



Recent Developments in Molecular Epidemiology

A Study of the Effects of Environmental Polycyclic Aromatic Hydrocarbons on Birth Outcomes in Poland

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The authors briefly review the current potential and limitations of molecular epidemiology. This approach uses biomarkers to measure the internal and bioeffective dose of toxicants, early biologic effects likely to be predictive of cancer, and variations in individual susceptibility. The most frequent application of biomarkers has been in assessment of exposure/dose and susceptibility due to genetic and nongenetic factors. More research is needed to establish the predictive significance of biomarkers in terms of disease risk. To illustrate that molecular epidemiology has potential in identifying etiologic factors in disease, this article presents data from a recent study of the developmental effects of fetal exposure to polycyclic aromatic hydrocarbons (PAH) via ambient pollution. The study was carried out in an industrialized area of Poland with relatively high levels of PAH pollution from coal burning. PAH-DNA adducts in leukocytes and plasma cotinine were measured in umbilical cord blood as dosimeters of transplacental PAH and cigarette smoke, respectively. The study subjects were 70 newborns from the industrialized city of Krakow and 90 newborns from Limanowa, a rural town with far greater use of coal for home heating. Newborns whose levels of PAH-DNA adducts were above the median (3.85/10⁸ nucleotides) had a significantly decreased birth length, weight, and head circumference. Cotinine was significantly inversely associated with birth weight and length. Although preliminary, these results provide a new molecular link between PAH exposure and developmental effects, generating initial data and hypotheses for further study. *Am J Epidemiol* 1998;147:309–14.

biological markers; environmental exposure; epidemiology, molecular; polycyclic hydrocarbons, aromatic; pregnancy outcome

Since 1982, with the initial article setting out the conceptual framework for integrating molecular measurements into epidemiologic research on cancer (1), molecular epidemiologic approaches have been applied to the study of many other diseases, including reproductive and developmental disorders (2–4). The 1982 paper discussed the potential of measuring biomarkers (molecular alterations or variants) in human samples to document individual exposure or dose, early biologic response or preclinical effect, and host susceptibility to carcinogens. It also stressed the need for validation of methods. Up to the present, the categorization of biomarkers has remained essentially

unchanged; but the need for adequate validation of molecular techniques prior to their use in epidemiologic studies or in screening has been more widely recognized (3, 5, 6). During the past 15 years, molecular epidemiology has been variously perceived as a new field, an advanced form of clinical epidemiology, or a subdiscipline of public health epidemiology (5, 7). Although the label has stuck, and many schools of public health and research institutes now have programs in “molecular epidemiology,” the most common view is that the approach represents a natural convergence of molecular biology and epidemiology and that it should remain broad in scope—owned by no one discipline—for the furtherance of disease prevention.

There are three major areas of disease prevention to which this approach can potentially contribute, working in conjunction with more established methods of laboratory testing, monitoring, and epidemiology. They are 1) the identification of etiologic factors and disease mechanisms, 2) modeling of the distribution of exposure and susceptibility within the population,

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Abbreviations: PAH, polycyclic aromatic hydrocarbons; B(a)P, benzo(a)pyrene.

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and 3) the design and monitoring of interventions (5, 8).

Most of the research carried out to date has used biomarkers to characterize exposure—or more specifically the internal or biologically effective dose of toxicants—or to control for confounding effects. Increasingly, biomarkers are being used to investigate the modulation of exposure and risk by genetic and other susceptibility factors. These studies have raised a host of methodological problems of a technological, epidemiologic, and ethical nature (6). While a goal is to substitute early preclinical response markers for disease endpoints in etiologic studies and interventions, there are few cases where the predictive significance of biomarkers has been established through the needed prospective or nested case-control studies. This remains a gap in the field.

