Congenital heart defects and maternal biomarkers of oxidative stress\textsuperscript{1–4}

Charlotte A Hobbs, Mario A Cleves, Weizhi Zhao, Stepan Melnyk, and S Jill James

ABSTRACT

Background: Women who have had pregnancies that were affected by nonsyndromic congenital heart defects have alterations in the homocysteine-methionine pathway that may indicate increased exposure to oxidative stress or reduced antioxidant defense or both.

Objective: Our goal was to establish a maternal metabolic risk profile for nonsyndromic congenital heart defects that would enhance current preventive strategies.

Design: Using a case-control design, we measured biomarkers of the transsulfuration pathway in a population-based sample of women whose pregnancies were affected by congenital heart defects (331 cases) and in a control group of women (125 controls). Plasma concentrations of reduced and oxidized glutathione, vitamin B-6, homocysteine, cysteine, cysteinylglycine (CysGly), and glutamylcysteine (GluCys) were compared between cases and controls after adjustment for lifestyle and sociodemographic variables.

Results: After covariate adjustment, cases had significantly lower mean plasma concentrations of reduced glutathione \((P < 0.0001)\), GluCys \((P < 0.0001)\), and vitamin B-6 \((P = 0.0023)\) and significantly higher mean concentrations of homocysteine \((P < 0.0001)\) and oxidized glutathione \((P < 0.0001)\) than did controls.

Conclusions: Biomarkers of oxidative stress involved in the transsulfuration pathway were significantly higher in women with pregnancies affected by congenital heart defects than in women without such a history. Further analysis of relevant biomarkers of oxidative stress and genetic and environmental factors is required to define the basis for the observed alterations. Identifying the nature and extent of alterations in biomarkers of oxidative stress may suggest primary intervention strategies and provide clues to a greater understanding of the pathogenesis of congenital heart defects. \textit{Am J Clin Nutr} 2005;82:598–604.

KEY WORDS Birth defects, congenital heart defects, oxidative stress, glutathione, homocysteine, folate

INTRODUCTION

Each year in the United States, \(\approx 150,000\) babies are born with birth defects, the leading cause of infant mortality (1). Among these birth defects, congenital heart defects (CHDs) are among the most prevalent and the most serious; they occur in \(\approx 8–10\) of every 1000 live births (2). Approximately 40% of babies affected by the most serious CHDs die in infancy (3). More than 85% of CHDs are thought to result from a complex interaction between maternal exposures and genetic susceptibilities (4).

Multiple studies have suggested that multivitamins containing folic acid may reduce the risk of CHDs (5–8). Efforts to elucidate the mechanism by which folic acid exerts a protective effect have evaluated the metabolism of 3 folate-dependent pathways—DNA nucleotide synthesis pathway, homocysteine-methionine pathway, and glutathione-transsulfuration pathway (Figure 1).

To better understand the role of folate-dependent pathways in the occurrence of CHDs, we measured plasma concentrations of metabolites of the homocysteine-methionine pathway in women who had pregnancies that were affected by CHD and in controls (9). We and others found that women with CHD-affected pregnancies had higher plasma homocysteine concentrations than did women with unaffected pregnancies (9, 10). We postulated that elevated homocysteine may either have a direct teratogenic effect or exert a negative effect on the developing embryonic heart through alterations in remethylation or transsulfuration or both (9).

The metabolic pathway from homocysteine to glutathione is referred to as the transsulfuration pathway. Approximately 50% of homocysteine generated from methionine is metabolized to cystathionine. This is an irreversible reaction that permanently removes homocysteine from the methionine cycle for the synthesis of cysteine and glutathione (11). Elevated homocysteine is associated with alterations in the transsulfuration pathway that lead to greater oxidative stress (12). Experimental models have suggested that, in addition to evidence of a direct teratogenic effect, elevated homocysteine may have an indirect embryotoxic effect.

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effect by increasing oxidative stress through excessive production of reactive oxygen species and by decreasing the glutathione-dependent antioxidant-defense mechanisms (12, 13). Chronic oxidative stress is also associated with low concentrations of vitamin B-6, reduced glutathione (GSH), and cysteine and high concentrations of oxidized glutathione (GSSG) (14, 15). The current study expands our previous study of folate-dependent pathways by examining micronutrients and metabolites in the transsulfuration pathway among women with CHD-affected pregnancies.

