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Caffeine, Selected Metabolic Gene Variants, and Risk for Neural Tube Defects

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Abstract

Background—Investigations of maternal caffeine intake and neural tube defects (NTDs) have not considered genetic influences. Caffeine metabolism gene effects were examined in the National Birth Defects Prevention Study.

Methods—Average daily caffeine was summed from self-reported coffee, tea, soda, and chocolate intake for mothers of 768 NTD cases and 4143 controls delivered from 1997–2002. A subset of 306 NTD and 669 control infants and their parents were genotyped for *CYP1A2*1F*, *NAT2 481C>T* and *NAT2 590G>A*. *CYP1A2*1F* was classified by fast or slow oxidation status and *NAT2* variants were categorized into rapid or slow acetylation status. Case-control logistic regression analyses, family-based transmission/disequilibrium tests and log-linear analyses, and hybrid log-linear analyses were conducted to produce odds ratios (OR) or relative risks (RR) and 95% confidence intervals (CI) for caffeine intake and maternal and infant gene variants, and to examine interaction effects.

Results—NTDs were independently associated with infant slow *NAT2* acetylator status (RR: 2.00, 95% CI: 1.10–3.64) and maternal *CYP1A2*1F* fast oxidation status (OR: 1.49 95% CI: 1.10–2.03). Caffeine-consuming mothers who were *CYP1A2*1F* fast oxidizers and *NAT2* slow acetylators had non-significantly elevated estimated risk for an NTD-affected pregnancy (OR: 3.10 95% CI: 0.86–11.21). Multiplicative interaction effects were observed between maternal caffeine and infant *CYP1A2*1F* fast oxidizer status ($p_{\text{interaction}} = 0.03$).

Conclusions—The association identified between maternal *CYP1A2*1F* fast oxidation status and NTDs should be examined further in the context of *CYP1A2*'s other substrates. Maternal caffeine and its metabolites may be associated with increased risk for NTD-affected pregnancies in genetically susceptible subgroups.

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Keywords

Caffeine; Gene-environment Interaction, Effect Modification; Cytochrome P450 CYP1A2; N-Acetyltransferase NAT2; Embryo, Mammalian; Metabolism; Maternal Exposure/*adverse effects; Neural Tube Defects; Spinal Dysraphism; Encephalocele

Neural tube defects (NTDs) are among the most common birth defects in the U.S. with etiologies that include genetic factors, environmental factors, and their joint effects. Previous studies of maternal caffeine intake during pregnancy and NTDs have been inconclusive. We recently demonstrated an association between maternal caffeine intake and increased risk for NTDs (odds ratio [OR]: 1.3, 95% confidence interval [CI]: 1.0–1.6), primarily for spina bifida (OR: 1.4, 95% CI: 1.1–1.9) and encephalocele (OR: 1.4, 95% CI: 0.7–2.7), in a large case-control study (Schmidt et al., 2009). Rates of caffeine metabolism vary greatly between individuals (McKusick, 1988) perhaps generating differences in susceptibility to caffeine and caffeine metabolites. Caffeine is primarily metabolized by the enzyme cytochrome P4501A2 (CYP1A2) in a phase I activation reaction (Campbell et al., 1987) but other enzymes, including N-acetyltransferase 2 (NAT2) participate in phase II conjugation reactions to further metabolize caffeine metabolites (Cascorbi et al., 1995) (Figure 1). CYP1A2 is an inducible enzyme that metabolizes numerous drugs and activates procarcinogens, with activity largely determined by genetic factors (Campbell et al., 1987; Eaton et al., 1995; Rasmussen et al., 2002). *CYP1A2*1F* is one of two known polymorphisms that determine CYP1A2 activity. The other polymorphism, *CYP1A2*1C*, is rare in Caucasian populations (<2%) (Wooding et al., 2002). Polymorphisms in *NAT2* determine whether individuals are rapid or slow acetylators of xenobiotics (Grant et al., 1997). Studies of maternal caffeine intake have demonstrated the importance of metabolic differences for other reproductive outcomes (Signorello et al., 2001; Sata et al., 2005). No study to date has examined the association between maternal caffeine intake and NTDs while taking into account variation in caffeine metabolism across individuals. Both maternal and fetal metabolism may influence levels of drugs and of their metabolites all of which are of potential risk to a developing fetus. Using a subset of participants from the National Birth Defects Prevention Study (NBDPS), this study explored associations between NTDs and maternal and infant gene variants involved in caffeine metabolism, as well as the role of such polymorphisms as potential modifiers of the effects of maternal caffeine intake.

Subjects and Methods

National Birth Defects Prevention Study (NBDPS)

The NBDPS is an ongoing multi-center population-based case-control study of birth defects that began in 1997 and is described in detail elsewhere (Yoon et al., 2001). Systematic clinical criteria were used to define and confirm case infants with major structural birth defects ascertained through population-based birth defects surveillance systems in each participating center (Yoon et al., 2001). Unaffected live born controls frequency matched to cases by birth year were randomly selected from either birth certificates or birth hospital records at each center.