The study described here illustrates the application of molecular epidemiology to gain a better understanding of the role of in utero environmental exposures (tobacco smoke and air pollution) in developmental impairment. It was intended to generate hypotheses for further research in an area that has proven elusive. To complement environmental monitoring and questionnaire data, biomarkers were used to estimate the individual dose of toxicants to the fetus (9). The biochemical and molecular dosimeters used to measure transplacental exposure were cotinine in umbilical cord blood, reflecting cigarette smoke exposure, and DNA adducts in cord blood leukocytes formed by PAH. The research illustrates both the advantages and the limitations of molecular epidemiology.

Krakow, Poland, is an industrialized city with relatively high levels of PAH in air pollution attributed to multiple sources, especially coal burning for industrial purposes and residential heating (10, 11). PAH exposure can result from inhalation of polluted air and from the diet (ingestion of grilled and smoked foods and vegetables grown in contaminated areas). Pollution levels are highest in the city center and decrease toward the periphery. We estimate that in 1991, the year preceding the birth of the newborns in this study, the women living in Krakow were exposed to annual average ambient concentrations of respirable particulates ranging from $37 \mu\text{g}/\text{m}^3$ for the least exposed group to $78 \mu\text{g}/\text{m}^3$ for the most exposed. The corresponding concentrations of benzo(a)pyrene (B(a)P), an indicator PAH, were estimated to be $7 \text{ ng}/\text{m}^3$ and $15 \text{ ng}/\text{m}^3$, representing approximately 0.02 percent of particulate matter (12). During 1995, annual average concentrations of B(a)P were estimated at $4.2 \text{ ng}/\text{m}^3$ and $9.2 \text{ ng}/\text{m}^3$ in the areas of Krakow with the lowest and highest particulate levels, respectively.

Limanowa, Poland, is in a rural agricultural district 70 km southeast of Krakow. In Limanowa, levels of respirable particulates were estimated to be two- and fivefold lower than those in the least and most polluted areas of Krakow, respectively (12). However, use of coal stoves for indoor home heating (which can emit substantial amounts of PAH (10)) was twice as frequent in Limanowa as in Krakow (12).

Experimental bioassays have shown a number of PAH to be transplacental carcinogens and to be associated with adverse reproductive outcomes, including decrements in fetal weight (13–16). For example, administration of B(a)P or coal liquefaction products containing high levels of PAH to pregnant rats caused significant decreases in fetal weight (15, 16), with effects capable of persisting into the postnatal period (14). PAH-DNA adducts represent the net effect of exposure, absorption, activation, detoxification, and repair and have been widely used to measure the individual biologically effective dose of PAH (8, 17). The adducts have an estimated half-life of approximately 9–23 weeks in leukocytes (18). Plasma cotinine is a validated internal dosimeter for tobacco smoke (19).

The current study was part of an ongoing research project evaluating multiple effects of environmental PAH on Polish women and newborns (12). As was reported previously, despite higher exposure to ambient PAH, mothers and newborns from Krakow did not differ significantly from those in Limanowa with respect to levels of PAH-DNA adducts in leukocytes, possibly because of greater coal use for home heating in Limanowa, after analyses controlled for dietary sources of PAH (20). However, among Krakow subjects for whom ambient exposure estimates were considered most reliable (those women not employed away from home), there was a significant dose-response relationship between estimated air pollution concentrations at the women's residences and PAH-DNA adduct levels in both maternal and newborn leukocytes (20). Newborn adduct levels were somewhat higher than paired maternal levels, despite experimental evidence that transplacental PAH exposures are 10-fold lower than paired maternal exposures. Here we have examined associations between fetal development and PAH-DNA adduct levels in the newborns as a dosimeter of transplacental PAH. Differences in birth outcomes between newborns from Krakow and Limanowa were also investigated.

MATERIALS AND METHODS

Subjects

Subjects were enrolled during January–March 1992 and included 70 mothers and newborns from Krakow

and 90 mothers and newborns from Limanowa. Enrollment alternated biweekly between Krakow and Limanowa to control for monthly variation in ambient pollutant levels, and was restricted to vaginal deliveries and to women who had resided in Krakow or Limanowa for at least 1 year. Detailed questionnaire and medical record data were collected on environmental exposures and known or potential risk factors for developmental impairment (12). A sample of umbilical cord blood was collected at delivery (20–60 ml) and was processed and stored as described previously (12).