SUBJECTS AND METHODS

Study design and participants

Cases were identified and ascertained through the Arkansas Reproductive Health Monitoring System, a statewide birth defects registry. Inclusion criteria for the cases were as follows: the woman lived in Arkansas at the time of completion of the index pregnancy and at the time of enrollment in the study; the outcome of the index pregnancy was a live-born infant, stillborn infant, or elective termination; the pregnancy ended between February 1998 and July 2004; a physician diagnosed a nonsyndromic septal, conotruncal, or right- or left-sided obstructive heart defect, which was confirmed by echocardiogram, surgery, or autopsy report, or all 3; the women were English or Spanish speaking; and the cases and controls had completed participation in the National Birth Defects Prevention Study (NBDPS) (16). Cases whose pregnancy was also affected by a known single-gene disorder, chromosomal abnormality, or syndrome were excluded; only cases with pregnancies with nonsyndromic CHDs were included. All cardiac cases were confirmed by echocardiogram, surgery, or autopsy report and reviewed by a single pediatric cardiologist before inclusion in the study.

Controls were English- or Spanish-speaking Arkansas residents who had live births that were unaffected by any birth defect; they were randomly chosen from all birth certificates registered at the Arkansas Department of Health with birth dates between June 1998 and April 2004. After ascertaining a person’s eligibility, a research nurse contacted the subject by mail and telephone, describing this study.

During scheduled home visits, the nurse obtained written informed consent and used routine venipuncture to obtain a blood sample. Cases and controls were excluded if they were pregnant at the time of the blood draw or were taking any known folate-antagonist medications. The study protocol and the provisions for informed consent were reviewed and approved by the Institutional Review Board at the University of Arkansas for Medical Sciences.

Covariates

Each participant’s ethnicity and educational level were self-reported during a structured computer-assisted telephone interview that was specifically designed for an ongoing multisite case-control study, the NBDPS; further details regarding the NBDPS were published previously (16). At the time of the home visit, a Block abbreviated food-frequency questionnaire (17) was administered, and information about the current use of multivitamins, cigarettes, alcohol, and caffeine intake was obtained.
Sample preparation and biomarker measurement

Blood samples were collected into evacuated tubes (B-D Biosciences, Dallas, TX) containing EDTA and immediately chilled on ice before being centrifuged at 4000 × g for 10 min at 4 °C. Plasma aliquots were transferred into cryostat tubes and stored at −80 °C until extraction and HPLC quantification.

To measure total homocysteine, cysteine, and GSH concentrations, 50 μL of a freshly prepared solution to reduce all sulfhydryl bonds (1.43 mmol sodium borohydride/L, 1.5 μmol EDTA/L, 66 mmol NaOH/L, and 10 μL isomyl alcohol was added to 200 μL plasma. The samples were incubated at 40 °C in a shaker for 30 min. To precipitate proteins, 250 μL ice-cold 10% metaphosphoric acid was added and mixed well, and samples were incubated for an additional 10 min on ice. After centrifugation at 18,000 × g for 15 min at 4 °C, the supernatant fluid was filtered through a 0.2-μm nylon membrane filter (PGC Scientific, Frederick, MD), and a 20-μL aliquot was injected into the HPLC system.

To measure oxidized glutathione (GSSG) concentrations, 100 μL of 10% metaphosphoric acid was added to 200 μL plasma to precipitate proteins; the solution was mixed well and incubated on ice for 30 min. After centrifugation at 15 min at 18,000 × g at 4 °C, supernatant fluids were passed through a 0.2-μm nylon membrane filter, and 20 μL was injected into the HPLC system. Plasma concentrations of pyridoxal-5′-phosphate (vitamin B-6) were measured by using an HPLC method developed by Lequeux et al (18) with slight modifications for the use of coulometric electrochemical detection.

Details of the method for HPLC elution and electrochemical detection were described previously (19, 20). Separation of metabolites was performed by using HPLC with a Shimadzu solvent-delivery system (model 580; ESA Inc, Chelmsford, MA) and a reverse-phase 5-μm C18 column (4.6 × 150 mm; MCM Inc, Tokyo, Japan) obtained from ESA Inc. A 20-μL aliquot of plasma extract was directly injected onto the column by using autosamplers from ESA Inc (model 542) or Beckman (model 507E; Beckman Instruments, Irvine, CA). All plasma metabolites were quantified by using Coulomichem II (model 5200A) and CoulArray (model 5600) electrochemical detection systems equipped with a dual analytic cell (model 5010), a 4-channel analytic cell (model 6210), and a guard cell (model 5020; all from ESA Inc). The unknown concentrations of plasma metabolites were calculated from peak areas and standard calibration curves with the use of HPLC software.

