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Final Report

Molecular Epidemiology of Severe Ambient Air Pollution on Women and the Developing Fetus

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I. Specific Aims

The research goal is validation of a number of promising biomarkers in two groups of Polish women and their newborn infants: 70 mother/child pairs from Krakow, a city with elevated air pollution and 90 pairs from Limanowa, a less polluted area. Specifically, we are evaluating:

1) the relationship between ambient exposures and the following markers of biologically effective dose and biologic response: PAH-DNA adducts by enzyme-linked immunosorbent assay (ELISA), aromatic-DNA adducts by ^{32}P-postlabeling and the frequency of gene mutation at the hprt locus in T-lymphocytes;

2) the relationship between markers of biologically effective dose (adducts) and markers of biologic response (gene mutation);

3) the comparison of the same biomarkers in maternal and fetal/newborn tissues to assess possible differences in response and susceptibility;

4) the ability of genetic/metabolic markers (CYP1A1 mRNA, CYP1A1 enzyme activity, CYP1A1 MspI polymorphism and glutathione-S-transferase (GSTM1) genotype) to modify markers of biologically effective dose and biologic response, including an estimate of host versus environmental contributions;

5) the relationship between biomarkers (PAH-DNA adducts and serum cotinine) to birth weight.

These specific aims remain essentially unchanged from those stated in our original application.

II. Study design:

The study included 320 subjects: 70 mother/newborn pairs from Krakow and 90 mother/newborn pairs from Limanowa, a small town in a rural agricultural district of Poland, with lower ambient pollution but heavier use of coal for residential heating (1). Enrollment was restricted to women who had resided in Krakow or Limanowa for at least 1 year and was limited to vaginal deliveries. Enrollment alternated on a bi- weekly basis between Krakow and Limanowa in the winter of 1992 to control for monthly variations in pollutant levels. Immediately after delivery, samples of umbilical cord blood and placenta were collected. A maternal blood sample was collected within 2 day postpartum. Samples were processed and stored as described (1).

A detailed validated questionnaire administered to the mother within 2 days postpartum included information on smoking, residential and employment histories, use of coal stoves for residential heating, and other environmental exposures. Subjects were asked to estimate the average number of weekly servings of specific foods consumed during pregnancy, such as smoked meats, cheese and fish, as potentially high dietary sources of PAH. In addition, subjects were asked about exposure to sources of PAH either at home or in the workplace (including coal tar, charcoal, tar roofing material and asphalt) as well as pesticides and other organic chemicals as potential inducers of CYP1A1. All interviews were conducted by 2 trained interviewers from the Department

of Epidemiology and Preventive Medicine, University Medical School, Krakow. Coded interview data were sent to Columbia University. Assessment of smoking status was based on questionnaire data, with plasma cotinine (a marker of recent cigarette smoke exposure) (2) used to verify questionnaire data (1).

Daily ambient monitoring data for Krakow (1990 - 1992) were provided by the Division of National Sanitary Inspection (15 monitoring stations) and by the U.S. EPA (5 monitoring stations). Each Krakow woman's exposure to ambient particulates was estimated by taking the average of PM_{10} measurements (in μg/m3) reported at the monitoring station closest to her residence for each of the past 2 years and the month prior to her delivery date. Ambient particulate data were available for 69/70 subjects from Krakow. As there was only 1 ambient monitoring station in Limanowa, individual ambient exposures could not be estimated for Limanowa subjects.

A stepwise approach was taken in analyzing the effects of ambient air pollution on biomarker levels (3). First, the difference in biomarker levels between residents of Krakow versus Limanowa was determined. Additionally, Krakow subjects were trichotomized into low, medium and high pollution groups based on ambient PM_{10} level at their place of residence. The difference in biomarker levels across pollution groups was determined for all Krakow subjects and for Krakow subjects not employed away from home during pregnancy. Estimates of exposure for the latter group are more reliable since unemployed women spend more of their time at their place of residence.