Birth defects surveillance systems in eight states (Arkansas, California, Georgia, Iowa, Massachusetts, New Jersey, New York, and Texas) provided data for these analyses. Each study center obtained institutional review board approval for the NBDPS. After computer-assisted telephone interviews were completed with eligible case and control mothers to collect demographic characteristics, pregnancy and health histories, and various other exposures, buccal swab kits were sent to families to obtain DNA samples. Established protocols were performed annually to assure genotyping proficiency, data quality, and high

concordance rates among NBDPS laboratories as described in Supplemental Digital Content 1 (Documentation, Sample Quality Assurance and Laboratory Proficiency)

Subject Selection

The current analyses included interview data for mothers of cases with NTDs and control mothers with estimated delivery dates from October 1, 1997 through December 31, 2002. Elective terminations and live- or stillborn infants diagnosed with anencephaly, spina bifida, or encephalocele comprised the case group. Both isolated NTD cases (infants with an NTD and no other unrelated major structural birth defect) and multiple NTD cases (infants with an NTD and one or more additional unrelated major structural birth defects) were included. Cases with known or strongly suspected chromosomal or single gene disorders (Rasmussen et al., 2003) or amnion rupture sequence were excluded from these analyses. Buccal sample data were included for all eligible case families and a subset of control families randomly selected using approximately twice the frequency of NTD families within each study center and birth year. Families with incomplete interviews, only paternal DNA samples available, or those reporting use of any fertility procedures, were excluded from genetic analyses.

Caffeine Exposure

In a previous report, we described our methods for assessing caffeine exposure for this study (Schmidt et al., 2009). An estimate of total maternal caffeine consumption was determined as the sum of the reported average caffeine per day from coffee, tea, soda, and chocolate intake for the year prior to pregnancy. Caffeine from medications was evaluated separately because information on medication intake was collected for a different time period. Caffeine-containing medications were not examined in this study because the number of women who reported their use was not adequate to assess gene-environment interaction effects. A standard amount of caffeine for each food or beverage was assigned based on previous literature and was multiplied by the reported average number of servings per day. Total caffeine exposure from all sources was categorized as “none” (0–9 mg/day) or “any” (10+ mg/day) for stratified analyses because associations between NTDs and each level of consumption were similar. Intake of caffeinated beverages during pregnancy was also determined as “none” versus “any” according to the methods described in Slickers et al. (2008) and examined for effect modification with genetic variants for mothers who intended to become pregnant when they did (defined as those who stopped using contraception to get pregnant or reported they wanted to be pregnant then) and those who recognized their pregnancy early (≤ 3 weeks or 1 month gestation).

Genotyping Methods and Categorization

Families of NTD-affected children and selected control families were genotyped for common variants of genes known to alter enzyme function within the caffeine metabolic pathway, including: *CYP1A2*1F* (*rs762551*), *NAT2 481C>T* (*rs1799929*), and *NAT2 590G>A* (*rs1799930*). The laboratory employed TaqMan SNP genotyping assays obtained through the Assay-on-Demand service (Applied Biosystems, 2009).¹³ Polymerase chain reaction (PCR) was performed on the 9700 GeneAmp® PCR System (Applied Biosystems, Foster City, CA) in a total volume of 3 μ l using TaqMan® Universal PCR Master Mix (part no. 4304437; Applied Biosystems) and Assay-on-Demand assay mix containing primers and probes. The PCR profiles consisted of an initial 10-minute incubation at 95°C for denaturation, and then 40 cycles of denaturation at 92°C for 15 seconds and extension at 60°C for 1 minute. Two microliters of a 1:10 DNA dilution from stock was used, or 1/5 microliter of the originating sample per genotype. Endpoint analysis was performed on the ABI PRISM 7900 HT system (Applied Biosystems, Foster City, CA).

The *CYP1A2*1F* variant was grouped into slow oxidizers (heterozygous C/A or homozygous C/C) and fast oxidizers (homozygous A/A). *NAT2* variants were classified as slow or rapid acetylators according to the methods of Cascorbi and colleagues (1995). More specifically, probable haplotypes were determined by genotypes at the *NAT2_481* and *NAT2_590* loci and the corresponding phenotypes were assigned to each of the allele combinations as shown in Table 1. These two *NAT2* polymorphisms represent about 91% of slow acetylator genotypes within Caucasians (Cascorbi et al., 1995). The methods used could not distinguish between the two haplotype combinations possible when subjects were heterozygous at both the *NAT2_481* and *NAT2_590* loci leading to either the rapid or the slow acetylator phenotype. The CG/TA haplotype (*4/*6E allele combination) leading to a rapid acetylator phenotype was expected to occur infrequently based on the very small percentage (<1%) of *6E alleles found in this and other populations. Thus, parents and infants who were heterozygous at both loci with indistinguishable haplotypes were classified as slow acetylators. Parental genotypes helped classify the majority of heterozygous infant haplotypes.

Statistical Analysis

The data were analyzed under two statistical frameworks applied to three study designs. Logistic regression analysis was used for case-control comparisons, and log-linear analysis was applied to both a case-parent triad design (Weinberg et al., 1998; Wilcox et al., 1998) and a hybrid design that combined the case-parent triad and case-control designs (Weinberg and Umbach, 2005). An extension of the original 2×2 transmission/disequilibrium test (ETDT) for multi-allele marker loci (Sham and Curtis, 1995) in case-parent triads was also used to verify the results of the log-linear analyses. Family-based case-parent triad designs provide inherent control for population stratification, or systematic differences in allele frequencies between subpopulations within a population, that can influence associations in case-control studies. On the other hand, case-control studies can provide greater power to detect lower risk estimates than family-based approaches, do not rely on Mendelian transmission, and allow for easy stratification and adjustment for multiple risk factors. The hybrid design has the advantages of both family-based and case-control studies. Consequently, primary findings for the genetic effects were decided *a priori* to come from the hybrid log-linear analyses, except when population structure bias was an issue and findings from the log-linear case-parent triad analyses were used instead. Caffeine by gene interaction findings were primarily tested using the case-control design. Primary findings were corrected for multiple testing using the Bonferroni procedure.