Laboratory assays

DNA was extracted from umbilical cord leukocytes, and PAH-DNA adduct levels were measured by a competitive enzyme-linked immunosorbent assay with fluorescence endpoint detection, essentially as described previously (21). The antiserum recognizes multiple, structurally related PAH diol-epoxide-DNA adducts (22). Values are expressed as the amount of B(a)P diol-epoxide-DNA that would cause similar inhibition in the assay. The quantity of DNA was adequate to measure PAH-DNA adducts in 135 umbilical cord blood samples (58 from Krakow and 77 from Limanowa). Plasma cotinine was measured in cord blood samples of all newborns by gas chromatography, as described elsewhere (12). The estimated half-life of plasma cotinine in nonsmoking adults is 48 hours; in the chronic exposure situation, this marker is a good reflection of daily uptake of nicotine (23, 24).

Statistical analysis

Associations between the outcome variables (birth weight, birth length, and head circumference) and newborn PAH-DNA adduct levels were analyzed by multiple linear regression and analysis of covariance in the Krakow and Limanowa groups, separately and combined. Levels of PAH-DNA adducts were analyzed both as a dichotomous variable—high (>median) versus low (\leq median)—and as a continuous measure. The regression models included covariates representing known or potential confounders of fetal development that were correlated with the birth outcomes ($p < 0.2$) and/or varied significantly between newborns from Krakow or Limanowa or with high versus low levels of adducts. Models controlled for maternal height, age, socioeconomic status (maternal educational level of ≤ 11 years vs. > 11 years), history of low birth weight (yes/no), gestational age (weeks), sex of the newborn, and whether or not the mother drank alcohol regularly during the pregnancy (≥ 1 drink/week during ≥ 1 trimester). Newborn plasma cotinine

level (ng/ml) was included rather than self-reported maternal smoking status, since the biomarker explained more of the variance in fetal development. When regression analyses were conducted controlling for maternal prepregnancy body mass index rather than maternal height, results were essentially unchanged from those reported here.

RESULTS

Birth outcomes and demographic data for Krakow and Limanowa subjects are presented in table 1. Compared with newborns from Limanowa, Krakow newborns had a lower mean birth weight and a significantly lower birth length and head circumference, before (table 1) and after data were controlled for maternal height, age, socioeconomic status, history of low birth weight, alcohol consumption, gestational age, sex of the newborn, and plasma cotinine.

Table 2 presents differences in birth outcomes for newborns with high versus low levels of PAH-DNA adducts. In both the Krakow and Limanowa groups, analyzed separately, all three measures (birth weight, birth length, and head circumference) were decreased in newborns with high adduct levels, although the differences were not significant in all cases. Among Krakow newborns, those with elevated PAH-DNA adduct levels had a significantly decreased birth length (by 1.8 cm, $p = 0.02$) and head circumference (by 0.9 cm, $p = 0.05$). Among Limanowa newborns, those

TABLE 1. Distribution of 160 newborns from two Polish cities according to birth outcomes and demographic variables, 1992

	Krakow	Limanowa
No. of mother-newborn pairs	70	90
Newborn birth weight (g)	3,328 (516)*	3,378 (464)
Newborn birth length (cm)	54.0 (3.0)†	55.5 (2.9)
Newborn head circumference (cm)	34.1 (1.5)†	34.7 (1.6)
Maternal age (years)	27.6 (5.3)†	25.4 (4.1)
Newborn plasma cotinine level (ng/ml), by maternal smoking status		
Current smoker	75.0 (n = 12)‡	63.7 (n = 4)
Ex-smoker	15.6 (n = 20)	20.1 (n = 18)
Nonsmoker	3.7 (n = 38)	4.1 (n = 68)
No. of mothers with ≤ 11 years of education (socioeconomic status)	23†	56
Maternal height (cm)	164.9 (5.0)†	162.5 (5.5)
Maternal prepregnancy weight (kg)	59.8 (8.8)	60.1 (10.3)
Maternal prepregnancy weight gain (kg)	15.5 (15.1)	13.0 (10.4)
No. of nulliparous mothers	31	41
No. of mothers with a prior history of low birth weight (<2,500 g)	4	4
Gestational age (weeks)	39.8 (1.5)	39.7 (1.4)
Sex of newborn (% female)	46	51
Maternal alcohol consumption (no. who consumed ≥ 1 drink/week in ≥ 1 trimester)	12	11

