BIOMARKERS OF EXPOSURE TO COMPLEX ENVIRONMENTAL MIXTURES

A Dissertation

by

ZIAD SAMI NAUFAL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Toxicology

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ABSTRACT

Biomarkers of Exposure to Complex Environmental Mixtures.

(May 2008)

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Maternal exposure to genotoxic chemicals may produce a variety of adverse birth outcomes. Depending on the dose and duration of exposure, adverse birth outcomes can range from premature or low-birth weight, to congenital abnormalities including neural tube defects (NTDs). The research described in this dissertation focused on several rural counties in Shanxi province, China. Shanxi has one of the highest rates of NTDs in the world. In 2005, the incidence of NTDs in the study counties ranged from 8 to 24 cases per 1,000 births. While some of these birth defects are likely to be related to nutrition, it is also suggested that environmental factors play a significant role. One such factor includes polycyclic aromatic hydrocarbon (PAH) exposure as a result of combustion of coal for indoor heating and cooking. Human populations in Shanxi depend heavily on coal as their main source of energy. This study determined the concentrations of PAHs in house dust, venous blood and placenta of study participants. Dust was collected from homes in the study site. Carcinogenic PAH levels in dust collected from kitchen floors ranged from 12 to 2,000 $\mu g/m^2$. The genotoxic potential of dust was confirmed by short-term bioassays. Median concentrations of total PAHs in placenta from children born with NTDs were elevated compared to matched controls and appeared to be associated with the risk of having a child with a NTD. Tobacco smoking was not associated with elevated levels of PAH biomarkers in this study population. Levels of bulky DNA adducts in placenta have also been quantified using ^{32}P-postlabeling. Adduct levels do not appear to be significantly different between cases and controls and were not associated with deletions in enzymes GSTM1 or GSTT1. These data suggest that children born with NTDs may be at increased risk due to exposure to genotoxic PAHs.

Studies with a larger number of subjects are needed to further elucidate the relationship between PAH exposure and adverse birth outcomes.

DEDICATION

To my mother, father and two brothers.

ACKNOWLEDGMENTS

I would like to express my deep gratitude to my advisor, Dr. Donnelly, for his guidance and support during my graduate studies. Dr. Donnelly not only taught me about Toxicology and research, but also gave me lessons in life. I would like, in addition, to thank Drs. McDonald, Safe and Carozza for serving on my graduate committee. Dr. McDonald was instrumental in helping me with the chemical analysis part of my research. Drs. Safe and Carozza were the instructors of two of the most fulfilling classes that I took as a graduate student.

I would also like to extend my gratitude to my friends, colleagues and the staff of the Veterinary Integrative Biosciences Department, School of Rural Public Health, and the Interdisciplinary Faculty of Toxicology at Texas A&M University. Specifically, I am indebted to people who helped me in this research: Dr. Ling-Yu He, Rebecca Lingenfelter, Dr. Leslie Cizmas, Dr. Annika Gillespie, Molly Richardson, Christine Naspinski, Dr. Tracie Phillips, Nancy White, Shirley Wang and Matt Kelley. I would like to thank Dr. Guo-Dong Zhou for the time he took to train me in his lab, his encouragement and supervision, as well as his expertise. I would also like to acknowledge Kim Daniel, Ben Morpurgo, Deborah Daniel, and Tammy Turner for their assistance with administrative issues. I would like to extend my thanks to Claire Bilberry and Dr. Daad Abi-Ghanem for their encouragement and help, especially when writing this document. I am also grateful to our collaborators at the Peking University Health Science Center in Beijing, China. Both the United States Environmental Protection Agency as well as the National Institute of Environmental Health Sciences' Superfund Basic Research Program Grant are acknowledged for funding this research.

Finally, I would like to thank my two Aggie brothers, Georges and Kamal for their support and help during the time we spent together away from home. Last, but certainly not least, thanks to my mother and father for all of their patience, encouragement, love and unconditional support throughout my graduate studies.

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NOMENCLATURE

ABBREVIATION	TERMINOLOGY
2NF	2-Nitrofluorene
3-MC	3-Methylcholanthrene
^{32}P	Radioactive Phosphorus Isotope
ABS	Assays for Chromosome Aberration
ADP	Adenosine Diphosphate
AKR	Aldo-Keto Reductase
ATP	Adenosine Tri-Phosphate
ATSDR	Agency for Toxic Substances and Disease Registry
B[a]P	Benzo[a]pyrene
BPDE	Benzo[a]pyrene Diol-Epoxide
COC	Contaminant of Concern
CPM	Counts per Minute
CYP	Cytochrome P450
DCM	Dichloromethane
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Estrogen Receptor
ERα	Estrogen Receptor Alpha
ETS	Environmental Tobacco Smoke
GC/MS	Gas Chromatography/Mass Spectroscopy
GST	Glutathione S-Transferase
HRS	Hazardous Ranking System
IRIS	Integrated Risk Information System
MEOH	Methanol
MFOs	Mixed Function Oxidases
MOLY	Mouse Lymphoma Assay

