Title

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Key Words

Cotinine; hydroxycotinine; high-resolution metabolomics; maternal pregnancy smoking; retinoblastoma; risk factors; childhood cancer epidemiology

Summary Statement

This study investigated associations between maternal smoking and retinoblastoma in offspring, using biomarkers of tobacco smoking in neonatal dried blood spots. The results indicate maternal smoking during pregnancy may be a risk factor for retinoblastoma, particularly among unilateral cases.

Abstract

Purpose: Prior studies examining the risk of retinoblastoma with maternal smoking were inconclusive, likely due in part to the reliance on self-reported maternal smoking. This study uses biomarkers of tobacco smoking in neonatal dried blood spots to investigate associations between maternal smoking and retinoblastoma in offspring.

Methods: We randomly selected 498 retinoblastoma cases and 895 controls born between 1983-2011 from a population-based case-control study in California. Maternal pregnancy-related smoking was measured using the following 3 metrics: provider or self-reported smoking during pregnancy, cotinine, and hydroxycotinine in neonatal blood. We employed multivariable logistic regression to estimate the effects of maternal tobacco smoking on retinoblastoma.

Results: Using all metrics (biomarkers or self-report), maternal smoking late in pregnancy or early postpartum was related to retinoblastoma [all types; Odds Ratio (OR)=1.44, 95% Confidence Interval (CI) 1.00, 2.09]. Relying on cotinine or hydroxycotinine to ascertain smoking, maternal smoking was related to unilateral retinoblastoma (OR=1.66, 95% CI 1.08, 2.57).

Conclusion: The results indicate that maternal smoking during pregnancy may be a risk factor for retinoblastoma, particularly among unilateral cases.

Introduction

Retinoblastoma is the most common eye cancer in childhood. Incidence peaks in infancy and declines thereafter, with nearly all cases diagnosed by age 6. Retinoblastoma results from the loss or mutation of the *RB1* gene. Familial retinoblastoma (<10% of cases) occurs due to the inheritance of a mutated gene from one parent, while sporadic disease that later becomes inheritable to children (sporadic heritable retinoblastoma, 30% of cases) results from a *de novo* mutation in parental gametes (most often of paternal origin [1]); in both of these instances the child most often develops bilateral, multifocal disease. The remaining 60% of cases result from somatic mutations in the same retinal cell which occur during the pregnancy or very early life, resulting in unilateral disease. Established causes of retinoblastoma are limited to ionizing radiation, while suspected causes include older paternal age, sunlight exposure, in-vitro fertilization, human papillomavirus, Hispanic ethnicity, and traffic related air pollution exposure.[1-4]

A limited literature suggests that maternal smoking in pregnancy, when smoking status was collected via midwife report or medical record review, increases risk of retinoblastoma (ORs~ 1.3-3.0 for any type of retinoblastoma).[5-8] Studies that relied on self-reported smoking, as ascertained via parental interview, mostly found weaker associations (ORs~ 0.6 – 3.3).[3, 4, 9, 10] Yet, there are concerns that tobacco use in research studies and in medical records is underreported by pregnant women,[11] though the measurement error is expected to be non-differential. To our knowledge, there have been no published studies relying on biomarkers of tobacco to ascertain maternal smoking status in late pregnancy and postpartum. In addition, most studies did not stratify by retinoblastoma subtype, despite the fact that the subtypes are expected to have varying etiologies.

In adults, 70-80% of nicotine is metabolized into cotinine and half of cotinine is metabolized into hydroxycotinine.[12] Nicotine and other tobacco smoke constituents cross the placenta and have been measured in the fetus and are also measurable in breast milk. Prior studies have suggested that cotinine in neonatal blood spots represents maternal active smoking, because passive smoking results in undetectable cotinine levels, e.g. detecting passive smoking would only be feasible with a much larger volume of blood

than is available in a dried blood spot. While hydroxycotinine is derived from smoking, it has other sources (e.g., dietary sources such as tomatoes and eggplant). The half-life of cotinine and hydroxycotinine are slightly longer in newborn's blood compared with that in maternal serum.[13] A study of 13 newborns at San Francisco General Hospital reported an elimination half-life for cotinine and hydroxycotinine of 16.3 hours and 18.8 hours in umbilical cord blood, respectively.[14] Neonatal dried blood spot cotinine has a high sensitivity (92.3%) and specificity (99.7%) of ascertaining maternal smoking close to the time of delivery with a threshold of 3.13-6 ng/mL suggested to distinguish smokers from nonsmokers (however biomarkers will only be present if the mother breastfed).[13] Using these biomarkers, the purpose of this paper is to examine associations between maternal late pregnancy smoking and retinoblastoma in offspring.

