Polycyclic aromatic hydrocarbons in maternal and cord blood plasma.

Paula G. Radmacher 1951-
University of Louisville

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POLYCYCLIC AROMATIC HYDROCARBONS IN MATERNAL AND CORD BLOOD PLASMA

By

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B.S., University of Louisville, 1973
M.S., University of Louisville, 1992
M.S.P.H., University of Louisville, 2000

A Dissertation
Submitted to the Faculty of the Graduate School of the University of Louisville
In Partial Fulfillment of the Requirements For the Degree of

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School of Public Health
University of Louisville
Louisville, KY

May 2007
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A Dissertation Approved on

March 30, 2007

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______________________________
Shirley A. Wilkerson, M.D., Ph.D.
DEDICATION

This dissertation is dedicated to my family, beginning with my husband, Michael, who has always been my better half. He keeps me afloat in troubled waters and is truly my “first mate.” My daughters, Andrea and Katie, have always believed I could do it, even when I wasn’t so sure. Their support means more than I can express. I am also grateful to my late parents, Charles and Doris Gill, who firmly believed that education was the key to the future.
ACKNOWLEDGMENTS

I would like to express my deep appreciation to my major professor, Dr. Stephen Looney, who persevered with me through distance, hurricane, and flood. His guidance and support were essential to the completion of this process. I would like to thank Dr. Steven Myers, who knew we could come up with a project (eventually) and opened his lab to me. I am also indebted to the other members of my committee, David Adamkin, MD, Susan Muldoon, Ph.D. and Shirley Wilkerson, MD, Ph.D., for their willingness to contribute their time and expertise to my education and growth.
ABSTRACT

A PILOT STUDY OF POLYCYCLIC AROMATIC HYDROCARBONS IN MATERNAL AND CORD BLOOD PLASMA

March 30, 2007

Polycyclic aromatic hydrocarbons (PAH) are chemicals generated from the incomplete combustion of organic materials, including tobacco smoke. Some PAH are known to be mutagenic and carcinogenic in humans, and of concern for the fetus when women smoke during pregnancy. Known consequences of smoking during pregnancy include low birth weight and preterm delivery. It is unknown if PAH are related to these outcomes. This pilot study was designed to measure concentrations of 3 PAH (anthracene, benzo(a)pyrene and 1-hydroxypyrene) in paired maternal and cord blood samples as well as any correlations between the two matrices. Plasma cotinine was used as a biomarker of tobacco exposure. Additionally, we asked if there is any relationship between the PAH concentrations and low birth weight or preterm delivery.

Results showed that all 3 PAH could be found in maternal and cord plasma. Anthracene was consistently shown to be significantly elevated in cord plasma compared to maternal plasma in subgroups based on increasing cotinine concentrations. However, none of the compounds studied were correlated with either birth weight or gestational age.
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INTRODUCTION

Environmental pollution has been around since the dawn of life on earth. Human-generated environmental pollution has been around at least since the discovery of fire in the Paleolithic Period. The advances and discoveries of civilization (the Renaissance, the Industrial Revolution, fossil fuels, the automobile, atomic energy, etc.) have increased the rate at which human-generated pollution has affected the health of the planet (air, water, soil) and the species that inhabit it. Environmental pollution is known to cause or contribute to a number of human diseases, including cancer, developmental disorders and immune, cardiovascular and pulmonary diseases. Among the many compounds that contribute to environmental pollution are the polycyclic aromatic hydrocarbons, a group of compounds with potential for carcinogenic and mutagenic activity in humans.

Polycyclic aromatic hydrocarbons (PAH) are products of incomplete combustion of organic matter. They are made up of fused benzenoid rings as well as unsaturated 4-, 5-, and 6-membered rings. Sources include automobile exhaust, coal-fired energy plants, tobacco and wood smoke, as well as grilled/smoked foods. (1) PAHs are highly lipid-soluble, readily absorbed from the gastrointestinal tract and rapidly distributed throughout the body, especially into fat tissue. (2) Metabolism occurs via the cytochrome P450-mediated mixed-function oxidase system, yielding, among other things, highly reactive epoxides
that are known to be carcinogenic, mutagenic and/or developmentally toxic. (3, 4) In studying the effects of PAH exposure, benzo[a]pyrene is widely used as a “representative PAH” because, at least in the urban setting, concentrations of individual PAHs are highly correlated. In addition to its carcinogenic potential, benzo(a)pyrene has also been shown to act as an endocrine disruptor due to its structural similarity to the cholesterol core of many hormones and/or by interfering with hormonal activity (5). Among other PAH compounds, 1-hydroxypyrene has often been followed in urine as a marker of PAH exposure. Anthracene, while not carcinogenic itself, has the potential for easy methylation at the 9- and 10- carbons on the middle ring to much more toxic compounds that are indeed carcinogenic (9-methyl anthracene and 10-methyl anthracene).

One common source of polycyclic aromatic hydrocarbons is tobacco smoke. It is ubiquitous in the environment and its use is recognized as detrimental to human health. It is especially problematic during pregnancy when the fetus is at great risk for significant harm from the chemicals that will cross the placenta and influence subsequent development. This study will focus on the presence of 3 specific PAH among the many compounds in tobacco smoke in maternal and cord blood (see Figure 1).
Figure 1.

Chemical structures of polycyclic aromatic hydrocarbons of interest in this study.

![Chemical structures](image)

**Benzo(a)pyrene**  **1-hydroxypyrene**  **Anthracene**

**BACKGROUND**

Concerns about atmospheric contamination are not new. Thirteenth century England was blanketed in coal smoke as Londoners switched from burning wood for fuel (as wood became scarce and expensive) to heating with sea-coal, a relatively plentiful (and cheap) but dirty alternative fuel. Such practice was banned by royal edict as far back as 1272, but citizens were not deterred. The term “smog” was coined in the early 1900s to describe the air of London, where naturally occurring fog combined with coal-burning smoke to envelope the city and surrounding areas. (6)

The early to mid-20th century saw a number of air stagnation events (periods of extraordinarily heavy pollution) that resulted in excess deaths and hospitalizations among citizens in the United States and elsewhere. (7, 8) These events provided well-defined intervals in which specific data (atmospheric...
conditions, hospital admissions and deaths) could be analyzed by scientists and governmental officials in order to better understand the short term effects of ambient outdoor air quality on public health. Epidemiological studies showed generally that the combination of increased concentrations of smoke and sulfur dioxide in the air, along with a given individual’s underlying biophysiological susceptibility (primarily cardiovascular or pulmonary disease), resulted in morbidity and mortality rates up to 10-fold higher than normal. (7)

Environmental pollution also contributes to respiratory hypersensitization, which has been on the increase in Western nations over the past several years. Allergic diseases are the 6th leading cause of chronic illness in the U.S., affecting up to 17% of the population and costing $18 billion annually. (9) Particulates, including soot, pollen, animal dander, insects and microbes, can trigger tissue inflammation/injury and airway reactivity (rhinitis, asthma, chronic bronchitis, etc.). (10, 11) Infants and children are especially sensitive to particulate pollution. Despite the fact that such chronic illnesses are not generally fatal (and consequently not reflected in mortality data), the social impact in terms of hospital admissions, missed school/work days, etc. is still significant. Studies from the Centers for Disease Control and Prevention have shown that up to 40 million Americans have chronic allergic rhinitis (hay fever) related to common airborne allergens, resulting in approximately 3.8 million lost days of work and school annually (12).

Although overall air quality in the late 20th and early 21st centuries is much improved over that in the 1950s and 1960s, indoor air pollution remains a
significant contributing factor to morbidity and mortality. The most ubiquitous indoor pollutant is tobacco smoke, which is known to contain over 4,000 chemicals (including PAHs) and has been classified as a carcinogen by the United States Environmental Protection Agency. Environmental tobacco smoke is made up of both mainstream smoke (that which has been inhaled by the smoker and then exhaled) and side-stream smoke (that emitted directly from a burning cigarette). Sidestream smoke is considered to be more toxic, gram for gram, than mainstream smoke since it contains chemicals and particulates in higher concentrations than smoke that has been scrubbed (filtered) by the smoker’s lungs. (13) Involuntary or passive smoking (a combination of mainstream and side stream smoke) by individuals sharing significant amounts of time and space with active smokers has been shown to increase the risk of serious lung disease (asthma, chronic bronchitis) as well as lung cancer. (14)

The developing fetus is the most vulnerable of involuntary smokers. Studies in reproductive epidemiology have shown that fetuses and infants are more likely to have a heightened sensitivity to any number of environmental toxicants due to their degree of exposure in relation to organ mass, physiologic immaturity to detoxify such compounds and critical windows of growth and development that may be negatively impacted by such exposures. (15-18) Convincing evidence already exists that relates maternal smoking during pregnancy with low birth weight (LBW, weight under 2.5 kg), intrauterine growth restriction (IUGR, weight for gestation less than 10th percentile), placenta previa (attachment of the placenta in the lower part of the uterus, often resulting in
excessive bleeding), placental abruption (precipitous detachment of the placenta from the uterus before birth of the infant), miscarriage and preterm delivery. (19-41) Life-long risk for postnatal or adult-onset disease in the infant whose mother smoked during pregnancy has not, so far, been quantified, primarily due to multiple family, socioeconomic and environmental confounding factors. However, measurable evidence of genetic damage from environmental tobacco smoke in non-smokers is mounting. (42-46)

Using paired maternal-cord blood specimens, this study will address the following research questions:

1. Are anthracene, benzo(a)pyrene and 1-hydroxypyrene present in measurable concentrations in maternal and cord blood plasma?
2. Do plasma concentrations of anthracene, benzo(a)pyrene and/or 1-hydroxypyrene correlate with cotinine among smoking and non-smoking women?
3. Do plasma concentrations of anthracene, benzo(a)pyrene and/or 1-hydroxypyrene in cord blood parallel levels in maternal plasma?
4. Are plasma concentrations of anthracene, benzo(a)pyrene and/or 1-hydroxypyrene correlated with birth weight or gestational age?

