011. There were too few black Hispanic mothers (n = 9) and Asian mothers (n = 37) to be included in further analysis. The demographics and frequencies of maternal behaviors for each type of CHD are provided in Table 2.

Among infants with an AVSD, there were more black and fewer Hispanic mothers than white mothers (Table 2). These differences reflect an increased OR for AVSD for blacks compared with whites of 1.86 (95% CI, 1.17–2.96; $p = 0.009$) and a decreased OR for AVSD for Hispanics compared with whites of 0.51 (95% CI, 0.34–0.76; $p < 0.001$), adjusting for proband sex. The racial or ethnic distribution among mothers of infants with ASD II differed from those of infants with no ASD II (Table 2): there were more black (OR, 2.00; 95% CI, 1.23–3.24; $p = 0.005$) and more Hispanic (OR, 1.44; 95% CI, 1.02–2.03, $p = 0.04$) mothers compared with white mothers, adjusting for proband sex. Regarding proband sex, there were more females compared to males among those with an AVSD compared to those without an AVSD (Table 2), leading to an OR of 2.08 (95% CI, 1.48–2.94, $p < 0.0001$), adjusted for race or ethnicity. The proband sex ratio did not differ significantly for infants with an ASD II (OR, 1.29; 95% CI, 0.94–1.77; $p = 0.12$) or a VSD (OR, 0.86; 95% CI, 0.63–1.17; $p = 0.33$). These patterns were consistent with those previously reported for all eligible infants (Freeman et al., 2008). Given these differences in association between AVSD, ASD II, and VSD with maternal race or ethnicity and proband sex, as well as the previously reported female bias between syndromic and nonsyndromic AVSD, these CHDs were analyzed separately. There were insufficient cases of TOF or other CHD to be included in this or subsequent analyses.

Maternal Supplement Use and CHDs in DS

We next investigated the relationship between maternal use of supplements containing folic acid and the frequency of CHD in DS probands, adjusting for race or ethnicity and sex. The proportion of white, black, and Hispanic mothers in the *uncertain* (21%, 18%, and 18%, respectively) and *missing* (2%, 1%, and 2%, respectively) groups did not differ ($p = 0.77$). A total of 407 mothers of infants in the *supplemented* group (white, n = 286; black, n = 35; Hispanic, n = 86) were compared to 392 mothers of infants in the *nonsupplemented* group (white, n = 109; black, n = 55; Hispanic, n = 228). Among these 799 mothers, a higher proportion of white mothers than black or Hispanic mothers used supplements containing folic acid (72%, 39%, and 27%, respectively; $p < 0.0001$).

AVSD

Logistic regression analysis regressing AVSD case-control status (reference: DS proband with no CHD, controlling for proband sex and maternal race or ethnicity) against folic acid use as the primary exposure demonstrated a statistically significant association between AVSD in probands with DS and mothers who did not take a supplement containing folic acid (OR, 1.69; 95% CI, 1.08–2.63; $p = 0.011$; Table 3). Hispanic maternal ethnicity and proband sex were significant in this model (Table 3). When folic acid use by race or ethnicity or by proband sex interaction terms were added to the model, they were not significant. Regardless, we stratified by these factors in a post hoc analysis. Among the three ethnic or racial groups, the association between lack of folic acid supplementation and AVSD reached significance only among Hispanic mothers (OR, 3.45; 95% CI, 1.36–8.71; $p = 0.014$; Table 4). In the stratified analysis by proband sex, there was a statistically significant association between lack of folic acid use and AVSD (OR, 2.32; 95% CI, 1.28–4.20; $p = 0.010$) among males adjusting for race or ethnicity. For females, there was no statistically