Logistic-Regression Framework

Logistic regression models were constructed to estimate odds ratios (OR) and 95% confidence intervals (CI) for total caffeine, and each maternal and infant gene variant using SAS, version 9.1 software (SAS Institute Inc., Cary, North Carolina). Risk factors for NTDs assessed as covariates were: maternal age at conception, race/ethnicity, pre-pregnancy body mass index (BMI), gravidity, parity, history of miscarriage, nausea or vomiting during pregnancy, periconceptional alcohol consumption, cigarette smoking (ever, in periconceptional period), oral contraceptive use, folic acid intake, and B vitamin consumption. Study center, family history of NTDs, and infant sex were also considered. Potential confounders identified in bivariate analyses and causing a 10% or more change in an exposure risk estimate were retained in the model. Stratified analyses and the significance of multiplicative interaction terms were used to examine the impact of potential effect modifiers.

Log-linear Framework

Main effects—The log-linear approach was used to assess the associations between NTDs and both maternal and infant genotypes with the case-parent triad as the unit of analysis (Weinberg et al., 1998; Wilcox et al., 1998). Infant genetic effects were tested by comparing the observed distribution of case genotypes with the expected distribution under Mendelian inheritance after stratification by parental genotypes. Tests of maternally-mediated genetic effects assumed symmetry of allele counts between the mothers and the fathers in the source population (mating symmetry) (Schaid and Sommer, 1993). The expectation-maximization (EM) algorithm was applied to allow the use of families with a missing parental genotype (Weinberg, 1999). In addition to providing likelihood-ratio tests (LRTs), the log-linear approach provided maximum-likelihood estimators of the genetic effects, allowing for different RRs corresponding to carrying one and carrying two copies of a susceptibility-related allele, relative to no copies (Weinberg et al., 1998). Under a dominant or a recessive model, the log-linear approach is more powerful than other transmission/disequilibrium tests in simulation studies (Starr et al., 2005).

Interaction effects—The LRT was used to examine the joint effects of caffeine exposure and each genotype on risk for NTDs based on a quantitative polytomous logistic model (QPL) (Kistner and Weinberg, 2004). This QPL method, originally designed as an extension of the log-linear approach to identify genes related to a quantitative trait, used case-parent triads to assess multiplicative gene $\times$ environment interaction effects by substituting the continuous maternal caffeine variable for the quantitative trait in the model. It was not necessary to assume Mendelian transmission under the null, or a normal distribution of caffeine intake. We confirmed that the assumption for no significant associations between caffeine intake and *NAT2* or *CYP1A2*1F* genotypes was met.

Hybrid Log-linear Framework

In the hybrid design, genotype information for case infants and their parents, was supplemented with genetic information for the parents of the control infants for additional power (Weinberg and Umbach, 2005). Relative risk parameters were estimated through log-linear, likelihood-based analysis. The hybrid log-linear model included six parameters that were proportional to the relative frequencies in the population of the six possible mating-types (based on parental genotype combinations) to allow stratification on parental mating types. Population structure bias was tested by adding an interaction term between NTD status and the mating type parameters, and testing the improvement in fit with both a 5-df LRT and a more sensitive 1-df test for trend across mating-type parameters. If p-values were low (<0.10), indicating vulnerability to population structure bias, results were confined to the case-parent triad study design. Asymmetry in parental mating was tested as described by Weinberg and Umbach (2005). Hybrid analyses were performed using the LEM (log-linear and event history analysis with missing data using the EM algorithm) software (van Den Oord and Vermunt, 2000).

Results

Characteristics of case and control mothers are reported elsewhere (Schmidt et al., 2009). As reported previously, of the 768 NTDs and 4143 controls with interview data, 761 (99.1%) cases and 4108 (99.2%) controls had data on total caffeine intake (Schmidt et al., 2009). The majority of mothers (87.3% case and 84.5% control) reported caffeine intake in the year prior to pregnancy. Additionally, 537 (70.7%) case and 2850 (69.8%) control mothers consumed caffeinated beverages during pregnancy. Caffeine consumption in the year prior to pregnancy was associated with slightly elevated risk for all NTDs combined (OR: 1.3,

95% CI: 1.0–1.6), that was strongest for spina bifida and encephalocele, and did not increase with higher levels of intake (Schmidt et al., 2009).

Of the families with interview data, a total of 328 (42.7%) case and 1320 (32.1%) control families returned buccal samples. Of these families, 306 (93.3%) case and 1271 (96.3%) control families met eligibility criteria and 669 control families were selected for genotyping. For both cases and controls, mothers in families who returned buccal samples were more likely to be non-Hispanic white and native born than mothers in families who did not return samples (Table 2). Mothers in case families who returned samples were more likely to be college-educated, to have household annual incomes above \$50,000, to report drinking alcohol during pregnancy, and to be of higher parity, and were less likely to be obese. Most maternal characteristics of families who were genotyped did not differ from those who returned samples. The frequency of case and control mothers reporting caffeine intake was similar among those with and those without samples returned and genotyped (Table 2), and the adjusted OR for any caffeine consumption among genotyped families was similar to that for all interviewed participants (OR: 1.42, 95% CI: 0.95–2.13).