Methods

As previously described, we developed a population-based case-control study of childhood cancers in California (births 1983-2011) by linking cases from the California Cancer Registry to California birth certificates; controls were frequency-matched on birth year and selected at random from birth records.[2] From this parent study, we randomly selected 501 retinoblastoma cases and 899 controls for metabolomics analyses.

The California Genetic Disease Screening Program collects blood samples from newborns by a heel-stick between 12 hours and 48 hours after a child's birth. Samples are stored on filter paper cards, air-dried at room temperature, and within 24 hours of collection, mailed to state-contracted laboratories for analysis. Over 99% of California infants are screened. After routine testing, remaining specimens are packed and stored at −20°C. Prior to screening, parents were provided with a privacy notification that described the possible research use of these specimens with the option to opt out of the research.

We conducted metabolomic profiling of blood spots according to methods previously developed.[15] Samples were extracted with 2:1 acetonitrile in water (containing a mixture of stable isotopic

internal standards) for 12 hours at 0-4 °C in the dark. Samples were then centrifuged to remove any particulate matter and were analyzed in triplicate using liquid chromatography interfaced to an ultra high-resolution mass spectrometer (Thermo Scientific Q-Exactive HF). Dried blood spots samples along with the NIST 1950 and QSTD (internal quality control) samples were analyzed in batches of 40 study samples using an acetonitrile gradient and two technical columns that include hydrophilic interaction liquid chromatography (HILIC) with positive electrospray ionization (ESI) and C18 hydrophobic reversed-phase chromatography with negative ESI, to enhance the coverage of metabolic feature detection. Raw data were extracted using apLCMS with modifications by xMSanalyzer, and batch corrected using ComBat. We extracted cotinine (*m/z* = 177.1023) and hydroxycotinine *(m/z* = 193.0973) [16] from the feature table generated by xMSanalyzer. After excluding 7 samples considered outliers during feature extraction, we included samples of 498 retinoblastoma cases and 895 controls in the current study.

Contemporaneous surveys of California women report that maternal pregnancy smoking prevalence ranged from approximately 50% in 1980 [17] to 14% from 1995-2002 [18], and 5% from 2008-2018.[19] Hydroxycotinine was present in 20% of samples but given that it has other sources than smoking, we followed the prevalence reported in the studies above and defined mothers as smokers if the newborn blood had hydroxycotinine intensities detected at the top 14%. Overall, this yielded an average prevalence of smoking of 17% across the study period from any of the smoking indicators.

We created 3 indicator variables based on 4 metrics for maternal late pregnancy smoking exposure to evaluate the association of interest and we also compared the estimated effect size. The first two were cotinine and hydroxycotinine, and the third was tobacco smoking as recorded on the birth certificate derived from 2 metrics of separate time periods. Smoking was recorded as such: from 1989 to 2005, the question "Were there pregnancy complications due to tobacco use during pregnancy?" was collected from medical providers; starting in 2007, the number of cigarettes per day in each trimester was collected from parents. We combined these 2 metrics to create a self or provider-reported pregnancy smoking variable as our final maternal smoking indicator if mothers had complications due to tobacco use

or reported to ever used cigarettes during pregnancy. As smoking is often underreported on the birth certificate,[11] we additionally examined agreement among these different smoking measures.

We examined the association between maternal smoking and retinoblastoma using unconditional logistic regression. Odds ratios (OR) and 95% confidence intervals were ascertained, with adjustment of birth year in the crude model (the matching factor). Selection of variables for adjustment was based on associations previously observed in our sample [2] and the literature.[1, 3] Final adjusted models included additional adjustment for maternal age, maternal race (White non-Hispanic, Hispanic any race, Black, Asian/Pacific Islander, other), maternal education, paternal age, and infection with any sexually transmitted disease (STD) in pregnancy. Adjustment for maternal ethnicity (Hispanic/non-Hispanic) and mothers' birth place (US/Mexico/other foreign born) did not change beta values by more than 10% and were left out of final models. We additionally stratified by laterality as a proxy for sporadic heritable vs. nonheritable disease.