ABBREVIATION	TERMINOLOGY
NAPL	Non-Aqueous Phase Liquid
NPL	National Priorities List
NRC	National Research Council
NTP	National Toxicology Program
OHP	Hydroxypyrene
PAH	Polycyclic Aromatic Hydrocarbon
PB	Phenobarbital
PCA	Polychlorinated Aromatic
PCB	Polychlorinated Biphenyl
PCP	Pentachlorophenol
PEI	Polyethyleneimine
PNK	Polynucleotide Kinase
RAL	Relative Adduct Labeling
RfD	Reference Dose
RPM	Revolutions per Minute
S9	Rat Liver Supernatant 9000
SAL	*Salmonella*/Microsome Mutagenesis Assay
SCE	Sister Chromatid Exchange
SCGE	Single Cell Gel Electrophoresis
SSC	Sodium Citrate + Sodium Chloride
TLC	Thin Layer Chromatography
USEPA	United States Environmental Protection Agency

CHAPTER I
INTRODUCTION

1.1 Overview

Humans may be exposed to hazardous chemicals at every stage of their lives. Hazardous chemicals in food or drinking water are almost always found as complex mixtures. One of the most common classes of chemical mixtures in the environment is polycyclic aromatic hydrocarbons (PAHs). These compounds are ubiquitous and have been detected in all environmental media, as well as biological receptors and settled dust. Extensive data exist to quantify concentrations of PAHs and other complex mixtures in environmental media. Studies have been conducted on a more limited basis to measure biomarkers of exposure in populations exposed to chemical mixtures in occupational and residential settings. This dissertation describes research to quantify PAHs in both environmental and biological samples; and, to investigate the relationship between DNA adducts and genetic polymorphisms in a population known to have an elevated risk of congenital malformations.

1.2. Complex Chemical Mixtures

High molecular weight organic chemicals are an environmental concern because they are persistent, prone to bioaccumulation, and because many compounds are known carcinogens. Every year several billion pounds of toxic chemicals are released into the environment from diverse sources. In 2003, almost 4.44 billion pounds of hazardous chemicals were released to the environment from industrial facilities operating in the United States (USEPA 2005). Hazardous chemicals may also be released into the environment from anthropogenic sources and the combustion of fossil fuels. These chemicals most often enter the environment as complex mixtures that include organic and inorganic compounds. PAHs and polychlorinated aromatic compounds (PCAs) are

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This dissertation follows the style of Environmental Health Perspectives.

among the most common classes of chemicals detected in environmental media (Mueller et al. 1991; Ramesh et al. 2004; Samanta et al. 2002). The composition of these mixtures varies greatly depending on the source and temperature at which the mixture was generated. Human exposure to chemical mixtures is common and could induce a variety of adverse health effects, including cancer, respiratory and cardiovascular disease.

A complex mixture may be described as a substance that contains from ten to more than one thousand components (Feron et al. 1995). In the case of most environmental mixtures, the composition is usually not qualitatively or quantitatively known (Groten et al. 2001). Often, less than 50% of the components of a complex mixture can be quantified. This is mostly due to matrix interferences and the close structural similarity of many of the components. In addition, compound interactions, both chemical and toxicological, may alter the properties of the components of a mixture.

The components of a complex mixture may induce synergistic, antagonistic, or inhibitory interactions. Mixture interactions may alter the toxicity of the components through changes in adsorption, metabolism, distribution and excretion. In most cases, chemicals cross cell membranes by passive diffusion. Depending on the water solubility of the components of a complex mixture, the rate of chemical transport into cells can be increased or inhibited. Also, depending on the composition of a chemical mixture, some compounds might enhance, inhibit or deplete metabolic enzymes which may alter chemical activation of certain components in the mixture. Moreover, components in a mixture can compete for binding sites on critical macromolecules within a cell which may alter the toxicity of this mixture.

Sources of complex mixtures include cooked foods, combustion byproducts, and releases associated with hazardous waste facilities. Hazardous chemicals released from industries may add to the burden of naturally occurring chemicals. Complex chemical mixtures containing PAHs have been detected at almost half of the 1,609 hazardous waste sites listed as Superfund sites in the United States (USEPA 2006). Brender et al. (2006) recently observed higher rates of adverse birth outcomes in populations living

within one mile of a Superfund site. PAH mixtures are commonly found at wood preserving sites, coal gasification sites, refineries, petroleum production facilities and other sites where petroleum products have been produced, stored or disposed. In addition, PAH mixtures are common combustion byproducts. Thus, sources of human exposure to PAH mixtures include not only the release of hazardous chemicals, but also ingestion of cooked foods and inhalation of cigarette smoke or other combustion byproducts. In fact, cigarette smoke is an example of a complex mixture that is well characterized in both animal and human studies. Cigarette smoke is a mixture of approximately 4,800 chemicals although some reports suggest that the actual number of chemical components is greater and can reach 100,000 chemicals (Green and Rodgman 1996; Rodgman et al. 2000; Wright 1956). It is possible to classify the components of cigarette smoke into four major functional classes: irritants, enzyme inducers, carcinogens and promoters. Irritant chemicals include acrolein, ammonia and formaldehyde. These chemicals can damage membranes and increase cell permeability to other mixture components. Enzyme inducers include nicotine and low molecular weight PAHs. Such compounds can increase the activity of xenobiotic metabolizing enzymes in an exposed organism, and thus increase activation of indirect carcinogens. Carcinogens or pro-carcinogens found in the mixture help initiate the process of carcinogenesis. These include organic chemicals such as benzo[a]pyrene (BaP), vinyl chloride, benzene, and other PAHs in addition to inorganic metals such as nickel. PAHs and catechol may also act as promoters by activating oncogenes and damaging tumor suppressor genes leading to abnormal cell proliferation, and ultimately neoplastic transformations. Thus, it is possible that the interactions of the components of cigarette smoke serve to increase the uptake and metabolism of the carcinogenic components, as well as to enhance genetic damage produced by binding with DNA. Animal studies with binary mixtures have generally observed additive interactions (Hughes and Phillips 1990; Tang et al. 2003; White 2002). Less information is available to characterize the interactions of complex mixtures.

The release of hydrocarbon mixtures to the environment may produce contamination of air, surface water, soil, sediment and/or groundwater. Such mixtures generally persist in the environment and could pose a threat to human and/or ecological health by accumulating in the food chain. PAHs in particular are insoluble in water and tend to concentrate in soil and sediment near industrial sources and hazardous waste sites. They also adsorb onto particulate matter in air and may settle in dust in indoor environments.

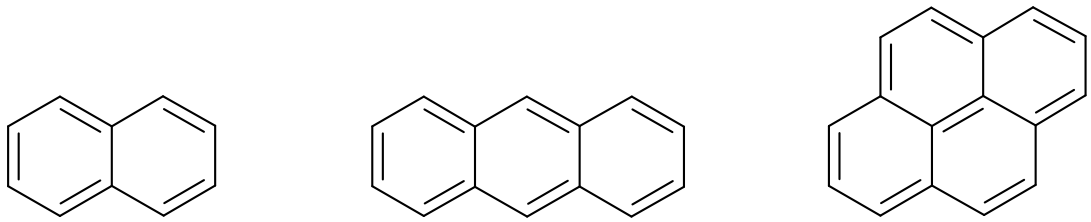
Humans are generally exposed to complex environmental mixtures capable of producing a broad range of biological effects (Gennings 1995; Teuschler and Hertzberg 1995). Environmental exposures are often repetitive, low dose exposures involving multiple pathways including inhalation, ingestion and dermal absorption. The severity of adverse health effects produced following such exposures varies greatly and depends not only on the dose and duration of exposure but also on intrinsic individual factors such as lifestyle exposures and genetic sensitivities.

1.3. Polycyclic Aromatic Hydrocarbons

1.3.1 Sources

PAHs are ubiquitous environmental contaminants. The incomplete combustion of virtually any type of organic material results in the production of these chemicals. PAHs share a similar chemical structure consisting of two or more fused benzene rings in linear, cluster or angular arrangements (Wilson and Jones 1993). The molecular structure of a representative 2-, 3- and 4-ring PAH is shown in Figure 1.1. PAHs are lipophilic non-polar chemicals that can adsorb to particles in the air or water and generally persist in the environment for extended periods of time (Brandt and Watson 2003).

PAHs have various natural and anthropogenic sources. High concentrations of PAHs are present in crude oil, coal and oil shale. These petroleum and petrochemical products are extensively used to produce fuels and synthetics (fibers and plastics) (Harvey 1997). The widespread use of petroleum products has increased the level of



Naphthalene Anthracene Pyrene

Figure 1.1. Structures and nomenclatures of representative 2-, 3- and 4-ring polycyclic aromatic hydrocarbons.

PAHs in the environment. Coke oven emissions and the combustion of fossil fuels and refuse generate approximately 50% of the total emissions of the model five-ringed PAH, BaP, in the United States. Vehicle emissions constitute another major source of PAHs, especially in urban areas where they generate approximately 35% of PAH emissions. Other sources include fumes from manufacturing industries and tobacco smoking. Natural sources of PAHs include forest fires and volcanic eruptions although anthropogenic sources are generally assumed to be more significant (Harvey 1997).

The chemical composition and concentration of PAH mixtures vary according to the temperatures at which they were generated. High temperatures in the absence of oxygen usually lead to formation of simple mixtures of unsubstituted PAHs. Intermediate temperatures such as smoldering wood will result in more complex mixtures including alkyl substituted PAHs. At lower temperatures, reaction rates are slow and predominant products are methyl and other alkyl substituted polyarenes (Harvey 1997).

Levels of PAHs in urban atmospheres depend on the density, sources of local emissions, temperature and local meteorological conditions among other factors. PAH levels for example, tend to be higher in cold winter months reflecting the increase in fossil fuel consumption. Atmospheric conditions, such as temperature inversions, may also increase PAH concentrations near ground level. The transition of PAHs from the gas phase into the solid phase (fly ash) occurs when temperatures are below 150°C

(Harvey 1997). Airborne PAHs are largely present as aerosols due to their low vapor pressure and high melting points. They either exist as more or less pure particles or are absorbed onto particulate matter (PM) such as soot and dust (ATSDR 1995). Particles less than 5 μm in diameter are respirable. Urban airborne particulates tend to be in this category (Harvey 1997). In remote areas, concentrations of airborne PAHs were estimated to be in the range of 1-50 ng/m^3 and have been detected at 100 to 1000-fold higher levels in urban or industrial areas depending on the season. In rural areas, background air levels of representative PAHs were reported to range between 0.02 and 1.2 ng/m^3, whereas in urban areas these levels ranged between 0.15 and 19 ng/m^3 (ATSDR 1995). Atmospheric PAH concentrations were also found to proportionally increase with population density as described by Hafner et al. (2005). PAHs in air from sites in developing countries were typically higher than those of developed countries which is most likely due to lack of regulation and technological innovation (Hafner et al. 2005).

A summary of airborne concentrations of PAHs reported in the literature is provided in Table 1.1. Air concentrations of carcinogenic PAHs in the city of Prague, one of the most polluted areas in the Czech Republic, were reported at 20 μg/m^3 in winter and 4 μg/m^3 in spring (Sram et al. 2007). In three sites in south of France described as urban, sub-urban and rural, the average air concentrations of 15 PAHs were reported to be 22, 4.5 and 16 ng/m^3 respectively (Albinet et al. 2007). PAHs in air samples collected in Beijing, China ranged in concentration between 29 and 362 ng/m^3 during the period from December 2004 to August 2005. Levels of PAHs were highest during winter and lowest during summer which is thought to be due to more frequent domestic coal-burning heaters use in winter and less photochemical degradation (Liu et al. 2007). In Vietnam and Japan, total PAH concentrations in air samples collected continuously for more than a year ranged between 4 and 9 ng/m^3. PAH levels were found to be highest during the rainy season in Vietnam and winter in Japan (Hien et al. 2007). Levels of airborne PAHs in a low-contaminated urban area in the Czech Republic were found to be associated with traffic volume. Air samples collected at high and low

traffic area in the study site had BaP concentrations of 0.6 ng/m^3 and 0.4 ng/m^3, respectively. Total PAH (tPAHs) concentrations from the high traffic area were 46 ng/m^3 whereas tPAHs were at 21 ng/m^3 in the low traffic area. The presence of elevated concentrations of benzo[ghi]perylene and coronene in the airborne samples confirmed that traffic emissions were the major source of PAHs in the study area (Ciganek et al. 2004). In a particulate speciation study from South Bronx, New York City with a heavy traffic volume, levels of tPAHs ranged between 110 and 375 ng/m3. Traffic on major highways around South Bronx was suggested to be the major source of air pollution in that area (Maciejczyk et al. 2004). Ambient atmospheric PAH concentrations in six Southern California communities including rural upwind and metropolitan downwind sites from Los Angeles ranged between 60 and 610 ng/m^3 over a one year sampling period. Significant seasonal differences in rural and urban communites were observed especially for particle-phased PAHs which increased with decreasing ambient temperatures. PAH levels at the rural site were significantly lower than communities located downwind from Los Angeles. PAHs were present mostly in the vapor phase and dominated by naphthalene, which was typically thousands of times higher in concentration than other measured PAHs. Exhaust emissions from motor vehicles played an important role in the observed particle-phase PAH levels (Eiguren-Fernandez et al. 2004). Concentrations of thirty different PAHs were measured in three heavily populated and highly industrialized regions of the United States; Los Angeles County, California and the cities of Houston, Texas and Elizabeth, New Jersey. Different PAH levels were found among the three different areas which reflected different dominant emission sources. The total PAH concentrations were 4.2 to 64 ng/m^3 in Los Angeles, 10 to 160 ng/m^3 in Houston, and 12 to 110 ng/m^3 in Elizabeth. Los Angeles County is dominated by mobile sources of PAHs such as motor vehicles, whereas Houston having a highly developed petrochemical industry had petrogenic and pyrogenic emission sources.

Table 1.1. Summary of BaP, carcinogenic (USEPA B2) and total PAH concentrations in outdoor air (ng/m^3) at different sites.

Site Location	BaP	Carcinogenic PAHs	Total PAH	Time Frame	Reference
Prague, Czech Republic	4300	19700	n/a*	January 2004	(Sram et al. 2007)
	800	3600	n/a	March 2004	
South of France	0.02-0.1	0.1-1	5-20	July 2004	(Albinet et al. 2007)
Beijing, China	2	20	30	August 2005	(Liu et al. 2007)
	25	175	360	December 2004	
Vietnam	0.6-0.7	n/a	7-10	January 2005-March 2006	(Hien et al. 2007)
Osaka, Japan	0.3	n/a	4	April 2005-May 2006	(Hien et al. 2007)
Brno, Czech Republic	0.4-0.6	2-3	20-50	October 2001	(Ciganek et al. 2004)

Table 1.1. (continued)

Site Location	BaP	Carcinogenic PAHs	Total PAH	Time Frame	Reference
South Bronx, New York	n/a	n/a	110-375	April 2001-February 2003	(Maciejczyk et al. 2004)
South California (Los Angeles area), USA	0.01-0.1	0.06-1	60-610	May 2001-July 2002	(Eiguren-Fernandez et al. 2004)
Los Angeles County, California, USA	0.1	n/a	4.2-64	June 1999-May 2000	(Naumova et al. 2002)
Houston, Texas, USA	0.04	n/a	10-160	June 1999-May 2000	(Naumova et al. 2002)
Elizabeth, New Jersey, USA	0.2	n/a	12-110	June 1999-May 2000	(Naumova et al. 2002)

*=not available

Emission sources in Elizabeth include a combination of the previously mentioned sources in addition to home heating with natural gas and oil which has been found to contribute to the atmospheric PAH concentrations during the cold months (Naumova et al. 2002).

PAHs may also be prevalent in indoor air at typically higher concentrations than outdoor air. Tobacco smoking is a major source of PAHs in residential environments. Cooking, heating and candle and incense burning are also among indoor PAH sources. Table 1.2 provides a summary of indoor air concentrations of PAHs reported in the literature. Zhu and Wang (2003) observed a mean total PAH concentration of 7,600 ng/m^3 in air samples collected from domestic kitchens in China consisting mainly of 2- and 3- ring PAHs, and 17,000 ng/m^3 in commercial kitchens predominantly comprised of 3- and 4- ring PAHs. Among the different cooking practices, boiling produced the least amount of PAHs. In kitchens of non-smoker homes located in Chicago, levels of tPAHs ranged between 13 and 2,454 ng/m^3. The correlation of indoor and outdoor concentrations of PAHs was found to be weak for low molecular weight PAHs but rather strong for high molecular weight PAHs. Thus, heavier PAHs are likely generated from outdoor sources (Li et al. 2005). In homes located in an urban area of Taipei, Taiwan, the mean concentration of tPAH in air samples was 267 ng/m^3. PAHs were more abundant in indoor rather than outdoor air, where they were present at average levels of 209 ng/m^3. Levels of PAHs were not significantly lower in summer than in winter. The most abundant PAH in the air of the sampled homes was naphthalene which is thought to be due to the common use of mothballs in wardrobes. Homes that burned incense had higher concentrations of BaP, fluoranthene, pyrene and benzo[ghi]perylene at 2.4, 6.2, 9.5, 7.6 ng/m^3, respectively. Air sampled in living areas of homes located in Shimizu, Japan was found to contain PAHs at levels ranging from 3,000 to 7,600 ng/m^3 (Ohura et al. 2004). In this study also, naphthalene was the most abundant PAH found in indoor air samples and was linked to the use of insect repellents. The median concentrations of indoor BaP in houses with smokers was higher than that in houses occupied by non-smokers, in winter and summer. Median levels of indoor BaP in smoker houses were

Table 1.2. Summary of BaP, carcinogenic (USEPA B2) and total PAH concentrations in indoor air (ng/m^3) at different sites.

Site Location	BaP	Carcinogenic PAHs	Total PAH	Time Frame	Reference
Domestic kitchens, Hangzhou, China	6-20	n/a*	3,600-9,800	December 2000	(Zhu and Wang 2003)
Commercial kitchens, Hangzhou, China	150-400	n/a	10,000-21,000	December 2000	(Zhu and Wang 2003)
Domestic kitchens, Chicago, Illinois, USA	n/a	n/a	13-2454	June 2000-August 2001	(Li et al. 2005)
Households, Shimizu, Japan	0.3-0.4	2.2-2.5	3,000-7,600	Summer 2000 and Winter 2001	(Ohura et al. 2004)
Households, Kuwait	0.2	2	7	February-April 2004	(Gevao et al. 2007)
Households, Taipei, Taiwan	2	24	267	August-September 2005 and December 1995-January 1996	(Li and Ro 2000)

Table 1.2. (continued)

Site Location	BaP	Carcinogenic PAHs	Total PAH	Time Frame	Reference
Households, Los Angeles County, California, USA	0.02-0.6	n/a	16-220	June 1999-May 2000	(Naumova et al. 2002)
Households, Houston, Texas, USA	0.003-1.1	n/a	21-310	June 1999-May 2000	(Naumova et al. 2002)
Households, Elizabeth, New Jersey, USA	0.006-0.2	n/a	22-350	June 1999-May 2000	(Naumova et al. 2002)
Households, Massachusetts, USA	n/a	n/a	8-31	July-August 1997	(Dubowsky et al. 1999)

*n/a=not available

0.5 ng/m^3 in winter and 0.25 ng/m^3 in summer whereas in non-smoker houses these values were 0.3 ng/m^3 in winter and 0.2 ng/m^3 in summer. Indoor air samples from non-smoking residences in three areas of the United States; Los Angeles County, Houston and Elizabeth, were analyzed for PAHs.PAH concentrations in these residences ranged from 8 to 350 ng/m^3. Data from these households suggest that indoor air concentrations of 5- to 7- ring PAHs, typically found in the particulate phase, are dominated by outdoor sources which were discussed in the previous section (Naumova et al. 2002). Indoor concentrations of PAHs from three nonsmoking households located in an urban, semi-urban and suburban area of Massachusetts were measured. Traffic was found to the major outdoor source of PAH levels indoors in all three locations. After adjusting for indoor sources, mean PAH concentrations were 31 ng/m^3 at the urban location almost two-fold that at the semi-urban location (19 ng/m^3) and more than three times than the suburban location (8 ng/m^3). During weekends, when traffic volume is low, indoor PAH levels were lower at all three locations; 10 ng/m^3 at the urban and semi-urban locations and 5 ng/m^3 at the suburban location. As for indoor sources, cooking seemed to be the major PAH source (Dubowsky et al. 1999). In residential settings in Kuwait, indoor air concentrations of PAHs ranged between 1.3 to 16 ng/m^3 and consisted predominantly of 3- and 4- ring PAHs. These levels are typically lower than the levels reported in the previously reviewed studies, which was suggested to be due to sampling gas-phase pollutants only. Indoor to outdoor ratios for individual PAHs indicated that no significant indoor sources exist for these compounds in the sampled homes (Gevao et al. 2007).

In sediment and soil, PAHs concentrations are often elevated in areas where coal, wood, gasoline, or other products have been burned. Levels of total PAHs in soils and sediments from different sites are summarized in Table 1.3. PAH concentrations in sediments from different sites in the United States ranged between 0.1 and 17,283 parts per million (ppm) (Gu et al. 2003; Kannan et al. 2005; Neff et al. 2005; Su et al. 1998). Two sediment samples, one heavily contaminated with creosote and the other contaminated with PAHs from urban runoff and deposition of pyrogenic PAHs from

combustion sources were collected from Wycoff/Eagle Harbor Superfund site in the state of Washington. Total PAH concentration in the creosote-contaminated sediment sample was 17,283 ppm as compared to 25 ppm in the other sample (Neff et al. 2005). Sediment cores were collected on the Black River located in north-central Ohio, in 1998 following closure of a coking facility in 1983 and remediation efforts during 1989 and 1990. Total PAH concentrations in the sediment cores ranged between 0.1 and 250 ppm (Gu et al. 2003). Sediment cores from 11 inland lakes in Michigan, had tPAH concentrations ranging from less than 0.25 to 17 ppm. The highest concentration of PAHs was detected in an urbanized watershed. The most abundant PAHs included 4- and 5- ring PAHs which constituted around 75% of the PAH content in sediment samples. Sources of PAHs contaminating the lakes include coke ovens, automobile traffic and wood burning (Kannan et al. 2005). Total PAH levels in sediment cores sampled in Green Bay, Wisconsin ranged between 0.84 and 8 ppm. Sources of PAHs in the study site included coke burning, highway dust and wood burning. The most abundant PAHs were heavy PAHs that included BaP, benzo[b]fluoranthene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene and benzo[g,h,i]perylene (Su et al. 1998). Sediment and soil samples were collected at Potter Cover near Jubany Station (Antarctica) a pristine area with PAHs generated mainly from diesel motor combustion and open-field garbage burning. Levels of PAHs in sediments ranged between 0.03 and 2 ppm but were lower in the soil samples where they ranged between 0.01 and 1 ppm (Curtosi et al. 2007). Significant levels of PAHs exist in soil in virtually all regions of the earth. PAH contamination tends to be higher in urban industrialized areas where levels of PAHs are usually 10 to 100 times more than those in undeveloped areas (Harvey 1997). Depending on sampling methods and locations of the sites the concentrations of PAHs can vary significantly. On a local scale, hazardous waste sites such as former manufactured-gas factory sites and wood-preserving facilities can be a concentrated source. PAH concentration from soil samples collected at a landfill in New York City ranged from 0.4 to 10 ppm (Black et al. 1989). Wang et al. (2007) collected

Table 1.3. Summary of total PAH concentrations in parts per million (ppm) in soil and sediments at different sites.

Source/Site Location	Total PAH	Medium	Reference
Wycoff/Eagle Harbor, Washington, USA	25-17,283	Sediment	(Neff et al. 2005)
Black River, Ohio, USA	0.1-250	Sediment	(Gu et al. 2003)
Various Inland Lakes, Michigan, USA	<0.25-17	Sediment	(Kannan et al. 2005)
Green Bay, Wisconsin, USA	0.8-8.0	Sediment	(Su et al. 1998)
Potter Cove (Antarctica)	0.03-2	Sediment	(Curtosi et al. 2007)
Potter Cove (Antarctica)	0.01-1	Soil	(Curtosi et al. 2007)
Fountain Avenue Landfill, New York City, USA	0.4-10	Soil	(Black W.V. 1989)
Former cokery site	2,600	Soil	(Eom et al. 2007)
Dalian, China	0.2-9	Soil	(Wang et al. 2007)
Glasgow, United Kingdom	12	Soil	(Morillo et al. 2007)
Torino, Italy	0.85	Soil	(Morillo et al. 2007)
Ljubljana, Slovenia	1	Soil	(Morillo et al. 2007)

surface soil samples from four sampling sites in Dalian, a city located in Northeast China.The four sites included one close to traffic, another in a park or residential area, a suburban and a rural site and PAH concentrations were reported to range between 0.2 and 9 ppm. Total PAHs showed an urban-suburban-rural gradient with the traffic site having around 18 times higher concentrations of tPAHs when compared to the rural site. Profiles of PAHs were also different among the different sites, with high molecular weight PAHs being more abundant in urban areas whereas low molecular weight PAHs were predominant in the suburban and rural areas. In a different study, urban soil samples were collected from three European cities; Glasgow located in the United Kingdom, Torino in Italy and Ljubljana in Slovenia. The highest levels of PAH contamination existed in the samples collected from Glasgow (12 ppm) which were ten-fold higher than the levels detected in soil samples from the other two cities. Phenanthrene, fluoranthene and pyrene were predominant in all samples and constituted 40% of total PAH content in soil samples. PAHs in the study's three cities were mainly of pyrogenic origins such as motor vehicle exhausts (Morillo et al. 2007).

Atmospheric dispersion of particle-bound PAHs often results in the deposition of these chemicals into surface waters. Approximately two-thirds of PAHs detected in surface water are particle-bound. Runoff of polluted ground sources or direct pollution of rivers and lakes by municipal and industrial effluents are also among the diverse sources of water contamination. Lower concentrations of PAHs may also be leached through soils into groundwater. The range of PAH concentrations detected in surface and ground water in various studies is summarized in Table 1.4. PAHs in water sampled near a bitumen field in Nigeria were found to range from 11 to 342 μg/L (Olajire et al. 2007). Water samples from a river located in northeast China had levels of PAHs that ranged from 1 to 14 μg/L. A wide range of PAHs at different concentrations were detected in these water samples indicating that there are potentially many different sources of PAHs in the river, possibly including industrial wastewater, sewage, spill oil, runoff and atmospheric fallout (Guo et al. 2007). Surface water samples from a major river in Hungary revealed that 2- and 3-ring PAHs comprised around 78% of total PAHs which

Table 1.4. Summary of total PAH concentrations in surface and ground water (μg/L) at different sites.

Source/Site Location	Total PAH	Medium	Reference
Bitumen Field, Nigeria	11-342	Surface Water	(Olajire et al. 2007)
Daliao River, China	1-14	Surface Water	(Guo et al. 2007)
Taihu Lake, China	0.2	Surface Water	(Ke et al. 2007)
Danube River, Hungary	0.01-3	Surface Water	(Nagy et al. 2007)
Former wood treatment site, Norway	5,200	Ground Water	(Hartnik et al. 2007)
Anoka Sand Plain, Minnesota, USA	<0.01	Ground Water	(Trojan et al. 2003)
Wood treatment sites, USA	10,051	Ground Water	Rosenfeld and Plumb 1991
Coal and oil gasification plant, Washington, USA	230-14,240	Ground Water	Turney and Goerlitz 1990

ranged in concentrations between 0.01 and 3 μg/L. In the study area, PAHs were mainly generated from the incomplete combustion of fossil hydrocarbons (Nagy et al. 2007). In a different study water samples have been collected from Taihu lake, a shallow freshwater lake in China. PAHs detected in these samples were predominantly composed of phenanthrene, fluoranthene and pyrene. Concentration of total PAHs was around 0.2 μg/L (Ke et al. 2007). Few recent data are available on the concentration of PAHs in ground water. In a study on ground water quality at Anoka Sand Plain Aquifer located in east central Minnesota, USA, a source of drinking water, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene were the most elevated PAHs, however their levels were in the ng/L range (Trojan et al. 2003). Ground water was sampled in a former wood treatment site located in Hommelvik, Norway. High molecular weight PAHs more specifically carcinogenic PAHs were mostly below detection limit. The most abundant PAH was naphthalene at 4,027 μg/L. Total PAH concentration was around 5,200 μg/L (Hartnik et al. 2007). Ground water collected near a coal and oil gasification plant and wood treatment facilities was found to have elevated levels of PAHs. Ground water sampled