Genotyping success rates for mother, father, and infant samples are presented with genotype and phenotype frequencies for each gene variant in Table 3. The observed genotype proportions for *CYP1A2*1F*, *NAT2 481C>T* and *590G>A* in controls were not significantly different from Hardy-Weinberg expectations ($\chi^2 = 2.3, 3.5$, and 1.0 , respectively).

CYP1A2

Maternal and infant *CYP1A2*1F* risk estimates near 1.0 for the heterozygous C/A genotypes supported grouping heterozygotes with homozygous C/C genotypes. An association was found between maternal *CYP1A2*1F* fast oxidation status and increased risk for NTDs (Table 4) which remained significant after a Bonferroni correction for multiple comparisons ($p=0.01$). No association was found for infant *CYP1A2*1F* fast oxidation status (Table 4). Findings for maternal and infant *CYP1A2*1F* in the case-control logistic regression and case-parent triad log-linear analyses were similar to those presented for the hybrid log-linear design (Supplemental Digital Content 2, Table S1). When stratified by reported race/ethnicity, the association between maternal *CYP1A2*1F* and NTDs was stronger for non-Hispanic white mothers (OR: 1.77, 95% CI: 1.20–2.61) than for other mothers (OR: 1.17, 95% CI: 0.69–2.00). The association for maternal *CYP1A2*1F* was also somewhat stronger when the infant was also a fast oxidizer (OR: 1.90, 95% CI: 0.87–4.17) compared to when the infant was a slow oxidizer (OR: 1.26, 95% CI: 0.64–2.50).

NAT2

Both tests for population structure bias indicated that for *NAT2* acetylator status the case-control component was vulnerable to bias due to population stratification and thus only the case-parent triad log-linear findings were reliable. The family-based log-linear approach, which controlled for this bias by stratifying on parental mating type, demonstrated an association between infant slow *NAT2* acetylation status and NTDs with adjustment for maternal *NAT2* acetylation status (RR: 2.00, 95% CI: 1.10–3.64). This association was also found using the ETDT ($\chi^2 = 13.0, p < 0.01$). There was a suggestion of an inverse association between slow maternal *NAT2* acetylation status and NTDs when compared to rapid acetylators (RR adjusted for infant *NAT2* acetylation status: 0.52, 95% CI: 0.27–1.03). This association was strongest for mothers who reported non-Hispanic white race/ethnicity (RR: 0.34, 95% CI: 0.14–0.81).

Caffeine by gene effect modification

Maternal gene variants—A slightly increased OR for the association between any maternal caffeine intake and NTDs was observed for children of mothers who were fast *CYP1A2*1F* oxidizers (A/A) (OR: 1.61, 95% CI: 0.87–2.98) compared to children of mothers who were slow oxidizers (OR: 1.31, 95% CI: 0.65–2.62). Slow maternal *NAT2* acetylation status was also associated with an increased OR between maternal caffeine intake and NTDs (OR: 1.85, 95% CI: 0.82–4.18) compared to those with rapid *NAT2* acetylators status (OR: 1.31, 95% CI: 0.72–2.40). When stratified across both maternal *CYP1A2*1F* oxidation and *NAT2* acetylation status, the risk estimate for the association between NTDs and caffeine intake in the year prior to pregnancy was most elevated for the offspring of mothers who were both fast oxidizers and slow acetylators (Table 5). For women with planned pregnancies, we similarly observed the highest risk estimate for maternal intake of caffeinated beverages during pregnancy for those who were fast *CYP1A2* oxidizers and slow *NAT2* acetylators (Supplemental Digital Content 3, Table S2).

Infant gene variants—Stratified case-control results revealed significantly greater estimated risk of NTDs associated with any maternal caffeine intake in the year prior to pregnancy for infants with fast oxidation status compared to those with slow oxidation status (Table 5; p for interaction = 0.03). This effect remained after adjusting for maternal *CYP1A2*1F* oxidation status (data not shown). A similar, though non-significant, effect was observed for caffeinated beverage intake during pregnancy for mothers with planned pregnancies (Table S2). The QPL analyses also demonstrated an interaction effect between infant *CYP1A2*1F* oxidation status and maternal caffeine intake as a continuous variable among case families. The interaction estimate for the association between each additional 100 mg caffeine and fast infant *CYP1A2*1F* oxidation status was 2.40 (95% CI: 1.10–5.21) using complete triads. The OR for maternal caffeine intake was slightly greater for rapid *NAT2* acetylator infants than for slow acetylator infants in both the case-control (Table 5) and family-based QPL analyses (data not shown).

Discussion

Study Strengths & Limitations

The NBDPS design provided a unique opportunity to examine the associations between NTDs and maternal caffeine exposure, maternal and infant gene variants, and their interaction effects in geographically and ethnically diverse populations. Further, it allowed for examination of associations through both case-control and family-triad analyses, with the exception of the anencephaly case families that were excluded from the family-based analyses because of missing infant genotypes. Using multiple analytic approaches allowed us to utilize the strengths of each approach. The family-based case-parent triad designs provided inherent protection against population structure bias, and supplementing these analyses with the case-control analyses provided additional statistical power. We were also able to test assumptions of the family-triad methods in controls and test whether population stratification could influence the case-control analyses. Finally, being able to replicate findings across multiple analytic approaches added credibility to consistent results.

