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Cigarette smoking during pregnancy: comparison of biomarkers for inclusion in epidemiological studies

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Prenatal exposure to tobacco smoke represents an important confounding factor in epidemiological studies addressing developmental effects and requires careful controlling by the use of biomarkers. We compared the following biomarkers of exposure to tobacco smoke during pregnancy and related biological effects in 23 smokers and 17 non-smokers: placental concentrations of heavy metals (cadmium, chrome, lead and zinc), cotinine concentration in meconium, placental CYP1A1 activity (EROD) and bulky DNA adducts. Cadmium was detected in all samples and found in higher concentration in placentas of smokers compared with non-smokers (geometric mean ± GSD: 56.1 ± 1.8 vs 27.4 ± 1.6 μg kg^{-1} dry weight; $p < 0.001$). Cotinine was not detected in meconium samples from the non-smoker group, while samples from the smoker group contained a mean concentration of 114.1 ± 2.9 μg kg^{-1}. Correlation analysis of biomarkers among smokers revealed that daily cigarette consumption was strongly correlated to placental cadmium (Pearson's $r = 0.83$, $p < 0.001$) and to cotinine ($r = 0.73$, $p < 0.001$). EROD activity was also higher in smokers than in non-smokers (9.4 ± 3.4 vs 2.5 ± 1.8 pmol resorufin min^{-1} mg^{-1} protein; $p < 0.001$) and values were correlated to cotinine concentration in meconium ($r = 0.80$, $p < 0.001$) and placental cadmium level ($r = 0.66$, $p < 0.001$). The amount of bulky DNA adducts in placenta was highly variable and poorly associated with smoking status. Because of their high sensitivity and specificity to detect women who smoke during pregnancy, cotinine concentrations in meconium and placental EROD activity should be incorporated in epidemiological studies that investigate adverse developmental effects induced by *in utero* exposure to environmental contaminants.

Keywords: smoking, placenta, cadmium, lead, cytochrome P450, DNA adducts.

Abbreviations: AHH, aryl hydrocarbon hydroxylase; GSD, geometric standard deviation; EROD, ethoxyresorufin-O-deethylase; PAHs, polycyclic aromatic hydrocarbons; TLC, thin-layer chromatography.

Introduction

Smoking during pregnancy is known to induce adverse developmental effects in children (Eskenazi and Bergmann 1995, Eskenazi and Trupin 1995). Perinatal health hazards associated with *in utero* exposure to tobacco toxicants include an increase in the incidence of premature birth, small birth weight and sudden death syndrome, a decrease in the infants' intellectual and motor development and abilities, as well as a higher prevalence of juvenile cancers (Walsh 1994, Gueguen

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et al. 1995). In addition to representing a public health concern in itself, maternal smoking during pregnancy constitutes a major confounding factor in epidemiological studies addressing developmental toxicity of chemical contaminants. Self-declared tobacco consumption is often used in epidemiological studies to determine the smoking status of individuals in a population. However, social desirability, variations in tobacco consumption, accuracy of self-estimated consumption over long periods of time and exposure to second-hand smoke may induce misclassification of smoking status (Coultas *et al* 1988, Klebanoff *et al.* 1998, Peacock *et al.* 1998).

Cotinine concentration in various body fluids constitutes a specific biomarker of tobacco exposure and has progressively replaced earlier biomarkers such as carboxyhaemoglobin and thiocyanate. While the short half-life of cotinine in maternal and neonatal body fluids (< 30 h) limits its interest as a biomarker of smoking during pregnancy (Jarvis *et al.* 1987, Haufroid and Lison 1998), cotinine accumulates in meconium and better reflects prenatal exposure to tobacco smoke (Ostrea *et al.* 1994). Several heavy metals found in tobacco smoke, such as cadmium, chromium, lead and nickel, also accumulate in tissues and fluids in relation to smoking (Edelman and Roggli 1989, Paakko *et al.* 1989, Shaham, *et al.* 1996, Rey *et al.* 1997). More specifically, cadmium accumulation was reported in the placenta of women who smoked during pregnancy (Roels *et al.* 1978, Kuhnert *et al.* 1982, 1987, Lagerkvist *et al.* 1996), suggesting its potential usefulness as a biomarker.

Smoking during pregnancy also triggers biochemical changes in the foeto-placental unit. Earlier studies have reported that tobacco smoking during pregnancy induced CYP1A1 activity in the placenta, a biotransformation enzyme in the P450 family (Pelkonen *et al.* 1979, Manchester and Jacoby 1981, Huel *et al.* 1989, Boden *et al.* 1995). Increases in CYP1A1 dependent aryl hydrocarbon hydroxylase activity (AHH) resulting from smoking were in turn associated with low birth weights (Pelkonen *et al.* 1979). Enzyme induction may also lead to the bioactivation of tobacco smoke toxicants (e.g. PAHs and aromatic amines) to reactive intermediates which can bind covalently to DNA and form adducts. The occurrence of DNA adducts was reported in various tissues of smokers (Randerath *et al.* 1989, Cuzick *et al.* 1990, Lewtas *et al.* 1993), including oral mucosa (Chacko and Gupta 1988), lungs (Phillips *et al.* 1988), bronchial epithelium and white blood cells (Phillips *et al.* 1990), and placenta (Everson *et al.* 1986, 1988, Arnould *et al.* 1997). Although the exact identification of these DNA adducts was not always achieved, a general increase in DNA adducts was suggested as a marker of early genotoxic effects due to a mixture of contaminants such as cigarette smoke (Randerath *et al.* 1988).

The present study was set out to compare the specificity and sensitivity of several biomarkers of foetal exposure to tobacco smoke toxicants, in order to identify those best suited for inclusion in epidemiological studies investigating developmental endpoints. Two types of markers were addressed in self-declared smokers and non-smokers: exposure markers and early effect markers. Exposure markers included heavy metal accumulation in the placenta, cotinine concentration in the meconium and self-declared tobacco consumption during pregnancy. CYP1A1 induction and DNA adducts in the placenta were investigated as biomarkers of early biochemical effects related to tobacco smoking during pregnancy. These markers provide information about the biologically effective

dose, or the level to which maternal smoking triggers a detectable biochemical change in the foeto-placental unit.

Methods

Study population and sample collection

Participants for this study were Caucasians from the town of Sept-Iles (Quebec, Canada), a coastal agglomeration comprising 26 000 residents and located on the North-Shore of the St Lawrence Estuary. The inhabitants' life-style is essentially urban and no exposure to pollutants above background environmental levels is known in this population. Following thorough explanation of the project and after signing a consent form, women entering the Sept-Iles hospital to give birth were recruited. The participating women answered a questionnaire addressing smoking and dietary habits. The population sample recruited for this project comprised 23 non-smokers and 17 smokers (based on self-declared tobacco consumption).