Results

The prevalence of maternal smoking in pregnancy among study participants measured by 4 metrics declined over the study period, from approximately 40% in the 1980s down to less than 5% in the 2000s (Figure 1). In total, we had 895 controls and 498 retinoblastoma cases, including 280 unilateral cases and 218 bilateral cases (Table 1). Cases were more often male, especially for bilateral disease. Maternal race/ethnicity was weakly associated with retinoblastoma, with children of Black mothers having a slightly higher risk of developing the disease. Case mothers tended to be more often multipara and cases had older mothers (>35 years) and fathers (>40 years). During the years when self- or provider-reported smoking was available on the birth certificate, pregnancy complications due to tobacco use during pregnancy were reported for 5 case mothers and 15 control mothers, while 7 case mothers and less than 5 control mothers reported smoking cigarettes during pregnancy.

Out of 1,393 mothers, 240 smoked in pregnancy, as measured by any of the smoking indicators: 29 (2.1%) according to self or provider reported smoking during pregnancy, 102 (7.3%) according to cotinine, and 195 (14.0%) according to hydroxycotinine. Detected intensities of cotinine and hydroxycotinine were highly correlated (Spearman's rho correlation coefficient = 0.77).

The prevalence of birth certificate-recorded smoking was much lower than when smoking was measured from blood spot biomarkers (Table 2). Among women who had pregnancy complications due to tobacco use reported on birth records, 65% had cotinine detected in their newborn blood and 55% hydroxycotinine. Cotinine and hydroxycotinine were present in 78% and 56%, respectively, of newborns' blood spots of children born to mothers who reported smoking during pregnancy.

We observed positive associations for retinoblastoma (all types) with maternal smoking using all smoking measures, but only the estimates for hydroxycotinine or a combination of all smoking markers had 95% CIs that excluded the null value (Table 3). The slightly smaller effect estimates for retinoblastoma seen with cotinine and self-reported smoking had wide confidence intervals that included the null value. Similar patterns were seen for unilateral cases. In contrast, none of the biomarker derived smoking measures were associated with the occurrence of bilateral cases, yet self or provider-reported smoking yielded a high odds ratio with wide 95% CIs that included the null value.

Discussion

This is the first investigation of maternal pregnancy smoking and retinoblastoma to use biomarker-derived measures of maternal smoking status. Our study observed positive associations when smoking was measured by the biologic markers, especially hydroxycotinine, or a combination of biomarkers, particularly for unilateral cases. Due to these different cancer development processes, the critical exposure windows for bilateral and unilateral cases are different. Unilateral cases are caused by two *RB1* gene mutations occurring in somatic cells at some point after conception, which makes pregnancy or the child's early life exposures more critical for unilateral cases.[1] Previous studies reported

increased risk of both bilateral and unilateral cases among mothers who smoked before or during pregnancy,[10] or had household tobacco exposure during pregnancy.[3] Yet, an earlier study by our group using a part of the same sample as the current study – limiting to the time period when self-reported smoking was collected on the birth certificate (2007-2011) – observed the strongest association with bilateral retinoblastoma only, consistent with the current results.[8]

Biological plausibility for the observations may involve several mechanisms. Nicotine metabolites can easily cross the placenta and are detected in the fetus. Newborns of smoking mothers had elevated frequencies of hypoxanthine-guanine phosphoribosyl transferase mutants, translocations, and DNA strand breaks.[20] In addition, nicotine and other tobacco smoke constituents are measurable in breast milk. Pregnancy and postpartum smoking is known to affect the pro-oxidant-antioxidant balance of breast milk. Human breast milk possesses antioxidant properties and can deactivate and scavenge reactive oxygen species, which play a role in the processes of carcinogenesis and chronic inflammation that in turn are implicated in diseases connected with retinopathy.[21] Although we did not observed increased risks for bilateral retinoblastoma cases with exposure to maternal smoking, cigarette smoke contains many well-established carcinogens and has been linked to an increased frequency of chromosomal abnormalities and aneuploidy in sperm.[22] Smoking causes increased free radical production which results in sperm DNA damage, possibly contributing to the etiology of sporadic heritable retinoblastoma.[22] This suggests that compared with maternal pregnancy exposure, paternal pre-conception smoking exposure may play a more important role of developing bilateral retinoblastoma.