A weakness of this study was the limited proportion of families with biologic samples available due to a delayed start for this aspect of the NBDPS and low participation rates. Because differences between families that did and did not contribute biologic samples were similar across case-status, bias of the genetic findings due to differential participation was unlikely. Case and control families who returned biologic samples and were genotyped were also similar to each other with regard to maternal race/ethnicity and most other characteristics, reducing the likelihood of confounding effects by these and perhaps other

unmeasured factors. Still, the low percentage of families on which the genetic findings are based could limit the generalizability of the findings. Success rates for genotyped buccal samples were also not optimal. Failures potentially could have distorted the genotype ratios observed, even though failures did not tend to be allele-specific, and case and control families were likely affected similarly. In addition, genotype, phenotype, and allele frequencies found in this study were similar to those reported in previous studies (Signorello et al., 2001; Lammer et al., 2004; Sachse et al., 1999; Welfare et al., 2000). The number of associations being tested raised the problem of chance findings generated from multiple tests. However, the findings for maternal CYP1A2 oxidation status and caffeine by child CYP1A2 interaction effects remained significant even after conservative Bonferroni corrections.

Given the importance of separating effects by NTD type, associations of gene effects were examined in the case-control analyses separately for anencephaly, spina bifida, and encephalocele, and by multiple versus isolated cases. Results for all types of NTDs combined were presented because results were similar across NTD type and power was limited within the smaller NTD groups. Family-based and gene-interaction analyses limited to isolated spina bifida cases were also conducted, and were again similar to combined results.

Potential limitations regarding the measure of caffeine intake were discussed in detail previously (Schmidt et al., 2009). The validity and reliability of caffeine assessment are high for the Willett food frequency questionnaire on which the food frequency for the current study was based (Munger et al., 1992), and classification of 'any' versus 'none' used for this study are likely more accurate than for the amount. However, information on caffeinated beverage intake was not specific to the time of neural tube closure and misclassification of caffeine intake during this time was possible, and probably at least partially explains the lack of a dose effect described previously (Schmidt et al., 2009). For caffeinated coffee, tea, and soda, we knew whether mothers consumed more, less, the same, or none during pregnancy, but not when they changed their intake or what their intake levels were during pregnancy. Nausea and aversion to caffeine, which can sometimes cause a woman to reduce her intake of caffeinated beverages during pregnancy, typically occurs after the time of gestation relevant to the development of NTDs (Gadsby et al., 1993). In addition, the majority of women recognized their pregnancy after the time relevant to neural tube closure, and thus might have continued their usual intake through the period critical to NTDs, especially if the pregnancy was not planned. On the other hand, women planning a pregnancy might have altered their caffeine consumption in the time prior to becoming pregnant. Finding similar, though attenuated results for these women when we examined whether genetic variants modified the effect of maternal caffeinated beverage intake *during* pregnancy lends some support for the robustness of the finding for effect modification by caffeine metabolism genes on the association between maternal caffeine intake and NTDs.

Gene Effects

The consistent evidence for a positive association between maternal *CYP1A2*1F* fast oxidation status and NTDs across study designs and analytic methods strengthens this finding. Observing a stronger risk estimate for maternal *CYP1A2*1F* in non-Hispanic white women may be a result of underlying genetic or cultural exposure differences. One such difference includes greater exposure to CYP1A2 inducers like caffeine and cigarette smoke for non-Hispanic white mothers.

The *CYP1A2*1F* AA genotype is associated with increased CYP1A2 inducibility (Lammer et al., 2004; Han et al., 2001; MacLeod et al., 1998). Previous studies support harmful effects of fast maternal *CYP1A2*1F* oxidation status on the developing fetus. High CYP1A2

activity might increase the risk of spontaneous abortion Signorello et al., 2001, recurrent pregnancy loss (Sata et al., 2005), and poor fetal growth (Grosso et al., 2006) independently, or by modifying the effect of caffeine. There are several potential pathways for fast maternal CYP1A2 activity to exert an independent effect. CYP1A2 is predominantly responsible for metabolizing numerous medications and environmental chemicals, potentially producing toxic metabolites. CYP1A2 also activates chemical carcinogens including heterocyclic and aromatic amines and nitroaromatic compounds. In addition to these exogenous metabolites, increased CYP1A2 activity can accelerate the metabolism of certain critical endogenous chemicals such as steroid hormones. Finally, *CYP1A2*1F* could be in linkage disequilibrium with other functional polymorphisms that are responsible for the association. The increased risk of NTD-affected pregnancies associated with maternal *CYP1A2*1F* fast oxidation status should be examined further in the context of its other substrates including estrogen, medications, or carcinogenic compounds. The lack of an association between infant *CYP1A2* and NTDs could have resulted from low power of detection, or could reflect the lack of CYP1A2 activity during neural tube formation, as CYP1A2 does not become active until the fourth to fifth post-fetal months (Oesterheld, 1998).

The evidence for the positive association between infant *NAT2* slow acetylation status and NTDs was strengthened by the increased effect with increasing gene dose and its verification in the ETDT analyses. Though levels of *NAT2* biotransformation are low in the neonate relative to the adult, the presence of *NAT2* mRNA in embryonic tissues and xenobiotic biotransformation has been detected as early as the preimplantation stage in mice (Filler and Lew, 1981), and evidence shows that the presence of *NAT2* activity differs by acetylator genotype in pre-term placentas such that *NAT2* activity was expressed only in those possessing the rapid *NAT2* acetylator allele (Smelt et al., 2000). At present, no other studies of infant *NAT2* acetylator status and NTDs are available to compare our results.

The association with maternal *NAT2* acetylation status for white mothers might be a chance finding. However, in one other study of maternal *NAT2* acetylation status and spina bifida, a slightly protective OR was similarly observed (van Rooij et al., 2002).

