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Evaluation of urinary *trans*-3′-hydroxycotinine as a biomarker of children's environmental tobacco smoke exposure

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Abstract

The utility of urinary *trans*-3′-hydroxy cotinine (3HC) as a biomarker of environmental tobacco smoke (ETS) exposure was investigated in comparison with urinary cotinine (COT), the sum (3HC + COT), and ratio of the two nicotine metabolites (3HC/COT). Participants were 150 ETS exposed children (aged 1–44 months) and their parents. Child urine samples were collected during 3 weekly baseline assessments and at interviews administered 3, 6, 12, and 18 months after baseline. Findings indicate that 3HC and COT can be measured reliably (rho = 0.96, 0.88) and show equivalent levels of repeated measures stability (rho = 0.71, 0.75). COT, 3HC, and 3HC + COT showed equally strong associations with air nicotine levels, reported ETS contamination, and reported ETS exposure ($r = 0.60–0.70$). The intraclass correlations of 3HC/COT were lower than those for COT or 3HC. Older children had a higher 3HC/COT ratio than younger children (3.5 versus 2.2), and non-Hispanic White children had a higher ratio than African-American children (3.2 versus 1.9). These findings suggest that COT, 3HC, and 3HC + COT are approximately equivalent and equally strong biomarkers of ETS exposure in children. Moreover, 3HC/COT may provide a useful indicator to investigate age- and race-related differences in the metabolism of COT and 3HC.

Keywords: *Tobacco smoke, exposure, children, cotinine,* trans-*3′-hydroxycotinine*

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Introduction

Environmental tobacco smoke (ETS) is classified as a known human carcinogen (US Department of Health and Human Services 2005), and exposure of children to ETS is associated with an increased number and severity of asthma attacks, respiratory tract and middle-ear infections, Sudden Infant Death Syndrome, and other illnesses (California Environmental Protection Agency 1997). Data from the 1999–2000

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National Health and Nutrition Survey reveal that more than 50% of children aged 3–11 years have been recently exposed to tobacco (US Centers for Disease Control and Prevention 2003). Because young children are particularly vulnerable to the negative health consequences of ETS exposure, reducing smoking in the home and ETS exposure by parents and caregivers is a major public health objective.

To protect non-smokers from ETS exposure, many states in the USA and many countries worldwide have passed legislation restricting smoking in public buildings and workplaces (Chapman 1996, Eisner et al. 1998, Fichtenberg & Glantz 2002, Kunzli 2002). Children, however, spend the majority of time indoors at home where these smoking restrictions do not apply, and household members smoking at home are the most important sources of ETS exposure (Gilpin et al. 2003). Because existing smoking restrictions have limited reach in private homes, interventions are needed to change smoking and exposure-related behaviours at home (Gehrman & Hovell 2003, Matt et al. 2004).

There is growing evidence that behavioural interventions can reduce ETS exposure at home (Hovell et al. 2000b, Roseby et al. 2003) To assess the effectiveness of such interventions, sensitive, reliable, and valid measures of children's exposure are needed (Benowitz 1996, Jaakkola & Jaakkola 1997, Hovell et al. 2000a,c). Among the major types of measures used to assess ETS exposure in children are (1) the reported smoking status of parents, caregivers, and other household members, (2) timeline-follow-back interviews and diaries detailing smoking and exposure behaviours over a certain period of time, (3) indoor air nicotine levels, and (4) biological measurements. Among biological markers, cotinine (COT) levels in urine, serum, saliva, hair, and toenails have been measured and are now a biomarker standard. COT is a metabolite of nicotine and is considered more accurate as a marker of exposure to ETS than is nicotine, due to its longer half-life in the body (Benowitz 1996). Using sensitive techniques in chemical analysis such as gas or liquid chromatography coupled with mass spectrometry, COT levels in the sub-part per billion range are quantifiable in serum and urine samples (Bernert et al. 1997). COT was selected as the single biological measure used to assess exposure to ETS in the Second National Report on Human Exposure to Environmental Chemicals conducted by the CDC (US Centers for Disease Control and Prevention 2003).

Some questions remain about the suitability of COT as the major biomarker for assessment of exposure to ETS. The excretion of COT in the urine accounts for, on average, only an estimated 9–15% of the nicotine dose (Benowitz et al. 1994, Byrd et al. 1994, Andersson et al. 1997, Hecht et al. 1999, Hukkanen et al. 2005). Other metabolites found in the urine include *trans*-3′-hydroxycotinine (3HC; an estimated 35–39% of nicotine dose), nicotine (8–11%), and the glucuronides of 3HC (8–23%) and cotinine (12–17%). Because 3HC is the most abundant metabolite in urine (Neurath et al. 1987), it may prove a more reliable marker of nicotine intake than COT (Benowitz & Jacob 1997), particularly at low levels of exposure. Because of its higher abundance, 3HC may also be a more sensitive urine marker than COT. This could be especially beneficial when studying young children and other vulnerable populations who may experience negative health consequences of ETS exposure at much lower exposure levels than healthy adults.