Table 3
Results from logistic regression models

Exposure		No CHD (%)	AVSD (%)	OR (95% CI)	p[a]	ASD II (%)	OR (95% CI)	p	VSD (%)	OR (95% CI)	p
Folic acid supplement	With	239 (54)	60 (48)	Ref.		64 (42)	Ref.		80 (48)	Ref.	
	Without	204 (46)	65 (52)	1.69 (1.08–2.63)	0.011[b]	88 (58)	1.69 (1.11–2.58)	0.007[b]	86 (52)	1.26 (0.85–1.87)	0.124[b]
Race or ethnicity	White	218 (49)	70 (56)	Ref.		64 (42)	Ref.		77 (46)	Ref.	
	Black	42 (9)	25 (20)	1.58 (0.88–2.86)	0.126	24 (16)	1.67 (0.92–3.05)	0.093	20 (12)	1.24 (0.68–0.2.29)	0.484
	Hispanic	183 (41)	30 (24)	0.41 (0.24–0.68)	0.0006	64 (42)	0.88 (0.56–1.39)	0.596	69 (42)	0.97 (0.64–1.46)	0.875
Proband sex	Male	246 (56)	49 (39)	Ref.	0.0009	73 (48)	Ref.		90 (54)	Ref.	0.706
	Female	197 (44)	76 (61)	2.02 (1.34–3.06)		79 (52)	1.38 (0.95–2.02)	0.094	76 (46)	1.07 (0.75–1.54)	
Smoking	No	399 (90)	—	—	—	144 (95%)	Ref.		—	—	—
	Yes	40 (9%)	—	—	—	6 (4%)	0.35 (0.14–0.86)	0.023	—	—	—
	MIssing	4 (1%)	—	—	—	2 (1%)	—		—	—	—

[a]Applying a Bonferroni correction for the three hypotheses originally tested adjusts the significance level to $p = 0.017$ (see Materials and Methods).
[b]One-sided p values are provided for the folic acid term.
CHD, congenital heart defect; ASD, atrial septal defect; OR, odds ratio; CI, confidence interval; VSD, ventral septal defect; Ref., referent group.

significant association (OR, 1.37; 95% CI, 0.84–2.24; $p = 0.146$). We observed a correspondingly lower male:female sex ratio among infants with an AVSD born to supplemented (male:female = 0.46) versus nonsupplemented mothers (male:female = 0.86). We had insufficient cases to stratify the analysis by both race or ethnicity and sex of the proband.

ASD II

Using the same logistic regression approach, we examined the influence of supplements containing folic acid among DS cases with an ASD II compared to DS controls with no CHD. Controlling for proband sex, maternal race or ethnicity, and maternal smoking demonstrated a significantly increased OR for lack of folic acid use (OR, 1.69; 95% CI, 1.11–2.58; $p = 0.007$; Table 3). Interaction terms with folic acid use by race or ethnicity or by proband sex were not significant. Again, as a post hoc analysis, when stratified by race or ethnicity this observation was significant only among Hispanic mothers (OR, 2.79; 95% CI, 1.32–5.87; $p = 0.004$; Table 4). When stratified by proband sex, the OR for lack of folic acid use was statistically significant among females (OR, 2.03; 95% CI, 1.11–3.72; $p = 0.011$) but not males (OR, 1.48; 95% CI, 0.81–2.70; $p = 0.106$), controlling for race and ethnicity.

VSD

We found no statistical evidence for an association between supplements containing folic acid and VSD among all individuals with DS (Table 3) or when we stratified by race or ethnicity or by sex of the proband (Table 4).

DISCUSSION

The high frequency of CHD is a significant cause of morbidity and mortality in DS (Frid et al., 2004; Ballweg et al., 2007; Shin et al., 2007). According to a 2005 report from the Center for Disease Control and Prevention National Center for Health Statistics, the birth rate among women older than 35 years has increased steadily since 1980 (Martin et al., 2007). Because increasing maternal age is strongly associated with increased risk for DS, understanding and preventing its associated birth defects are of great importance. We demonstrate that a lack of maternal folic acid supplementation is associated with an approximately 1.7-fold increased frequency of AVSD and of ASD II in DS, but no statistically significant increased frequency of VSD (Table 3). Because our data represent a diverse population, we were able to explore the relationship between maternal folic acid supplementation and DS-associated CHD in the context of race or ethnicity and proband sex.