For statistical analyses, biomarkers were log-transformed as needed to stabilize the variance and obtain a more symmetrical distribution. Associations were initially evaluated by Chi-square and Student's t-test followed by multivariate analyses. The regression models included cigarette smoke exposure, place of residence (Krakow versus Limanowa) or ambient pollution group (high, middle low; Krakow only), average number of servings per week during pregnancy of foods high in PAH (smoked meat, cheese and fish), use of coal stoves for residential heating and home/occupational exposures to PAH and other organics.

III. **Results**

1. Analysis of the relationship between ambient exposures and biomarkers:

a. Adducts and HPRT: After controlling for smoking status and other potential confounders, overall there was no significant difference in PAH-DNA adduct levels measured by ELISA in mothers and infants from Krakow compared to Limanowa, possibly because of higher indoor air concentrations of PAH from coal burning in Limanowa. While coal use (yes/no) was controlled in the multivariate analyses, more precise measures of use (duration, amount of time spent in rooms heating by coal stoves) were not obtained. When we restricted analysis to the non-coal users, adduct levels in maternal white blood cells (WBC) were significantly higher (about 2-fold) in Krakow compared to Limanowa ($P=0.03$, Student's t-test).

In Krakow, among subjects for whom the exposure estimations were most reliable (those women not employed away from the home) a dose-response relationship was seen between PAH-DNA adduct levels in maternal and infant WBC (but not placental tissue)

and increasing ambient PM_{10} pollution at the woman's residence ($p< 0.05$, controlling for smoking status and other potential confounders) (4-5). Specifically, the trend for increasing maternal WBC adduct levels with increasing ambient air pollution groups was statistically significant ($p = 0.02$). Further, adduct levels were significantly increased in women residing in the high compared to low pollution area ($\beta = 1.77$, $p = 0.05$). Among infants of women not employed away from home, adduct levels in infant cord WBC were also significantly increased among those residing in both the middle ($\beta = 1.3$, $p = 0.05$) and high ($\beta = 1.7$, $p = 0.03$) compared to the low pollution area.

Maternal WBC PAH-DNA adduct levels were also significantly associated with active cigarette smoking status ($\beta = 0.85$, $p < 0.01$) and, among nonsmokers, were significantly higher in subjects reporting ETS exposure compared to those reporting no ETS exposure ($\beta = 0.58$, $p = 0.01$) (4-5). In addition, maternal adducts were positively associated with self-reported number of cigarettes per day of passive exposure during pregnancy ($\beta = 0.02$, $p = 0.07$. The relationship between cigarette smoke exposures and adduct levels in infant cord WBC and placental tissue were nonsignificant or inverse, possibly because of modulation by placental enzymes (4-6). There was significant interindividual variability in adduct levels among the women and their newborns (30-40 fold) consistent with other studies.

Although the adduct levels measured by postlabeling were significantly higher in the infants compared to the mothers ($P<0.01$), the other relationships seen with PAH-DNA adducts by ELISA were not significant for adducts measured by ^{32}P-postlabeling. Further, carcinogen-DNA adducts measured by the ^{32}P-postlabeling method were not correlated with those by ELISA, suggesting that the methods detect a different spectrum of adducts. These results are in contrast to those from our previous study in Polish adults, in which a significant correlation was seen between adduct levels measured by ELISA and adducts measured by ^{32}P-postlabeling using a different postlabeling methodology (7). The current postlabeling method uses lower concentrations of radiolabeled ATP (8).

Preliminary analyses of results for HPRT mutation on 67 mothers and 64 infants, including 47 mother/infant pairs, have been completed. There was a significant correlation between cloning efficiency and mutant frequency in both mothers and infants. Possibly due to differing lifetimes of the biomarkers, HPRT mutation frequency was not correlated with adduct levels measured by either postlabeling or ELISA in mothers or infants. Results of the multiple regression analyses are being reviewed.

