Mid-Pregnancy Cotinine and Risks of Orofacial Clefts and Neural Tube Defects

GARY M. SHAW, DrPH, SUZAN L. CARMICHAEL, PhD, STEIN EHL. VOLSET, DrPH, WEI YANG, MD, RICHARD H. FINNELL, PhD, HENK BLOM, PhD, ØIVIND MÖTTUN, PhD, and PER M. UELAND, MD

Objective  Past studies of cigarette smoking as a contributor to orofacial clefts and neural tube defects (NTDs) used self-reports of smoke exposures. We have correlated measurements of cotinine (a nicotine metabolite) in mid-pregnancy sera with clefts and NTDs.

Study design  From a repository of >180 000 mid-pregnancy serum specimens collected in California from 2003 to 2005 and linked to delivery outcome information, we identified 89 orofacial cleft-associated pregnancies, 80 NTD-affected pregnancies, and randomly selected 409 pregnancy specimens that corresponded to infants without malformations as control subjects. Cotinine was measured by liquid chromatography-mass spectrometry. No smoke exposure was defined as cotinine values <2 ng/mL, and any exposure was defined as ≥2 ng/mL.

Results  We observed odds ratios of 2.1 (95% CI, 1.0-4.4) for clefts and 0.4 (95% CI, 0.1-1.7) for NTDs associated with exposure. After adjusting for race/ethnicity, age, and serum folate levels, odds ratios were 2.4 (95% CI, 1.1-5.3) and 0.6 (95% CI, 0.1-2.5). We explored 2 cotinine levels, 2 to 10 ng/mL and >10 ng/mL for clefts (data were too sparse for NTDs). Odds ratios for these levels were 3.3 (95% CI, 0.9-11.9) and 1.7 (95% CI, 0.7-4.2), respectively.

Conclusion  Smoking exposures, as measured with cotinine levels during mid-pregnancy, were associated with increased risks of clefts and possibly reduced risks of NTDs. (J Pediatr 2009;154:17-9)

Numerous studies have explored potential teratogenic effects of periconceptional cigarette smoking. The evidence associated specifically with orofacial clefts, and smoking tends to support modest effects of approximately 1.5-fold for any versus no smoking in early pregnancy and evidence to indicate an exposure-response effect. The evidence associated with NTDs is far less clear. Findings of reduced risks, elevated risks, and no risks have appeared in the literature.

Past studies of cigarette smoking as a contributor to etiologies of orofacial clefts and neural tube defects (NTDs) have involved self-reports of active smoking and secondhand smoke exposures. In this prospective study, we attempt to improve on the challenges of collecting such exposures gathered study retrospectively by measuring cotinine (a nicotine metabolite) in mid-pregnancy sera from a large population in California.

METHODS

This study included data from a repository of mid-pregnancy sera. Specimens were collected from approximately 70% of women during the 15th to 18th week of pregnancy in selected regions of California (Orange, San Diego, and Central Valley counties) as part of the California Expanded AFP (alpha fetoprotein) program that screens for NTDs and cytogenetic abnormalities. The collection and processing of specimens was as follows: 1) samples were taken at draw stations with BD Vacutainer 3.5-mL serum separator tubes with no anticoagulants or preservatives and centrifuged; 2) samples were received by designated clinical laboratories from draw stations at room temperature, on average 3.0 days after draw; 3) AFP assays were run on samples, usually on the day received; 4) samples were refrigerated as long

<table>
<thead>
<tr>
<th>AFP</th>
<th>Alpha fetoprotein</th>
<th>OR</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTD</td>
<td>Neural tube defect</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See editorial, p 4 and related article, p 10

From the March of Dimes California Research Division, Children’s Hospital Oakland Research Institute, Oakland, CA (G.S., S.C., W.Y.); Medical Birth Registry of Norway, Norwegian Institute of Public Health, Bergen, Norway (S.V.); Institute of Biosciences and Technology, The Texas A&M University System Health Science Center, Houston, TX (R.F.); Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands (H.B.); Bevital AS, Armauer Hansens Hus, Bergen, Norway (Ø.M., P.U.); and Section for Pharmacology, Institute of Medicine, University of Bergen and Haukeland University Hospital, Armauer Hansens Hus, Bergen, Norway (P.U.).

Supported by funds from the Centers for Disease Control and Prevention, Center of Excellence Award U50/CCU913241 and by NIH/NNINDS RO1 NS050249. The authors declare no potential conflicts of interest.

Submitted for publication Apr 21, 2008; last revision received Jun 30, 2008; accepted Aug 5, 2008.

