Cord Serum Cotinine as a Biomarker of Fetal Exposure to Cigarette Smoke at the End of Pregnancy

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This study investigated the association between biomarkers of fetal exposure to cigarette smoke at the end of pregnancy, cotinine in cord serum and in maternal and newborn urine samples, and quantitative measurement of smoking intake and exposure evaluated by maternal self-reported questionnaire. Study subjects were 429 mothers and their newborns from a hospital in Barcelona, Spain. A questionnaire including smoking habits was completed in the third trimester of pregnancy and on the day of delivery. Cotinine concentration in cord serum was associated with daily exposure to nicotine in nonsmokers and with daily nicotine intake in smokers. The geometric mean of cotinine concentration in cord serum statistically discriminated between newborns from nonexposed and exposed nonsmoking mothers, and between these two classes and smokers, and furthermore was able to differentiate levels of exposure to tobacco smoke and levels of intake stratified in tertiles. Urinary cotinine levels in newborns from nonsmoking mothers exposed to more than 4 mg nicotine daily were statistically different from levels in two other categories of exposure. Cotinine concentration in urine from newborns and from mothers did not differentiate between exposure and nonexposure to environmental tobacco smoke (ETS) in nonsmoking mothers. Cord serum cotinine appeared to be the most adequate biomarker of fetal exposure to smoking at the end of pregnancy, distinguishing not only active smoking from passive smoking, but also exposure to ETS from nonexposure. *Key words*: **cord blood, cotinine, daily exposure to nicotine, fetal exposure to cigarette smoke, nicotine daily intake, questionnaire, urine.** *Environ Health Perspect* **108:1079–1083 (2000). [Online 25 October 2000]**
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Prenatal exposure to smoking has consequences both in childhood and in adulthood (*1–5*). Fetal exposure to cigarette smoke is usually assessed by questionnaires administered to mothers during or after pregnancy (*1,6*). However, difficulties in recognizing smoking behavior or recalling smoking exposure, or changes in smoking habits during gestation could bias these assessments.

In a country such as Spain, with a high prevalence of young female smokers (*7*) and a high passive intake through social events (*8*), questionnaires could be even less valid. In addition, pregnant women, conscious of the risks of tobacco smoke products for the fetus, may be reluctant to admit active smoking or passive exposure due to social pressure (*9*).

In recent years there has been increasing interest in the use of biomarkers of smoking exposure to improve the validity of assessment by questionnaire (*9–13*). At present, cotinine measured in blood, saliva, or urine appears to be a reliable marker of recent smoking status in population studies (*14–16*). However, collection of saliva or urine in newborns is rather unfeasible.

Two recent studies have measured cotinine in cord blood (*9,11*) and compared it to self-reported maternal smoking. The first study (*9*) included 27 newborns from smoking mothers, whereas the other study (*11*) was carried out in a population with a low intensity of smoking during pregnancy and without measurement of exposure to environmental tobacco smoke (ETS).

Within the framework of a cohort study on the effects of prenatal and postnatal environmental exposures in the inception of atopy and asthma [Asthma Multicenter Infant Cohort Study; AMICS (*17*)], we aimed to measure prenatal exposure to tobacco smoke. For this purpose, cotinine was measured in cord serum samples and maternal and newborn urine collected on the day of delivery and compared to maternal self-reported questionnaires. The objective of the present analysis was to assess the association between these biomarkers of exposure, particularly cotinine in cord blood, and the quantitative measurement of smoking intake and exposure at the end of pregnancy measured through questionnaire.

Materials and Methods

Subjects. Mothers and their newborns were recruited for the AMICS study. Pregnant women (n = 638; median age = 29 years) attending the Hospital del Mar in Barcelona, Spain, during 1997 and 1998 were invited to participate if they anticipated living in the city during the study years and had a telephone. A total of 573 women had a term newborn, accepted enrollment in the study, and gave informed consent. Both parents signed informed consent for their newborns.

Eligibility criteria included only single births and a sufficient amount (> 1 mL) of one of the three biological samples collected, which led to collection and analysis of 404 (70.5%) samples of cord serum, 226 (39.4%) maternal urine samples, and 164 (29.0%) newborn urine samples. In cases of cord serum and maternal urine samples, no differences ($p > 0.2$) in maternal age, smoking habits, or social class (based on occupation) were found between included and excluded participants. In the case of newborn urine samples there was a lower percentage of nonexposed nonsmokers in included participants (27.4%) than in excluded participants (37.4%), even if not statistically significant ($p = 0.07$).