2. Analysis of the ability of genetic/metabolic markers to modify markers of biologically effective dose.

a.CYP1A1 MRNA levels and EROD activity in placental tissue: Both placental CYP1A1 MRNA and EROD activity were significantly associated with active cigarette smoking status and highly correlated with infant plasma cotinine levels ($p < 0.001$) (1-3). Ex-smokers also had significantly increased placental CYP1A1 MRNA levels compared to nonsmokers. Among nonsmokers placental EROD activity was increased with exposure ($p = 0.05$). A significant association between ambient pollution at the women's place of residence within Krakow and placental CYP1A1 MRNA was seen among subjects not employed away from home. Placental CYP1A1 MRNA, but not EROD

activity, also increased significantly with dietary PAH. PAH-DNA adduct levels in both placental tissue and infant WBC were not significantly associated with placental CYP1A1 MRNA. In samples with detectable EROD activity the relationship between EROD activity and WBC adduct levels was significant and inverse ($r = -0.34$, $p = 0.05$). The correlation between CYP1A1 MRNA and EROD activity was positive and significant ($r = 0.36$, $p < 0.001$) (3-4). These results suggest that CYP1A1 activity in placental tissue reduces DNA damage in the infant.

b.Analysis of genetic variants and adducts:

GSTM1 is expressed rarely and only at low levels in fetal tissues, so infant GSTM1 genotype was not analyzed. Genotyping at the GSTM1 locus was completed for 143/160 maternal samples. Of these, 72 (50%) were homozygous deleted (GSTM1-/-). The remaining 71 (50%) of the women had 1 or 2 copies of the gene (GSTM1+/+, +/-).

Determination of the CYP1A1 MspI RFLP was completed for 142/160 maternal samples. Of these, 24 (17%) were heterozygous for the restriction site (CYP1A1 MspI+/-) and 118 (83%) did not have the restriction site (CYP1A1 MspI-/-). None of the women were homozygous for the restriction site (CYP1A1 MspI+/+). Determination of the CYP1A1 MspI RFLP was completed on 158/160 infants (140/160 umbilical cord DNA samples and 149/160 placental DNA samples). Of these, 29 (18%) were heterozygous (CYP1A1 MspI-/+) and 3 (2%) were homozygous (CYP1A1 MspI+/+). The remaining 126 (80%) of the infants did not have the restriction site (CYP1A1 MspI-/-).

There was no significant association between maternal WBC adduct levels and either the CYP1A1 MspI RFLP or GSTM1 genotype, before or after controlling for potential confounders (4). Nor was there any indication of an interaction between the genotypes or between each genotype separately and exposure on maternal adduct levels.

However, adjusting for potential confounders, PAH-DNA adduct levels in both placental tissue and infant WBC were 1.65 fold higher in infants who were heterozygous or homozygous for the CYP1A1 MspI RFLP compared to infants without the restriction site. The difference was significant for placental tissue ($\beta = 0.5$, $p < 0.01$, $n = 158$) and of borderline significance for infant WBC ($\beta = 0.5$, $p = 0.06$, $n = 135$), controlling for potential confounders (3-4). The effect of the CYP1A1 genotype on adduct formation was greater than that of CYP1A1 activity.

The differing relationship between CYP1A1 and adducts in the women and the newborns may result in part from reduced detoxification capabilities via phase II enzymes in fetal tissues (9-10) rendering the fetus more susceptible to the effects of the CYP1A1 genotype. The public health implication of this finding is that a subset of infants (those with the CYP1A1 polymorphism) may be at heightened risk from environmental exposures to PAH.

3. Comparison of the biomarkers in mother/infant pairs and in different specimens from the same donor.

Of the 160 mothers and infants in the study, 112 pairs (224 subjects) had adequate

amounts of DNA for adduct analyses. Comparison of PAH-DNA adducts levels in these 112 paired maternal and infant WBC samples showed that adduct levels were higher in infants than in mothers (7.9±0.93 versus 5.9±0.77 per 10^8 nucleotides). Although the difference was not significant ($p = 0.13$, Wilcoxon matched-pairs signed-ranks test), the results are noteworthy since the transplacental dose of PAH is estimated to be 10-fold lower in the fetus compred to the mother. There was only a modest correlation between maternal and infant WBC PAH-DNA adduct levels ($r = 0.14$, $p = 0.13$). Adduct levels in placental tissue were significantly higher than those in paired maternal WBC samples (8.2±0.57 versus 6.4±0.81 per 10^8 nucleotides, $p < 0.01$, Wilcoxon Signed-ranks Test). There was no correlation between maternal WBC and placental tissue adduct levels ($r = 0.06$, $p = 0.5$). PAH-DNA adducts in paired placental tissue and infant cord WBC samples were available for 134 infants. Adduct levels in placental tissue were higher than those in paired infant cord WBC samples (8.8±0.6 versus 7.7±0.8, $p < 0.05$, Wilcoxon matched-pairs signed-ranks test). Placental and infant cord PAH-DNA adduct levels were weakly but significantly correlated ($r = 0.18$; $p = 0.03$).