**GENERAL ENVIRONMENTAL CONCERNS**

Air pollution is a public health issue with political/social underpinnings. A number of epidemiological studies have shown associations between morbidity/mortality rates and air quality all across the globe. (1, 5, 7, 11, 16, 17,
This research has examined risks in urban vs. rural communities, white collar vs. blue collar neighborhoods, indoor vs. outdoor pollution, industrialized vs. developing countries and across all age groups. There is little question that the general health of any community is highly dependent on the quality of the air its citizens breathe. However, one of the challenges of interpreting epidemiological studies is to quantify and control for the effects of multiple cofactors (social, cultural, economic). Public policy must be a driving force for pollution abatement, as pollution is a product of the activities of individuals, industries and governments, all of which must be balanced for the good of the community.

An increasing prevalence of asthma/respiratory hypersentization has been noted across the globe in recent years. Bascom (10) reviewed a number of studies from North and South America supporting the hypothesis that environmental factors contribute to the development of respiratory allergy, asthma, and chronic bronchitis. In addition to the usual triggers (pollen, dust mites, animal dander) the author discusses how ambient air pollutants (fossil fuel emissions, particulates, etc., both stationery and mobile) and unfavorable climatic conditions can compound the allergic response in individuals that have a genetic susceptibility to this heightened sensitivity. While “air pollution” is often used to imply a singular entity, it is actually variable in character depending on the sources, the climate, the season, and the chemicals in the mix. Human responses to elevations in specific pollutants can range from cough and shortness of breath to asthma exacerbation, lung injury and decreased lung
function. (56) Ozone, a secondary atmospheric pollutant formed by the photochemical reaction between nitrogen oxides and hydrocarbons, is often increased in summer months when fossil fuel emissions react with sunlight. Exercising in an elevated ozone environment can lead to decreased lung function, tissue injury and inflammation, all of which are measurable for hours after exposure ceases. Epidemiologic studies show that such conditions are associated with increased hospitalizations and emergency department visits for individuals with respiratory risk factors. (56)

Particulate matter (PM) has been shown increasingly in recent years to be an important factor in defining air quality, due to effects on heart and lung health. PM is a complex mixture of small particles and liquid droplets, with particle size directly linked to potential for negative health effects. (57) Particulates that are 10 micrometers (microns, µ) or smaller are of the strongest concern because they are generally not filtered out by the upper airway (nose and throat), and pass on into the lungs. Coarse particles, 2.5 – 10 µ in diameter) are often detected in dusty areas/regions and near industrial sites. Fine particles, 2.5 µ or smaller) are common components of smoke and haze and found in the emissions of power plants, industries and auto exhaust.

Dockery et al conducted a prospective, cohort study of air pollution and mortality among adults in 6 United States cities from 1974-1991. (48) At the time of enrollment, subjects underwent baseline spirometric testing and completed questionnaires detailing medical and smoking history, as well as occupational exposures. During the intervening years, subjects were contacted annually for
updates on medical and vital status. Ambient air quality data were obtained from governmental sources. Death certificates were obtained for 98% of those who died during the study period; causes of death were independently verified. After adjusting for smoking behavior and other risk factors, there remained a statistically significant association between air pollution (especially fine particulates) and mortality (adjusted rate ratio 1.26 (95% C.I. 1.08-1.47).

Epidemiological studies from South Korea, (51) Czechoslovakia (47) Mexico (53) and California (58) have shown that even among infants, after controlling for premature birth and underlying respiratory disease (e.g. bronchopulmonary dysplasia), there is credible evidence that fine particulate matter (PM$_{2.5}$) air pollution contributes to respiratory-related deaths in the post-neonatal period.

In the Louisville, KY region of the Ohio Valley, air pollution control is a constant battle, especially in the summer months when heat, humidity and stagnant weather patterns exacerbate baseline pollution conditions. While air quality is much improved over the mid- late- 20$^{th}$ century, the region still saw 8 days of excessive ozone (U.S. Environmental Protection Agency standard $\geq$ 85 parts per billion for 8 hours), in the year 2005. (59) In terms of PM$_{2.5}$, a more recently implemented pollution control measure, the Louisville Metro area continues to struggle with significant elevations, primarily in the summer months. In each year from 1999-2005, concentrations of PM$_{2.5}$ exceeded the acceptable standard ($15 \, \mu g/m$) in the months of June, July and August from 1.1-2.0 fold. (60) Reports from the first 5 months of 2006 showed that PM$_{2.5}$ pollution was significantly down from previous years and met/exceeded the national standard.
Data from the summer months of 2006 were not available at the time of this paper.

Environmental pollutants can also impact the orderly development of organisms as well as normal cellular processes. Pesticides, (61-63) radiation, (64, 65) and heavy metal compounds (17, 66-68), whether in the water, soil or air, all have the potential to cause disruptions in the normal maturational sequence. During embryogenesis and even beyond, the presence (or absence) of chemical signals can permanently alter the structure and function of cells, tissues and organs. Slotkin et al have shown the disruptive effect of nicotine, a neurologically active chemical with specific receptors, on the developing brain in a rat model. (69) When a pregnant rat was exposed to an infusion of nicotine comparable to that found in (human) heavy smokers, there were 4 major neurological effects in the post-natal rat pup, including presence of markers of cell damage, decreased numbers of cells, inhibition of DNA synthesis and blunted synaptic activity in the forebrain. These effects indicate that, at least in the rat, nicotine, one of many chemicals humans acquire primarily through cigarette smoking, is a nervous system teratogen associated with significant short and long-term alterations in normal brain development.

In the human brain, nicotine reacts with nicotinic acetylcholine receptors, (70) which appear as early as 4-5 weeks’ gestation. As in the rat model, these receptors are involved in modulating dendritic outgrowth, establishment of neuronal connections and synaptogenesis during development. (71) In the
immature brain, stimulation of these receptors leads to interaction with the genes that control cell replication, differentiation, growth and death. (72)

Epidemiological studies have suggested that *in-utero* exposure to nicotine is associated with negative neurobehavioral effects later in childhood and adolescence. (73-76) However, the confounding effect of socioeconomic factors on childhood behavior have made direct linkage between smoking and later behavior difficult to interpret. Recently, Maughan et al, using the Longitudinal Twin Study of Britain, showed that prenatal smoking had a strong and statistically significant dose-response relationship to childhood conduct disorders at 5 and 7 years of age, especially in boys. (77) The researchers found that approximately two-thirds of the variation in the reported conduct problems at each age was found to be attributable to genetic factors. However, after controlling for the genetic influences, prenatal smoking in the mother continued to predict such problems. In an effort to account for more potentially confounding factors, the researchers measured social-environmental factors that may have contributed to the observed behaviors. Once parental antisocial behavior, maternal depression and social deprivation were controlled in the analysis, in addition to the genetic risk, the strong initial effect of smoking on early childhood conduct was reduced by about 75%. For children of women that were light-moderate smokers, the effect was non-significant. For children of women that were heavy smokers, the effects could still be detected, but the magnitude was greatly reduced.
SMOKING EFFECTS AND CONCERNS

Among adults in the U.S., tobacco smoke has been officially linked to cancer and cardiovascular disease in active smokers since the first Surgeon General’s Report on Smoking and Health in 1964. (78) This report summarized the results of more than 7,000 published articles that correlated smoking with specific diseases. Since 1964, there have been 28 additional supplements and reports from the Office of the Surgeon General that further describe the health risks from exposure to environmental tobacco smoke. Other scientists from the fields of medicine and public and environmental health continue to report on the dangers of environmental tobacco smoke to users and those who share common breathing space. Recently (June 2006), the current Surgeon General, Richard H. Carmona, issued a comprehensive scientific report that concludes “there is no risk-free level of exposure to secondhand smoke.” (14)

As noted in Table 1, adapted from “Cigarette Secrets,”(79) tobacco smoke contains a broad array of chemicals from multiple classes that are present in both mainstream and side stream smoke. All of those listed are considered by the International Agency for Research on Cancer (IARC, a part of the World Health Organization) to have sufficient scientific evidence of carcinogenicity or tumorigenesis in animals. Those with “sufficient” evidence of carcinogenicity in humans are noted with an S. Those with “limited” evidence are noted with an L.
Table 1.