Caffeine by gene effect modification

The results of this study suggest that maternal caffeine consumption might be associated with increased risk for a NTD-affected pregnancy in women who are *CYP1A2*1F* fast oxidizers and *NAT2* slow acetylators, and whose infants are fast oxidizers. These findings are consistent with harmful effects of caffeine metabolites, rather than caffeine itself. Estimated risk was elevated for fast *CYP1A2* oxidizers who would convert caffeine parent molecules into metabolites quickly. Then, if the mother was a slow acetylator, these metabolites would accumulate, lengthening the time both she, and presumably the fetus, were exposed to these metabolites. Though not studied for other birth defects, the pattern of greater risk estimates for maternal caffeine in *CYP1A2* rapid oxidizers has been demonstrated for other pregnancy outcomes, including spontaneous abortions (Signorello et al., 2001) and recurrent pregnancy loss (Sata et al., 2005). Signorello et al. (2001) found caffeine effects for *CYP1A2* fast, but not slow, oxidizers, and for both rapid and slow *NAT2* acetylators; they did not report results for *NAT2* genotype-*CYP1A2* phenotype combinations.

Infant *CYP1A2*1F* was the only gene variant demonstrating a multiplicative interaction effect with maternal caffeine intake. This interaction effect was comparable between the QPL analysis with total caffeine in continuous form and the none-versus-any stratified case-control analysis, adding strength to the evidence for an effect. Although this interaction effect resulted from stratified data with small cell sizes, its consistency across approaches and the OR of nearly 4.0 for fast oxidizing infants with mothers who consume caffeine

warrant further study. In terms of caffeine metabolism, if caffeine metabolites are hazardous and the infant is a fast oxidizer, caffeine molecules the infant is exposed to through the mother would break down into unsafe metabolites more quickly. One would also predict that effects of increased enzyme activity associated with infant *CYP1A2*1F* would be stronger in the presence of an inducer such as caffeine (Goasduff et al., 1996). Even though CYP1A2 is not likely to be active in fetuses during neural tube development, the fetus can demethylate caffeine *in vitro* Cazeneuve et al., 1994). It is possible that a polymorphism in another gene in linkage disequilibrium with *CYP1A2*1F* is responsible for the interaction effect observed. *CYP1A2* is a gene of modest size (7 kb) that lies within a single large linkage disequilibrium block that extends from the *CYP1A1* gene that is centromeric of *CYP1A2* to the *CSK* gene that is telomeric. Although there is not complete correlation of all SNPs within this region, shared associations are particularly likely between *CYP1A2* and *CYP1A1* genes. These genes are separated by a 23-kb segment containing no other open reading frames (Corchero et al., 2001), and share a common 5-prime flanking region and regulatory elements. *CYP1A1* is expressed in the placenta and CYP1A1 is present during organogenesis. It metabolizes exogenous toxins, including procarcinogens (Oestergeld, 1998) and possibly caffeine in the absence of CYP1A2 function. Expression of *CYP1A1* is also induced by caffeine (Goasduff et al., 1996).

Conclusions

Our findings suggest that genetic variants for phase I and II metabolic enzymes might influence susceptibility to NTDs and should be examined in future studies as independent risk factors and as effect modifiers of other exposures. Because CYP1A2 and NAT2 play a role in the metabolism of several substrates, additional studies of their interaction with environmental, pharmacological, and nutritional exposures could elucidate mechanisms involved in neural tube development. Importantly, this study provided evidence that maternal caffeine intake could be associated with increased risk for NTDs in genetically susceptible subgroups, and that caffeine metabolites might be contributing to this increased risk. Differences in the prevalence of susceptible genotypes between populations might explain some of the inconsistencies between findings from previous studies of caffeine as a risk factor for NTDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Major Biotransformation Pathways of Caffeine Metabolism

Boxes represent enzymes. 84% of caffeine (1,3,7-trimethylxanthine) is metabolized into paraxanthine (1,7-dimethylxanthine) through phase I reactions by cytochrome P4501A2 (CYP1A2). N-acetyltransferase 2 (NAT2) then metabolizes an undetermined intermediate [?] into 5-Acetylamino-6 formylamino-3- methyluracil (AFMU) for excretion in the urine. Figure adapted from Signorello et al. (2001). CYP2A6 = Cytochrome P4502A6; 1,7U = 1,7-Dimethylurate; 1X = 1-Methylxanthine; 1U = 1-Methylurate.

TABLE 1**NAT2 Genotype, Haplotype, and Phenotype Assignment**

Genotype at Nucleotide		Haplotypes	Phenotype Assignment^a
481	590		
CC	GG	CG/CG	Rapid
CT	GG	CG/TG	Rapid
CC	GA	CG/CA	Rapid
CT	GA	(CG/TA) ^b	Slow
		TG/CA	Slow
TT	GG	TG/TG	Slow
CC	AA	CA/CA	Slow
TT	GA	TG/TA	Slow
CT	AA	CA/TA	Slow
TT	AA	TA/TA	Slow

^aClassification methods derived from Cascorbi, et al., 1995⁴

^bCG/TA should be classified as rapid but because this phase occurs only rarely, when the phase was unknown, those heterozygous at both loci were classified as slow

TABLE 2
 Characteristics of Case and Control Mothers by Availability of Biologic Samples and Selection for Genotyping