4.Analysis of the association between PAH-DNA adduct levels and fetal development:

Infants from Krakow had lower mean birth weight (by 125.1 grams, $p = 0.08$), birth length (by 1.8 cm, $p = 0.0002$) and head circumference (by 0.8 cm, $p = 0.0007$) than infants from Limanowa, controlling for known risk factors (maternal height, age, educational levels, pregnancy weight gain, parity, prior history of LBW, gestational age, gender of the infant and plasma cotinine). When current smokers were removed from the analysis, all 3 measures of fetal development were significantly lower ($p \leq 0.05$) for Krakow compared to Limanowa infants (birth weight by 167.9 grams; length by 1.9 cm; head circumference by 1 cm). PAH exposure does not appear to explain this difference since PAH-DNA adduct levels were comparable in the two populations. This finding suggests that some unmeasured factor(s) that differ between the two areas may be responsible.

However, after controlling for known risk factors, a significant inverse association was seen in both cohorts combined between infant WBC PAH-DNA adducts (dichotomized into ≤ the median versus > the median) and infant birth weight ($\beta = -146.9$ gm, $p = 0.05$) and birth length ($\beta = -1.1$ cm, $p < 0.02$). There was also a highly significant inverse association between infant adduct levels and infant head circumference ($\beta = -0.9$ cm, $p < 0.0005$). The associations between infant adduct levels and measures of fetal development were of a similar magnitude when Krakow and Limanowa infants were analyzed separately. When infant PAH-DNA adduct levels were included in the regression model as a continuous variable, adduct levels remained significantly inversely correlated with head circumference ($p=0.004$ after controlling for birth weight), suggesting asymmetrical growth retardation. The relationship between infant adduct levels and all three measures of fetal development was of a similar magnitude and remained significant after current smokers had been removed from the analysis.

The magnitude of growth retardation seen in the Polish study (160 gm, 1.04 cm and 0.95 cm in birth weight, length and head circumference respectively) has been linked in other research to adverse sequelae. Although the mechanisms are not necessarily similar, reductions of a similar magnitude have been seen in studies of prenatal exposure to alcohol, cigarette smoking, opiates and cocaine (11-19). For example, maternal smoking is associated with an average reduction in birth weight of 200 grams and a doubling in the risk of having a LBW baby (14). Weight at birth is a predominant determinant of infant health; and LBW is a major cause of infant mortality (14,20). Some but not all studies have linked prenatal cocaine exposure to a 1-2 centimeter reduction in infant head circumference (12,15,19). Although mechanisms by which cocaine affects head growth are probably different from those operating in the Polish cohort, a similar reduction was seen in the Polish newborns with elevated PAH-DNA adducts. Most head growth occurs during the prenatal period, with >60% of adult head circumference attained at birth (21). In adults, head circumference is correlated with brain size and is estimated to account for 10% of intelligence (21). A number of studies have found that reduction in infant head circumference at birth or during the first year of life correlates with lower I.Q. as well as poorer cognitive functioning and school performance in childhood (19,22,23).

The findings are suggestive of a causal relationship between PAH and adverse birth outcomes; they are strengthened by the observation of the same effect (with similar effect size) in both of the populations studied. However, they do not prove causality; nor do they indicate that adduct formation is necessarily the mechanism by which PAH may be affecting fetal growth and development. We consider the extent of DNA binding by PAH in newborn WBC to be a useful dosimeter of the PAH that has 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 benzo(a)pyrene (B[a]P) may interfere with intrauterine growth during pregnancy due to its anti-estrogenic effects, thereby disrupting the endocrine system (24). Similar to PCBs, which are associated with deficits in fetal growth and IQ (25-26), PAH bind to human Ah receptor to induce P450 enzymes (27). Additionally, the developing central nervous system appears particularly sensitive to DNA damaging agents (28) and may respond by activating apoptotic pathways (29-30). For example, in humans, fetal microcephaly has been seen following exposure to ionizing radiation (10 - 20 rads) (31) and anticonvulsant drugs (32). Risk from anti-convulsants was most pronounced in infants deficient in enzymes that detoxify the DNA-binding intermediate (33-34). We found that PAH-DNA adduct levels were more strongly associated with reduction in head circumference than with birth weight or length; and the data are suggestive of asymmetrical growth retardation related to DNA binding. However, they do not prove causality or mechanism.