Folic Acid and Proband Sex

We previously observed a twofold increased risk for an AVSD and a 1.3-fold increased risk for an ASD II among live-born DS females compared with males (Freeman et al., 2008). At the time, we suggested that the slight albeit statistically significant increased risk for an ASD II among females may be due to misclassification of AVSD cases; therefore, our a priori hypothesis focused on AVSD.

Although the folic acid use by sex interaction term was not statistically significant for any CHD, we decided to stratify the data by proband sex because of the different patterns we observed among the three major CHD groups. For AVSD, the association of lack of maternal folic acid supplementation was significant among male probands (OR, 2.32; 95% CI, 1.28–4.20; $p = 0.010$) but not female probands (OR, 1.37; 95% CI, 0.84–2.24; $p = 0.146$). Among ASD II, a statistically significant association was observed among females (OR, 2.03; 95% CI, 1.11–3.72; $p = 0.011$) but not males (OR, 1.48; 95% CI, 0.81–2.70; $p = 0.106$). No difference in OR was observed for VSD by proband sex (Table 4). At this point, it is unclear whether

Table 4
Results from stratified logistic regression models

Exposure	Folic acid supplement	No CHD (%)	AVSD (%)	OR (95% CI)	p^a	ASD II (%)	OR (95% CI)	p	VSD (%)	OR (95% CI)	p
Race or ethnicity											
White	With	161 (74)	47 (67)	Ref.		45 (70)	Ref.		56 (73)	Ref.	
	Without	57 (26)	23 (33)	1.39 (0.84–2.28)	0.139	19 (30)	1.21 (0.64–2.29)	0.275	21 (27)	1.06 (0.59–1.90)	0.426
Black	With	17 (40)	9 (36)	Ref.		9 (38)	Ref.		6 (30)	Ref.	
	Without	25 (60)	16 (64)	1.49 (0.60–3.70)	0.238	15 (62)	1.72 (0.54–5.50)	0.179	14 (70)	1.71 (0.53–5.50)	0.183
Hispanic	With	61 (33)	4 (13)	Ref.		10 (16%)	Ref.		18 (26)	Ref.	
	Without	122 (67)	26 (87)	3.45 (1.36–8.71)	0.014	54 (84)	2.79 (1.32–5.87)	0.004	51 (74)	1.41 (0.76–2.63)	0.138
Sex											
Male	With	128 (52)	19 (39)	Ref.		31 (42)	Ref.		41 (46)	Ref.	
	Without	118 (48)	30 (61)	2.32 (1.28, 4.20)	0.010	42 (58)	1.48 (0.81–2.70)	0.106	49 (54)	1.27 (0.73–2.19)	0.199
Female	With	111 (56)	41 (53)	Ref.		33 (42)	Ref.		39 (51)	Ref.	
	Without	86 (44)	35 (46)	1.37 (0.84–2.24)	0.146	46 (58)	2.03 (1.11, 3.72)	0.011	37 (49)	1.26 (0.71–2.22)	0.215

[a]One-sided p values are provided for these models.
CHD, congenital heart defect; AVSD, atrioventral septal defect; OR, odds ratio; CI, confidence interval; VSD, ventral septal defect; Ref., referent group.

these patterns are biologically significant or simply random effects.