Reprint requests: Dr Gary Shaw, March of Dimes California Research Division, Children’s Hospital Oakland Research Institute, 5700 Martin Luther King Jr Way, Oakland, CA 94609. E-mail: gshaw@marchofdimes.com.

0022-3476/$ - see front matter
Copyright © 2009 Mosby Inc. All rights reserved.
10.1016/j.jpeds.2008.08.006
as 7 days when further testing was necessary, 5) samples were sent on cold packs via overnight mail to the serum storage bank; and 6) samples were aliquoted, labeled with barcodes, and frozen at $-70^\circ$C within an average of 3.5 days of receipt at the serum repository.

Each woman’s serum specimen was linked with delivery outcome information to determine whether her fetus had an orofacial cleft, NTD, or another structural malformation as defined elsewhere or was born without malformation. The study included deliveries that were live-born, stillborn (fetal deaths at >20 weeks gestation), or electively terminated on the basis of prenatal diagnoses. Among the >180 000 pregnancy specimens collected for testing in the period 2003 to 2005, we identified 89 orofacial cleft-associated pregnancies and 80 NTD-affected pregnancies. All the 89 cases with clefts had cleft lip with or without cleft palate. Of the 80 NTD cases, 31 had spina bifida, and 49 had anencephaly. To serve as a referent group (control subjects), we randomly selected pregnancy specimens among the total number of available specimens at a ratio of approximately 5 control subjects/case. These potential control subjects were further reviewed for presence of a structural malformation in the delivered infant. Infants with malformations or infants identified with insufficient specimen to analyze were excluded. This sampling scheme resulted in 409 control specimens. Thus, this was analyzed as a nested case-control study. The collection of samples and the study protocol were approved by the California Health and Welfare Agency Committee for the Protection of Human Subjects.

Serum specimens for the 578 cases and control subjects were sent on cold packs to the University of Bergen for analyte measurements. To assess cigarette smoking exposures, the metabolite cotinine was measured with LC-MS/MS. No smoke exposure was defined as values <2 ng/mL, and any smoke exposure was defined as $\geq 2$ ng/mL. Similar cutoff points have been used in earlier studies of reproductive outcomes. That is, cotinine levels <2 ng/mL indicate no exposure, whereas levels of 2 to 10 ng/mL are indicative of environmental tobacco smoke exposure, and levels $>10$ ng/mL are indicative of active smoking. Serum folate was also measured. Details about the laboratory assays can be found elsewhere. All analyte measurements were done with researchers blind to case or control status.

We estimated risks with odds ratios (ORs) and 95% CIs (SAS software version 9.1). Models were constructed to assess effects associated with the 2 defined categories of cotinine. Covariates in some analyses included maternal race/ethnicity (Hispanic; white, non-Hispanic; Asian; black; other), age (<25 years; 25-29 years; 30-34 years; and $\geq 34$ years), and a 2.4-fold risk when adjusted for race/ethnicity, age, and serum folate levels. Although data were limited, we attempted to estimate the effects associated with higher cotinine levels. We explored 2 cotinine levels, 2 to 10 ng/mL and 2 to 10 ng/mL, for cleft lip with/without cleft palate. The ORs for these levels were 3.3 (95% CI, 0.9-11.9) and 1.7 (95% CI, 0.7-4.2), respectively. We also explored whether observed effects differed by the phenotypic distinction of unilateral versus bilateral cleft lip. Of the 89 cases, 74% were unilateral, 26% were bilateral, and 10% were of unknown laterality.

## RESULTS

Table I provides background characteristics of the study population showing an expected greater frequency of Hispanic subjects in the NTD case group. NTD and cleft cases tended to be slightly more represented in the 25- to 29-year age group and slightly less represented in the 30- to 34-year age group.

Table II shows a 2.1-fold increased risk for cleft lip with/without cleft palate associated with smoke exposures, and a 2.4-fold risk when adjusted for race/ethnicity, age, and serum folate levels. Although data were limited, we attempted to estimate the effects associated with higher cotinine levels. We explored 2 cotinine levels, 2 to 10 ng/mL and $>10$ ng/mL, for cleft lip with/without cleft palate. The ORs for these levels were 3.3 (95% CI, 0.9-11.9) and 1.7 (95% CI, 0.7-4.2), respectively. We also explored whether observed effects differed by the phenotypic distinction of unilateral versus bilateral cleft lip. Of the 89 cases, 74% were unilateral, 26% were bilateral, and 10% were of unknown laterality.