A partial list of toxic and carcinogenic compounds in tobacco and tobacco smoke. (79)

<table>
<thead>
<tr>
<th>Polycyclic Aromatic Hydrocarbons</th>
<th>Aldehydes</th>
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<tbody>
<tr>
<td>Benz[a]anthracene</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>Benzo(a)pyrene&lt;sup&gt;S&lt;/sup&gt;</td>
<td><strong>Miscellaneous inorganics:</strong></td>
</tr>
<tr>
<td>Chrysene</td>
<td>Arsenic&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>Di-benzo(a,h)anthracene</td>
<td>Cadmium&lt;sup&gt;L&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>N-Nitrosamines</strong></td>
<td>Chromium&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-Nitrosodimethylamine</td>
<td>Lead</td>
</tr>
<tr>
<td>N-nitrosoethylmethylamine</td>
<td>Nickel&lt;sup&gt;L&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-nitrosonornicotine</td>
<td>Polonium 210&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
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Tobacco smoke is one of the most ubiquitous indoor air pollutants. In addition to the chemicals and carcinogens known to be contained in tobacco smoke, it also contains significant amounts of particulates, including small, respirable ones (PM$_{2.5}$). (80, 81) By virtue of the differences in respiratory rate and minute ventilation between infants and adults, infants and toddlers inhale a larger dose of air pollutants per unit of body mass than adults breathing the same air; they also appear to be more vulnerable to the effects. Using data from the Third National Health and Nutrition Examination Survey (1988-1994), Gergen et al (58) reported that respiratory complications from exposure to environmental tobacco smoke (asthma, chronic bronchitis, and wheezing) were more common in children 2-20 months than in children 3-5 years of age. After adjusting for relevant confounding factors, the authors determined that the excess attributable risk for these respiratory complications showed that 40-60% of the cases could be linked to environmental tobacco exposure.

A causal association between involuntary smoking (passive tobacco smoke exposure) and lung cancer appears to exist, as the risk among non-smoking spouses has been shown to be increased 20-30% over non-smokers in general. (13, 82, 83) In the United States, a meta-analysis of 11 epidemiological studies showed that environmental tobacco smoke may be responsible for as many as 3,000 lung cancers annually in non-smokers (35 and over) and up to 35,000 deaths. (13) Studies have shown that adults exposed passively to tobacco smoke in the workplace often develop respiratory symptoms such as wheezing, cough, etc. However, it is unclear if, or to what extent, this exposure
explains chronic obstructive pulmonary disease or cardiovascular disease in non-smokers.

In Kentucky, where tobacco has been a prominent cash crop throughout the Commonwealth’s history, over 8,000 citizens die of tobacco-related diseases each year. In 2005, Medicare and Medicaid costs related to the health effects of tobacco were estimated to be $1.2 billion, or $300/Kentuckian. (84) Kentucky exceeds the national average in prevalence of tobacco use in all age categories (adult, high school and middle school), racial/ethnic groups (whites, African-Americans), gender and during pregnancy. By far, annual deaths from cancers of the trachea, lung and bronchus are the leading causes of smoking-attributable mortality (SAM).
Table 2.

Comparisons of tobacco use and consequences in Kentucky and in the U.S. (84, 85)

<table>
<thead>
<tr>
<th></th>
<th>Kentucky</th>
<th>U.S.</th>
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<tbody>
<tr>
<td>Any tobacco (%)</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>Males (%)</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Females (%)</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Caucasians (%)</td>
<td>29</td>
<td>22</td>
</tr>
<tr>
<td>African-Americans (%)</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Hispanics (%)</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Middle school (%)</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>High school (%)</td>
<td>44</td>
<td>28</td>
</tr>
<tr>
<td>During pregnancy (%)</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>Years of potential life lost (per 100,000 persons)</td>
<td>5,597</td>
<td>3,805</td>
</tr>
<tr>
<td>Smoking Attributable Mortality (per 100,000 persons)</td>
<td>385</td>
<td>273</td>
</tr>
</tbody>
</table>

SMOKING CONCERNS AMONG MOTHERS AND INFANTS

For infants and children, it has been shown that living in homes with tobacco smoke clearly results in more coughing, wheezing and respiratory illness than living in smoke-free homes. (13, 58) In the United States, it is estimated that 35-40% of children live in homes where others smoke. (86) Diagnoses of asthma, chronic bronchitis and wheezing increase with increasing environmental tobacco smoke exposure and with decreasing postnatal age. (58)
Among women of childbearing age, the negative effects of environmental tobacco smoke can reach beyond personal health risks to those of a developing fetus. Multiple studies have addressed the consequences of maternal smoking during pregnancy both for the mother and the infant: increased risk for premature rupture of membranes, (87, 88) premature birth and low birth weight (birth weight <2.5 kg), (89-91) placenta previa/abruption, (92) miscarriage, and stillbirth. (93, 94)

The link between smoking during pregnancy and LBW was first reported by Simpson in 1957 (35) and has been confirmed in numerous reports since. This effect is independent of other factors that influence birth weight, including gestational age, gender, parity, race, pre-pregnancy anthropometrics, and socioeconomic status. A series of 14 studies from the United States, Canada, the United Kingdom, Italy, Norway and Sweden during the years 1959-91 were compared in the 2001 Surgeon General’s Report on Women and Smoking. (36) Smoking intensity was defined by either maternal report or cotinine concentrations. Infants of smokers were 66-320 grams lighter at birth when compared to infants of non-smokers. In general, the more a woman smoked, the larger the birth weight deficit when compared to the infant of a non-smoker.

The primary mechanism of reduced fetal growth due to smoking is presumed to be a combination of relative hypoxia due to placental vasoconstriction and carbon monoxide binding to fetal hemoglobin oxygen. (95-97) However, it is possible that other mechanisms, including altered nutrient metabolism, contribute as well. (98-101) Studies by D’Souza et al (26) and
Harrison et al (102) showed that the reduction in birth weight was related to loss of lean mass rather than reduced fat deposition, which is consistent with an hypoxic or other non-nutritional mechanism. Smoking during the first trimester of pregnancy is temporal with cellular hyperplasia and organogenesis. Any of these mechanisms may function to alter or restrict the normal developmental pattern or influence a critical window that cannot be recovered.

Smoking cessation during pregnancy can lead to gains in birth weight and reductions in the likelihood of low birth weight. Secker-Walker et al conducted clinical trials examining smoking cessation and relapse prevention. (103) They ascertained cigarette consumption by both maternal report and urinary cotinine levels and found that women who stopped smoking early in pregnancy (before the first prenatal visit) and remained abstinent could reduce the expected birth weight loss by as much as 300 grams.

England and colleagues compared the effects of 3 different patterns of cigarette exposure during pregnancy (quit, reduced, increased) with women whose smoking behavior did not change. (104) Patterns were determined by personal report and urinary cotinine measurements. Women that reduced/quit smoking before or soon after study enrollment delivered infants that were, on average, ~32 grams heavier than babies of women that continued the same level of smoking. However, when birth weight was stratified by maternal cigarette use at enrollment, infants of women with low exposure who then reduced their cigarette consumption were ~200 grams heavier than infants of women who were light, but consistent, smokers.
In Kentucky, the incidence of low birth weight has been higher than the national average for the past decade. This is accompanied by a high prevalence of smoking during pregnancy, with rates in Kentucky usually the highest in the nation.

Table 3.

Prevalence of low birth weight (% live births) and smoking during pregnancy in the United States and Kentucky in 3 time periods. (105)

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>% LBW</td>
<td>% Smokers</td>
<td>% LBW</td>
</tr>
<tr>
<td>Kentucky</td>
<td>7.8</td>
<td>24.5</td>
<td>8.2</td>
</tr>
<tr>
<td>U.S.</td>
<td>7.4</td>
<td>13.6</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Specific studies have addressed the effects of smoking on indices of newborn health. Symptoms of neonatal nicotine withdrawal, including jitteriness and irritability, have been described by multiple authors. (106-109) Alterations in body composition and growth have been known and studied for decades. (23, 92, 110-113) Evidence suggests that there is a dose-response as well as a temporal relationship between smoking and reduced birth weight. (22, 28, 104, 114-116) The earlier in her pregnancy a woman quits, the smaller the effect on her infant’s birth weight. (104, 117) Smoking also is associated with lower levels of markers of bone metabolism in infants of smoking mothers when compared to those of women that do not smoke. (118-121) The biological explanations for these effects have centered on immunologic (100) as well as physiologic
pathways. (122) Postnatally, an increased risk for Sudden Infant Death Syndrome (SIDS), (123, 124) upper respiratory tract infection, and asthma has been linked to smoking by adults in the child’s environment (58, 125, 126).