The possibility of a sex-specific association between CHD and maternal folic acid use should be considered based on observations of a folic acid-related developmental disorder in which the frequency differs by sex. NTDs, in particular anencephaly, were observed more frequently in female than in male fetuses (Martinez Frias et al., 1986; Seller, 1986). Intriguingly, as the prevalence of anencephaly has declined over time, a steeper decline in female frequency has narrowed this sex ratio difference (Besser et al., 2007; Canfield et al., 2009). These results suggest that folic acid supplementation was sufficient to reduce the risk of anencephaly in both the higher-risk female fetuses and male fetuses. The effectiveness of maternal folic acid supplementation in reducing female NTD risk differs from the trend of our AVSD in DS data. We found that the nonsignificant pattern of our ASD II associations with maternal folic acid supplementation by sex followed the same pattern observed among NTDs. These findings may suggest a difference in tissue-specific or sex-specific thresholds of folic acid effects on heart development.

The potential for a true difference in sex-specific patterns of AVSD and ASD II associated with maternal folic acid supplementation is intriguing, because this would suggest a different etiology, rather than misclassification as we originally proposed (Freeman et al., 2008). The sex-specific AVSD and ASD II patterns must be replicated. For VSD, we have not observed a sex-specific influence of folic acid use in DS, an observation which also must be replicated.

Folic Acid and Race or Ethnicity

In this study, we observed that the use of maternal supplements containing folic acid varied by race and ethnicity. Among the 1011 mothers included, folic acid supplementation before the fourth week of pregnancy was lower in black and Hispanic mothers compared with white mothers (32% and 22% vs. 56%). Our findings are consistent with data from the National Health and Nutrition Examination Surveys (Yang et al., 2007), indicating that among nonpregnant women a smaller percentage of Hispanic and black women compared with white women consumed a minimum of 0.4 mg of folic acid per day as recommended by the U.S. Public Health Service (Centers for Disease Control and Prevention, 1992). Although serum and red blood cell (RBC) folate levels have improved since mandatory fortification, persistent low RBC folate levels, and lower reported folic acid intake have been reported in non-Hispanic blacks (Ganji and Kafai, 2006; Kant and Graubard, 2007).

In this study, infants born to Hispanic mothers showed the most pronounced difference in AVSD and ASD II risk associated with maternal folic acid supplementation (Table 4), despite having the lowest overall risk for AVSD compared with whites and blacks and a comparable risk for ASD II compared with blacks (Freeman et al., 2008). This finding suggests a greater effect of folic acid supplementation on the Hispanic population. Interestingly, population-based studies have demonstrated an increased risk of NTD-affected pregnancies among Hispanic women compared with white women (Williams

et al., 2005; Velie et al., 2006; Carmichael et al., 2008; Canfield et al., 2009). The risk of NTD-affected pregnancies was highest among foreign-born Hispanic mothers, suggesting that environmental influences contribute to NTD risk in this population (Carmichael et al., 2008; Velie et al., 2006). In our study, the majority of Hispanic mothers (83%) were born outside of the United States. It would be interesting to determine whether the association between lack of maternal folic acid supplementation and AVSD and ASD II in this population differs in a second- or third-generation Hispanic-American population.

In addition, we suggest that the absence of a skewed sex ratio in our original report of Hispanic AVSD cases (Freeman et al., 2008) is consistent with comparable frequencies of AVSD and ASD II in males and females with DS whose mothers did not take a supplement containing folic acid. Because the majority of Hispanic mothers did not take a supplement containing folic acid, there was no overall paucity of male AVSD cases. Among the small number of Hispanic male probands whose mothers took a supplement containing folic acid, 0 in 42 (0%) had an AVSD compared with 14 in 126 (11%) whose mothers did not take a supplement containing folic acid. As a result, the overall frequency of AVSD in Hispanic probands with DS was only 10%, similar to other reports (de Rubens Figueroa et al., 2003; Vida et al., 2005) suggesting a lower inherent risk for AVSD in the Hispanic population.