The predominant source of serum cotinine is tobacco use. Based on this measure, we observed a higher smoking rate than seen with self- or provider reports. When using the variables available on the birth certificate, only 20 (2.1%) women were identified as smokers due to pregnancy complications from tobacco use (1983 to 2005), and 9 (4.3%) women reported smoking cigarettes during pregnancy (2007+). Not surprisingly, identifying smokers solely by the presence of related pregnancy complications leads to substantial underreporting of smoking, both compared to offspring measures of serum cotinine - as done

here - and to surveys of pregnant California women.[17] Although results were based on a small number of smoking mothers, self-reported smoking on the birth certificate captured only 54% and 56% of all smokers identified by cotinine and hydroxycotinine, respectively.

Underreporting on birth certificates is expected for several data elements, but smoking may be especially vulnerable to underreporting [11] due to the stigma of smoking during pregnancy, which may lead women to not disclose their smoking behavior to health care providers. This is reflected in a study of California, Michigan, New York, and Washington mother-child pairs that reported while 12% of the newborn dried blood spots contained cotinine $\geq$ 9.0 ng/g, indicative of active smoking of the mother, 41% of these mothers reported no smoking on the birth certificate.[23]

Nevertheless, the pregnancy smoking prevalence derived from blood spot biomarkers is still an imperfect measure. Neonatal dried blood spots are typically collected from the infant during the postpartum hospital stay, or by midwives for out-of-hospital births, though the latter accounts for less than 1% of California births.[24] Only if the mother had smoked recently and attempted breastfeeding would we have captured smoking with these biomarkers. In the US, 60%-75% of women initiated breastfeeding in the 1980s to 2010s,[25, 26] and in California, 67%-86% of mothers attempted any breastfeeding during 2004 to 2008, while 90% initiated breastfeeding in 2010.[26, 27] One US study found that mothers who smoked had half the breastfeeding rate of non-smoking mothers;[28] the impact on our results would be expected to be nondifferential and bias to the null. Also only about 10% of nicotine and its metabolites (cotinine, hydroxycotinine, and cotinine *N*-oxide) pass through the epithelial cells of mammary glands into breast milk.[29]

Due to the limitations of untargeted metabolomics, the absolute concentration of tobacco metabolites could not be determined as a measure for active smoking. Instead, we utilized cotinine and hydroxycotinine and selected a plausible cutoff that was in line with the pregnancy smoking prevalence reported in other studies during our study period. In a validation study, we observed stable intensities of cotinine, hydroxycotinine, and other metabolites despite the long time period of storage.[30] A limitation is

that we do not have information on whether a retinoblastoma case is familial and having familial cases would weaken our association between retinoblastoma and maternal smoking. However, we expect the impact to be minor because familial cases account for less than 10% of all retinoblastoma. Our stratified analysis by laterality for all exposure metrics further confirmed this assumption, where we considered bilateral cases as a proxy of sporadic heritable cases.

In summary, an elevated risk of retinoblastoma was observed with maternal smoking in pregnancy, especially for unilateral retinoblastoma. Even though capturing maternal smoking with biomarkers in neonatal dried blood spots was dependent on breastfeeding, our study suggests that they have great utility in identifying late pregnancy smokers compared to self- or provider-reported smoking on birth certificates that may be affected by under-reporting.