Rather than measuring ETS exposure through either COT or 3HC, there are potential advantages of assessing ETS exposure as the sum of both metabolites. This is the case because interindividual and interethnic variability in the rate of conversion of

nicotine to COT and of COT to 3HC have been reported (Benowitz et al. 1999, Benowitz & Jacob 2001, Dempsey et al. 2004), and the sum of the two metabolites COT+3HC may prove more stable than the measurement of either COT or 3HC alone. In a study of smokers and snuff users, exposure metrics combining 3HC+COT and 3HC+COT plus glucuronides were more highly correlated with reported exposure than COT alone (Boswell et al. 2000).

Most data on levels and performance of 3HC relative to COT have been determined in adult smokers (Benowitz et al. 1999, 2003, Tuomi et al. 1999, Benowitz & Jacob 2000, 2001, Boswell et al. 2000, de Leon et al. 2003, Dempsey et al. 2004). In contrast, much less is known about 3HC levels in adults or children resulting from ETS exposure (Tuomi et al. 1999, Kohler et al. 2001). No studies have examined the longitudinal stability of 3HC relative to cotinine in ETS exposed children. Because 3HC may be a particularly useful biomarker in low-exposure populations of children, data on the performance of this biomarker in children are needed.

Determining both levels of COT and 3HC metabolites may yield another potential advantage. Because 3HC has been metabolized from COT, the ratio of 3HC to COT (3HC/COT) can shed light on the efficiency of the metabolic processes converting COT to 3HC (Dempsey et al. 2004). This is of particular interest to answering questions about the role of interindividual differences in nicotine metabolism and the extent to which such differences may confound COT and 3HC as biomarkers of ETS exposure.

The present study used data from a longitudinal randomized trial of an ETS counselling and smoking cessation intervention with 150 children of smoking mothers. We first examined ETS contamination in the homes of these children and the distributions of COT, 3HC, 3HC+COT, and reported ETS exposure. Second, we examined the reliability and stability of nicotine metabolites. Third, we investigated the intercorrelations of the biomarkers and their association with measures of ETS contamination at home and reported ETS exposure. Fourth, we examined the 3HC/COT ratio and investigated its reliability, stability, and associations with measures of ETS contamination and exposure. Finally, we examined age, gender, and ethnicity differences in COT, 3HC, the 3HC+COT sum, and the 3HC/COT ratio.

Materials and methods

Design

Data for this study came from a longitudinal randomized trial of a counselling intervention aimed at lowering passive smoke exposure in young children of low-income smoking mothers and assisting mothers and other adults in the home with quitting smoking. Participants were randomly assigned to the counselling intervention or to a treatment-as-usual control group after the collection of baseline data. To establish a stable baseline of ETS exposure, three urine samples were collected 1 week apart during scheduled interview assessments. The median interval between the first and third urine collection was 15 days, and in 80% of instances, the time interval was 21 days or fewer.

Interviews and child urine collections were repeated at 3, 6, 12, and 18 months after the last baseline assessment. For participants in the intervention arm, the 6 month assessment was the post-test measure conducted after completion of counselling. In

summary, this design yielded seven urine samples: three baseline samples collected at 1-week intervals and four additional samples collected at 3, 6, 12, and 18 months after baseline.

Subjects

One hundred-and-fifty children and their mothers were recruited from seven Women, Infants, and Children Supplemental Food and Nutrition Program (WIC) offices in San Diego County. To qualify for this study, the participating child had to be reportedly exposed to at least ten cigarettes by the mother during the week before the third baseline assessment. Mothers had to speak English and at the time of enrolment could not be breastfeeding the participating child, to avoid the confounding of biomarkers from the child's secondhand smoke exposure by those from contaminated breast milk.

The children's age ranged from 1 to 48 months (median = 22 months), and 56% were female. A total of 10.0% of the children were Latino/Hispanic, 10.7% were African-American, 54.7% were non-Hispanic White, and 24.7% were of mixed or other ethnic-racial backgrounds. Recruitment and study protocols were approved by the Institutional Review Board of San Diego State University.

Measures

Personal interviews were conducted by trained staff in the residence of the participant or at another location of her choosing. Responses were recorded on paper copies by the interviewer. Interviews were audio-recorded, and all interviews were reviewed for quality control.