---

**Table I. Characteristics of cleft lip/palate-affected, neural tube defect-affected, and unaffected (control subjects without malformations) deliveries, California 2003 to 2005**

<table>
<thead>
<tr>
<th>Race/Ethnicity</th>
<th>Cleft lip/palate cases (n = 89)</th>
<th>NTD cases (n = 80)</th>
<th>Controls (n = 409)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %*</td>
<td>n %*</td>
<td>n %*</td>
</tr>
<tr>
<td>Hispanic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White non-Hispanic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\geq 34$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Table II. Tobacco smoke exposures as estimated with cotinine levels among cleft lip/palate-affected, neural tube defect-affected, and unaffected (control subjects without malformations) deliveries, California 2003 to 2005**

<table>
<thead>
<tr>
<th>Smoke exposure*</th>
<th>Cleft lip/palate cases (n = 89)</th>
<th>NTD cases (n = 80)</th>
<th>Controls (n = 409)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
<td>OR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95% CI</td>
</tr>
<tr>
<td>No</td>
<td>78</td>
<td>77</td>
<td>383</td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>Adjusted OR†</td>
<td></td>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>1.1-5.3</td>
<td>0.1-2.5</td>
</tr>
</tbody>
</table>

*Ref, Reference.
†Adjusted for maternal age, race/ethnicity, and serum folate levels.

---

*Percentages may not equal 100 because of missing data or rounding.*
18% bilateral, and 8% unknown. The ORs associated with smoke exposures were 1.7 (95% CI, 0.7-4.2) for unilateral clefts and 3.4 (95% CI, 0.9-12.7) for bilateral clefts.

Table II shows an OR of 0.4 for NTDs associated with smoke exposures. This estimated effect was imprecise because of a small number of subjects exposed. This small sample size precluded further detailed analyses.

DISCUSSION

We observed that smoking exposures, as defined with measured cotinine levels during mid-pregnancy, were associated with increased risks of cleft lip with or without cleft palate and possibly reduced risks of NTDs. Adjustment for potential effects of race/ethnicity, age, and serum folate levels did not yield substantially different estimates. Although the number of cases considered here was relatively modest, it investigated the unbiased measure of smoking exposure (cotinine) on risks for NTDs and orofacial clefts. Indeed, a recent paper that quantified the reliability of smoking reporting in studies of orofacial clefts indicated that, to be informative, future studies will need to reduce the effects of exposure misclassification from self-reported information on smoking.13

Observing a positive relation between cotinine and risk of cleft lip with/without cleft palate extends the relatively consistent findings in the literature that periconceptional smoking increases clefting risk.1,14,15 We observed limited evidence to suggest that the effect may be higher for bilateral clefts, an observation made earlier.14 Several hypothesized mechanisms have been put forward to explain associations observed between cigarette smoke exposures and orofacial clefts. These include hypoxia from carbon monoxide,16 teratogenic effects of nicotine, cadmium, or myriad amines,17-19 and alterations to folate metabolism.20,21 However, estimated effects were only minimally influenced by adjusting for serum folate levels in these data. The reduced risk of NTDs with measurable cotinine levels adds to a literature that conveys mixed findings. Studies have shown reduced risks,2,22 no risks,7,8 or increased risks4-6 associated with maternal smoking during the periconceptional period and NTDs. Our finding is quite imprecise, (ie, the confidence intervals were consistent with an interpretation of both increased risk and protective effects).

This study investigated cotinine levels measured during pregnancies that subsequently resulted in the delivery of infants/fetuses with cleft lip with/without cleft palate and NTDs. These data extend our inferential ability because of its prospective design and the sensitivity and specificity of cotinine as an index of tobacco smoke exposure. Cotinine measurements overcome the criticisms of biased exposure reporting.23 This study, however, is not without its limitations. First, although the study involved nearly 200 000 women, the number of affected pregnancies was relatively small, resulting in a fairly imprecise estimation of effects. Second, the collection of women's specimens was on average 12 weeks after closure of the neural tube and 8 weeks after the closure of the lip and palate processes. The half-life of cotinine is short, 8 to 16 hours, in pregnancy.24 Although it seems likely that bias caused by this single point-in-time measurement would tend to result in an underestimate of effects, we cannot be certain. Third, the results for cleft lip with/without cleft palate did not indicate a dose-response relationship. Our ability to observe such a relationship was hampered by the sample size. Thus, these observations should be replicated in other prospective designs in pregnant women who may become available in places such as Norway and Denmark.

REFERENCES