Placentas were collected within 1 h from expulsion. Connective tissue and blood vessels were removed and 10–g samples were cut from the pink portion of the tissue. Samples were placed in polycarbonate vials and frozen at –80 °C until shipping to the laboratory on dry ice. Meconium was collected from the first nappy, transferred into a conical polypropylene tube and kept frozen at –20 °C until analysis.

Analyses of heavy metals

Placenta samples (0.5 g wet weight) were homogenized to a slurry and acid digested overnight at 120 °C in 1% (v/v) nitric acid (HNO_3). A dilute aliquot (1:10) of the tissue digest was processed. Concentrations of heavy metals (^{114}Cd, ^{53}Cr, ^{60}Ni, ^{208}Pb, ^{68}Zn) were simultaneously determined by inductively coupled plasma mass spectrometry (Houk 1986, Nuttal *et al*. 1995). ^{103}Rh was used as the internal standard. Metal concentrations were expressed in μg metal kg^{-1} dry weight tissue and the limit of detection was 10 μg kg^{-1} for all metals. Bias and coefficient of variation for Cd , Cr, Ni and Zn were determined using DOLT-2 standard (National Research Council Canada). The SRM 1577 standard (US National Bureau of Standards) was used for Pb. Coefficients of variation for Cd, Cr, Pb, Ni and Zn were respectively 6.9%, 19%, 6.2%, 3.3% and 4.8%; biases were +4.3%, –13.5%, +5.9%, +10.0% and +7.0%, respectively.

Cotinine analyses

Cotinine was extracted from meconium according to the procedure developed by Lewis (1994). Meconium samples were homogenized to a fine powder in glacial acetic acid and acetone/diphenylamine solution (1:2). The solvents were evaporated under vacuum and the sample dissolved in phosphate buffer (60 mM, pH 6.0). Cotinine was extracted by solid phase extraction and measured by mass spectrometry. The detection limit for cotinine in meconium was 5 μg kg^{-1} dry weight. In the absence of standard reference material for cotinine in meconium, meconium samples from non-smokers (cotinine below the detection limit) were spiked with 50 ng of cotinine and average recoveries varied from 95 to 122%. One meconium sample (No. 3127) was analysed seven times on different days and the coefficient of variation was 8.8%.

CYP1A1 activity

Microsomes were prepared from placental tissue by differential centrifugation as described in Lagueux *et al*. (1997, 1999). Ground placenta (3 g) was homogenized in a buffered sucrose solution (50 mM Tris–0.25 M sucrose; pH 8.0). The homogenate was clarified by a first centrifugation at 10 000 *g* (4 °C) and the supernatant was collected and centrifuged at 100 000 *g* for 1 h (4 °C). The microsome pellets were re-suspended in 500 μl of microsome buffer with a glass Dounce homogenizer (Blaessig Glass, Rochester, New York, USA), aliquoted (100 μl) and stored at –80 °C.

Ethoxyresorufin-O-deethylase (EROD) activity was assayed in 96-well plates according to a modification of the method of Kennedy *et al*. (1995) described in Lagueux *et al*. (1997, 1999). Each sample was analysed in quadruplicate (2 × 25 μl and 2 × 10 μl microsomal suspension), and the same sample was tested on different plates to control for intra- and inter-plate variability. Microsomes from cells expressing CYP1A1 (Gentest Corp. Woburn, Massachusetts, USA) were also used as positive controls, and six blank wells were performed on each plate to determine the detection limit of resorufin and fluorescamine fluorescence. A dual standard curve for protein (bovine serum albumin) and resorufin concentrations was always performed on each plate. Briefly, the reaction was carried out in sodium phosphate buffer (50 mM, pH 8.0) in the presence of NADPH (1 mM final concentration). Microsomes were incubated with ethoxyresorufin (10 mM final concentration) at 37°C for 30 min. The reaction was

terminated by adding acetonitrile containing 300 μg ml^{-1} fluorescamine. Fluorescence was measured using a Cytofluor™ 2350 (Millipore™) with a 395 nm excitation filter and 460 nm emission filter (fluorescamine) and with a 530 nm excitation filter and 590 emission filter (resorufin). EROD activity was expressed in pmoles of resorufin formed per minute per mg of microsomal protein.

DNA adducts

DNA was extracted from placental tissue following a standard phenol–chloroform method and detection of bulky DNA adducts was performed by ^{32}P-post-labelling (Randerath *et al*. 1981 1988, Gupta 1996) according to the modifications described in Lagueux *et al*. (1999). Briefly, 10 μg DNA was digested with micrococcal nuclease and spleen phosphodiesterase. Aliquots were kept for HLPC quantification of the digested DNA. Nuclease P1 enrichment of adducts was performed on the remaining DNA digest and samples were labelled with 100 μCi γ-^{32}P-ATP with T4 polynucleotide kinase. Adducts were separated by multi-dimensional thin layer chromatography on PEI–cellulose plates using the following solvents: D1: 1 M sodium phosphate, pH 6.0; D3, 8.0 M lithium formate, 8.5 M urea, pH 3.5; D4, 2.0 M lithium chloride, 1.0 M Tris–HCl , 8.5 M urea, pH 8.0 and D5, 1.7 M sodium phosphate, pH 6.0. D2 was omitted throughout. Radioactive spots were counted using the InstantImager™ electronic autoradiography device (Canberra Packard™) and relative adducts labelling was calculated as described by Gupta (1996) and Lagueux *et al*. (1999). In each experiment, untreated calf thymus DNA, benzo[*a*]pyrene-treated calf thymus DNA and ATP were used as controls.

Statistical analyses

Data for several biomarkers followed a log-normal distribution and therefore geometric mean and geometric standard deviation were presented in descriptive statistics. Differences in selected characteristics and biomarker levels between smokers and non-smokers were tested using Student's *t*-tests on $\log_{10}$-transformed values. A value equal to half the detection limit was attributed to values under the detection limit. Fisher's exact tests were used to compare proportions of women in each group showing selected characteristics. Correlations between biomarkers were assessed using Pearson's correlation coefficients on $\log_{10}$-transformed values. Sensitivity, specificity and weighted Kappa were computed to evaluate the validity of biomarkers to predict smoking status during pregnancy. *Sensitivity* is the ratio of the number of true positive results with a biomarker over total the number of positives (i.e. total number of smokers including false negatives). *Specificity* is the ratio of the number of true negative results over the number of total negatives (i.e. total number of non-smokers including false positives). The level of significance was 0.05. All statistical tests were performed using the SPSS 8.0 for Windows statistical package (SPSS Inc., Chicago, IL).