A bogus-pipeline procedure (Murray et al. 1987, Matt et al. 1999) was implemented to increase the validity of mother reported smoking and exposure behaviour. The procedure consisted of installing inactive (i.e. bogus) air monitors in the home for the duration of the study in areas of highest exposure. These inactive dosimeters were in addition to nicotine monitors placed for a sample of the participating families.

Air nicotine concentration. Air nicotine monitors were installed in the homes of a randomly selected 40% sample of study participants. Monitors were installed in the room of greatest exposure 1 week before the third baseline assessment (pretest) and collected at that assessment. The monitoring period therefore corresponded to the time period covered by the interview questions (i.e. the 7 days). Similarly, new monitors were installed 1 week before the 6-month (post-test) assessment and collected at that assessment. The number of hours monitors were in place in the home was recorded. The monitors consisted of a modified 37-mm diffusive sampling cassette with a sodium bisulfate treated Teflon-coated glass-fibre filter. The monitors were stored at room temperature and sent to K. Hammond (University of California, Berkeley) for analysis (Hammond & Leaderer 1987). The level of detection for one full week of exposure is 0.01 $\mu g\ m^{-3}$.

Reported overall secondhand smoke exposure of child. To determine the child's overall exposure to secondhand smoke, the mother was asked about cigarettes smoked during the previous 7 days indoors by any person in the same room as the child, or in a car

with the child present. This included cigarettes smoked (1) in the home and car by the mother and the other parent (husband, live-in boyfriend, etc.), (2) in the home by other residents and visitors, (3) away from home at public and private sites (e.g. sporting event, daycare), and (4) in a car by people other than the parents. The reported overall ETS exposure of the child is the total number of cigarettes smoked in the presence of the child during the previous 7 days.

Reported secondhand smoke contamination at home. To determine the secondhand smoke contamination of the child's home, the mother was asked about all cigarettes smoked in the home during the previous 7 days. This included cigarettes smoked in any room of the home by the mother, the other parent, other residents, or visitors. The reported overall ETS contamination of the home is the total number of cigarettes smoked at home during the previous 7 days.

Weekday and weekends. Previous research has shown that smoking and exposure behaviours at home differ between week/work days and weekend/non-work days. Therefore, working mothers were asked questions about workdays and non-workdays separately, and non-working mothers were asked about weekdays and weekend days separately.

Urine collection. Children's urine samples were collected during three weekly baseline assessments, and at interviews administered approximately 3, 6, 12, and 18 months after the third baseline assessment. If a sample could not be collected at the time of the interview, staff returned to pickup samples collected by the mother up to 1 week later. For children who were not toilet-trained, two panty shields (Natracare LLC; Denver, CO, USA) were placed in diapers and removed when wet. Using a sterile 20-ml syringe (Becton, Dickinson & Co., Franklin Lakes, NJ, USA), the urine was expressed from the cotton pads into a 5-ml cryovial. Samples from toilet-trained children were collected with a standard urine collection cup. Matt et al. (1999) showed that expression from cotton rolls did not affect urine cotinine levels.

Urine samples were kept refrigerated in portable coolers for transportation from residences to the certified Biosafety 2 Research Center laboratory, and frozen within 24 h of collection. Batches of samples were packed in Styrofoam insulation containers with dry ice and express shipped to the Clinical Pharmacology laboratory at the University of California San Francisco for analysis. Data collection information was removed from each vial and recorded, and the vial labelled only with a sequential ID number to keep the laboratory blind to the identity of the contributor of each sample. For approximately 10% of urine samples, a second (split-half) sample was analysed as a reliability check on reported laboratory values. As a check on possible drift in laboratory values over time, some of the second half samples were staggered into later shipments. Statistical analyses comparing earlier and later laboratory analyses showed no evidence of drift.

Cotinine (COT) and trans-*3′-hydroxycotinine (3HC)*. Urine samples were analysed by high-performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry (LC-MS/MS), a method similar to the one described in Bernert et al. (1997), modified for the simultaneous determination of both COT and

3HC. The limit of quantitation (LOQ) was 0.2 ng ml^{-1} for each metabolites. Urine COT and 3HC levels were expressed in nanograms of metabolite per millilitre of urine (ng ml^{-1}). COT and 3HC level were not correlated with the time of day when they were collected (data not shown).

Combined nicotine metabolites (3HC+COT) and ratio of 3HC to COT (3HC/COT). The sum (3HC+COT) and ratio (3HC/COT) were computed based on the molar equivalents of 3HC and COT. The sum is expressed in picomoles of metabolites per millilitre of urine. The 3HC+COT level and the 3HC/COT ratio were not correlated with the time of day when they were collected (data not shown).