According to the Centers for Disease Control and Prevention (CDC), the prevalence of smoking during pregnancy in the United States in 2002 was estimated to be 11.4%, a decrease of 38% from 1990. (127) During that same 12-year period, the rate in Kentucky decreased only 14.4% to 24.4%, a rate exceeded only by pregnant women in West Virginia (26.2%). Among females aged 15-19, the rate of smoking during pregnancy in Kentucky increased from 32.9% to 34.1% (1990-2002). Only Vermont, New Hampshire and West Virginia had teen rates that were higher during that period. Figure 2 shows that the 24% overall rate for Kentucky, while alarming in and of itself, does not convey that there are regions of extremely high tobacco use in certain parts of the Commonwealth where the prevalence is 3-4 times that across the United States. Coupled with the increased rates of prematurity and low birth weight, the financial burden on Kentuckians related to smoking during pregnancy is significant. CDC analysis of data from pregnancy risk surveillance and birth certificates resulted in an estimate of smoking-attributable neonatal expenditures (in 1996 US dollars) of $704 per maternal smoker (range $519-1,334) both nationally and in Kentucky. (128)
Figure 2.

Prevalence of smoking during pregnancy in Kentucky by county. (84)
The impact of personal cigarette smoking in the short term (pregnancy and the neonatal period) on the health of mothers and infants has been discussed. However, passive exposure to tobacco smoke at home or in the work place can also be detrimental to a woman and her infant during pregnancy and postnatally. Side stream smoke represents approximately 85% of total environmental tobacco smoke and contains higher concentrations of some carcinogens than does mainstream smoke. (13) Outcome studies comparing birth weight of infants of pregnant non-smokers passively exposed to tobacco smoke to infants of non-exposed women have had varied results. Generally, though, they show that the birth weight reduction in infants of passively exposed women was smaller than that seen in infants of active smokers. (38, 129) Horne et al. and others have reported that infants exposed pre- and post-natally to tobacco smoke have decreased arousability from sleep, (130-133) which may be a contributing factor to sudden infant death syndrome (SIDS). Although the specific cause of SIDS remains elusive, since the introduction of the “Back to Sleep” program in the early 1990’s, deaths from SIDS have declined by almost 50%, leaving exposure to environmental tobacco smoke as one of the strongest remaining (and modifiable) risk factors for this event. (134) Infants and children chronically exposed to tobacco smoke in the home also have a significantly increased risk of developing asthma, allergies and upper respiratory infections (bronchitis, ear infections, pneumonia). (58, 135-137).

Tobacco smoke is a toxicant to the embryonic, fetal and postnatal lung and can exert varying degrees of harm throughout gestation and beyond, based
on the stage of lung development (proliferation, differentiation, branching) at the
time of the exposure. (17) Enzyme systems necessary for intrapulmonary
metabolism as well as the detoxification of foreign molecules (epoxide hydrolase,
cytochrome P450 mono-oxygenases, glutathione-S-transferase, and other anti-
oxidant systems) mature at differing rates throughout gestation and after birth.
Exposure to damaging compounds prior to the availability of these enzymes may
permanently alter their developmental profile. Molecular signals orchestrate the
process temporally and spatially throughout the continuum from embryogenesis
through adolescence, when lung growth is generally complete. Substances that
interfere with these signals may result in deviant lung development. Although a
normal term pregnancy (40±2 weeks) allows for development that is adequate to
sustain life, fully 80% of lung development occurs after birth. (17) Continued
exposure to pollutants such as tobacco smoke perpetuates the risk for altered
development.

Nicotine (a component of the tobacco leaf and tobacco smoke) is readily
transported to the fetal compartment via the placenta. (15) Luck et al. showed
that the serum nicotine ratio (umbilical vein/maternal) was 1.12±0.3, evidence
that it is concentrated in the fetus compared to the mother. (138) The authors
also found that nicotine in the amniotic fluid was increased up to 88% relative to
maternal serum values. Nicotine’s effects include decreased uterine artery blood
flow, variable changes in umbilical artery flow, changes in fetal oxygenation (due
to the binding of carbon monoxide to fetal hemoglobin) and acid base balance,
as well as decreased fetal heart rate and increased mean arterial pressure.
Nicotine has been shown to be toxic in adults with doses as small as 2 mg. (140)

Amniotic fluid, which bathes, nourishes and cushions the fetus during gestation, actually represents fetal urine. During the first trimester of pregnancy, it is derived from maternal blood plasma that diffuses through the tissues of the fetus into the surrounding fluid. From approximately 10-11 weeks (simultaneous with the formation of fetal kidneys), the major component of amniotic fluid is fetal urine, supplemented with growth factors and secretions from the lungs, oral and nasal cavities, as well as the fetal surface of the placenta. This fluid is constantly circulated as the baby "inhales" (swallows) existing fluid and replaces it through "exhalation" (urination). The fetal skin is highly permeable prior to keratinization (about mid-gestation) and readily absorbs compounds present in the surrounding fluid. Thus, from around 10 weeks of gestation, the fetus of a smoking mother is chronically exposed to tobacco products by multiple routes: maternal circulation, placental transport, dermal and gastrointestinal absorption. (141, 142)

Cotinine is the major metabolite of nicotine and it has been used for a number of years as a biomarker of exposure to cigarette smoke due to its chemical stability, persistence in the blood stream (half-life ~24 hours) and freedom from interfering substances. Jauniaux et al detected cotinine in fetal fluids as early as 7 weeks’ gestation. (141) Donnenfeld et al measured simultaneous serum cotinine concentrations in a small number of maternal/fetal pairs undergoing clinically-indicated percutaneous umbilical blood sampling between 21 and 36 weeks gestation, (143) finding a mean fetal/maternal cotinine

(139).
ratio of 0.9 (95% CI 0.83-0.97). Eliopoulos et al measured nicotine and cotinine in hair of newborn infants, finding a dose response based on maternal smoking behavior and a linear relationship between cotinine levels in individual mothers and babies. (144) It is not clear if the cotinine measured in fetal tissues/fluids is the product of fetal metabolism of maternal nicotine or if it is maternal cotinine passed to the fetus via the placenta. (143)

In pregnancy, the placenta is the organ of exchange between the fetal and maternal circulations. It is made of fetal (chorionic) and maternal (endometrial) components and serves to provide nutrition, respiration and excretion for the fetus. These functions are carried out through metabolic, transfer, endocrine and immunologic activities. Compounds that enter the mother’s body (nutrients, medications, toxins, etc.) may travel directly to the fetus or may be modulated by maternal metabolism or placental activity. While the human placenta can metabolize many foreign chemical compounds, the breadth of metabolizing enzymes is more limited than in the liver which is the primary detoxifying organ. Hakkola et al showed that there may be a gestational influence on the appearance and duration of effect of some of the cytochrome-P (CYP) family enzymes. (145, 146) Their studies revealed that mRNA for several CYP enzymes was present in human placental tissue as early as the first trimester, when mitotic frequency is high and there is rapid functional and structural development. They also showed that the number of different mRNAs that were detectable decreased as the pregnancy reached term. The presence of toxic substances, such as PAH, in placental tissues or fluids during critical windows of development is
alarming in view of the high rates of hyperplasia and hypertrophy in the placenta and the fetus and the potential for disruption of normal maturational processes. PAH are known to cross the placenta, although animal experiments have indicated that the fetal dose is probably an order of magnitude less than that presented to maternal organs and tissues. (147-149) Yet, further animal studies have shown PAH administration/exposure in the pregnant female can lead to tumor development in the offspring in a multitude of organ systems, including liver, lung, lymphatics and central nervous system (150-152).

The presence of PAH compounds in placental tissues from both smoking and non-smoking women has been demonstrated by Gladen et al (153). Placentae were drawn from births in two Ukrainian cities participating in the European Longitudinal Study of Pregnancy and Childhood (ELSPAC) a program of the World Health Organization. The Ukraine ELSPAC study was conducted by the Ukrainian Institute of Pediatrics, Obstetrics and Gynecology in cooperation with the University of Illinois School of Public Health. The cities were known to have a history of significant industrial air pollution problems. Smoking status of the mothers was determined by questionnaire at 20 weeks gestation. Seven specific PAH were measured (including anthracene and benzo(a)pyrene). Levels were generally higher in smokers when compared to non-smokers. However, of 178 samples analyzed, only 8 (4.5%) were from women that admitted to being current smokers; 21% classified themselves as ex-smokers.