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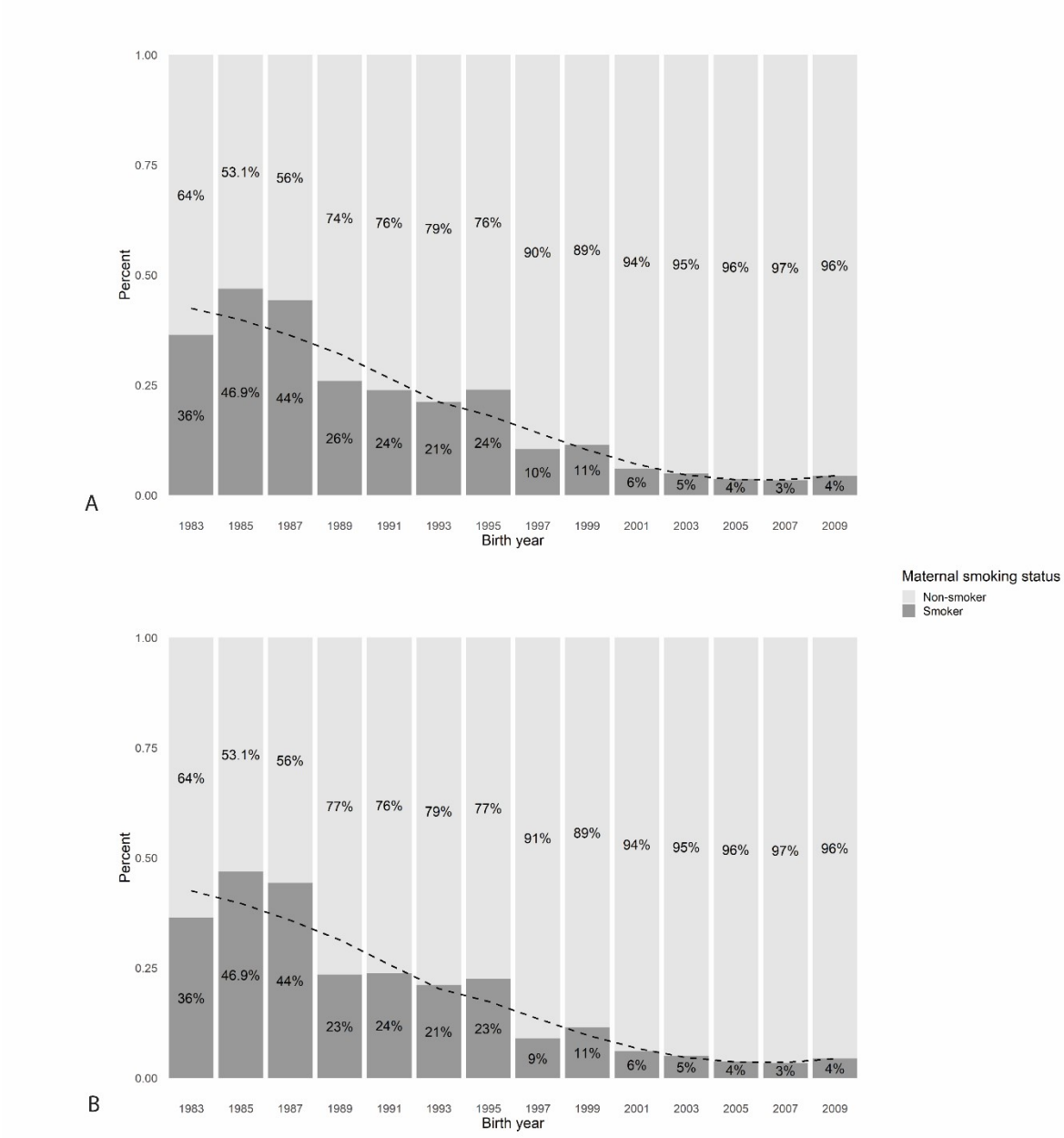

Figure 1. Peripartum smoking rate by year among California mothers, as measured by 3 indicators. (A) Smoking prevalence (self-reported, cotinine, or hydroxycotinine), 1983-2011. (B) Biomarker-derived smoking prevalence (cotinine or hydroxycotinine), 1983-2011. If the mother did not breastfeed, smoking would not be ascertained in the biomarker-derived prevalence.