Missing values. For nine urine samples, the COT level was reported as below the 0.2 ng ml^{-1} limit of quantitation (LOQ). For one of these nine, the 3HC level was also below the 0.2 ng ml^{-1} level of quantitation. In each instance, a value of 0.1 ng ml^{-1} was substituted for estimating 3 HC and COT. For all analyses of 3HC/COT ratios, cases with COT or 3HC levels below the level of quantitation were defined as missing. For nine urine samples, a 3HC value could not be determined because of analytic interference. For two samples, the quantity of the urine collected was insufficient for analysis.

Extreme values. Out of 932 urine samples collected across all time points and analysed, 48 (5%) had COT values exceeding 100 ng ml^{-1} or 3HC values exceeding 300 ng ml^{-1}, which are considered high for non-smoking children. The pattern of COT and 3HC values across all seven measurement points was inspected for each child who had high values on either metabolite. Urine samples were considered uncharacteristic for that child, and therefore questionable, if a metabolite value (1) exceeded 100 ng ml^{-1} for COT and 300 ng ml^{-1} for 3HC and (2) was more than five times as large as the next highest value among all the repeated measures for that individual. All 15 values identified in this way as suspect were collected after the baseline assessments. It was suspected that incentives for completing measures may have influenced some mothers to substitute other than the child's urine for these samples. These 15 samples were omitted from analyses.

Statistical analysis

Analyses were performed using SPSS version 11.5.0 and STATA version 8 for Windows (SPSS, Inc. 2002, StataCorp 2003). To correct for positively skewed distributions and to stabilize heterogeneous variances, reported smoking, reported exposure, COT, 3HC, 3HC+COT, 3HC/COT, and air nicotine levels were subjected to logarithmic transformation (i.e. $x' = \ln(x+1)$) for all correlation and regression analyses. To take advantage of the longitudinal design, all correlation and regression analyses were conducted using STATA's xtreg procedure for mixed-model cross-sectional time series data. This procedure controlled for the within-subject dependence of repeated measures, making it possible to utilize data from all available data points from baseline to the 18-month follow-up.

Results

Distribution of ETS exposure and contamination measures

Table I shows centiles of the distributions of measures of ETS exposure and contamination, geometric means, and coefficients of variation. Over the course of 7 days, an average of 37 cigarettes (median = 34) were reportedly smoked in the presence of the children. The mean COT and 3HC levels were 11.4 and 32.7 ng ml^{-1}, respectively. The average of the combined nicotine metabolites was 236.5 pmol ml^{-1}. Coefficients of variation using the log-transformed measures ranged from 30% (3HC + COT) to 37% (COT).

Mothers reported that on average 31 cigarettes/week (median = 43) had been smoked indoors at home during the previous week. The average air nicotine concentration for that week was 1.72 μg/m^3 (median = 1.44 μg m^{-3}).

Intraclass correlations: split-half samples

Split-half samples were analysed for 10% of all biomarkers collected. Of those, 65 pairs came from different children and provided the basis for the split-half intraclass coefficients reported in Table II. This coefficient captures measurement unreliability due to differences between subsamples in handling, preparation, storage, or chemical assays.

The split-half reliability was 0.88 for COT, 0.96 for 3HC, and 0.96 for the sum of the metabolites. That is, single subsamples of COT, 3HC, and the sum of the metabolites provided consistent measures of the metabolites in the entire urine sample.

Intraclass correlations: stability of three baseline measures

To examine the stability of the two nicotine metabolites and their sum, intra-class correlation coefficients were computed based on the three repeated weekly baseline measures that were collected for each child. The single measure intra-class correlation coefficients for COT, 3HC, and COT + 3HC are given in Table II. These coefficients indicate how well single measurements represent the overall level observed in three weekly assessments. These coefficients reflect differences between replications due to change in actual exposure levels over time as well as potential differences in the procedures for collecting and preparing samples. Table II shows that the intraclass stability coefficients were consistently lower than split-half intraclass coefficients, indicating that exposure levels likely changed during the baseline assessment period. This suggests that single COT or 3HC measures provide a fairly but not highly accurate measure of the overall exposure level over a 3-week period.

Relationship between biological, environmental, and reported measures of contamination and exposure

Correlations among nicotine metabolites. Urine COT and 3HC correlated very highly with each other ($r = 0.85$) and with the sum, of which each is a part. The correlation between COT and 3HC, corrected for unreliability based on their split-half intraclass correlations, is $r_{corr} = 0.93$. This indicates that the two metabolites share approximately 86% of their variance.