	NTD Families				Control Families			
	Without Biologic Samples (n = 440)		With Biologic Samples (n = 328)		Without Biologic Samples (n = 2823)		With Biologic Samples (n = 1320)	
	No.	(%) ^a	No.	(%) ^a	No.	(%) ^a	No.	(%) ^a
Age at conception (years)								
12-19	75	(17.0)	47	(14.3)	423	(15.0)	181	(13.7)
20-29	233	(52.9)	171	(52.2)	1359	(48.1)	671	(50.8)
30-34	81	(18.4)	68	(20.7)	712	(25.2)	319	(24.2)
35 or older	51	(11.6)	42	(12.8)	329	(11.7)	149	(11.3)
Race/ethnicity								
Non-Hispanic White	192	(43.6)	207	(63.3)	1617	(57.5)	860	(65.3)
Non-Hispanic Black	59	(13.4)	20	(6.1)	381	(13.5)	117	(8.9)
Hispanic	165	(37.5)	86	(26.3)	652	(23.2)	288	(21.9)
Other	24	(5.5)	14	(4.3)	164	(5.8)	53	(4.0)
Birthplace								
United States	321	(73.8)	270	(82.3)	2239	(80.4)	1107	(83.9)
Other	114	(26.2)	58	(17.7)	546	(19.6)	212	(16.1)
Education								
< High school graduate	110	(25.3)	57	(17.4)	480	(17.3)	199	(15.1)
High school graduate	147	(33.8)	83	(25.3)	695	(25.0)	337	(25.5)
College	178	(40.9)	188	(57.3)	1607	(57.8)	783	(59.4)
Household income								
≤ \$50,000	291	(77.6)	222	(71.2)	1532	(64.3)	802	(64.9)
> \$50,000	84	(22.4)	90	(28.8)	850	(35.7)	434	(35.1)
Pre-pregnancy obesity								
BMI < 30 kg/m ²	307	(74.9)	246	(77.6)	2306	(85.6)	1080	(84.5)
BMI ≥ 30 kg/m ²	103	(25.1)	71	(22.4)	389	(14.4)	198	(15.5)

	NTD Families						Control Families					
	Without Biologic Samples (n = 440)			With Biologic Samples (n = 328)			Without Biologic Samples (n = 2823)			With Biologic Samples (n = 1320)		
	No.	(%) ^a	No.	(%) ^a	No.	(%) ^a	No.	(%) ^a	No.	(%) ^a	No.	(%) ^a
Cigarette smoking ^b												
No	353	(81.1)	274	(83.5)	256	(83.7)	2230	(20.2)	1085	(82.2)	539	(80.6)
Yes	82	(18.9)	54	(16.5)	50	(16.3)	566	(79.8)	235	(17.8)	130	(19.4)
Alcohol intake ^b												
No	301	(69.2)	210	(64.4)	199	(65.5)	1677	(60.2)	820	(62.4)	413	(61.9)
Yes	134	(30.8)	116	(35.6)	105	(34.5)	1107	(39.8)	495	(37.6)	254	(38.1)
Intake of folic acid-containing supplement ^c												
No	223	(52.6)	137	(42.9)	134	(45.1)	1336	(48.6)	598	(46.1)	331	(50.7)
Yes	201	(47.4)	182	(57.1)	163	(54.9)	1412	(51.4)	698	(53.9)	322	(49.3)
Parity												
0	140	(31.9)	131	(39.9)	125	(40.8)	1149	(40.8)	506	(38.3)	263	(39.3)
≥ 1	299	(68.1)	197	(60.1)	181	(59.2)	1665	(59.2)	814	(61.7)	406	(60.7)
Caffeine Intake												
None ^d	56	(12.9)	41	(12.5)	38	(12.4)	424	(15.2)	211	(16.0)	111	(16.6)
Any	378	(87.1)	286	(87.5)	268	(87.6)	2368	(84.8)	1105	(84.0)	556	(83.4)

^aNumbers of cases and controls are less than the total due to missing data. Percentages might not add up to 100 due to rounding

^bDuring the month before through the first three months of pregnancy

^cDuring the three months before through the first month of pregnancy

^dTotal average caffeine intake < 10 mg/day

TABLE 3

Genotypes and Phenotypes for Genotyped Case and Control Family Members

	NTD Families						Control Families					
	Mothers (n = 305)		Fathers (n = 244)		Infants (n = 160)		Mothers (n = 634)		Fathers (n = 510)		Infants (n = 600)	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
<i>CYP1A2 *1F^a</i>	243	(79.7)	190	(77.9)	109	(68.1)	534	(84.2)	416	(81.6)	448	(80.2)
CC	23	(9.5)	17	(8.9)	9	(8.3)	59	(11.0)	36	(8.7)	40	(8.9)
CA	80	(32.9)	75	(39.5)	46	(42.2)	208	(39.0)	171	(41.1)	171	(38.2)
AA	140	(57.6)	98	(57.6)	54	(49.5)	267	(50.0)	209	(50.2)	237	(52.9)
<i>CYP1A2</i> Oxidizer Status	243	(79.7)	190	(77.9)	109	(68.1)	534	(84.2)	416	(81.6)	448	(80.2)
Slow (CC, CA)	103	(42.4)	92	(48.4)	55	(50.5)	267	(50.0)	207	(49.8)	211	(47.1)
Fast (AA)	140	(57.6)	98	(51.6)	54	(49.5)	267	(50.0)	209	(50.2)	237	(52.9)
<i>NAT2 48I</i>	250	(82.0)	188	(77.0)	109	(68.1)	541	(85.3)	409	(80.2)	435	(79.4)
CC	89	(35.6)	68	(36.2)	4	(3.7)	198	(36.6)	138	(33.7)	163	(37.5)
CT	122	(48.8)	95	(50.5)	53	(48.6)	241	(44.5)	193	(47.2)	202	(46.4)
TT	39	(15.6)	25	(13.3)	16	(14.7)	102	(18.9)	78	(19.1)	70	(16.1)
<i>NAT2 590</i>	237	(77.7)	193	(79.1)	110	(68.8)	505	(79.7)	396	(77.6)	432	(76.4)
GG	126	(53.2)	100	(51.8)	59	(53.6)	249	(49.3)	211	(53.3)	209	(48.4)
GA	92	(38.8)	75	(38.9)	45	(40.9)	215	(42.6)	144	(36.4)	181	(41.9)
AA	19	(8.0)	18	(9.3)	6	(5.5)	41	(8.1)	41	(10.4)	42	(9.7)
<i>NAT2</i> Haplotype	218	(71.5)	159	(65.2)	89	(55.6)	459	(72.4)	355	(69.6)	374	(68.1)
CG/CG	26	(11.9)	26	(16.4)	14	(15.7)	57	(12.4)	40	(11.3)	41	(11.0)
CG/TG	60	(27.5)	41	(25.8)	21	(23.6)	98	(21.4)	83	(23.4)	89	(23.8)
CG/CA	38	(17.4)	19	(11.9)	15	(16.9)	76	(16.6)	48	(13.5)	60	(16.0)
CG/TA ^b	NA		NA		0	(0.0)	NA		NA		3	(0.8)
TG/CA	46	(21.1)	42	(26.4)	22	(24.7)	116	(25.3)	82	(23.1)	92	(24.6)
TG/TG	29	(13.3)	17	(10.7)	12	(13.5)	72	(15.7)	60	(16.9)	49	(13.1)
CA/CA	15	(6.9)	8	(5.0)	5	(5.6)	29	(6.3)	31	(8.7)	33	(8.8)
TG/TA	2	(0.9)	5	(3.1)	0	(0.0)	9	(2.0)	5	(1.4)	6	(1.6)
CA/TA	1	(0.5)	1	(0.6)	0	(0.0)	2	(0.4)	4	(1.1)	1	(0.3)

	NTD Families						Control Families					
	Mothers (<i>n</i> = 305)		Fathers (<i>n</i> = 244)		Infants (<i>n</i> = 160)		Mothers (<i>n</i> = 634)		Fathers (<i>n</i> = 510)		Infants (<i>n</i> = 600)	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
TA/TA	1	(0.5)	0	(0.0)	0	(0.0)	0	(0.0)	2	(0.6)	0	(0.0)
NAT2 Acetylator Status	218	(71.5)	159	(65.2)	89	(55.6)	459	(72.4)	355	(69.6)	374	(68.1)
Rapid	124	(56.9)	86	(54.1)	50	(56.2)	231	(50.3)	171	(48.2)	193	(51.6)
Slow	94	(43.1)	73	(45.9)	39	(43.8)	228	(49.7)	184	(51.8)	181	(48.4)

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TABLE 4

Odds Ratios (OR) for Associations between *CYP1A2 *1F* Gene Variants and NTDs Using a Hybrid Log-Linear Method Combining Case-control and Case-Parent Trio Study Designs

	OR ^a	(95% CI)
Maternal <i>CYP1A2 *1F</i>		
CC	1.0	
CA	1.06	(0.62–1.78)
AA	1.55	(0.89–2.70)
Slow Oxidizer (CC, CA)	1.0	
Fast Oxidizer (AA)	1.49	(1.10–2.03)
Infant <i>CYP1A2 *1F</i>		
CC	1.0	
CA	1.06	(0.52–2.13)
AA	0.87	(0.42–1.80)
Slow Oxidizer (CC, CA)	1.0	
Fast Oxidizer (AA)	0.84	(0.57–1.23)

^aMaternal *CYP1A2 *1F* adjusted for infant *CYP1A2 *1F*; Infant *CYP1A2 *1F* adjusted for maternal *CYP1A2 *1F*

TABLE 5

Association between Maternal Caffeine Intake and NTD-affected Pregnancies Stratified by Maternal and Infant *CYP1A2***IF* Oxidizer and *NAT2* Acetylator Status

	Maternal Caffeine Intake	No. of Cases	No. of Controls	OR ^a	(95% CI)
Maternal <i>CYP1A2</i> * <i>IF</i> Oxidizer / <i>NAT2</i> Acetylator Status					
Slow / Rapid	None	8	17	1.0	
	Any	46	91	1.04	(0.41–2.59)
Slow / Slow	None	3	12	1.0	
	Any	27	87	1.21	(0.32–4.63)
Fast / Rapid	None	9	22	1.0	
	Any	49	79	1.49	(0.63–3.52)
Fast / Slow	None	3	16	1.0	
	Any	50	83	3.10	(0.86–11.21)
Infant <i>CYP1A2</i> * <i>IF</i> Oxidizer Status					
Slow (CC, CA)	None	10	32	1.0	
	Any	45	176	0.80	(0.37–1.75)
Fast (AA)	None	3	48	1.0	
	Any	49	188	3.96	(1.18–13.3)
Infant <i>NAT2</i> Acetylator Status					
Rapid	Any	6	35	1.0	
	None	44	158	1.63	(0.64–4.12)
Slow	None	7	29	1.0	
	Any	32	148	0.85	(0.34–2.13)

^a Adjusted for maternal education (<16, 16+ yrs), maternal alcohol consumption one month before through the third month of pregnancy