5. Analyses of the Relationship Between Cotinine and Biomarkers/ Development:

The Polish study demonstrated the value of cotinine as an internal dosimeter of cigarette smoke during pregnancy. Maternal and infant cotinine levels were highly

correlated ($r = 0.83$, $p < 0.001$) and, as expected, were increased significantly by active cigarette smoking of the mother ($p < 0.0001$). Additionally, for women who reported not smoking during pregnancy, cotinine levels in both mothers and infants were significantly increased with ETS exposure ($p < 0.01$). A highly significant inverse correlation was seen between infant plasma cotinine and birth weight (partial $r = -0.33$, $p < 0.0001$ controlling for potential confounders) (35). Further, cotinine provided a better fit (i.e., explained more of the variance in birth weight) than did other measures of maternal smoking derived from the questionnaire, none of which were significantly associated with birth weight (36).

When analyses were restricted to infants of women who reported that they did not smoke during pregnancy, plasma cotinine remained inversely correlated with birth weight (partial $r = -0.17$, $p = 0.07$). Cotinine levels in infant samples were higher than in paired maternal samples, a difference that was significant both for the total cohort (14.2 ± 2.8 vs 8.3 ± 2.0, $p < 0.0001$) and after analyses were restricted to mother/infant pairs for whom blood samples were collected within 6 hours of one another (average of 0.9 ± 1.2 hours SD) (10.6 ± 3.5 vs 8.0 ± 2.6, $p = 0.0001$).

6. Summary:

In conclusion, these results indicate that there is significant transplacental transfer of PAH and ETS constituents from mother to fetus; that maternal and infant WBC PAH-DNA adduct levels may be increased with environmental exposure to PAH from ambient pollution; and that plasma cotinine is increased by ETS. The finding of higher adduct levels in the infant compared to the mother suggests increased susceptibility of the developing fetus to DNA damage, while the finding of higher cotinine levels suggests reduced capability of the developing fetus to detoxify and clear cigarette smoke constituents. The study also provided **preliminary** molecular evidence that transplacental PAH as well as ETS exposure to the fetus may be compromising fetal development. If confirmed, these findings could have significant public health implications. However, the results are limited by lack of source-specific individual exposure assessments and by lack of information on postnatal neurodevelopmental status of the infants.

IV. List of Publications Resulting from the Grant

Perera, F.P., Whyatt, R.M. Biomarkers and molecular epidemiology in mutation/cancer research. Mutation Res, 313:117-129, 1994.

Whyatt, R.M, Garte, S.J., Cosma G., Bell, D.A., Jedrychowski, W. Wahrendorf, J., Randall, M.C., Cooper, T.B., Ottman, R., Tang, D. Tsai, W.Y. Dickey, C.P., Manchester, D.K., Crofts, F., Perera, F.P. CYP1A1 mRNA levels in placental tissue as a biomarker of environmental exposure. Cancer Epi Biom Prev, 4:147-154, 1995.

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Perera, F.P., Blaner, W., Mooney, L.A. Molecular epidemiologic methods in cancer chemoprevention. Eur Journal of Cancer Prev, 5(Suppl 2):1-6, 1996.

Published Abstracts

Whyatt, R.M., Garte, S.J., Cosma, G., Jedrychowski, W., Wahrendorf, J., Perera, F.P. Environmental and genetic determinants of CYP1A1 mRNA levels in placental tissue. AACR Annual Meeting, San Francisco, California, 1994.

Yin, B.Y., Whyatt, R.M., Perera, F.P., Santella, R.M. Immunoaffinity isolation and ELISA quantitation of 8-hydroxy-2'deoxyguanosine. AACR Annual Meeting, San Francisco, California, 1994.