The reduced number of urine samples was due to difficulties experienced in their collection (e.g., contemporary presence of meconium in the collection bag, loss of sealing in the bags during urine collection, frequent irritations in female newborns, etc.). General information on delivery (e.g., birth weight, gestational age, etc.) was recorded from hospital files.

Questionnaire information. An exhaustive questionnaire including smoking habits was completed at the first antenatal care visit to the hospital, usually during the third trimester of pregnancy. Mothers were asked if they were nonsmokers, occasional smokers, or daily smokers. If they were daily smokers, they were asked the average number of cigarettes currently smoked per day and the brand of cigarette. None of the mothers who

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smoked reported smoking on a less-than-daily basis (occasional smokers) or consumption of cigars. Women who reported cessation of smoking at the time of interview (20%) were considered nonsmokers. Regarding exposure to ETS, nonsmoking mothers were asked if they were regularly exposed to ETS, where and by whom (husband or other people in the family or/and at work), the average number of cigarettes and the brand of cigarette smoked by these people, and the average hours of exposure. Tobacco consumption and exposure to ETS were calculated as milligrams nicotine daily intake (NDI) and milligrams daily exposure to nicotine (DEN), respectively. In the case of smoking mothers, the NDI was obtained from the average number of cigarettes smoked per day multiplied by nicotine content (in milligrams) of each cigarette (*15*). In the case of nonsmoking mothers with a passive exposure, DEN was calculated as NDI of the active smoker in the environment of the mother multiplied by the hours during which exposure was reported to occur (as a fraction of 24 hr) (*18*). If nonsmoking mothers declared contact with more than one smoker, the different exposures were added. A shorter version of the AMICS questionnaire (one page only) was administered the day of delivery as a confirmation of the previous one. If there was disagreement between the two questionnaires (n = 15, 3.5%), the second one was considered to be the most reliable.

Urinary cotinine. Urine samples were collected on the day of delivery. Samples from newborns were obtained using a special adhesive collection bag.

Samples were stored at –80°C until analysis. Urinary cotinine was measured in duplicate using a double antibody radioimmunoassay according to a method described by Van Vunakis et al. (*19*). The level of cross-reactivity of the cotinine antibody with other nicotine metabolites was less than 5%. The detectable range of measurement from the standard curve was 0.2–20 ng/mL cotinine, with an interassay coefficient of variation of 6–10%. Assays were performed without knowledge of questionnaire response. Samples, which at first assay fell outside the calibration curve, were opportunely diluted.

Creatinine was measured by enzymatic colorimetric test (CREA, MPR1, creatinine PAP; Boehringer Mannheim, Mannheim, Germany), and urinary creatinine levels were used to estimate urinary dilution. A cutoff of 50 ng/mL was employed for urinary cotinine to distinguish active smokers from nonsmokers (*20*).

Cord serum cotinine. Umbilical cord blood was obtained at delivery and immediately centrifuged. Serum was collected and stored at –80°C until analysis. Cord serum cotinine was analyzed in duplicate by radioimmunoassay as described above for urine samples. A cutoff of 14 ng/mL was used for cord serum cotinine to distinguish active smokers from nonsmokers (*11*) and one of 1 ng/mL cord serum cotinine to distinguish nonexposed nonsmokers from exposed nonsmokers (*4*).

Statistical methods. We compared sociodemographic and clinical data of mothers and newborns in terms of self-reported smoking status using chi-square tests for categorical variables and analysis of variance (ANOVA) statistics for continuous variables. Because cotinine values did not follow a normal distribution (distribution skewed to the right), cotinine concentrations were log transformed to fit a normal distribution. To assess a dose–response relationship between smoking habits and cotinine levels, groups of DEN in nonsmoking mothers and groups of NDI in smoking mothers were stratified according to tertiles. Furthermore, to compare cotinine concentrations among nonexposed nonsmokers, exposed nonsmokers, and smokers, adjusting for potential confounders such as maternal age, sex of the child, or creatinine levels, a multiple linear regression analysis was conducted. The same process was used to relate cotinine amounts in cord blood, maternal and newborn urine, and birth weight. To assess the form of the association between cotinine levels in cord serum and NDI, a nonparametric smooth function was assessed using the Lowess method. In addition, although out of the scope of the study, we attempted to calculate our optimal cutoff value to separate between nonexposed and exposed nonsmokers in relation to self-reported smoking status using the receiver operating characteristic (ROC) curve. An ROC curve was calculated considering the sensitivity as the percentage of exposed nonsmokers detected and specificity as the percentage of nonexposed nonsmokers correctly classified.