Statistical analysis

Because of the skewed distribution of these measurements, Wilcoxon’s Mann-Whitney rank-sum test was used to compare alcohol consumption, cigarette smoking, caffeine intake, and interval between pregnancy and participation. All plasma biomarkers exhibited positively skewed distributions; therefore, to achieve normality, biomarker data were log transformed (natural log) before analysis. Mean log-transformed biomarker concentrations of cases and controls were compared by using a Student’s t test, whereas linear regression was used to adjust these comparisons for age, race, education level, breastfeeding status, multivitamin supplement intake, smoking, alcohol consumption, caffeine intake, and interval between the end of pregnancy and the blood draw. Crude and adjusted odds ratios (ORs) and corresponding 95% CIs for the association between plasma biomarkers and case or control status were computed by using logistic regression. Analyses were performed with SAS statistical software (version 9.1; SAS Institute, Cary, NC).

RESULTS

As shown in Table 1, of the 331 cases and 125 controls included in this study, 77.0% were white. At the time of the blood draw, 63.7% of the cases were <30 y old, and less than half (45.0%) of the cases reported alcohol consumption. There was no significant difference between the percentage of cases (44.4%) and the percentage of controls (44.8%) who reported regular multivitamin use at the time of the blood draw. Smoking was significantly (P = 0.0401) more prevalent among cases (28.1%) than among controls (18.4%), but caffeine intake did not vary significantly between cases and controls (P = 0.2838).

We previously reported on the plasma concentrations of homocysteine and folate among a subset of these women (9). In the current study, we included homocysteine and folate and also measured the plasma concentrations of GSH and GSSG, cysteineylglycine (CysGly), glutamylcysteine (GluCys), and cysteine (Table 2). We compared log-transformed mean concentrations between cases and controls after adjustment for lifestyle and sociodemographic variables. Compared with controls, cases had significantly lower mean plasma concentrations of GSH (P < 0.0001), GluCys (P < 0.0001), and vitamin B-6 (P = 0.0023); significantly higher mean concentrations of homocysteine (P < 0.0001) and GSSG and GSSG:GSH were further compared between plasma concentrations of GSSG above the 70th percentile in controls; 178 cases (54.1%) had GSSG above the 70th percentile in controls, and 39.3% had concentrations above the 70th percentile in controls; 178 cases (54.1%) had plasma concentrations of GSSG above the 70th percentile in controls, and 151 cases (46.0%) had GSSG-GSH above the 90th percentile of the ratio distribution among controls. More than half of the cases had GSH concentrations that were below the 30th percentile of GSH in controls. These distributional differences remained important after covariate adjustment. The ORs for each metabolite increase or decrease in a dose-dependent manner, which suggests that risk increases as the metabolite value moves toward the extremes. Specifically, the OR at the most extreme values of GSSG: GSH was 9.08 (95% CI: 4.6, 17.9). Thus, if a woman had GSSG: GSH > 0.055, she was 9 times as likely to have a CHD-affected pregnancy as an unaffected pregnancy.

DISCUSSION

In a previous publication (9), evidence of impairment in remethylation of homocysteine was shown by lower methionine and S-adenosylmethionine concentrations and higher S-adenosylhomocysteine concentrations among women with CHD-affected pregnancies. Our current findings indicate that the higher homocysteine observed among women with CHD-affected pregnancies may extend beyond impairments in remethylation of homocysteine to impairments in the transsulfuration of homocysteine. Specifically, in comparison to controls, cases with CHD-affected pregnancies had significantly lower concentrations of vitamin B-6, GluCys, and GSH and significantly higher concentrations of GSSG.
We found that cases were 2.9 times as likely as were controls to have plasma vitamin B-6 concentrations in the lowest percentile. Despite this difference in plasma vitamin B-6 concentrations, dietary and total intake of vitamin B-6 from diet plus supplements did not differ significantly between cases and controls (adjusted $P = 0.9740$ and 0.9520, respectively). Vitamin B-6 is necessary to convert homocysteine to cystathionine and then to cysteine (15, 21). Limited evidence is available regarding