Perera, F.P. Molecular epidemiology in cancer prevention. Extended abstract, AACR Annual Meeting, San Francisco, California, 1994.

Perera, F.P. Molecular epidemiology in environmental carcinogenesis. Keystone Symposia "Molecular Toxicology", Copper Mountain, Colorado, 1995.

Whyatt, R.M., Santella, R., Garte, S.J., Ottman, R., Gladek-Yarborough, A., Young, T., Jedrychowski, W., Cosma, G., Perera, F.P. Effects of environmental exposures on DNA damage in women and the developing fetus and its modulation by genetic/metabolic factors. AACR Annual Meeting, Toronto, Canada, 1995.

Perera, F.P., Tang, D., Whyatt, R., Dickey, C., Mooney, L.A. DNA damage, antioxidants and environmental carcinogenesis. U.S.-Japan Joint Conference on Antimutagens and

Anticarcinogens, NIEHS, Research Triangle Park, North Carolina, 1995.

Perera, F.P. Molecular epidemiology in environmental carcinogenesis. 2nd International Conference on Environmental Mutagens in Human Populations, Prague, Czech Republic, 1995.

Whyatt, R.M., Santella, R.M., Bell, D.A., Garte, S.J., Jedrychowski, W., Yarborough, A., Young, T., Perera, F.P. Biomarkers of exposures and susceptibility in newborns. AACR Annual Meeting, Washington, D.C.,1996.

Whyatt, R.M., Jedrychowski, W., Santella, R.M., Rauh, V.A., Perera, F.P. Application of biologic markers to assess effects of ambient air pollution on fetal development in Poland. APHA Annual Meeting, New York, N.Y., 1996.

Submitted/In Press

Perera, F.P., Santella, R.M., Whyatt, R.M. Molecular epidemiology: Genetic damage in adults and newborns related to environmental exposures in Eastern Europe. Env Health Persp, in press.

Whyatt, R.M., Bell, D.A., Santella, R.M., Garte, S.J., Jedrychowski, W., Gladek-Yarborough, A., Cosma, G., Manchester, D.K., Young, T.-L., Wahrendorf, J., Cooper, T.B., Ottman, R., Perera, F.P. Environmental exposures, polycyclic aromatic hydrocarbon-DNA in human placenta and their modulation by CYP1A1 genotype and enzyme induction. Submitted.

Whyatt, R.M., Santella, R.M., Jedrychowski, W., Garte, S.J., Bell, D.A., Ottman, R., Gladek-Yarborough, A., Cosma, G., Young, T.-L., Wahrendorf, J., Cooper, T.B., Randall, M.C., Manchester, D.K., Perera, F.P. Environmental and genetic factors in procarcinogenic DNA damage in Polish mothers and newborns, submitted.

Whyatt, R.M., Jedrychowski, W., Rauh, V., Santella, R.M., Ottman, R., Young, T.-L., Wahrendorf, J., Cooper, T.B., Randall, M.C., Perera, F.P. A molecular link between polycyclic aromatic hydrocarbons and impaired development in newborns, submitted.

Jedrychowski, W., Whyatt, R.M., Cooper, T.B., Flak, E., Perera, F.P. Exposure misclassification error in studies on prenatal effects of tobacco smoking in pregnancy and the birth weight of children. submitted.

Perera, F.P., Weinstein, I.B. Recent Developments in Molecular Epidemiology. American Journal of Epidemiology, submitted.

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4. Whyatt RM, Santella RM, Jedrychowski W, Garte SJ, Bell DA, Ottman R, Gladek-Yarborough A, Cosma G, Young TL, Wahrendorf J et al. Polycyclic aromatic hydrocarbon-DNA adducts in Polish mothers and newborns and modulation by GSTM1 and CYP1A1 genotype and CYP1A1 enzyme induction. In Preparation

5. Whyatt R, Santella R, Garte SJ, Ottman R, Gladek-Yarborough A, Young T, Jedrychowski W, Cosma G, Perera FP. Effects of environmental exposures on DNA damage in women and the developing fetus and its modulation by genetic/metabolic factors. Proc AACR 1995;36:284

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