Table I. Number of baseline observations, centiles, geometric means, coefficients of variation of measures of environmental tobacco smoke (ETS) exposure, ETS contamination, and the ratio of nicotine metabolites.

			Centiles						
Measures	*n*	*n* <LOQ	10	25	50	75	90	Geometric mean	CV
ETS exposure									
COT (ng ml^{-1})[a]	429	5	2.67	5.62	11.39	23.56	38.91	11.37	.37
3HC (ng ml^{-1})[a]	428	0	5.70	14.29	32.73	74.17	152.33	32.69	.34
3HC+COT (pmol ml^{-1})[a]	428	5	51.34	111.69	245.36	545.16	995.51	236.53	.30
Reported ETS exposure of child (cigarettes/week)[b]	135	n.a.	12	20	34	80	123	36.54	.29
ETS contamination									
Air nicotine concentration (μg m^{-3})[c]	50	0	0.03	0.26	1.44	3.09	8.11	1.72	.83
Reported ETS contamination — home (cigarettes/week)[b]	139	n.a.	0	16	43	100	204	31.16	.48
Ratio of nicotine metabolites 3HC/COT (pmol ml^{-1})[d]	423	0	1.12	1.72	2.86	4.15	6.25	2.87	.36

COT, cotinine; 3HC, *trans*-3′-hydroxycotinine; 3HC+COT, sum of *trans*-3′-hydroxycotinine and cotinine; 3HC/COT, ratio of *trans*-3′-hydroxycotinine to cotinine; LOQ, level of quantitation (0.2 ng ml^{-1}); CV, coefficient of variation.

[a]Three urine samples were collected per child during three baseline assessments.

[b]Reported exposure and contamination were assessed at the third baseline assessment reflecting the previous 7 days.

[c]Passive air nicotine monitors were placed for 1 week in 40% of the homes for the 7 days before the interview, the same period of time about which mothers were reporting.

[d]Missing if 3HC or COT were <LOQ.

Table II. Intraclass correlations and intercorrelations for measures of environmental tobacco smoke (ETS) contamination and exposure.

	Intraclass correlations (95% confidence interval)			Pearson correlations					
	Single sample split-half	Three samples stability	48-h delayed recall	3HC	3HC+COT	3HC/COT	Reported contamination	Reported exposure	Air nicotine
COT	0.875 (0.803; 0.922)	0.750 (0.684; 0.807)	n.a.	0.848	0.924	−0.109[n.s.]	0.703	0.617	0.605
3HC	0.959 (0.934; 0.975)	0.709 (0.635; 0.774)	n.a.		0.983	0.454	0.657	0.616	0.553
3HC+COT	0.956 (0.928; 0.973)	0.715 (0.642; 0.779)	n.a.			0.298	0.692	0.632	0.593
3HC/COT	0.779 (0.660; 0.860)	0.458 (0.354; 0.559)	n.a.				0.102[n.s.]	0.167	0.010[n.s.]
Reported contamination	n.a.	n.a.	0.825 (0.718;.895)					0.784	0.626
Reported exposure	n.a.	n.a.	0.857 (0.763;.916)						0.398

All correlations are significantly larger than zero ($p < 0.05$) except those marked with n.s.
n.s., Correlation is not significantly larger than zero ($p > 0.05$); COT, cotinine; 3HC, *trans*-3′-hydroxycotinine; 3HC+COT, sum of *trans*-3′-hydroxycotinine and cotinine; 3HC/COT, ratio of *trans*-3′-hydroxycotinine to cotinine.

Correlations between nicotine metabolites and child's reported exposure. The correlations between nicotine metabolites and the number of cigarettes to which the child was reportedly exposed were 0.62 (COT), 0.62 (3HC), and 0.63 (3HC+COT), indicating that 38–40% of the variance of each nicotine metabolite in the urine was accounted for by reported number of cigarettes to which a child was exposed. Corrected for unreliability, the shared variances ranged from 46 to 49%.

Correlation between air nicotine levels and reported home contamination. The correlation between air nicotine levels during the past week and reported contamination as measured by the number cigarettes smoked in the home during that week was 0.63. This indicates that 39% of variance in air nicotine levels, as assessed by a passive monitor in a single room, was accounted for by reported ETS contamination.

Correlations between nicotine metabolites and reported contamination. The correlations between nicotine metabolites and reported contamination as measured by the number of cigarettes reportedly smoked in the home were also similar, 0.70 (COT), 0.66 (3HC), and 0.69 (COT+3HC), indicating that 43–49% of the variance in nicotine metabolites is accounted for by the reported number of cigarettes smoked at home during the previous week. Corrected for unreliability, the proportions of shared variance range from 71 to 83%.