TABLE 1
Selected characteristics of cases and controls at entry in the study

<table>
<thead>
<tr>
<th></th>
<th>Cases ($n = 331$)</th>
<th>Controls ($n = 125$)</th>
<th>$P^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Race [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>252 (76.1)</td>
<td>98 (78.4)</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>45 (13.6)</td>
<td>19 (15.2)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>34 (10.3)</td>
<td>8 (6.4)</td>
<td>0.4633</td>
</tr>
<tr>
<td><strong>Age [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$&lt;30$ y</td>
<td>211 (63.7)</td>
<td>74 (59.2)</td>
<td></td>
</tr>
<tr>
<td>$\geq30$ y</td>
<td>120 (36.3)</td>
<td>51 (40.8)</td>
<td>0.3870</td>
</tr>
<tr>
<td><strong>Drinking status [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>182 (55.0)</td>
<td>56 (44.8)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>149 (45.0)</td>
<td>69 (55.2)</td>
<td>0.5857</td>
</tr>
<tr>
<td><strong>Vitamin supplementation [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>183 (55.3)</td>
<td>69 (55.2)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>147 (44.7)</td>
<td>56 (44.8)</td>
<td>1.0000</td>
</tr>
<tr>
<td><strong>Smoking status [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>238 (71.9)</td>
<td>102 (81.6)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>93 (28.1)</td>
<td>23 (18.4)</td>
<td>0.4010</td>
</tr>
<tr>
<td><strong>Breastfeeding status [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>294 (88.8)</td>
<td>107 (85.6)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>37 (11.2)</td>
<td>18 (14.4)</td>
<td>0.3820</td>
</tr>
<tr>
<td><strong>Education [n (%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than high school</td>
<td>45 (13.6)</td>
<td>17 (13.6)</td>
<td></td>
</tr>
<tr>
<td>Completed high school</td>
<td>111 (33.5)</td>
<td>34 (27.2)</td>
<td></td>
</tr>
<tr>
<td>13–15 y</td>
<td>77 (23.3)</td>
<td>35 (28.0)</td>
<td></td>
</tr>
<tr>
<td>$\geq16$ y</td>
<td>75 (22.7)</td>
<td>39 (31.2)</td>
<td>0.2547</td>
</tr>
<tr>
<td><strong>Drinks/wk among drinkers</strong></td>
<td>0.36 (0.16–1.16)$^3$</td>
<td>0.33 (0.13–1.09)</td>
<td>0.9953</td>
</tr>
<tr>
<td>Cigarettes/d among smokers</td>
<td>10.0 (6.0–19.0)</td>
<td>10.0 (5.0–15.0)</td>
<td>0.2738</td>
</tr>
<tr>
<td>Daily caffeine intake (mg)</td>
<td>28.1 (4.4–93.6)</td>
<td>20.6 (5.02–54.7)</td>
<td>0.2838</td>
</tr>
<tr>
<td>Interval between end of pregnancy and participation (mo)</td>
<td>14.9 (8.3–21.7)</td>
<td>22.8 (14.2–32.7)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

1 Percentages may not equal 100 because of missing data or rounding.

2 Calculated by Fisher’s exact test for categorical variables and by Mann-Whitney $U$ test for continuous variables.

3 Median; interquartile ranges in parentheses (all such values).

We found that cases were 2.9 times as likely as were controls to have plasma vitamin B-6 concentrations in the lowest percentile. Despite this difference in plasma vitamin B-6 concentrations, dietary and total intake of vitamin B-6 from diet plus supplements did not differ significantly between cases and controls (adjusted $P = 0.9740$ and 0.9520, respectively). Vitamin B-6 is necessary to convert homocysteine to cystathionine and then to cysteine (15, 21). Limited evidence is available regarding

TABLE 2
Summary statistics for plasma biomarker concentrations and crude and adjusted values for comparison of log-transformed plasma biomarker concentrations between cases and controls

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Cases ($n = 331$)</th>
<th>Controls ($n = 125$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>9.44 ± 2.41</td>
<td>7.86 ± 1.57</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GSH (µmol/L)</td>
<td>6.93 ± 1.78</td>
<td>7.50 ± 1.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CysGly (µmol/L)</td>
<td>43.63 ± 6.60</td>
<td>44.21 ± 6.0</td>
<td>0.3120</td>
</tr>
<tr>
<td>GluCys (µmol/L)</td>
<td>2.75 ± 1.04</td>
<td>3.28 ± 1.26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cysteine (µmol/L)</td>
<td>233.92 ± 22.25</td>
<td>233.57 ± 19.94</td>
<td>0.9626</td>
</tr>
<tr>
<td>Vitamin B-6 (nmol/L)</td>
<td>34.22 ± 11.40</td>
<td>37.23 ± 12.00</td>
<td>0.0066</td>
</tr>
<tr>
<td>Folate (µg/L)</td>
<td>10.23 ± 4.25</td>
<td>11.12 ± 4.95</td>
<td>0.0527</td>
</tr>
<tr>
<td>Vitamin B-12 (ng/L)</td>
<td>482.30 ± 196.6</td>
<td>498.38 ± 201.08</td>
<td>0.3985</td>
</tr>
<tr>
<td>GSSG (µmol/L)</td>
<td>0.373 ± 0.173</td>
<td>0.274 ± 0.090</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ratio of GSSG to GSH</td>
<td>0.055 ± 0.023</td>
<td>0.037 ± 0.012</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 Two hundred twenty-four of the cases and 90 of the controls were included in a previously published report of biomarkers of the homocysteine-methionine pathway. GSH, reduced glutathione; CysGly, cysteinylglycine; GluCys, glutamylcysteine; GSSG, oxidized glutathione.