Results

Biomarkers of exposure to tobacco toxicants during pregnancy

Characteristics of participants according to their smoking status during pregnancy are presented in table 1. The proportion of primiparous women was greater among non-smokers than smokers. Smokers were on average 3.8 years younger and gave birth to smaller and shorter newborns than non-smokers. Only two women from the non-smoker group smoked cigarettes prior to their pregnancy. Smokers reduced their cigarette consumption by 37% on average during pregnancy.

Table 2 presents placental heavy metals concentrations and meconium cotinine levels in the non-smoker and smoker groups. Tobacco consumption in the smoker group ranged from 3 to 25 cigarettes per day. Cotinine was not detected in non-smokers (< 5 μg kg^{-1} wet weight), but meconium samples from the smoker group contained a mean (geometric) concentration of 114.1 μg kg^{-1}, with values ranging from 17 to 831 μg kg^{-1}. Cadmium, lead and zinc were always present at levels above the detection limit (10 μg kg^{-1} dry weight). Chromium was detected in 43% and 24% of placenta samples obtained from non-smokers and smokers respectively ($p = 0.315$; Fisher's exact test). Nickel was detected in 39% of samples from the non-smoker group and 77% of samples from the smoker group ($p = 0.02$; Fisher's

Table 1. Characteristics of participants according to smoking status during pregnancy.

Characteristics	Non-smokers (n=23)	Smokers (n = 17)	p-value[a]
Maternal			
Age, years	28.3 ± 3.4[b]	24.5 ± 6.0	0.027
Weight (prior to pregnancy), kg	61.8 ± 12.1	58.3 ± 11.8	0.363
Smoked before pregnancy	8.7%	100%	<0.001
Cigarettes per day before pregnancy	–[c]	18.5 ± 8.7	–
Cigarettes per day during pregnancy	0	11.6 ± 6.5	–
Parity (primiparous)	52.2%	11.8%	0.017
Newborn			
Male	52.2%	52.9%	1.000
Weight, kg	3.6 ± 0.5	3.1 ± 0.4	0.003
Length, cm	51.9 ± 2.1	50.3 ± 2.5	0.036

[a] p-value for Student's t-test (comparison of means) or Fisher's exact test (comparison of proportions).
[b] Arithmetic mean ± standard deviation (SD).
[c] Only two women in the non-smoker group reported smoking before pregnancy (= 3 cigarettes per day).

Table 2. Concentrations of heavy metals in placenta and cotinine in meconium according to cigarette smoking during pregnancy.

	Non-smokers (n = 23)		Smokers (n = 17)	
Biomarkers	Geo. mean ± GSD[a]	% detected[b]	Geo. mean ± GSD	% detected
Heavy metals in placenta[c]				
Cadmium	27.4 ± 1.6	100	56.1 ± 1.8[d]	100
Chromium	13.0 ± 3.3	43	<10	24
Lead	38.4 ± 1.6	100	43.0 ± 1.8	100
Nickel	11.1 ± 3.4	39	28.8 ± 3.0[d]	77
Zinc	66.9 ± 1.1	100	69.2 ± 1.1	100
Cotinine in meconium[e]	<5	0	114.1 ± 2.9[c,d]	100

[a] Geometric mean ± geometric standard deviation.
[b] Percentage of samples in which the biomarker was detected.
[c] Concentration in μg kg^{-1} dry weight.
[d] Mean value for smokers is significantly greater than that for non-smokers (p<0.05).
[e] Concentration in μg kg^{-1} wet weight; meconium samples were available for 15 non-smokers and 15 smokers.

exact test). Mean concentrations of all metals but chromium in placenta samples from smokers were higher than in samples from non-smokers, but statistically-significant differences were observed only for cadmium (2-fold, p<0.001) and nickel (2.6-fold, p=0.015). Given the low percentages of detection for nickel in placenta, this metal was not further considered as a potential marker of tobacco smoke exposure. Furthermore, the lack of statistically significant differences for lead and zinc concentrations ruled-out the use of these metals as biomarkers of smoking during pregnancy. Hence, only cadmium was considered in further analyses.

The relationship between self-declared tobacco consumption and placental cadmium concentration or cotinine content in meconium was examined among smokers (figure 1). A significant correlation was found between placental cadmium concentration and daily self-declared cigarette consumption (r=0.83, p<0.001, n=17) (figure 1(A)). A slightly weaker correlation was noted between cotinine in meconium and self-declared cigarette consumption (r=0.73, p=0.002, n=15)

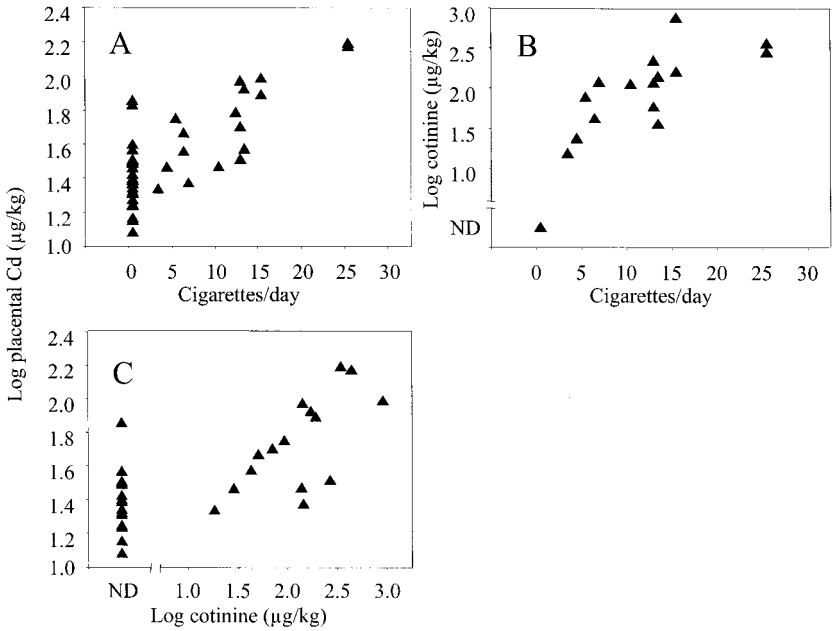

Figure 1. Correlations between biomarkers of fœtal exposure to tobacco smoke during pregnancy. (A) Daily self-declared cigarette consumption vs placental cadmium concentration; (B) daily self-declared cigarette consumption vs cotinine concentration in meconium; (C) cotinine level in meconium vs placental cadmium concentration.