All analyses were performed using Stata, version 5.0 (StataCorp, College Station, TX).

Results

Of the 429 pregnant women included in the study, 34% reported daily smoking during the last trimester of pregnancy, 32% declared they were exposed to ETS, and 34% declared they were nonsmokers not exposed to ETS (Table 1). None of the demographic or socioeconomic characteristics obtained by questionnaire was significantly related to maternal smoking status, whereas the percentage of previous abortions and birth weight of the newborns were statistically different between smokers and nonsmokers.

Distribution of cotinine levels in different biological matrices under examination are reported in Table 2 as percentiles.

Table 3 presents cord serum cotinine according to the cutoffs encountered in the international literature used to distinguish newborns of smoking mothers from newborns of nonsmoking mothers, and also to indicate passive exposure in newborns of nonsmokers (*4,11*) in relation to the self-reported questionnaire. In addition, a cutoff of 1.78 ng/mL obtained in this study from the ROC curve to discriminate newborns from nonexposed and exposed nonsmokers (sensitivity and specificity of 60%) was also included in

Table 1. Characteristics of the study population according to self-reported smoking habits.

	Nonexposed nonsmokers (n = 146)	Exposed nonsmokers (n = 139)	Smokers (n = 144)
Mothers			
Age (mean ± SD)	28.7 ± 5.67	29.2 ± 5.57	28.6 ± 5.20
Father's social class (%)	n = 116	n = 124	n = 119
Professional	12.1	3.2	5.9
Managerial and technical	12.1	12.9	9.2
Skilled (nonmanual)	33.8	46.0	40.3
Skilled (manual)	19.8	20.2	23.5
Partly skilled	19.9	17.7	18.5
Unskilled	3.3	0	2.6
Previous abortions (%)			
≥ 1	33.3	37.9	46.7*
Child			
Male (%)	50.0	50.4	54.6
Order in family (%)			
1	48.4	43.1	35.9
2	37.6	37.9	50.0
> 2	14.0	19.0	14.1
Birth weight in grams (mean ± SD)	3288.7 ± 495.2	3319.9 ± 433.1	3104.7* ± 405.0
Length in centimeters (mean ± SD)	49.3 ± 2.3	49.5 ± 2.2	49.0 ± 2.8
Cranial perimeter in centimeters (mean ± SD)	34.5 ± 1.9	34.6 ± 1.4	34.1 ± 1.5
Low weight (< 2,500 g) (%)	4.8	2.2	6.3
Premature (< 37 weeks) (%)	5.7	5.1	5.0

*p< 0.05 in relation to nonexposed nonsmokers.

the table and compared with the value from the literature. Urinary cotinine levels higher than 50 ng/mL were observed in 14% of the self-reported nonexposed nonsmokers and in 28% of self-reported exposed nonsmokers. These values decreased to 2% and 17% when urinary cotinine from newborns of nonexposed and exposed mothers, respectively, were considered (data not shown).

Table 4 shows cotinine levels in cord serum and in newborn and maternal urine in relation to self-reported smoking habits. The median level of cord serum cotinine among the newborns of smoking mothers was more than 30 times higher than that of newborns from exposed nonsmokers, and almost 50 times higher than that of newborns from nonexposed nonsmokers. In addition, an increasing trend of median level of cord serum cotinine was observed when stratifying in tertiles in both milligrams DEN in exposed nonsmokers and milligrams NDI in smoking mothers. Results were confirmed when considering geometric means of cord serum cotinine in the three groups of newborns, adjusting for confounding factors. Indeed, geometric mean of cotinine concentration in cord serum was able to statistically discriminate between newborns from nonexposed and exposed nonsmokers, and between these two classes and smokers, and furthermore was able to statistically differentiate levels of exposure to tobacco smoke and levels of intake stratified in tertiles.