Madhavan et al (154) reported the presence of benzo(a)pyrene, dibenzo(a, c)anthracene and chrysene in maternal blood, cord blood, breast milk
and placental tissue from non-smoking Indian women. Except for benzo(a)pyrene, the other PAH compounds were higher in concentration in cord blood than other matrices; benzo(a)pyrene was highest in maternal milk. The authors believed the primary source to be grilled/fried foods common in the diet in that population. The lipophilic nature of PAH helps to explain the elevated concentrations in human milk but also reveals human milk to be a significant source of exposure for the breast-feeding infant.

**Programming In-Utero and Chronic Disease in Adults**

By definition, an embryo, later a fetus, is expected to spend approximately 280 days in a developmental continuum: hyperplasia and hypertrophy, cellular replication and organ maturation. Periods of extreme cellular activity may justifiably be called critical windows in which the absence of an essential factor or the presence of a noxious substance may result in altered development. Strong evidence exists that environmental exposures of various kinds during pregnancy can have profound effects that may predispose an infant to vulnerabilities in the postnatal period and beyond, (155) a concept that has been termed “programming” by some researchers.

The theory of “programming” is based on the hypothesis that in-utero or early childhood events/exposures that occur at sensitive or critical periods of development may alter the structure, physiology and/or metabolism of an individual for a lifetime. (156) Barker and others have written extensively about the evidence for this theory and have shown that the risk for a number of adult,
chronic diseases can be associated with sub-normal size at birth (low birth weight). (156-159) The list of candidate diseases includes coronary artery disease (CAD), (160-162) insulin resistance/type II diabetes, (163-165) hypertension, (22, 166, 167) and obesity. (168) The exact mechanism for expression of in-utero events decades after they occurred is not known, but speculation has centered on altered programming of the hypothalamic-pituitary adrenal axis (169, 170) which may manifest as alterations in secretion of cortisol (171, 172) and/or insulin-like growth factor. (173) Whether the alteration in cortisol is an increase (171, 172) or a decrease (174-176) is unclear. What is intriguing is that thinness at birth, coupled with an increased “tempo of growth” in childhood appears to translate into an increased risk for heart disease in later adulthood. (161) The classic outcome in infants of mothers that smoke is lower birth weight than in infants of women that do not smoke. Is there any reason to believe that the high prevalence of such adult morbidities as coronary artery disease, diabetes and obesity in Kentucky can be linked, at least to some degree, with the historic pattern of maternal smoking during pregnancy?

This pilot study was designed to investigate the presence of 3 specific PAH, all with carcinogenic/mutagenic potential, in maternal-cord blood specimens from women living in the Jefferson County Kentucky region. An additional objective was to determine if there was any relationship between those PAH and birth weight or gestational age.
MATERIALS AND METHODS

This study was approved by the University of Louisville Institutional Review Board and the Norton Hospital Research Office prior to initiation.

Blood samples were collected at Norton Hospital, an urban delivery center in Louisville, KY. The maternal population has been shown in past studies to be comprised of 30-40% smokers (from light to heavy) as well as multiple ethnicities. Anonymous paired specimens (maternal and cord) were drawn by the labor and delivery staff into tubes containing the anti-coagulant lithium heparin. Maternal specimens were drawn with other ordered labs at the time of admission. Cord blood specimens were drawn at the time of delivery. Tubes were identified only by an ID code, M (maternal) or C (cord), with birth weight (BW) and estimated gestational age (GA) provided on the label. Tubes were refrigerated at 4-8°C in the hospital blood bank and retrieved within 24-72 hours of collection.

Tubes were centrifuged for 15 minutes at 3500 rpm to separate plasma from packed cells. Plasma was aliquoted into low temperature freezer tubes, labeled with the code number and then stored frozen (-75°C) in the Neonatal Research Laboratory until analyzed. Packed red cells were stored separately in the original tube for other analyses. Plasma was thawed at room temperature and sonicated for 5 minutes prior to analysis.
BIOCHEMICAL ANALYSIS

Reference standards were obtained from Dr. Steven R. Myers in the Department of Pharmacology and Toxicology at the University of Louisville School of Medicine. Stock solutions of anthracene, 3,4,8,9,-dibenzo(a)pyrene and 1-hydroxypyrene were prepared in HPLC-grade dichloromethane (Burdick Jackson).

Chromatography was performed with a Waters High Pressure Liquid Chromatography system (HPLC) consisting of an M600E delivery system, M717 autosampler, M996 photodiode array detector (PDA) all of which were controlled by Waters Empower Build 1154 software (version 5.00.00.00) running on a Dell Dimension 8100 computer with Windows XP Professional operating system. The analytical column was a 25 cm x 10 mm C18 (Waters) packed with Ultrasphere ODS (5µ pore size). The carrier solvent was 100% acetonitrile (Burdick Jackson HPLC grade) running isocratically at 1 mL/minute. The injection volume was 10 µL. PDA detection was full spectrum (190-600 nm) with resolution of 1.2 nm. Each standard was run in multiple dilutions to establish linear reference curves for each compound. See figures 3-5.
Figure 3.

Standard curve of anthracene. Waters HPLC System with Photodiode Array detection 190-600 nm.
Figure 4.

Standard curve of benzo(a)pyrene. Waters HPLC System with Photodiode Array detection 190-600 nm.
Figure 5.

Standard curve of 1-hydroxypyrene. Waters HPLC System with photodiode array detection 190-600 nm.
Chromatograms for each compound were extracted at 254 nm prior to integration which was performed with the same Waters software, using individual PAH standard curves as references (see figure 6).

**Figure 6.**

**Chromatogram of anthracene, 1-hydroxypyrene and benzo(a)pyrene.**

Waters HPLC System with photodiode array detection, extracted at 254 nm.

Plasma (~500 µl) was pipetted into 13x100 mm borosilicate tubes. One mL of HPLC-grade ethyl acetate was added, tubes were agitated vigorously on a Vortex Jr. platform for 1 minute and then centrifuged for 15 minutes at 3500 rpm.
If a plasma volume less than 500 µl of plasma was available for analysis, the sample was still extracted with 1 mL of ethyl acetate, noting the plasma volume used. The supernatant was transferred to a clean tube and the extraction process repeated. The supernatants were combined and dried under nitrogen in a chemical hood at room temperature. When the solvent had evaporated, tubes were capped and refrigerated at 4°C until analyzed. The residue was warmed to room temperature and then reconstituted with a volume of dichloromethane (Fisher Scientific) equal to the original plasma volume. Tubes were sonicated for 1 minute and the reconstituted extract transferred to numbered sample vials before placing on the autosampler for analysis.

**COTinine**

Enzyme Linked Immunoassay (ELISA) kits for cotinine determination were obtained from Cozart Bioscience, Ltd. (Oxfordshire, UK). Kits included 96-well plate, standards, wash buffer, enzyme, substrate, and stop solution.

Twenty microliters (µL) of standards (0 to 50 ng/mL) or unknowns (in duplicate) were added to individual wells coated with anti-cotinine antibody. Cotinine enzyme conjugate (100 µL) was added to each well and the plate incubated at room temperature for 30 minutes. Wells were then washed three times with 350 µL of wash buffer to remove any non-specific plasma components. One hundred µL of 3,3′,5,5′ tetra methyl benzidine (color reagent) were added to each well and the samples incubated again at room temperature for 30 minutes. One hundred µL 3 N HCl were added as a stop solution. Final
absorbance was measured at 450 nm on a Biotek ELX-800 microplate reader controlled via Dell Dimension 8100 computer with Windows XP Professional and Biotek KC4 software. Concentrations of the unknowns were calculated against the standard curve.

**STATISTICAL PLAN AND ANALYSIS**

**POWER AND SAMPLE SIZE CONSIDERATIONS**

There were no preliminary data or published studies available from which we could extract reliable estimates of the anticipated effect sizes in this study. Since the primary focus of the statistical analysis for this study will be a comparison of the PAH and cotinine levels in the matched maternal serum-cord blood samples, we based our sample size calculation on the value of n required to achieve 80% power for detecting a small to medium effect size when using the paired t-test to compare the mean PAH and cotinine levels between the matched maternal serum and cord blood samples. Using a two-tailed paired t-test with a significance level of 0.05, a sample size of 60 matched samples would yield 80% power for detecting a true effect size of 0.35. (177) [Under Cohen’s classification scheme for effect sizes, 0.2 is considered small and 0.5 is considered medium.] This sample size of 60 will also yield 80% power for detecting a true correlation as small as 0.35 between any two analytes (e.g., maternal cotinine vs. cord anthracene) using a significance level of 0.05. A correlation of this magnitude is considered to represent a medium effect size according to Cohen’s scheme. A total sample size of 60 (corresponding to 30 in
each of two independent groups) will yield 80% power for detecting a large sample size (0.80 in Cohen’s scheme) when using the independent samples t-test with a significance level of 0.05. However, independent group comparisons will not be a major focus of the analyses in this study.