(figure 1(B)). Figure 1(C) depicts the significant relation noted between placental cadmium and cotinine levels in meconium ($r=0.69$, $p=0.005$, $n=15$).

Biomarkers of early response to tobacco smoke during pregnancy

Placental CYP1A1 activity data and the relation of this biomarker of early biological effect to biomarkers of tobacco exposure are presented in figure 2. In figure 2(A), dot plots show the skewed distributions of placental EROD activity in smokers and non-smokers. Most smokers displayed high values but in some cases low activities were found. All but one self-declared non-smokers showed low EROD activities. Mean (geometric) placental EROD activity in smokers was nearly 4-fold higher than that of non-smokers ($p<0.001$). Figure 2(B) shows the moderate correlation observed between EROD activities and placental cadmium concentrations ($r=0.66$, $p<0.001$, $n=40$). A stronger correlation was noted with cotinine level in meconium ($r=0.80$, $p<0.001$, $n=30$) (figure 2(C)), and self-declared consumption was also correlated to EROD activity ($r=0.77$, $p<0.001$, $n=40$; data not shown). When the analysis was restricted to smokers, similar correlations were noted between EROD activity and placental cadmium ($r=0.67$, $p=0.003$, $n=17$), cotinine in meconium ($r=0.71$, $p=0.003$, $n=15$) and self-declared cigarette consumption ($r=0.70$, $p=0.002$, $n=17$).

In figure 3, examples of typical chromatograms obtained after post-labelling of DNA adducts in placenta of smokers and non-smokers are shown. In each experiment, a negative control using untreated calf-thymus DNA (figure 3(A)) was processed to assess cross-contamination and background radioactivity. A positive

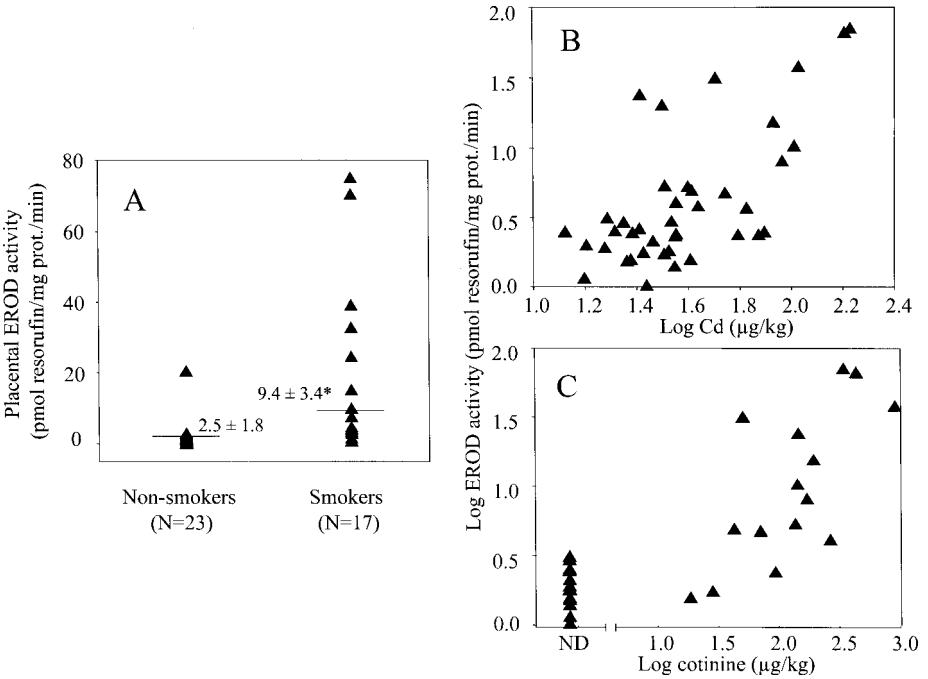

Figure 2. CYP1A1 activity (EROD; pmol min^{-1} mg^{-1} protein) in placenta of smokers and non-smokers and correlations with biomarkers of exposure. (A) Dot plot of EROD activities showing data distribution and geometric mean ± GSD; (B) correlation between placental cadmium concentration and EROD activity; (C) correlation between cotinine concentration in meconium and EROD activity. *Mean of log_{10}-transformed values for smokers is greater than that of non-smokers ($p < 0.001$).

control using benzo[*a*]pyrene-treated calf thymus DNA was also processed (figure 3(B)) to verify labelling efficiency and adequacy of chromatography conditions. Some artifactual spots are seen on the negative control chromatogram (endogenous adducts of CT-DNA, background radioactivity), but nothing is detected in the central part of the TLC plate. The major benzo[*a*]pyrene related adducts on the positive control plate migrate diagonally from the lower left corner of the plate to the upper right corner, as expected, thus confirming the efficiency of the labelling assay and the adequacy of chromatography conditions. The TLC plate for the non-smoker sample (figure 3(C)) does not show any major spots in the central region, although more radioactivity remained in the diagonal radioactive zone and some weak spots were detectable. However, the smoker sample (figure 3(D)) shows a much stronger diagonal radioactive zone as well as many distinct spots around it. All radioactive spots were counted and the relative adduct labelling was computed.

Figure 4 shows distributions of bulky DNA-adduct levels in placenta samples from smokers ($n=6$) and non-smokers ($n=18$). The distribution of values in smokers was skewed with three subjects showing low levels and three displaying high values. Mean (geometric) DNA-adduct levels in smokers (8.3 ± 5.9) was 3.6-fold higher than that of non-smokers (2.3 ± 2.5) ($p=0.03$). Relationships between bulky DNA adducts levels and tobacco smoke exposure markers are illustrated in figures 4(B) (placental cadmium) and 4(C) (cotinine in meconium). DNA adducts were not correlated to placental cadmium ($r=0.19$, $p=0.38$, $n=24$), cotinine in