Table 1. Characteristics of the study population, 1983-2011 (N=1393)

	Controls (N=895)	All cases (N=498)	Unilateral cases (N=280)	Bilateral cases (N=218)
	N (%)	N (%)	N (%)	N (%)
Child's sex				
male	439 (49.1)	267 (53.6)	143 (51.1)	124 (56.9)
female	456 (50.9)	231 (46.4)	137 (48.9)	94 (43.1)
Mother's age (years)				
19 or less	100 (11.2)	48 (9.6)	25 (8.9)	23 (10.6)
20-24	224 (25.0)	111 (22.3)	66 (23.6)	45 (20.6)
25-29	240 (26.8)	155 (31.1)	94 (33.6)	61 (28.0)
30-34	214 (23.9)	110 (22.1)	57 (20.4)	53 (24.3)
35 and older	117 (13.1)	74 (14.9)	38 (13.6)	36 (16.5)
Maternal Race/Ethnicity				
White Non- Hispanic	303 (33.9)	152 (30.5)	83 (29.6)	69 (31.7)
Hispanic US born	140 (15.6)	84 (16.9)	51 (18.2)	33 (15.1)
Hispanic Foreign born	282 (31.5)	152 (30.5)	80 (28.6)	72 (33.0)
Black	53 (5.9)	37 (7.4)	23 (8.2)	14 (6.4)
Asian/Pacific Islander	93 (10.4)	59 (11.8)	36 (12.9)	23 (10.6)
Other	24 (2.7)	14 (2.8)	7 (2.5)	7 (3.2)
Maternal education (years)				
8 or less years	92 (11.8)	50 (11.0)	27 (9.6)	23 (10.6)
9-11 years	160 (20.6)	89 (19.6)	41 (14.6)	48 (22.0)
12 years	234 (30.1)	132 (29.0)	80 (28.6)	52 (23.9)
13 to 15 years	156 (20.1)	91 (20.0)	56 (20.0)	35 (16.1)
16 more years	135 (17.4)	93 (20.4)	47 (16.8)	46 (21.1)
Paternal age (years)				
19 or less	32 (3.8)	20 (4.2)	10 (3.6)	10 (4.6)
20-24	175 (20.8)	76 (16.1)	48 (17.1)	28 (12.8)
25-29	214 (25.4)	108 (22.8)	68 (24.3)	40 (18.3)
30-34	198 (23.5)	118 (24.9)	62 (22.1)	56 (25.7)
35-39	135 (16.0)	87 (18.4)	51 (18.2)	36 (16.5)
40+	89 (10.6)	64 (13.5)	28 (10.0)	36 (16.5)
Paternal education (years)				
8 or less years	108 (14.8)	58 (13.5)	28 (10.0)	30 (13.8)
9-11 years	107 (14.7)	67 (15.6)	38 (13.6)	29 (13.3)
12 years	227 (31.1)	132 (30.7)	78 (27.9)	54 (24.8)
13 to 15 years	146 (20.0)	71 (16.5)	40 (14.3)	31 (14.2)
16 more years	142 (19.5)	102 (23.7)	53 (18.9)	49 (22.5)
Parity				
0	371 (41.5)	192 (38.6)	101 (36.1)	91 (41.7)
1	267 (29.9)	142 (28.5)	78 (27.9)	64 (29.4)
2	147 (16.4)	107 (21.5)	64 (22.9)	43 (19.7)
3	70 (7.8)	37 (7.4)	27 (9.6)	10 (4.6)
4 or more	39 (4.4)	20 (4.0)	10 (3.6)	10 (4.6)
Maternal smoking, as listed on the birth certificate				
Pregnancy complications due to tobacco use during pregnancy, 1989-2005				
Yes	15 (2.4)	5 (1.4)	<5	<5
No	603 (97.6)	350 (98.6)	194 (98.5)	156 (98.7)
Reported number of cigarettes per day (3 months before or during pregnancy), 2007+				
Yes	<5	7 (8.1)	<5	6 (15.4)
No	123 (98.4)	79 (91.9)	46 (97.9)	33 (84.6)
Either of the above				
Yes	17 (2.3)	12 (2.7)	<5	8 (4.1)

No	726 (97.7)	429 (97.3)	240 (98.4)	189 (95.9)

Table 2. Percent agreement among the smoking metrics *

	Pregnancy complications due to tobacco use during pregnancy, 1989-2005	Self-reported smoking, 2007+	Cotinine present	Hydroxycotinine	Total (%)
Pregnancy complications due to tobacco use during pregnancy, 1989-2005	100%	---	65%	55%	20 (2.1%)
Self-reported smoking, 2007+	---	100%	78%	56%	9 (4.3%)
Cotinine			100%	63%	102 (7.3%)
Hydroxycotinine				100%	195 (14.0%)
Total (smoking rate indicated by this measure)	20 (2.1%)	9 (4.3%)	102 (7.3%)	195 (14.0%)	240

* Metrics listed in each row are the reference when calculating the percentage of agreement

Table 3. Risk of retinoblastoma in relation to maternal smoking

		Retinoblastoma (all)			
		All Births (N =1393)			
		Total N	Smoker N (%)	Crude OR (95% CI) [1]	Adjusted OR (95% CI) [2]
Cotinine	**Cases**	498	40 (8.0)	1.25 (0.83, 1.90)	1.32 (0.78, 2.23)
	Controls	895	62 (6.9)	Reference	Reference
Hydroxycotinine	**Cases**	498	76 (15.3)	1.37 (0.99, 1.90)	1.58 (1.05, 2.37)
	Controls	895	119 (13.3)	Reference	Reference
Either cotinine or hydroxycotinine	**Cases**	498	88 (17.7)	1.24 (0.91, 1.68)	1.41 (0.98, 2.05)
	Controls	895	147 (16.4)	Reference	Reference
Self-reported or provider-reported smoking (1989+)	**Cases**	442	12 (2.7)	1.20 (0.57, 2.54)	1.81 (0.68, 4.82)
	Controls	745	17 (2.3)	Reference	Reference
Any of the 3 measures	**Cases**	498	90 (18.1)	1.25 (0.92, 1.69)	1.44 (1.00, 2.09)
	Controls	895	150 (16.7)	Reference	Reference
		Unilateral retinoblastoma			
		All Births (N =1175)			
		Total N	Smoker N (%)	Crude OR (95% CI) [1]	Adjusted OR (95% CI) [2]
Cotinine	**Cases**	280	25 (8.9)	1.36 (0.84, 2.22)	1.47 (0.79, 2.73)
	Controls	895	62 (6.9)	Reference	Reference
Hydroxycotinine	**Cases**	280	53 (18.9)	1.69 (1.16, 2.46)	1.92 (1.21, 3.06)
	Controls	895	119 (13.3)	Reference	Reference
Either cotinine or hydroxycotinine	**Cases**	280	59 (21.1)	1.48 (1.03, 2.11)	1.66 (1.08, 2.57)
	Controls	895	147 (16.4)	Reference	Reference
Self-reported or provider-reported smoking (1989+)	**Cases**	245	<5	0.73 (0.24, 2.18)	---
	Controls	745	17 (2.3)	Reference	Reference
Any of the 3 measures	**Cases**	280	59 (21.1)	1.44 (1.01, 2.06)	1.67 (1.08, 2.58)
	Controls	895	150 (16.8)	Reference	Reference
		Bilateral retinoblastoma			
		All Births (N =1113)			
		Total N	Smoker N (%)	Crude OR (95% CI) [1]	Adjusted OR (95% CI) [2]
Cotinine	**Cases**	218	15 (6.9)	1.11 (0.62, 2.01)	1.09 (0.53, 2.23)
	Controls	895	62 (6.9)	Reference	Reference
Hydroxycotinine	**Cases**	218	23 (10.6)	0.98 (0.59, 1.60)	1.17 (0.65, 2.10)
	Controls	895	119 (13.3)	Reference	Reference
Any biomarker	**Cases**	218	29 (13.3)	0.98 (0.59, 1.60)	1.17 (0.65, 2.10)
	Controls	895	147 (16.4)	Reference	Reference
Self-reported or provider-reported smoking (1989+)	**Cases**	197	8 (4.1)	1.82 (0.77, 4.28)	2.79 (0.96, 8.10)
	Controls	745	17 (2.3)	Reference	Reference
Any of the 3 measures	**Cases**	218	31 (14.2)	1.02 (0.66, 1.58)	1.17 (0.70, 1.97)

	Controls	895	150 (16.8)	Reference	Reference

[1]Adjusted for birth year

[2]Additionally adjusted for maternal age, maternal race, maternal education, paternal age, and any sexually transmitted disease