The correlations between nicotine metabolites and contamination as measured by air nicotine levels in the home were similar for each metabolite, 0.61 (COT), 0.55 (3HC), and 0.63 (COT+3HC), indicating that 31–37% of the variance in nicotine metabolites is accounted for by air nicotine levels in the home during the past week.

Gender, age, and ethnicity differences in nicotine metabolites

To investigate gender, age, and ethnicity differences, each of the two metabolites and their sum were regressed on child gender, age, and ethnicity, controlling for reported ETS contamination at home and reported ETS exposure of the child. Thus, observed differences for demographic factors were statistically independent of the number of cigarettes smoked at home and the number of cigarettes to which the child was reportedly exposed. The air nicotine dosimeter measure of contamination was not included as a covariate because of the small number of data points for that variable, which would have unduly restricted the sample size for analyses.

Table III shows the unstandardized regression coefficients for the models of COT and 3HC, and the combined metabolites from mixed-model regression analyses. Because age, metabolite levels, reported exposure, and reported contamination varied within- and between-subjects, regression coefficients are reported for both within- and between-subjects relationships.

Across all three models, reported contamination accounts for a significant proportion of variance in COT, 3HC, and COT+3HC. This is the case for both within- and between-subjects effects for these variables. In the models for 3HC and the sum of the metabolites, reported exposure accounts for an independent additional amount of variance.

With respect to COT, 3HC, and COT+3HC, we observed no statistically significant differences by race/ethnicity or gender. However, there were marginally significant age effects for COT (within-subjects) and 3HC (between-subjects).

Table III. Mixed-model multiple regression analyses of *trans*-3′-hydroxycotinine, cotinine, the sum of the metabolites, and the ratio of the metabolites.

	Response variable			
Explanatory variables	COT	3HC	3HC+COT	3HC/COT
Reported exposure				
Between-subjects	0.0786	0.1916*	0.1481^{+}	0.0677^{+}
Within-subjects	0.0576	0.1179**	0.0916*	0.0277
Reported contamination				
Between-subjects	0.3803**	0.3598**	0.3740**	−0.0097
Within-subjects	0.1631**	0.1505**	0.1537**	−0.0055
Child ethnicity[a]				
Latino	−0.2929	−0.2880	−0.3042	−0.0137
African-American	0.0535	−0.2900	−0.1763	−0.2415*
Other	−0.0172	−0.1588	−0.1138	−0.0825
Child gender	−0.0863	0.0734	0.0395	0.1060
Child age				
Between-subjects	−0.0378	0.1115^{+}	0.0618	0.0965**
Within-subjects	−0.1112^{+}	0.0457	0.0081	0.1528**
Model fit				
R^2	0.3879	0.3465	0.3688	0.1037
$F(7,140)$	23.22**	19.29**	22.16**	3.77**

COT: cotinine; 3HC, *trans*-3′-hydroxycotinine; 3HC+COT, sum of *trans*-3′-hydroxycotinine and cotinine; 3HC/COT, ratio of *trans*-3′-hydroxycotinine to cotinine.
[a]Reference group: non-Hispanic White.
$^{+}p<0.10$; *$p<0.05$; **$p<0.001$.

3HC/COT ratio

Table I presents descriptive information concerning the distribution of the ratio, showing the geometric mean and median to be approximately 2.9 with an interquartile range from 1.70 to 4.18. This indicates that 50% of the urine samples had 3HC levels that were about two-to-four times those of COT.

Table II shows that the split-half and stability intraclass correlations of the ratio are 0.76 and 0.44, respectively, indicating that the ratio can be reliably measured and tends to be the same over time within an individual. Note that the stability and reliability of the ratio are lower than those for the component measures, indicating that the metabolic ratios vary more over time than either COT or 3HC levels.

Table II also shows the correlations of the ratio with each of the other measures of ETS contamination and exposure. The correlations of the ratio with air nicotine levels, reported contamination, reported exposure, and urine COT are not significantly different from zero. The ratio is significantly correlated with 3HC and with the combined metabolite measure, indicating that higher levels of 3HC are associated with higher levels of the ratio ($r=0.387$). This is not a result of instability due to very low COT values in the denominator, but is seen over the range of all values.