Urinary cotinine levels in newborns from nonsmoking mothers exposed to more than 4 mg nicotine daily were statistically different from levels within the other two categories of exposure. The same result was obtained when examining urinary cotinine levels of the mothers. However, whereas urinary cotinine levels in newborns from smoking mothers showed a statistical difference with increasing values of NDI, geometric means of cotinine concentration in maternal urine showed a lower intensity of association and did not display any statistical difference among tertiles of NDI.

Figure 1 shows the association between cotinine levels in cord blood and NDI in smokers, which appears to be linear except for a plateau at high levels of smoking. Similar functions were obtained using urinary cotinine.

The relationship between birth weight of the newborns and concentration of cotinine in cord serum, and in urine from newborns and their mothers is presented in Table 5. The median levels of the biomarker in each of the biological fluids under examination showed a decreasing trend with increasing birth weight. In the highest tertile of birth weight (> 3,500 g), the geometric mean of cotinine in cord serum and newborn urine adjusted for confounding factors was statistically different from those of newborns with a weight < 3,000 g. There was no association between cotinine in biological fluids from newborns and mothers and length and cranial perimeter of the baby (data not shown).

Discussion

Results from this study show that measurement of cotinine levels in body fluids was useful in predicting smoking habits at the end of the pregnancy and hence the quantitative fetal exposure to tobacco smoke. For the first time, the population was categorized not only as smokers and nonsmokers, but also with consideration of exposure to ETS among nonsmokers.

Levels of cotinine in cord serum and urine from newborns and mothers were generally higher than those observed in other studies dealing with both active smoking and exposure to ETS (*9,11,21–23*). In particular, if we consider the cutoff of 1 ng/mL reported by Bearer at al. (*4*) for cord serum to discern between exposure and nonexposure to ETS, less than 20% of newborns of nonsmoking mothers who did not report any exposure presented values of cord serum in the range of 0.2–1 ng/mL, whereas the majority (75%) of these newborns showed cord serum cotinine in the interval of passive exposure (1–14 ng/mL). Conversely, this last percentage decreases to 35% if using the cutoff of 1.78 ng/mL obtained from the ROC curve. The higher cutoff found for cotinine in cord blood, in comparison with that of Bearer at al. (*4*), might be explained mainly with the technique used in this study for cotinine measurement. In fact, a radioimmunoassay was used that showed a certain cross-reactivity with trans-3′-hydroxycotinine, the other urinary metabolite of nicotine in smokers and nonsmokers (*24*). However, exposed nonsmokers were classified better in the interval of 1–14 ng/mL (79% within the interval) than in the interval of 1.78–14 (52% within the interval). Indeed, this study was not designed for cutoff calculation, and because cord serum cotinine levels between nonexposed and exposed nonsmokers are extremely close if compared with intervals between smokers and nonsmokers, it was only possible to obtain a sensitivity and specificity of 60%. Finally, 4% of newborns from nonexposed nonsmokers and 8% of newborns from exposed nonsmokers had cord serum cotinine in the interval corresponding

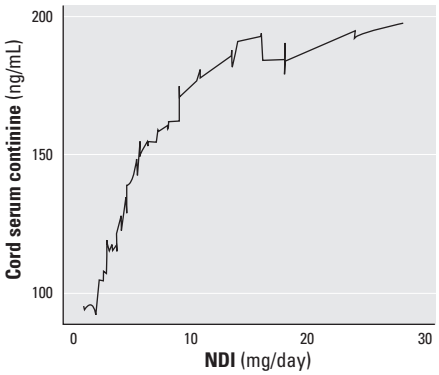

Figure 1. Smoothed graph of NDI (mg/day) against cord serum cotinine (ng/mL).

Table 2. Cotinine in different biological matrices.

	No.	ND (%)	Min	Percentile 5th	25th	50th	75th	95th	Max
Cord blood (ng/mL)	404	1.98	< 0.20	0.65	1.45	3.08	41.15	325	910
Newborn urine (ng/mL)	164	0	0.81	1.97	6.59	16.89	207.75	893	2,000
Maternal urine (ng/mL)	226	0	0.84	3.04	6.75	19.10	714.00	2,000	2,000

Abbreviations: Max, maximum; Min, minimum; ND, nondetectable.

Table 3. Maternal smoking status by questionnaire and cord serum cotinine levels according to the cutoff established to distinguish exposition to ETS and active smoking.

	Cord serum cotinine (ng/mL)					
	Nondetectable levels	Absence of exposition		Passive smoking		Active smoking
	≤ 0.2	0.2–1[a]	0.2–1.78[b]	1–14[a]	1.78–14[b]	> 14
Self-reported smoking habit	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Nonexposed nonsmokers	5 (3.7)	23 (17.2)	77 (57.5)	101 (75.4)	47 (35.1)	5 (3.7)
Exposed nonsmokers	3 (2.2)	14 (10.5)	50 (37.3)	106 (79.1)	70 (52.2)	11 (8.2)
Smokers	0 (0.0)	1 (0.7)	4 (2.9)	17 (12.5)	14 (10.3)	118 (86.8)
Total	8 (2.0)	38 (9.4)	131 (32.4)	224 (55.4)	131 (32.4)	134 (33.2)

[a]Cutoff by Bearer et al. (*4*). [b]Cutoff by our study.

to newborns from active smokers. Therefore, even considering different cutoffs and thus different degrees of nondetected exposure, maternal unawareness and underestimation of ETS exposure, as well as an unwillingness to declare active smoking during pregnancy have to be considered, in agreement with other authors (*9,11*). These results were confirmed when urine samples were considered. Indeed, not only did a high percentage of mothers who did not report active smoking nor exposure to ETS present urinary levels of cotinine > 50 ng/mL [the cutoff to distinguish active smokers from nonsmokers according to Jarvis et al. (*20*)], but a certain percentage of newborns also had these high levels. Furthermore, it must be stressed that these cotinine levels could be conservative, as cord serum and urine from mothers and newborns were collected at the time of delivery. On the other hand, urinary dilution, which can be greatly affected at delivery time, must be taken into account as a bias for cotinine levels in urine. In this study, however, geometric means adjusted for log creatinine consistently matched median values.

Nonetheless, a self-reported smoking habit was a significant determinant of cotinine levels in the biological fluids under examination. NDI was used as the measure of tobacco consumption. This parameter is a better indicator of active smoking than the number of cigarettes smoked per day, as the latter measure does not take into account different tar and nicotine content in different brands of cigarettes.

Exposure to ETS in nonsmoking mothers was measured by DEN as exposure to NDI of surrounding smokers. Although this measure probably overestimates exposure to ETS if compared to consumption, its use in this study may be seen as an attempt to objectively measure source strength (number of smokers and their smoking pattern) (*12*) independent from different individual perceptions. Clearly, DEN did not take into account possible differences in air flow, ventilation, and proximity to the smokers. Indeed, the determination of these parameters should have required the objective measurement of indoor air nicotine in all possible microenvironments of exposition or the use of personal monitoring of vapor-phase nicotine, which was beyond the scope of the present study (*12*). Furthermore, NDI and DEN did not consider individual differences in both maternal and fetal metabolism and elimination of tobacco smoke products. For this reason and due to problems of underreporting and misreporting, the calculation of direct correlations between questionnaire information and levels of cotinine in different biological fluids was considered worthless, given that previous attempts have not succeeded in obtaining reasonable results (*6,9,11*).

It is well known that fetal growth may be adversely affected by both maternal active and passive smoking, and birth weight has been found to be inversely correlated with both neonatal and postneonatal mortality (*25,26*). In this study birth weight was inversely associated to cotinine levels in biological fluids under investigation. In particular, cord serum and newborn urinary cotinine were able to reliably discriminate different birth weight tertiles.

Some authors have questioned the use of cotinine in body fluids as a biomarker to validate questionnaires on smoking habits due to limitations related to its use. These include a generally short half-life so that only recent exposure or consumption can be represented; considerable intersubject variability in uptake, metabolism, and elimination; and the fact that cotinine is not the active agent in causing adverse health effects (*12*). In addition, there is a lack of linear relationship between cord serum cotinine and nicotine daily intake at high smoking levels (ceiling effect). However, in our study cord serum cotinine was able to indicate the quantity of both active and passive maternal exposure. Furthermore, the ceiling effect in measures of smoking intensity does not constitute a problem in epidemiologic follow-up studies, where the aim is to refine the detection at low levels of smoking consumption or of passive smoking, and to detect deceivers.

The use of a biomarker at delivery would certainly underestimate smoking consumption or exposition earlier in pregnancy and this period could be relevant with regard to a

Table 4. Differences of cotinine levels (ng/mL) in biological matrices and self-reported smoking habits during pregnancy.

	Cord serum cotinine			Newborn urinary cotinine			Maternal urinary cotinine		
Self-reported smoking habit	No.	Median	Adjusted GM[a]	No.	Median	Adjusted GM[b]	No.	Median	Adjusted GM[b]
Nonexposed nonsmokers	134	1.62	1.72	45	8.56	8.26	66	8.76	11.81
Exposed nonsmokers	134	2.40	2.76*	59	10.26	12.37	79	14.33	17.22
DEN[c] (tertiles)									
≤ 2	53	1.63	1.90	17	6.70	6.19	25	9.93	11.61
2–4	46	2.48	3.03**	24	9.97	9.92	29	13.83	13.51
> 4	35	3.80	4.43**	18	17.32	30.87**	25	19.33	34.35**
Smokers	136	73.80	59.33*	60	440.25	307.44*	81	1155.00	543.28*
NDI[d] (tertiles)									
≤ 3.6	49	40.00	31.16	22	153.30	160.65	33	650.00	336.26
3.6–9	63	81.90	73.07***	29	633.00	514.80***	33	1373.00	799.10
> 9	24	154.80	137.44***	9	611.30	568.36***	15	1970.00	925.57

GM, geometric mean.
[a]Adjusted for maternal age and sex of child. [b]Adjusted for log creatinine, maternal age, and sex of child. [c]DEN (mg nicotine) = $\sum_{\text{smokers}} \left(\text{NDI}_{\text{smoker}} \times \frac{\text{hr spent with the smoker}}{24 \text{ hr}} \right)$

[d]NDI (mg nicotine) = mg nicotine/cigarette × number cigarettes smoked/day.
*$p < 0.05$ in relation to nonexposed nonsmokers. **$p < 0.05$ in relation to first tertile of DEN. ***$p < 0.05$ in relation to first tertile of NDI.

Table 5. Association between birth weight and cotinine (ng/mL) measured in cord serum and in maternal and newborn urine.

	Cord serum cotinine			Newborn urinary cotinine			Mother urinary cotinine		
Birth weight (g)	No.	Median	Adjusted GM[a]	No.	Median	Adjusted GM[b]	No.	Median	Adjusted GM[b]
< 3,000	97	3.90	10.23	45	70	58.28	55	202.00	92.30
3,000–3,499	153	3.07	7.53	63	16.57	36.17	91	19.35	63.79
≥ 3,500	90	2.61	3.47*	38	12.45	17.38*	54	16.73	30.71

GM, geometric mean.
[a]Adjusted for maternal age and sex of child. [b]Adjusted for log creatinine, maternal age, and sex of child. *$p < 0.05$ in relation to first tertile of birth weight.

number of outcomes. Indeed, 20% of the women in our study reported quitting smoking cigarettes during pregnancy (usually first trimester). Determination of nicotine in hair of mothers could clarify this issue, evidencing eventual changes in smoking habits during pregnancy, expecially when performing analyses of different hair sections corresponding to different periods of gestation. In addition, nicotine in the hair of mothers can be compared to newborn hair nicotine, which could account for all periods of fetal exposure (*12*). In any case, if mothers who gave up smoking produced any bias in the association between cotinine levels and the eventual effects in children (birth weight in the present analysis), this bias would be conservative.

We believe that the accurate assessment of fetal exposure to smoking through the objective measure of a biomarker could be of major importance in a cohort such as this one, in which the ultimate goal is the investigation of the effects of prenatal and postnatal environmental exposures to pollutants, including tobacco smoke, in the inception of atopy and asthma. In conclusion, both cord serum cotinine and newborn urinary cotinine appeared to be adequate biomarkers due to their ability to be associated with levels of active smoking, levels of passive maternal exposure to smoking, and to neonatal effects such as birth weight. However, only cord serum cotinine was able to discriminate active smoking from exposure to ETS and exposure from nonexposure, possibly because of higher statistical power due to the higher number of samples collected. Finally, the ease of collection of cord blood compared to the collection of urine from newborns, and the need to adjust for an eventual dilution in the case of urine samples, which requires a further creatinine assay, advocate for the extension of the use of cord serum cotinine as a biomarker of prenatal exposure to smoking.

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