* Numbers in parentheses, standard deviation.

† $p \leq 0.02$ for Krakow vs. Limanowa (Student's *t* test for birth length, head circumference, age, and height; χ^2 for smoking status and educational level).

‡ Number of smokers.

TABLE 2. Difference in birth outcomes for Polish newborns with high (>median) versus low (≤median) leukocyte levels of PAH*-DNA adducts, 1992†

	Birth weight (g)		Birth length (cm)		Head circumference (cm)	
	Difference	<i>p</i> value	Difference	<i>p</i> value	Difference	<i>p</i> value
Krakow newborns (<i>n</i> = 58)	-205	0.11	-1.8	0.02	-0.9	0.05
Limanowa newborns (<i>n</i> = 77)	-129	0.16	-0.8	0.17	-1.2	0.0004
All newborns	-147	0.05	-1.1	0.02	-0.9	0.0005

* PAH, polycyclic aromatic hydrocarbons.

† Analyses controlled for maternal height, age, socioeconomic status (educational level), history of low birth weight, maternal alcohol consumption, gestational age (weeks), sex of the newborn, and plasma cotinine (ng/ml). Median adduct level, 3.85/10⁶ nucleotides.

with elevated adduct levels had a significantly decreased head circumference (by 1.2 cm, $p = 0.0004$). Considering all of the newborns combined (Krakow and Limanowa), those with high levels of PAH-DNA adducts had a significantly lower birth weight ($p = 0.05$), birth length ($p = 0.02$), and head circumference ($p = 0.0005$) than newborns with low adduct levels (table 2).

After combining the two groups and removing current smokers in order to investigate the effects of PAH in the absence of active smoking by the mother, we found that all three measures of fetal development remained significantly lower among those with high adduct levels versus low levels ($p \leq 0.03$). To remove the effect of both active and passive smoking, we further restricted the analyses to the 53 newborns of nonsmokers without detectable plasma cotinine. The newborns with elevated adduct levels had a decreased birth weight (by 217 g, $p = 0.1$) and length (by 0.8 cm, $p > 0.2$) and a significantly decreased head circumference (by 1.3 cm, $p = 0.006$).

When levels of PAH-DNA adducts among all of the newborns were included in the regression models as a continuous variable, they were inversely, though not significantly, correlated with birth weight and length. Adduct levels were inversely correlated with head circumference both before ($p = 0.006$) and after data were controlled for birth weight ($p = 0.003$), suggesting asymmetrical growth retardation.

Consistent with prior research (25), an inverse correlation was seen between newborn plasma cotinine level (ng/ml) and birth weight ($p = 0.0001$, after controlling for place of residence and the other potential confounders). In this study, cotinine was also inversely associated with birth length ($p = 0.003$). The effect of cotinine on birth outcomes was independent of that of PAH-DNA adducts.

DISCUSSION

These findings suggest that transplacental exposure to PAH from multiple environmental sources may

compromise fetal development. They are consistent with previous ecologic data indicating that birth outcomes are worse in industrialized, contaminated regions of Poland (26) and with experimental studies indicating that certain PAH are developmental toxicants (13, 15).

The results do not imply that developmental damage is necessarily mediated by DNA binding, although that is one possible mechanism. Here the extent of DNA binding by PAH in newborn leukocytes was used as a dosimeter of PAH that have reached the fetus. Neither the mechanisms by which PAH exert developmental toxicity nor the target sites have been identified. Indeed, it is possible that PAH act by more than one mechanism. For example, it has been hypothesized that B(a)P exposure may interfere with uterine growth during pregnancy because of its antiestrogenic effects, thereby disrupting the endocrine system (15). Similar to polychlorinated biphenyls, which are associated with deficits in fetal growth and intelligence quotient (27, 28), PAH bind to the human Ah receptor to induce P450 enzymes (29). Additionally, the developing central nervous system appears to be particularly sensitive to DNA-damaging agents (30), and it may respond by activating apoptotic pathways (31, 32). For example, in humans, fetal microcephaly has been seen following exposure to ionizing radiation (33) and anticonvulsant drugs (34). Risk from anticonvulsants was most pronounced in infants deficient in enzymes that detoxify the DNA-binding intermediate (35, 36). It is of interest that, in the present study, PAH-DNA adduct levels were most strongly associated with reduction in head circumference; and the data were suggestive of asymmetrical growth retardation related to DNA binding.

The growth retardation associated in this study with high levels of PAH-DNA adducts (147 g in weight, 1.1 cm in length, and 0.9 cm in head circumference) is in the range of that which has been linked to adverse human health sequelae of exposure to other developmental toxicants, including maternal smoking, cocaine, and polychlorinated biphenyls (19, 27, 28, 37).

Weight at birth is a predominant determinant of infant health, and low birth weight is a major cause of infant mortality (19). Head circumference has been correlated with brain size, intelligence quotient, and cognitive function (38). A reduction in head circumference of 1–2 cm at birth has been associated with reduced mental and psychomotor development in early childhood (37, 39).

In the present study, the combination of known risk factors and PAH-DNA adducts does not explain the observed difference in birth outcomes between Krakow and Limanowa. It is possible that unmeasured factors of urbanization, including perhaps constituents of air pollution other than PAH, are contributing to developmental deficits in Krakow. For example, in northern Bohemia (Czech Republic), airborne sulfur and nitrogen oxides have been associated with low birth weight and small size for gestational age (40), while in China, an association was seen with sulfur oxides and particulates (41).

Strengths of this study include the detailed questionnaire monitoring data, the ability of biomarkers to measure individual doses of PAH and tobacco smoke, and the control of known risk factors associated with fetal development, including smoking and alcohol drinking by the mother. Limitations include the one-time-only measurement of biomarkers, the lack of personal monitoring of the women during pregnancy, and our inability to draw conclusions about mechanisms. Although we were unable to evaluate the relation of the molecular dose of PAH to leukocytes versus the molecular dose of PAH to target tissue, prior research indicates that, in humans, PAH are distributed systemically and form adducts in numerous tissues, including those in the developing fetus (42, 43). We were also unable to evaluate the effect of timing of exposure on the associations observed in this study. Given their estimated half-life in leukocytes of 9–23 weeks (18), PAH-DNA adducts measured in newborns will largely reflect exposures incurred during the latter half of pregnancy.

To our knowledge, this is the first molecular epidemiologic evidence that transplacental PAH adversely affect fetal development. However, the molecular link seen here does not prove causality and should be considered preliminary. There exists the potential for confounding by other unmeasured constituents of air pollution and diet. Further study is warranted by the potential implications for public health, given widespread exposure to PAH during pregnancy and earlier results from this study (20) indicating that infants receive a high biologic dose of PAH relative to mothers. PAH levels comparable to those seen in Poland are found in other industrialized areas of the world, but

in most Western countries they are considerably lower. Although prior data do not indicate a threshold for PAH-DNA adduct formation (10), the effect of lower levels of PAH on human fetal development is unknown.

In conclusion, although the results are preliminary and require confirmation, the study illustrates the potential benefits of using biomarkers in the identification of environmental etiologic factors. They support continued commitment to the development and use of this approach in disease prevention.

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