2 Two-sample $t$ test using log-transformed data.

3 Adjusted for age, race, education, number of cigarettes smoked per day, alcohol consumption, vitamin intake, caffeine intake, breastfeeding status, and the interval between the end of pregnancy and study participation (multivariate linear regression).
Evidence for oxidative embryopathy is based largely on animal studies that allow experimental manipulation of the timing and dose of oxidant exposure that is not possible in human studies (31, 32). In animal models, glutathione depletion and oxidative stress have been strongly implicated in the etiology of multiple birth defects (31, 33). Depending on the timing and duration of exposure, the functional consequences of oxidative injury may induce embryopathy and dysmorphogenesis (31, 32). The developing fetus may be exposed to potentially teratogenic reactive oxygen species, and normal organogenesis may depend on a critical balance of antioxidant activity and prooxidant exposures that are modifiable by genetic or environmental factors, or both. Despite abundant supportive evidence from animal studies, limited human studies have been conducted on the association between birth defects and glutathione-mediated oxidative stress. Genetic epidemiologic studies support an association between orofacial clefts and phase II detoxification enzymes that protect cells from toxicants by conjugation with glutathione. Risk estimates of orofacial clefts were high among women exposed to hydrocarbons whose infants had a glutathione-S transferase M1 homozygous variant genotype (34). Mothers and infants with the glutathione-S transferase T1 null genotype had 5 times the risk of nonsyndromic oral facial clefting as did mother-infant pairs with the wild-type genotype (35).

Important methodologic limitations of our study should be considered. The blood obtained to measure biomarkers was collected well after the index pregnancies had ended. As we previously postulated (9), plasma concentrations of biomarkers in our study may not represent biomarker concentrations at the time of organogenesis, but they may identify women who have persistently elevated homocysteine concentrations among pregnancies that are affected by orofacial clefts and phase II detoxification enzymes that protect cells from toxicants by conjugation with glutathione. Risk estimates of orofacial clefts were high among women exposed to hydrocarbons whose infants had a glutathione-S transferase M1 homozygous variant genotype (34). Mothers and infants with the glutathione-S transferase T1 null genotype had 5 times the risk of nonsyndromic oral facial clefting as did mother-infant pairs with the wild-type genotype (35).

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antioxidant defense mechanism that may place women at risk of having a CHD-affected pregnancy.

We did not examine the relation between cardiac phenotypes and biomarkers. Some particular cardiac phenotypes may be more influenced by altered glutathione metabolism than are others. The medical charts of control infants were thoroughly reviewed and abstracted by trained health information management professionals, and the findings were reviewed by study physicians. Any control infant diagnosed with a congenital birth defect was excluded. However, echocardiograms were not routinely performed on control infants to definitively rule out a cardiac lesion. Thus, it is possible that some of the control infants may have had minor cardiac lesions that were never detected and documented in their medical record.

The basis for the abnormal metabolic profile observed in the current study cannot be defined without further analysis of relevant genetic and lifestyle factors. Alterations in the transsulfuration pathway among women whose infants had CHDs provide an informative metabolic map for candidate gene exploration. For example, we observed that GSH and GluCys were significantly lower in cases than in controls, but cysteine and CysGly did not differ significantly between the groups (Figure 1). This observation suggests that polymorphisms in the GluCys ligase gene, which encodes an enzyme that is rate-limiting for glutathione synthesis, may contribute to the complex etiology of non-syndromic CHDs. Further studies are needed to determine whether polymorphisms in the GluCys ligase gene and other genes in the transulfuration pathway contribute to the development of CHDs.

To our knowledge, this is the first study to show abnormal glutathione metabolism among women with a history of a pregnancy affected by CHDs. If our findings are replicated by other investigators, further investigations into the association between CHDs and the glutathione antioxidant defense mechanism may provide new etiologic clues about the underlying mechanism of non-syndromic CHDs. Efforts that combine information from metabolic studies with genetic and epidemiologic studies may identify novel primary prevention strategies.

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CAH was responsible for the experimental design and analysis of data; MAC was responsible for the design of the statistical data analysis; WZ was responsible for statistical analyses; SM was responsible for laboratory biomarker analyses; SJJ was responsible for analysis and interpretation of data; and all authors were responsible for writing the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES