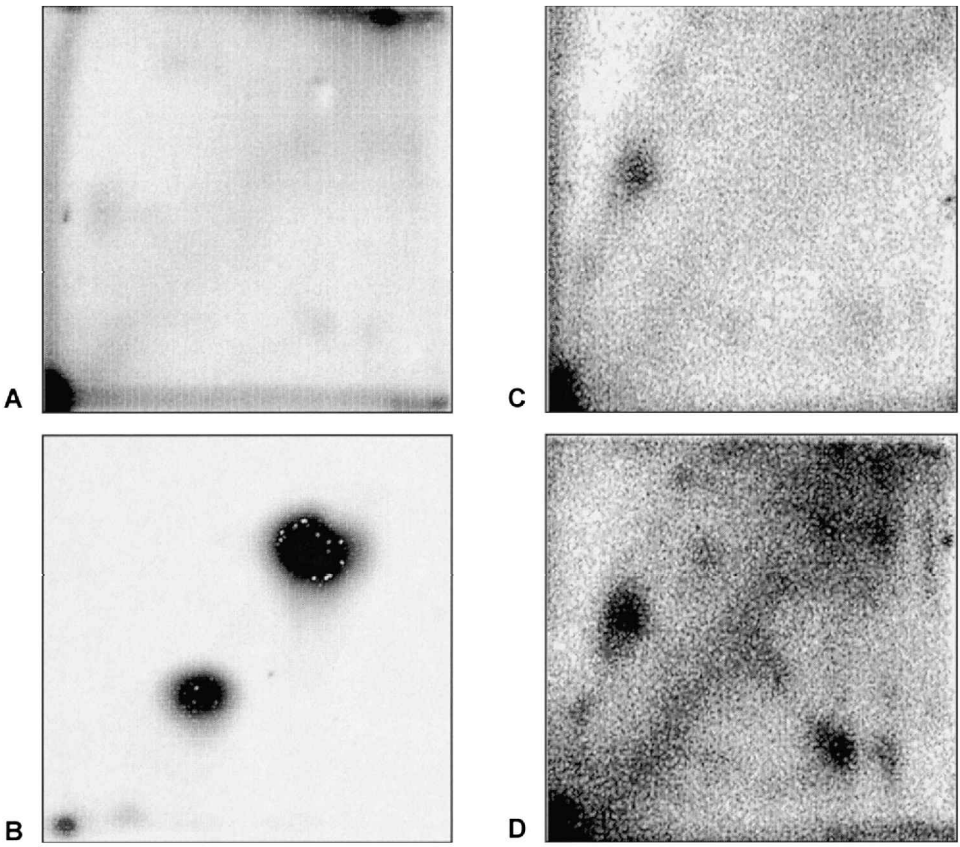

Figure 3. Examples of typical chromatograms obtained by ^{32}P-post-labelling. (A) Negative control, untreated calf thymus DNA; (B) positive control, benzo[*a*]pyrene-treated calf thymus DNA; (C) placental DNA from a non-smoker; (D) placental DNA from a smoker.

Table 3. Validity of various biomarkers to predict cigarette consumption during pregnancy (≥ 10 cigarettes per day).

Biomarker	N	Sensitivity	Specificity	Kappa (95% CI)	*p*-value
Cotinine in meconium ≥ 86 μg kg^{-1}	30	0.90	0.90	0.78 (0.54-1.00)	< 0.001
Cadmium in placenta ≥ 42 μg kg^{-1}	40	0.73	0.83	0.53 (0.24-0.82)	0.001
Placental EROD activity ≥ 5 pmol resorufin min^{-1} mg^{-1} protein	40	0.82	0.86	0.64 (0.38-0.90)	< 0.001

meconium ($r=0.39$, $p=0.13$, $n=16$) or daily cigarette consumption ($r=0.25$, $p=0.25$, $n=24$). There was no significant correlation between CYP1A1 induction (EROD activity) and bulky adducts levels ($r=0.27$, $p=0.20$, $n=24$).

The validity of potential biomarkers to predict a consumption of 10 cigarettes or more during pregnancy was tested using dichotomised variables with the 70th percentile as the cut-off point (table 3). Both placental EROD activity and

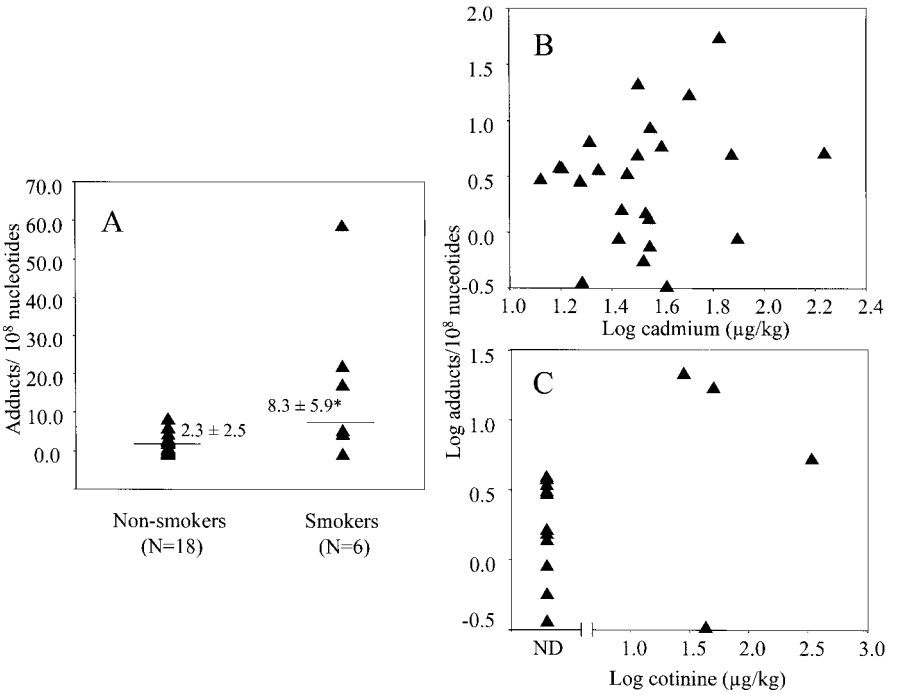

Figure 4. DNA adducts in placenta of smokers and non-smokers. (A) Dot plot of relative adducts labelling (adducts 10^{-8} nucleotides) showing data distribution and geometric mean ± GSD; (B) correlation between placental cadmium concentration and adduct levels; (C) correlation between cotinine concentration in meconium and adduct levels. *Mean of $\log_{10}$-transformed values for smokers is greater than that of non-smokers ($p = 0.03$).

meconium cotinine concentration displayed high sensitivity and specificity values (> 80%; $p<0.001$). A lower specificity to predict cigarette consumption during pregnancy was noted for placental cadmium concentration (73%).

Discussion

This study was designed to compare various biomarkers of tobacco consumption during pregnancy, in order to identify those best suited for inclusion in epidemiological studies investigating adverse developmental effects induced by prenatal exposure to environmental contaminants. Confounding by tobacco smoking during pregnancy is a major problem in such studies. Self-declared tobacco consumption may not be reliable and could result in misclassification of smoking status, as it may be affected by several factors. These include variations in cigarette consumption through time, underestimation due to social desirability (especially in the case of pregnant women), inter-individual variation in the number of puffs per cigarette and the quantity of smoke inhaled at each puff, or exposure to second-hand smoke (Coultas *et al*. 1988, Klebanoff *et al*. 1998, Peacock *et al*. 1998).

Cotinine is a direct metabolite of nicotine and plasma levels constitute a highly specific marker of tobacco consumption (Jarvis *et al*. 1987, Haufroid and Lison

1998). In contrast, few studies have investigated the use of cotinine concentration in meconium as a marker of foetal exposure to tobacco products. In the present study, cotinine was not detected in meconium samples from the non-smoker group (limit of detection: 5 μg kg^{-1}). The mean concentration in smokers was 114.1 μg kg^{-1} and dose-dependent increases in cotinine levels were noted with the number of cigarettes smoked per day. Using a radioimmunoassay, Ostrea *et al*. (1994) reported the following mean concentrations of nicotine metabolites in nanograms per millilitre: non-smokers, 10.9; passive smokers, 31.6; light active smokers; 34.7, and heavy active smokers, 54.6. These authors also noted a statistically-significant correlation between meconium nicotine metabolite concentration (cotinine and *trans*-3'-hydroxycotinine) and the degree of maternal smoking ($r = 0.54$; $p < 0.001$). Baranowski *et al*. (1998) found concentrations of cotinine ranging from 20 to 86 ng g^{-1} in 11 positive samples, using solid-phase extraction and a high-performance liquid chromatographic method with diode-array detection. It is not clear why concentrations reported in the latter two studies are lower than those reported here. This difference could be due to higher nicotine content in Canadian cigarettes. The mean nicotine concentration determined in 23 Canadian brands of cigarettes was found to be 32% greater than that measured in 35 American brands (Kozlowski *et al*. 1998). Differences as regards procedures for meconium sampling, extraction and analysis may also explain discrepancies in cotinine levels among studies.

Several metals were considered as potential biomarkers of tobacco smoking during pregnancy. Our results show that cadmium accumulated to significantly higher levels in placental tissue of smokers compared with non-smokers. When expressed on a wet weight basis (assuming a 82% water content in placenta samples), mean (arithmetic) concentrations documented in the present study in smokers and non-smokers were respectively 12 μg kg^{-1} and 6 μg kg^{-1}. Similar concentrations of 12 μg kg^{-1} and 8 μg kg^{-1} were reported by Kuhnert *et al*. (1987) in 65 smokers and 84 non-smokers from Cleveland (US). An early study by Roels *et al*. (1978) documented higher concentrations of respectively 17 μg kg^{-1} and 12 μg kg^{-1} in 109 smokers and 333 non-smokers from Belgium. In contrast, Lagerkvist *et al*. (1996) noted lower mean concentrations of respectively 5.0 μg kg^{-1} and 2.6 μg kg^{-1} in 28 smokers and 43 non-smokers from Northern Sweden. The strong correlation noted in the present study between placental cadmium and cotinine concentration in meconium indicates that cadmium exposure is indeed closely related to cigarette smoking during pregnancy. Watanabe *et al*. (1987) reported that cigarettes produced in 20 different countries around the world contained a mean (geometric) concentration of 1.31 μg g^{-1}, with values ranging between 0.29 and 3.38 μg g^{-1}. Cadmium exposure from cigarette smoking is by far the major contributor to cadmium body burden, even in the Inuit population that has high dietary sources of exposure (Rey *et al*. 1997).

Mean placental EROD activity in smokers was increased nearly 4-fold compared with that of non-smokers. Several studies have reported an increase in CYP1A1 activity (AHH or EROD) in placenta samples of pregnant women who smoke (Pelkonen *et al*. 1979, Manchester and Jacoby 1981, Pasanen *et al*. 1988, Huel *et al*. 1989, Boden *et al*. 1995). PAHs found in cigarette smoke are potent aryl hydrocarbon receptor agonists and therefore regulate the expression of CYP1A1 in human placenta (Okey *et al*. 1994a, b, Hakkola *et al*. 1998). The range of values reported for smokers within a study is large and mean values are also quite different from one study to another. Furthermore, poor correlations have been reported

between placental EROD activity and either self-declared tobacco consumption or biomarkers of tobacco smoking such as thiocyanate or cotinine blood levels. In our study, we noted moderate to strong correlations between EROD activity and biomarkers of exposure such as placental cadmium concentration and meconium cotinine level. This may result from these markers being more specific of foeto-placental unit exposure than maternal blood markers.

Some women in the smoker group showed higher bulky DNA-adduct levels than non-smokers and mean values differed by close to a 4-fold factor between those groups. Several authors previously reported that total bulky DNA adduct levels are increased in placenta of women who smoked during pregnancy (Everson *et al*. 1986, 1988, Hansen *et al*. 1993, Topinka *et al*. 1997). The high variability in adduct levels observed in the smoker group as well as the small number of samples analysed preclude meaningful conclusions regarding the usefulness of this biomarker. Large inter-individual differences in the formation of DNA adducts may be due to numerous factors including genetic polymorphisms (biotransformation enzymes), dietary factors or antioxidant levels. The lack of standards allowing the positive identification of specific adducts related to compounds found in cigarette smoke further complicates the interpretation of total placental DNA adduct levels as a marker of exposure to cigarette smoke. Additional studies of larger sample size are needed to explore the usefulness of this marker in epidemiological studies.

Factors such as sensitivity, specificity, invasiveness of biological sampling and cost of analysis must be considered when selecting a biomarker for inclusion in epidemiological studies. Meconium and placenta are easily available biological materials compared with maternal or neonatal blood. Furthermore, levels of toxicants in meconium and placenta should better reflect foetal exposure than levels in maternal body fluids. Both placental EROD activity and cotinine level in meconium showed high specificity and sensitivity to identify women who smoked more than 10 cigarettes per day during pregnancy. Although placental cadmium concentration displays a lower sensitivity compared with the other two markers, methods to assay cadmium in biological tissues are relatively inexpensive and straightforward, which may represent an interesting trade-off in epidemiological studies conducted on very large samples.

In summary, cotinine in meconium was more sensitive and specific than placental cadmium as a biomarker of cigarette smoking during pregnancy. EROD activity in placenta was strongly correlated to both biomarkers of exposure. In addition, it was also a sensitive and specific biomarker of cigarette smoking during pregnancy. Furthermore, considering that this biomarker of early biological effect was previously linked to small birth weights in women who smoked during pregnancy, we recommend that placental EROD activity should be incorporated in epidemiological studies investigating developmental outcomes.

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References

ARNOULD, J. P., VERHOEST, P., BACH, V., LIBERT, J. P. and BELEGAUT, J. 1997, Detection of benzo[*a*]pyrene-DNA adducts in human placenta and umbilical cord blood. *Human and Experimental Toxicology,* **16**, 716–721.

BARANOWSKI, J., POCHOPIEN, G. and BARANOWSKA, I. 1998, Determination of nicotine, cotinine and caffeine in meconium using high-performance liquid chromatography. *Journal of Chromatography B Biomedical Sciences Application,* **707**, 317–321.

BODEN, A. G., BUSH, P. G., BURKE, M. D., ABRAMOVICH, D. R., AGGETT, P., MAYHEW, T. M. and PAGE, K. R. 1995, Human placental cytochrome P450 and quinone reductase enzyme induction in relation to maternal smoking. *Reproduction, Fertility and Development,* **7**, 1521–1524.

CHACKO, M. and GUPTA, R. C. 1988, Evaluation of DNA damage in the oral mucosa of tobacco users and non-users by ^{32}P-adduct assay. *Carcinogenesis*, **9**, 2309–2313.

COULTAS, D. B., HOWARD, C. A., PEAKE, G. T., SKIPPER, B. G., and SAMET, J. M. 1988, Discrepancies between self-reported and validated cigarette smoking in a community survey of New-Mexico Hispanics. *American Review of Respiratory Disease,* **137**, 810–814.

CUZICK, J., ROUTLEDGE, M. N., JENKINS, D. and GARNER, C. 1990, DNA adducts in different tissues of smokers and non-smokers. *International Journal of Cancer,* **45**, 673–678.

EDELMAN, D. A. and ROGGLI, V. L. 1989, The accumulation of nickel in human lungs. *Environmental Health Perspectives*, **81**, 221–224.

ESKENAZI, B. and BERGMANN, J. J. 1995, Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and post-natal smoke exposure. I. Effects on physical growth at age 5 years. *American Journal of Epidemiology*, **142**, S10–S18.

ESKENAZI, B. and TRUPIN, L. S. 1995, Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and post-natal smoke exposure. II. Effects on neurodevelopment at age 5 years. *American Journal of Epidemiology,* **142**, S19–S29.

EVERSON, R. B., RANDERATH, E., SANTELLA, R. M., CEFALO, R. C., AVITTS, T. A. and RANDERATH, K. 1986, Detection of smoking-related covalent DNA adducts in human placenta. *Science,* **231**, 54–57.

EVERSON, R. B., RANDERATH, E., SANTELLA, R. M., AVITTS, T. A., WEINSTEIN, I.B. and RANDERATH, K. 1988, Quantitative associations between DNA damage in human placenta and maternal smoking and birth weight. *Journal of the National Cancer Institute*, **80**, 567–576.

GUEGUEN, C., LAGRUE, G. and JANSE-MAREC, J. 1995, Retentissement, sur le fetus et l'enfant, du tabagisme pendant la grossesse. *Journal de Gynécologie, Obstétrique, et Biologie de la Reproduction,* **24**, 853–859.

GUPTA , R. C. 1996, ^{32}P-postlabeling for detection of DNA adducts. In *Technologies for Detection of DNA Damage and Mutations*, G.P. Pfeifer, ed. (New York: Plenum Press), pp. 45–61.

HAKKOLA, J., PELKONEN, O., PASANEN, M. and RAUNIO, H. 1998, Xenobiotic-metabolizing cytochrome p450 enzymes in the human feto-placental unit: role in intrauterine toxicity. *Critical Reviews in Toxicology,* **28**, 35–72.

HANSEN, C., ASMUSSEN, I. and AUTRUP, H. 1993, Detection of carcinogen–DNA adducts in human fetal tissues by the 32P-postlabeling procedure. *Environmental Health Perspectives,* **99**, 229–231.

HAUFROID, V. and LISON, D. 1998, Urinary cotinine as a tobacco-smoke exposure index: a minireview. *International Archives of Occupational and Environmental He*alth, **71**, 162–168.

HOUK, R S. 1986, Mass spectrometry of inductively coupled plasma. *Analytical Chemistry*, **58**, 97A-105A

HUEL, G., GODIN, J., MOREAU, T., GIRARD, F., SAHUQUILLO, J., HELLIER, G. and BLOT, P. 1989, Aryl hydrocarbon hydroxylase activity in human placenta of passive smokers. *Environmental Research,* **50**, 173–187.

JARVIS, M. J., TUNSTALL-PEDOE, H., FEYERABEND, C., VESEY, C. and SALOOJEE, Y. 1987, Comparison of tests used to distinguish smokers from non-smokers. *American Journal of Public Health,* **77**, 1435–1438.

KENNEDY, S. W., JONES, S. P. and BASTIEN, L. J. 1995, Efficient analysis of cytochrome P4501A catalytic activity, porphyrins and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Analytical Biochemistry,* **226**, 362–370.

KLEBANOFF, M. A., LEVINE, R. J., CLEMENS, J. D., DERSIMONIAN, R. and WILKINS, D. G. 1998, Serum cotinine concentration and self-reported smoking during pregnancy. *American Journal of Epidemiology,* **148**, 259–262.

KOZLOWSKI, L. T., MEHTA, N. Y., SWEENEY, C. T., SCHWARTZ, S. S., VOGLER G. P., JARVIS, M. J. and WEST, R. J. 1998, Filter ventilation and nicotine content of tobacco in cigarettes from Canada, the United Kingdom, and the United States. *Tobacco Control*, **7**, 369–375.

KUHNERT, P. M., KUHNERT, B. R., BOTTOMS, S. F. and ERHARD, P. 1982, Cadmium levels in maternal blood, fetal cord blood, and placental tissues of pregnant women who smoke. *American Journal of Obstetrics and Gynecology*, **142**, 1021–1025.

KUHNERT, B. R., KUHNERT, P. M., DEBANNE, S. and WILLIAMS, T. G. 1987, The relationship between cadmium, zinc and birthweight in pregnant women who smoke. *American Journal of Obstetrics and Gynecology,* **157**, 1247–1251.

LAGERKVIST, B. I., SANBERG, S., FRECH, W., JIN, T. and NORDBERG, G. F. 1996, Is placenta a good indicator of cadmium and lead exposure? *Archives of Environmental Health*, **51**, 389–394.

LAGUEUX, J., AFFAR, E. B., NADEAU, D., AYOTTE, P., DEWAILLY, E. and POIRIER, G. G. 1997, A microassay for the detection of activities with alkoxyresorufin substrates. *Molecular and Cellular Biochemistry,* **175**, 125–129.

LAGUEUX, J., PEREG, D., AYOTTE, P., DEWAILLY, E. and POIRIER, G. G. 1999, Cytochrome p450 CYP1A1 enzyme activity and DNA adducts in placenta of women environmentally exposed to organochlorines. *Environmental Research*, 80, 369–82.

LEWIS, D. E. 1994, Forensically acceptable determination of gestational fetal exposure to drugs and other chemical agents. United States Patent 5326708.

LEWTAS, J., MUMFORD, J., EVERSON, R. B., HULKA, B., WILKOSKI, T., KOZUMBO, W., THOMPSON, C., GEORGE, M., DOBIAS, L., SRAM, R., LI, X. and GALLAGHER, J. 1993, Comparison of DNA adducts from exposure to complex mixtures in various human tissues and experimental systems. *Environmental Health Perspectives*, **99**, 89–97.

MANCHESTER, D. K. and JACOBY, E. H. 1981, Sensitivity of human placental monooxygenase activity to maternal smoking. *Clinical Pharmacology and Therapeutics*, **30**, 687–692.

NUTTAL, K. L., GORDON, W. H. and ASH, K. O. 1995, Inductively coupled plasma mass spectrometry for trace element analysis in the clinical laboratory. *Annals of Clinical and Laboratory Science*, **25**, 264–271.

OKEY, A. B., RIDDICK, D. S. and HARPER, P. A. 1994a, Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends in Pharmacological Sciences*, **15**, 226–232.

OKEY, A. B., RIDDICK, D. S. and HARPER, P. A. 1994b, The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicology Letters*, **70**, 245–248.

OSTREA, E. M., KNAPP, D. K., ROMERA, A., MONTES, M. and OSTREA, A. R. 1994, Meconium analysis to assess fetal exposure to nicotine by active and passive maternal smoking. *Journal of Pediatrics,* **124**, 471–476.

PAAKKO, P., KOKKONEN, P., ANTTILA, S. and KALLIOMAKI, P. 1989, Cadmium and chromium as markers of smoking in human lung tissue. *Environmental Research,* **49**, 197–207.

PASANEN, M., VAHAKANGAS, K., SOTANIEMI, E. A. and PELKONEN, O. 1988, The effect of cigarette smoking on drug metabolism in the liver and placenta: the use of cotinine in verifying smoking status. *European Journal of Drug Metabolism and Pharmacokinetics,* **13**, 41–45.

PEACOCK, J. L., COOK, D. G., CAREY, I. M., JARVIS, M. J., BRYANT, A. E., ANDERSON, H. R. and BLAND, J. M. 1998, Maternal cotinine level during pregnancy and birthweight for gestational age. *International Journal of Epidemiology*, **27**, 647–656.

PELKONEN, O., KARKI, N. T., KOIVISTO, M., TUIMALA, R. and KAUPPILA, A. 1979, Maternal cigarette smoking, placental aryl hydrocarbon hydroxylase and neonatal size. *Toxicology Letters*, **3**, 331–335.

PHILLIPS, D. H., HEWER, A., MARTIN, C. N., GARNER, R. C. and KING, M. M. 1988, Correlation of DNA adduct levels in human lung with cigarette smoking. *Nature,* **336**, 790–792.

PHILLIPS, D. H., SCHOKET, B., HEWER, A., BAILEY, E., KOSTIC, S. and VINCZE, I. 1990, Influence of cigarette smoking on the levels of DNA adducts in human bronchial epithelium and white blood cells. *International Journal of Cancer*, **46**, 569–575.

RANDERATH, K., REDDY, M. V. and GUPTA, R. C. 1981, ^{32}P-Labelling test for DNA damage. *Proceedings of the National Academy of Sciences of the United States of America*, **78**, 6126–6129.

RANDERATH, K., MILLER, R. H., MITTAL, D. and RANDERATH, E. 1988, *Monitoring human exposure to carcinogens by ultrasensitive postlabelling assays: application to unidentified genotoxicants.* IARC Scientific Publications, No.89 (Lyon: IARC), pp. 361–367.

RANDERATH, E., MILLER, R. H., MITTAL, D., AVITTS, T. A., DUNSFORD, H. A. and RANDERATH, K. 1989, Covalent DNA damage in tissues of cigarette smokers as determined by ^{32}P-postlabelling assay. *Journal of the National Cancer Institute,* **81**, 341–347.

REY, M., TURCOTTE, F., LAPOINTE, C. and DEWAILLY, E. 1997, High blood cadmium levels are not associated with consumption of traditional food among the Inuit of Nunavik. *Journal of Toxicology and Environmental Health,* **51**, 5–14.

ROELS, H., HUBERMONT, G., BUCHET, J. P. and LAUWERYS, R. 1978, Placental transfer of lead, mercury, cadmium and carbon monoxide in women. *Environmental Research*, **16**, 236–247.

SHAHAM, J., MELTZER, A., ASHKENAZI, R. and RIBAK, J. 1996, Biological monitoring of exposure to cadmium, a human carcinogen, as a result of active and passive smoking. *Journal of Occupational and Environmental Medicine*, **38**, 1220–1228.

TOPINKA, J., BINKOVA, B., MRACKOVA, G., STAVKOVA, Z., PETERKA, V., BENES, I., DEJMEK, J., LENICEK, J., PILCIK, T. and SRAM, R. J. 1997, Influence of GSTM1 and NAT2 genotypes on placental DNA adducts in an environmentally exposed population. *Environmental and Molecular Mutagenesis,* **30**, 184–195.

WALSH, R. A. 1994, Effects of maternal smoking on adverse pregnancy outcomes: examination of the criteria of causation. *Human Biology,* **66**, 1059–1092.

WATANABE, T., KASAHARA, M., NAKATSUKA, H. and IKEDA, M. 1987, Cadmium and lead contents of cigarettes produced in various areas of the world. *Science of the Total Environment,* **66**, 29–37.