Table III gives the results of regression analyses, examining gender, age, and ethnicity differences in the ratio after controlling for reported ETS contamination and exposure. Neither reported contamination nor reported exposure accounted for variance in the ratio. However, child age was significantly related both within-subjects

(as a child ages the ratio tends to become higher) and between-subjects (older children tend to have higher ratios than younger children). Based on the marginally significant age effects for COT and 3HC, the age effect for the ratio may have two separate components. First, the negative association between COT and age suggests that COT levels decline with age, controlling for gender, ethnicity, reported contamination, and reported exposure. Second, the positive relationship between 3HC and age suggests that 3HC levels increase with age. In combination, these two effects yield a positive relationship between age and the 3HC/COT ratio.

Further investigations revealed that children below the median age of 22 months at baseline had a median 3HC/COT ratio of 2.24, whereas older children had a median ratio of 3.47. There were no statistically significant quadratic or cubic relationships between age and the ratio.

Findings from the regression analyses also revealed a statistically significant difference in the mean ratios of African-American and non-Hispanic White children. Specifically, African-American children had significantly lower ratios than non-Hispanic White children (median = 3.15 for non-Hispanic White; median = 1.90 for African-American children).

Discussion

This study investigated the potential utility of urinary *trans*-3′-hydroxy cotinine as a biomarker of ETS exposure over and in addition to urinary cotinine. To the best of our knowledge, this is the first study to compare the split-half reliability, stability, and intercorrelation of the two major metabolites of nicotine (i.e. COT, 3HC), their sum, and their ratio in a sample of young children exposed to secondhand smoke. In addition, this is the first study to examine in detail the within-subject variability of these metabolites based on multiple urine samples taken over a 2-week period. Briefly put, urinary COT and 3HC show high reliability, equivalent levels of temporal stability over a 2-week period, and similarly strong correlations with air nicotine levels at home and parent-reported smoking and secondhand smoke exposure. While the sum of the two metabolites did not appear to add significantly to the reliability and validity of its components, the ratio of the two metabolites revealed two major novel findings. The first concerns between- and within-subjects effects of age, indicating larger 3HC/COT ratios in older than in younger children. The second finding concerns significantly lower 3HC/COT ratios in African-American than non-Hispanic White children. In the following, we review and discuss the implications of these findings.

Urinary trans-*3′-hydroxycotinine versus urinary cotinine*

Contrary to expectations, 3HC in addition to COT did not significantly add to our ability to assess exposure to ETS. 3HC and COT levels in urine were correlated very highly with each other (0.85). Correlations with non-biological assessments of exposure were similar in magnitude for COT and 3HC. 3HC and COT correlated highest with reported ETS contamination (0.66 and 0.70), followed by reported ETS exposure (0.62 and 0.62) and air nicotine levels at home (0.55 and 0.61) (Table II). In combination, these findings suggest that 3HC and COT do not differ in important ways for assessing exposure to ETS in children. The sum of the two nicotine metabolites does not appear to provide additional benefits over the individual

metabolites as a biomarker of ETS exposure. Split-half reliability, stability, and validity coefficients of the sum are virtually indistinguishable from its constituent components (Table II). If variability in metabolism of nicotine to COT and COT to 3HC contributed greatly to variability in COT and 3HC levels, the sum of the metabolites would presumably be more stable than individual levels (Boswell et al. 2000). Our lack of a finding that the sum provided additional useful information was to be expected given the high level of correlation between the two metabolites.

Mixed-model multiple regression analyses showed that, after controlling for reported exposure and reported contamination, there were no significant differences between gender or ethnic groups with respect to 3HC or COT. There were, however, marginally significant relationships ($p < 0.10$) with age for both 3HC and COT. While these relationships should not be over-interpreted, they are noted here, because in combination they play a role in the relationship between age and the 3HC/COT ratio. Age and COT showed a negative association within-subjects, i.e. as children grew older over the course of the study, cotinine levels tended to decline. In contrast, age and 3HC showed a positive association between-subjects, i.e. older children tended to have higher 3HC levels than younger children.

We did not adjust the 3HC levels for urine dilution. Although COT levels appear to be independent of urine dilution, 3HC is likely somewhat dependent on urine dilution due to its polar nature. Kohler et al. (2001) reported a significant increase in correlation for 3HC in mothers and newborns when the urine of the mother, though not the newborn, was adjusted for creatinine concentration. In addition, urinary 3HC was more highly correlated with salivary 3HC when adjusted for creatinine in samples from adult smokers (Kohler et al. 1999). It is not known if adjustment of our 3HC levels for creatinine would have increased the precision of 3HC as a biomarker. However, time of urine sample collection did not significantly influence 3HC levels, and creatinine concentrations are shown to vary by time of urine collected (Barr et al. 2005). Furthermore, there is no reason to correct for urine dilution with respect to 3HC/COT. Because this is a ratio, correcting the analytes for creatinine would cancel out and the ratio would remain unchanged.

One potential benefit of determining 3HC rather than COT levels is the greater abundance of 3HC and therefore more analytical sensitivity. However, we found a trivial difference in the ability to detect the two metabolites in the present study. Fewer than 0.2% of urine samples had 3HC and less than 1% had COT levels below the level of quantitation. Quantitative levels of 3HC and COT can be determined in the laboratory at very low exposure levels (LOQ $= 0.20$ ng ml^{-1}). The present findings are in contrast to Kohler et al. (2001), who could detect 3HC but not COT in the urine of the neonates from ETS exposed mothers. This group used a different analytical procedure (HPLC) with a reported limit of detection of approximately 0.9 ng ml^{-1}, about five times higher than in the current study. It should also be noted that in 9 ($<1\%$) of the urine samples from the present study, 3HC levels could not be determined because of analytic interference. Analytic interference did not contribute to missing values for COT.

Reported ETS contamination and exposure

The present findings indicate that parent reports of ETS contamination at home (i.e. number of cigarettes smoked in the home) provide reliable and valid measures of ETS

contamination as measured by air nicotine dosimeter levels ($r = 0.63$). Similarly, parent reports of the child's ETS exposure (i.e. the number of cigarettes smoked in the same room or car with the child) provide reliable and valid measures of ETS exposure as measured by nicotine metabolites ($r = 0.62$ for 3HC and COT). In fact, the correlations between parent-reported ETS contamination at home and nicotine metabolites were higher than those between nicotine metabolites and air nicotine dosimeter measures (0.70 versus 0.61 for COT; 0.66 versus 0.55 for 3HC). Overall, these findings confirm the reliability and validity of carefully designed parent reports of child ETS (Matt et al. 1999, 2000, 2004) that can provide extensive contextual information on exposure. Such contextual information is critical to interventions designed to reduce ETS exposure in children's homes.

Trans-*3′-hydroxycotinine to cotinine ratio (3HC/COT)*

The present findings indicate that in young children levels of 3HC in urine are on average three times higher than COT levels. The split-half intraclass correlation of 3HC/COT (0.76) was lower than that of 3HC (0.96) or COT (0.88). Over a 3-week period, the 3HC/COT ratio showed significant stability (0.46), though at a lower level then either 3HC or COT.

The ratio of the two metabolites was unrelated to cotinine levels, air nicotine levels, and reported contamination. The ratio was significantly correlated with 3HC ($r = 0.45$), 3HC + COT ($r = 0.30$), and reported exposure ($r = 0.17$). Both correlations are likely to be an artefact of the part–whole relationship of biological and reported exposure measures with the ratio.

Mixed-model multiple regression analyses revealed that, independent of reported exposure and reported contamination, 3HC/COT was significantly related to child age and ethnicity (Table III). Older children (cross-sectional and longitudinal) had a higher median 3HC/COT ratio than younger (cross-sectional: 3.5 versus 2.2) children, and non-Hispanic White children had a higher median ratio than and African-American (3.2 versus 1.9) children. As mentioned above, the association between age and the 3HC/COT ratio may be the result the differential association between age and COT (negative) and age and 3HC (positive). That is, compared with younger children, older children tended to have lower COT but higher 3HC levels.

The finding that the 3HC/COT ratio increased with age suggests that the activity of the enzyme CYP2A6, which is primarily responsible for the metabolism of cotinine, may be increasing with age (Dempsey et al. 2004). Age-related increases in the activity of other drug-metabolizing enzymes have been reported (Tanaka 1998), but this is the first observation on CYP2A6. These data are consistent with reports that the half-life of cotinine decreases in older children as compared with younger children (US Environmental Protection Agency 1992).

The finding that the 3HC/COT ratio is higher in non-Hispanic White than in African-American children is consistent with data showing faster nicotine and cotinine metabolism in adult non-Hispanic Whites compared with African-Americans (Benowitz et al. 1999). Moreover, this is also consistent with the finding that African-American children have higher levels of cotinine than White children even after accounting for differences in exposure and variables such as size of residence (Wilson et al. 2005).

In summary, the present study suggests that COT, 3HC, and the sum of the two metabolites are approximately equivalent and equally strong biomarkers of ETS exposure in children. The two metabolites show similarly strong patterns of split-half reliability, temporal stability, and associations with air nicotine levels, reported ETS contamination at home, and reported ETS exposure. This study also adds novel findings concerning the 3HC/COT ratio and its potential use as an indicator to study differential age-related changes and race-related differences in the metabolism of COT and 3HC.

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