All statistical analyses were conducted with SPSS v.14. Graphs were produced with Sigma Plot 10.0. Comparison of maternal and matched cord plasma samples was conducted with paired T-tests. Sub-group comparisons were conducted with the independent t-test. Logarithmic transformations were applied prior to examining associations between maternal serum and cord blood concentrations to improve interpretability of scatterplots. Spearman correlations were used to measure associations between maternal and cord blood concentrations. Statistical significance was set at p<0.05.
RESULTS

Eighty-nine paired specimens were acquired from labor and delivery for this cross-sectional study. Of those, 25 had incomplete data, lacking either birth weight, gestational age or both. Sixty-four specimens were considered complete and included in the study. Infants with birth weight <2.5 kg were considered low birth weight (LBW). The proportions of LBW infants (12.5%) in this study sample exceeded the expected Kentucky rate (~8%) by 60%. Infants born before 38 completed weeks’ gestation were considered to be preterm (PT); those completing 38 or more weeks were considered term (T). The proportion of PT infants (27.7%) in this sample exceeded the expected rate (~12%) by over 200%. No infants were considered to be post-term (>42 weeks). Among term infants, one was large for gestational age (>90\textsuperscript{th} percentile) and 2 were small for gestational age (<10\textsuperscript{th} percentile). None of the preterm infants demonstrated aberrant fetal growth. Table 4 describes the specimen pool.
Table 4.

Demographics of maternal-infant pairs. (Mean, 95% C.I.)

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Term</th>
<th>Preterm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>64 (100%)</td>
<td>46 (72%)</td>
<td>18 (28%)</td>
</tr>
<tr>
<td><strong>Birth Weight (kg)</strong></td>
<td>3.08 (2.92, 3.23)</td>
<td>3.22 (3.09, 3.34)</td>
<td>2.07 (1.67, 2.47)</td>
</tr>
<tr>
<td><strong>Gestational Age (weeks)</strong></td>
<td>38.1 (37.6, 38.6)</td>
<td>38.5 (38.1, 38.8)</td>
<td>35.6 (32.5, 38.6)</td>
</tr>
<tr>
<td><strong>Low Birth Weight (&lt;2.5 kg)</strong></td>
<td>8 (12.5%)</td>
<td>3 (6.5%)</td>
<td>5 (27.7%)</td>
</tr>
</tbody>
</table>

The mean cotinine concentration in maternal plasma (47.5 ± 17.2 ng/mL) was strongly suggestive of high proportions of smoking women among this cohort. There were no statistically significant differences in concentrations of anthracene, 1-hydroxypyrene or benzo(a)pyrene between maternal and cord plasma (Table 5).
Table 5.

Concentrations of anthracene, benzo(a)pyrene, 1-hydroxypyrene and cotinine in maternal and cord blood plasma. (Mean, 95% C.I.)

<table>
<thead>
<tr>
<th></th>
<th>Maternal Plasma</th>
<th>Cord Blood Plasma</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=64</td>
<td>N=64</td>
<td></td>
</tr>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>7.0 (4.2, 9.8)</td>
<td>8.7 (5.6, 11.9)</td>
<td>0.338</td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td>4.9 (3.3, 6.6)</td>
<td>3.6 (2.8, 4.4)</td>
<td>0.174</td>
</tr>
<tr>
<td>1-Hydroxypyrene (pmol/mL)</td>
<td>274.2 (233.4, 315.1)</td>
<td>279.0 (242.0, 315.9)</td>
<td>0.864</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>47.5 (43.2, 51.8)</td>
<td>46.2 (39.5, 52.8)</td>
<td>0.732</td>
</tr>
</tbody>
</table>

When groups were distinguished by low birth weight (LBW) or not, there were no significant differences for either maternal or cord plasma concentrations (See Table 6).
Table 6.

Concentrations of anthracene,* benzo(a)pyrene,** 1-hydroxypyrene# and cotinine by low birth weight status. (Mean, 95% C.I.)

<table>
<thead>
<tr>
<th></th>
<th>Maternal Plasma</th>
<th>Cord Plasma</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LBW N=8</td>
<td>Not LBW N=56</td>
<td></td>
</tr>
<tr>
<td>ANTH* (pmol/mL)</td>
<td>4.9 (0, 14.4)</td>
<td>7.3 (4.3, 10.3)</td>
<td>0.585</td>
</tr>
<tr>
<td>BP** (pmol/mL)</td>
<td>3.5 (2.0, 4.9)</td>
<td>5.2 (3.2, 7.1)</td>
<td>0.139</td>
</tr>
<tr>
<td>1-HP# (pmol/mL)</td>
<td>238.8 (109.3, 368.4)</td>
<td>279.3 (235.0, 323.6)</td>
<td>0.510</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>47.7 (28.4, 67.1)</td>
<td>47.5 (43.1, 51.9)</td>
<td>0.975</td>
</tr>
</tbody>
</table>
The mean concentration of anthracene in term cord plasma was significantly higher than in maternal plasma (Table 7). There was no significant difference in concentrations of anthracene in plasma from mothers and infants that delivered preterm. Concentrations of cotinine, benzo(a)pyrene and 1-hydroxyptrene were similar in maternal and cord plasma, regardless of the length of pregnancy.
Table 7.

Concentrations of anthracene,* benzo(a)pyrene,** 1-hydroxypyrene# and cotinine in maternal and cord blood plasma by term or preterm delivery. (Mean, 95% C.I.)

<table>
<thead>
<tr>
<th></th>
<th>Term N=46</th>
<th></th>
<th>Preterm N=18</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maternal</td>
<td>Cord</td>
<td>p</td>
<td>Maternal</td>
</tr>
<tr>
<td>ANTH* (pmol/mL)</td>
<td>7.8 (4.2, 11.5)</td>
<td>9.4 (5.5, 13.3)</td>
<td><strong>0.029</strong></td>
<td>4.8 (1.0, 8.5)</td>
</tr>
<tr>
<td>BP** (pmol/mL)</td>
<td>5.5 (3.2, 7.8)</td>
<td>3.9 (2.8, 5.1)</td>
<td>0.372</td>
<td>3.5 (3.2, 7.8)</td>
</tr>
<tr>
<td>1-HP# (pmol/mL)</td>
<td>285.3 (232.3, 338.3)</td>
<td>272.4 (239.9, 304.8)</td>
<td>0.981</td>
<td>246.1 (188.1, 304.1)</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>47.7 (43.0, 52.4)</td>
<td>44.1 (36.3, 51.9)</td>
<td>0.675</td>
<td>46.9 (36.6, 57.3)</td>
</tr>
</tbody>
</table>
When comparing women who delivered preterm and those delivering at term (Table 8), all maternal plasma PAH values from women that delivered PT trended to be lower than those from women that delivered at term, but there were no significant differences. The mean cord plasma concentration of benzo(a)pyrene from infants who delivered prior to 38 weeks was significantly lower than that from infants delivered at term.
Table 8.

Concentrations of anthracene,* benzo(a)pyrene,** 1-hydroxypyrene# and cotinine by term or preterm delivery. (Mean, 95% C.I.)

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th></th>
<th>Cord</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Term</td>
<td>Preterm</td>
<td>p</td>
<td>Term</td>
</tr>
<tr>
<td>ANTH* (pmol/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.8 (4.2, 11.5)</td>
<td>4.8 (1.0, 8.5)</td>
<td>0.232</td>
<td>9.4 (5.5, 13.3)</td>
</tr>
<tr>
<td>BP** (pmol/mL)</td>
<td>5.5 (3.2, 7.8)</td>
<td>3.5 (2.5, 4.6)</td>
<td>0.120</td>
<td>3.9 (2.8, 5.1)</td>
</tr>
<tr>
<td>1-HP# (pmol/mL)</td>
<td>285.3 (232.3, 338.3)</td>
<td>246.1 (188.1, 304.1)</td>
<td>0.309</td>
<td>272.4 (239.9, 304.8)</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>47.7 (43.0, 52.4)</td>
<td>46.9 (36.6, 57.3)</td>
<td>0.888</td>
<td>44.1 (36.3, 51.9)</td>
</tr>
</tbody>
</table>
Visual inspection of the PAH and cotinine data suggested severe skewness, with several extreme values, so a logarithmic transformation was applied to each analyte. Figures 7-10 are scatter plots showing the least squares regression of the log transformed concentrations of cord plasma PAH and cotinine on the corresponding maternal analytes. LN anthracene: maternal vs. cord plasma shows a significant positive trend of increasing cord concentrations. LN benzo(a)pyrene: maternal vs. cord plasma shows a downward trend but the linear regression is not statistically significant. The graphical representation of LN 1-hydroxypyrene: maternal vs. cord shows a slight downward trend but with several extreme observations and the regression line for LN cotinine: maternal vs. cord shows a slight positive trend but, again, there is a wide distribution of values. Neither of the latter two linear regressions are statistically significant.
Figure 7.