Strengths and Weaknesses of This Study

In this study, both DS case and control infants were drawn from a live-born population. Families whose infants were stillborn or died shortly after birth were not recruited (Freeman et al., 2007). By excluding early infant deaths, infants with more severe birth defects, including some heart defects, may have been excluded. However, because the number excluded owing to infant death was small (<5% of those identified), and because this group did not differ proportionally in maternal race or ethnicity or proband sex, this exclusion is not likely to significantly affect our findings. However, it is important to note that up to 80% of conceptions with DS are lost before birth (Hassold and Jacobs, 1984), resulting in the live-born population being highly selected. In addition, we have no data on the number of DS conceptions electively terminated and how this group varies by race or ethnicity. An effect of maternal folic acid use on fetuses with trisomy 21 that do not survive gestation cannot be addressed in this study.

Our results differ from those of Meijer et al. (2006), who used a similar study design based on live-born infants with DS and CHD (case) and DS and no CHD (control). There was no statistically significant association between the use of supplements containing folic acid and CHD in DS. Their study included primarily white mothers, whereas our study included a more racially and ethnically diverse sample. In addition, the ascertainment period differed; Meijer et al. (2006) identified probands prior to the 1998 mandate for dietary folic acid fortification (1978–1997), whereas our study sample was ascertained after that mandate (2001–2004). In both studies, mothers were interviewed using a standardized questionnaire and cases were diagnosed using hospital records that allowed classification of CHD. Both studies paid particular attention to defining the use of folic acid supplementation during the time of heart development, although there were differences in inclusion and exclusion based on that definition. Thus, the strengths of both studies were similar. Variability of folic acid exposure (mothers in our study had exposure to folic acid through both fortification and supplementation), racial or ethnic differences, and small sample sizes (once subtypes of CHD were studied) may all contribute to these conflicting findings. We strongly suggest that stratification of CHD subtypes is important given the differences in developmental mechanisms. We have shown that lack of maternal folic acid supplementation was statistically associated with specific DS-associated CHD, namely AVSD and ASD II. This observation underscores the importance of ensuring phenotypic homogeneity within a study population and the utility of studying a sensitized population.

Despite the large sample size, the number of DS probands with the specific types of CHD was relatively modest, particularly among infants of black or Hispanic mothers. The size of this sample was insufficient to determine the effects of genetic and environmental factors known to influence folate pathway function, such as the *MTHFR* c.677C>T and c.1298A>C, *MTR* c.2756C>G, and *MTRR* c.66A>G gene polymorphisms, which are common and affect enzyme activity (Frosst et al., 1995; van der Put et al., 1997; Weisberg et al., 1998; Harmon et al., 1999). We recently reported an association between *SLC19A1* gene variants and AVSD in probands with DS (Locke et al., 2010). Brandalize et al. (2009) observed a higher rate of CHD in probands with DS whose mothers had a CT or TT *MTHFR* c.677 genotype when the mother did not take a supplement containing folic acid. These studies suggest that maternal and proband gene-environment interactions should be explored further. Maternal use of cigarettes was inversely associated with ASD II; however, the number of mothers exposed was small and none were Hispanic. Maternal alcohol use was not associated with AVSD, ASD II, or VSD in our study. Our study did not explore maternal diet; therefore, we were unable to account for a potentially significant source of variability in folic acid consumption. Continued ascertainment of a racially and ethnically diverse population is key to further understanding the role that maternal folic acid supplementation plays in the risk of AVSD and ASD II, and potentially VSD. Moreover, confirming the differential sex-specific patterns of risks will provide insight into the etiology of these different CHDs.

Future Directions

We are unable to determine whether the associations observed are causal, nevertheless the associations are significant and provide a basis for future studies. Despite a growing body of evidence that folic acid supplementation reduces the risk of NTDs, nonsyndromic CHDs, and other birth defects, the majority of women in this study did not take a supplement containing folic acid before the 4th week of pregnancy. If maternal age in the United States continues to increase, more pregnancies will be

at risk for DS. Although we have found that folic acid supplementation was associated with fewer DS-associated CHDs, we have not explained the differences in frequency of AVSD and ASD II among racial and ethnic groups. More studies to identify other environmental and genetic risk factors for CHD will help clinicians educate their patients on the best preventative measures before pregnancy.

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