Scatter plot with linear regression of LN anthracene: maternal vs. cord plasma

\[
y = 1.16 + 0.23(x)
\]

\[
r = 0.25
\]

\[
p = 0.047
\]
Figure 8.

Scatter plot with linear regression of LN benzo(a)pyrene: maternal vs. cord plasma

\[ y = 1.25 - 0.125x \]
\[ r = -0.15 \]
\[ p = 0.232 \]
Figure 9.

Scatter plot with linear regression of LN 1-hydroxypyrene: maternal vs. cord plasma

\[ y = 5.80 - 0.10x \]
\[ r = -0.08 \]
\[ p = 0.556 \]
Figure 10.

Scatter plot with linear regression of LN cotinine: maternal vs. cord plasma

\[ y = 3.57 + 0.25x \]

\[ r = 0.01 \]

\[ p = 0.917 \]
Given the lack of linear association between log-transformed maternal and cord plasma concentrations and the presence of so many extreme values, Spearman correlations were used to measure the associations between maternal and cord plasma concentrations. There were no significant correlations between LN benzo(a)pyrene: cord and any maternal PAH or cotinine, or between LN cotinine: cord and any maternal analyte. None of the data are shown. Tables 9-10 list the remaining Spearman correlations. Anthracene: cord was significantly correlated with anthracene: maternal and cotinine: maternal (Table 9). 1-hydroxypyrene: cord was significantly correlated with 1-hydroxypyrene: maternal (Table 10).
Table 9.

**Spearman correlations between maternal concentrations of anthracene, benzo(a)pyrene, 1-hydroxypyrene, cotinine and cord plasma anthracene.**

<table>
<thead>
<tr>
<th>Maternal</th>
<th>Cord Plasma Anthracene (pmol/mL)</th>
<th>( r_s )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>0.38</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td>-0.09</td>
<td>0.507</td>
<td></td>
</tr>
<tr>
<td>1-Hydroxypyrene (pmol/mL)</td>
<td>0.13</td>
<td>0.302</td>
<td></td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>0.40</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
Table 10.

Spearman correlations between maternal concentrations of anthracene, benzo(a)pyrene, 1-hydroxypyrene and cotinine and cord plasma 1-hydroxypyrene.

<table>
<thead>
<tr>
<th>Maternal</th>
<th>Cord Plasma 1-hydroxypyrene (pmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$</td>
</tr>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>0.01</td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td>0.11</td>
</tr>
<tr>
<td>1-Hydroxypyrene (pmol/mL)</td>
<td>0.25</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>-0.13</td>
</tr>
</tbody>
</table>
Plasma cotinine concentrations were individually subjected to Spearman correlation analysis with each PAH compound separately for the maternal and cord concentrations. In maternal plasma, cotinine was positively correlated with 1-hydroxypyrene (Table 11). In cord plasma, cotinine was negatively correlated with 1-hydroxypyrene as seen in Table 12.

**Table 11.**

**Correlations between maternal plasma concentrations of cotinine and individual PAH**

<table>
<thead>
<tr>
<th>MATERNAL COTININE</th>
<th>rs</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>0.09</td>
<td>0.487</td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td>-0.05</td>
<td>0.690</td>
</tr>
<tr>
<td>1-Hydroxypyrene (pmol/mL)</td>
<td>0.30</td>
<td>0.016</td>
</tr>
</tbody>
</table>
Table 12.

Correlations between cord plasma concentrations of cotinine and individual PAH

<table>
<thead>
<tr>
<th></th>
<th>rs</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>0.01</td>
<td>0.912</td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td>-0.01</td>
<td>0.915</td>
</tr>
<tr>
<td>1-Hydroxypyrene (pmol/mL)</td>
<td>-0.26</td>
<td>0.038</td>
</tr>
</tbody>
</table>
Maternal plasma PAH compounds correlated significantly with each other (Table 13). However there was no correlation between maternal cotinine and the individual PAH (data not shown).

**Table 13.**

**Correlations among maternal plasma PAH and cotinine**

<table>
<thead>
<tr>
<th>MATERNAL</th>
<th>1-Hydroxypyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo(a)pyrene</td>
<td>0.28</td>
</tr>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>0.32</td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Unlike what was seen in the maternal plasma, cord plasma PAH compounds did not show significant correlations. However, cotinine: cord did correlate significantly with 1-hydroxypyrene. (Table 14).

**Table 14.**

Correlations among cord plasma PAH and cotinine.

<table>
<thead>
<tr>
<th>CORD</th>
<th>Benzo(a)pyrene</th>
<th>1-Hydroxypyrene</th>
<th>Cotinine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
<td>rs</td>
</tr>
<tr>
<td>Anthracene (pmol/mL)</td>
<td>0.08</td>
<td>0.518</td>
<td>0.03</td>
</tr>
<tr>
<td>Benzo(a)pyrene (pmol/mL)</td>
<td></td>
<td>0.23</td>
<td>0.065</td>
</tr>
<tr>
<td>1-Hydroxypyrene (pmol/mL)</td>
<td></td>
<td></td>
<td>-0.26</td>
</tr>
</tbody>
</table>
After reviewing studies from Jauniaux (178) Ziegler (179) and Perera (3), a maternal cotinine concentration of 35 ng/mL was chosen as a cut-point to distinguish between two groups of women: those whose tobacco smoke exposure was limited (none-light) and those whose smoke exposure was more significant (moderate-heavy). Differences in maternal PAH concentrations within those sub-groups are shown in Table 15. The concentration of anthracene in the moderate-heavy group was more than twice that of the none-light group and was statistically significant; the other PAH were not.
Table 15.

Maternal anthracene, benzo(a)pyrene and 1-hydroxypyrene by maternal cotinine subgroups (Mean, 95% C.I.)

<table>
<thead>
<tr>
<th></th>
<th>Maternal cotinine</th>
<th>Maternal cotinine</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;35 ng/mL</td>
<td>≥35 ng/mL</td>
<td></td>
</tr>
<tr>
<td>N=8</td>
<td>N=56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthracene</td>
<td>3.0 (0.6, 5.4)</td>
<td>7.6 (4.4, 10.7)</td>
<td>0.019</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>3.3 (1.9, 4.7)</td>
<td>5.2 (3.3, 7.1)</td>
<td>0.105</td>
</tr>
<tr>
<td>1-Hydroxypyrene</td>
<td>232.7 (154.0, 311.4)</td>
<td>280.2 (234.4, 326.0)</td>
<td>0.259</td>
</tr>
</tbody>
</table>

When birth weight, gestational age and cord plasma concentrations of PAH and cotinine were compared in the two maternal smoking groups, mean anthracene remained significantly higher in the moderate-heavy smoking group (Table 16). Cord plasma benzo(a)pyrene was lower in the none-light group, but did not reach statistical significance. There was no statistically significant difference in concentrations of 1-hydroxypyrene between the groups. Interestingly, cord plasma cotinine was similar between the groups (43.0±24.7 vs. 46.6±27.3 ng/mL, none-light compared to moderate-heavy, respectively) despite an almost 2-fold difference in the maternal cotinine concentrations.
(27.2±7.7 vs. 50.5±16.2 ng/mL, none-light compared to moderate-heavy, respectively).

Table 16.

**Birth weight, gestational age, cord plasma PAH and cotinine by maternal cotinine subgroups (mean, 95% C.I.)**

<table>
<thead>
<tr>
<th>Maternal cotinine</th>
<th>Maternal cotinine</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;35 ng/mL N=8</td>
<td>≥35 ng/mL N=56</td>
<td></td>
</tr>
<tr>
<td>BW (kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.94 (2.4, 3.5)</td>
<td>3.09 (2.9, 3.3)</td>
<td>0.533</td>
</tr>
<tr>
<td>GA (wks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36.9 (35.0, 38.8)</td>
<td>38.3 (37.7, 38.8)</td>
<td>0.151</td>
</tr>
<tr>
<td>ANTH* (pmol/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.2 (1.0, 7.4)</td>
<td>9.4 (5.8, 12.9)</td>
<td>0.026</td>
</tr>
<tr>
<td>BP** (pmol/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.7 (2.3, 3.2)</td>
<td>3.7 (2.8, 4.7)</td>
<td>0.061</td>
</tr>
<tr>
<td>1-HP# (pmol/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>262.9 (156.8, 369.0)</td>
<td>281.3 (240.7, 321.9)</td>
<td>0.717</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43.0 (22.3, 63.7)</td>
<td>46.6 (39.3, 53.9)</td>
<td>0.713</td>
</tr>
</tbody>
</table>

*Anthracene

**Benzo(a)pyrene

#1-Hydroxypyrene
To summarize the results of this study, there were significant correlations between:

- Maternal and cord concentrations of anthracene,
- Maternal and cord concentrations of 1-hydroxypyrene,
- Anthracene, benzo(a)pyrene and 1-hydroxypyrene in maternal plasma,
- Maternal cotinine and cord anthracene,
- Maternal cotinine and cord 1-hydroxypyrene,
- Cord cotinine and cord 1-hydroxypyrene.

There were significant differences between:

- Cord concentrations of benzo(a)pyrene in term vs. preterm
- Cord concentrations of anthracene in subgroups based on maternal cotinine above or below 35 ng/mL
- Cord concentrations of anthracene in subgroups of maternal low, moderate and heavy smoking prior to delivery
DISCUSSION

Smoking during pregnancy remains a significant public health concern despite considerable evidence of the dangers in scientific literature and lay press. The physical evidence for maternal harm from smoking during pregnancy has been known for decades and has been discussed earlier in this paper:

- Miscarriage/spontaneous abortion
- Placenta previa, placental abruption
- Preterm delivery

Infant effects, both immediate and in the months after birth, are also well known and have been discussed:

- Low birth weight (even after a full term gestation)
- Nicotine withdrawal during the first hours after birth
- Decreased arousability from sleep
- Increased risk for sudden infant death syndrome (SIDS)

The data related to learning delays and behavioral disturbances are less clear because of the confounding effects of social and genetic factors. However, concerns remain because of data from animal studies that show the disruptive effects of nicotine on neural development.
This study began with 4 research questions.

1. Are anthracene, benzo(a)pyrene and 1-hydroxypyrene present in measurable concentrations in maternal and cord blood plasma?

Yes. Each PAH was measured in both maternal and cord blood plasma, although in some individuals, the concentrations were very low.

2. Do plasma concentrations of anthracene, benzo(a)pyrene and/or 1-hydroxypyrene correlate with cotinine among smoking and non-smoking women?

Maternal cotinine was correlated only with 1-hydroxypyrene in maternal plasma. Maternal cotinine did, however, correlate significantly with cord anthracene and 1-hydroxypyrene. Within cord plasma samples, as was seen in maternal plasma, cotinine was correlated only with 1-hydroxypyrene.

3. Do plasma concentrations of anthracene, benzo(a)pyrene and 1-hydroxypyrene in cord blood parallel levels in maternal plasma?

Again, the answer is no. Extremes of concentrations existed within paired maternal and cord plasma samples in which very high concentrations in maternal plasma were coupled with low concentrations in cord plasma and vice versa. Mean anthracene concentrations always trended to be higher in cord plasma than in maternal plasma regardless of the subgroup being studied (term, preterm, low birth weight), but without reaching statistical significance. This suggests that there may either be a concentrating effect on the fetal side, as has been observed with nicotine (138), or that the fetus lacks the enzyme machinery to metabolize anthracene to other compounds, perhaps leading to an accumulation
over time. The effect of labor, especially its duration, on the processes involved in PAH and cotinine metabolism are not known.

Benzo(a)pyrene trended to be higher in maternal plasma than in cord while 1-hydroxypyrene was not consistently higher or lower in one or the other even though it did correlate with maternal and cord cotinine.

4. Are plasma concentrations of anthracene, benzo(a)pyrene and 1-hydroxypyrene correlated with birth weight or gestational age?

Although our sample had more low birth weight and preterm infants than would be expected, it did not appear that concentrations of any of the measured PAH or cotinine were correlated with those outcomes. This study has shown, however, that anthracene, benzo(a)pyrene and 1-hydroxypyrene are present in cord blood in measurable concentrations.

Given the thousands of chemicals found in tobacco smoke, it is difficult to attribute any one effect to a single chemical or group of chemicals. Much research has focused on nicotine and carbon monoxide and the resulting hypoxia and vasoconstriction that are believed to be responsible for intrauterine growth restriction and low birth weight (95, 122, 139, 180, 181). Other recent studies have linked components of tobacco smoke, especially PAH, to a variety of enzymatic, inflammatory and cellular changes in the placenta and the fetus. (43, 182-190)

Unlike a singular event that may or may not coincide with a critical epoch, maternal smoking during pregnancy represents chronic exposure of the fetus to the thousands of compounds in tobacco smoke many times a day over multiple
days or weeks. Repeated exposure across numerous critical windows may amplify the resulting effect from very early in gestation. Although it is impossible to know how many fetal organs, cells, organelles and genes are altered each time a woman smokes a cigarette, it clear that these compounds are reaching the fetus in measurable quantities.

Kentuckians are caught in a vortex of poor health: excessive and increasing numbers of low birth weight and preterm infants, rising numbers of overweight/obese children and adults with many dying from heart-related disease. A large proportion of these problems can be attributed to the "Kentucky 3-point lifestyle:"

- Cigarettes
- Junk food
- Inactivity

If programming theory is correct, we face an even more difficult task to break out of that cycle given the numbers of infants that experience sub-optimal gestational conditions which are then reinforced by the Kentucky life style. The unacceptably high numbers of infants that experience the ill effects of their mothers' smoking before and after birth are but one facet of the two-pronged public health challenge before us: 1) to convince children and adolescents to avoid smoking at all and 2) to encourage and support smokers in their efforts to quit. This second task is especially important for women who are or who are thinking about becoming pregnant.
LIMITATIONS OF THE STUDY

There were several limitations in this study.

1. This was a pilot study, and as such, was designed to gather preliminary data for a larger study rather than to test a specific hypothesis. There are over 4,000 chemicals in tobacco smoke; this study only attempted to study 3. There are many more that remain unstudied.

2. Maternal blood specimens were drawn at the time of admission to the labor and delivery service in order to avoid an extra blood draw for the mother. Thus, there was a time lapse between the maternal specimen and the cord blood specimen drawn at delivery. While cotinine has a half-life of 18-20 hours, the time difference between specimens is a variable that was not quantified.

The prevalence of high concentrations of cotinine suggest there were over 85% moderate to heavy smokers in this convenience sample, a condition that is highly unlikely. While cotinine is a good screening method, there are factors that could affect cotinine concentrations at the time of the blood draw, including the time lapse since the last cigarette, routine smoking patterns of the women, plasma volume, the duration and intensity of labor, as well as the level of environmental exposures other than personal tobacco smoking that could increase cotinine levels. All of these items are unknowns due to the anonymity of the blood samples. A better marker of true tobacco
smoke exposure would be hemoglobin-protein adducts, such as 4-amino-biphenyl. (191) Assays for these adducts were not performed for this study but will be included in future extensions of this research.

3. These were anonymous samples, lacking data as to the mother’s smoking history, diet, physical parameters of the pregnancy, etc. This decision was based on early experience that women smokers were quite reluctant to participate in a study that was focused on effects of smoking on the fetus. Ultimately this would lead to a bias toward non-smokers in the sampled population.

4. While personal smoking is presumed to be the major source of PAH in this sample, there are no data as to PAH exposure resulting from air pollution or from the woman’s local environment. A woman’s home or work environment that provided excessive exposure to air-borne particulates and/or PAH could have a significant effect on the concentrations we saw even if she, herself, was not a smoker.

**Future Research**

This study suggests several additional investigations. Prospective studies of women, smokers and non-smokers, that follow them in detail throughout their pregnancies with periodic blood samples and activity diaries, augmented by amniotic fluid studies, if warranted by normal clinical care, and delivery studies (maternal and cord) would create a more complete picture of gestational exposure and effects in the infant. These studies could include genotyping for
enzyme polymorphisms and biomarkers of exposure (DNA or hemoglobin protein adducts) as well as quantitation of a wider panel of PAH.

Monitoring of PAH or levels of other compounds in women that decrease or actually quit smoking during pregnancy would provide insight into the persistence of these compounds once smoking is no longer a factor. Postnatal studies of infants, especially those that experienced growth restriction \textit{in-utero}, should focus on early growth recovery (catch-up) and neurodevelopmental progress. Another approach to combating the \textit{in-utero} growth restriction could involve nutritional intervention to promote growth recovery.

The same plasma samples from this study can be further analyzed for other PAH and their metabolites as well as hemoglobin adducts. Further study of anthracene and its metabolites/adducts may enhance current investigations.

Body composition studies of infants that do and do not experience \textit{in-utero} growth restriction from maternal smoking may be useful in helping to determine what facet of body growth is constrained by the action of smoking. New technology makes these studies rapid, non-invasive and reliable.

A study that is underway involves quantitating these compounds in the milk of women that deliver preterm and correlating the results with growth outcomes in the nursery. Due to the benefits of human milk for human babies, breastfeeding will almost always be the preferred method of feeding. However, it is important to understand the level to which these compounds in mother’s milk may add to an infant’s \textit{in-utero} body burden.
These and many other studies will continue as tobacco smoking and air pollution are here to stay. Basic science investigators will continue to use improving technology to focus on the why and how tobacco impacts human health. Public health professionals will continue to educate policy makers and the general public to the importance of eliminating tobacco smoke from the environment for the good of everyone, but especially infants and children.
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163. Lithell HO, McKeigue PM, Gerglund L, Mohsen R, Lithell UB, Leon DA. Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years. British Medical Journal 1996;312(406-10.).