near a coal and oil gasification in Seattle, Washington, USA that ceased operation in 1956 was found to contain total PAH levels ranging from 230 to 14,240 µg/L. PAH levels were highest where ground water was in contact with a non-aqueous phase liquid (NAPL) in the soil (Tumey and Goerlitz 1990). PAHs in ground water from five wood treatment sites in the United States had an average concentration of 10,051 µg/L. Low molecular weight PAHs, which are typically more water soluble, were the most abundant in ground water samples. Less soluble 3- and 4- ring PAHs were present at level lower than 200 µg/L (Rosenfeld and Plumb 1991).

Exposure to carcinogenic PAHs from human ingestion of drinking water is minor compared to other potential routes of exposure. According to water quality data from the World Health Organization (WHO), levels of PAHs in drinking water were found to vary between 1 and 11 µg/L (Skupinska et al. 2004). PAH levels in selected water supplies in four U.S. cities were measured previously and found to be ranging between 0.01 and 0.6 µg/L (Basu and Saxena 1978).

PAH contamination of surface water may result in distribution of these compounds into the food chain. Because PAHs, especially heavier congeners, are water insoluble or lipophilic, they are likely to enter and bioaccumulate in the food chain. PAHs can be taken up by planktons, mollusks, fish and eventually consumed by humans (Harvey 1997). PAHs bioaccumulated in plant and animal tissues may reach higher levels than those found in air or water (ATSDR 1995).

Direct contamination of food with PAHs has also been reported. Intake of PAH-rich foods was linked with cancer of the stomach and esophagus (Ward et al. 1997) as well as cancer of the colon and rectum in humans (Sinha et al. 1999). PAHs in food items can have plant or animal origins. Vegetables with large leaves accumulate PAHs on their surface and to a lesser extent in their internal tissue. Grazing cattle and poultry can accumulate PAHs in their tissues. PAH contamination was detected in leafy plants such as lettuce, spinach, tea, tobacco and in smoked meats and fish. PAHs present in plants are most likely due to atmospheric contamination. In fresh meats and seafood, PAHs exist due contamination of air, water or animal feed. Cooking methods such as

frying, charcoal broiling tend to increase the level of PAHs as well as other potentially carcinogenic chemicals such as heterocyclic amines in foods (HCAs). Depending on the method of food preparation, levels are highly variable but can reach parts per billion (ppb) levels (ATSDR 1995). PAHs are formed from organic compounds by the recombination of smaller organic compounds originally fragmented at high cooking temperatures. PAHs are generally produced in lesser amounts at temperatures below 400^{o}C but increase linearly in the range between 400 and 1000^{o}C (Jagerstad and Skog 2005). Barbecued meat for example, can have a PAH level as high as 10 to 20 ppb (Phillips 1999). Epidemiological studies revealed a positive association between consumption of red meat cooked by deep frying and risk of breast cancer (Dai et al. 2002).

Food may be considered a main source of exposure in human populations. Contamination of food by PAHs can be environmental or through cooking or processing. Average daily intake of PAHs in humans has been estimated to be 0.2 μg from air, 0.03 μg from water (ATSDR 1995) and 2 to 3 μg from food. The average daily intake of PAHs from food compares to 2 to 5 μg PAHs per pack of cigarettes in a regular smoker (Jagerstad and Skog 2005). Exposure from dietary sources can account for more than 70% of PAH exposure in non-smokers and also contributes significantly to non-occupational exposure to PAHs. Cereals, oils and vegetables are some of the main sources of PAHs in the diet (Phillips 1999). In plants, PAH contamination occurs by soil to root uptake or through the atmosphere. Uptake through the atmosphere occurs by deposition of airborne particles containing PAHs on the plant leaves which is especially important in plants with broad leaves such as lettuce (Ramesh et al. 2004). PAHs levels found in leafy vegetables were reported in several studies (Jakszyn et al. 2004; Kulhanek et al. 2005). Kulhanek et al. (2005) described bioconcentration factors in leafy vegetables from the Czech Republic. The factor for BaP was 5 x 10^{-6} without attached soil and 0.01 with soil. In foods from animal origin, accumulation of PAHs occurs in grazing cattle and poultry feeding on contaminated pastures and vegetation (Crepineau et al. 2003). PAH occurrence in fish and other seafood is due to contamination of fresh and

coastal waters (Ramesh et al. 2004). Food content was analyzed for potential carcinogens including PAHs. Levels of BaP and total PAHs in several food items are summarized in Table 1.5. For products from plant origin, the following values were reported: vegetables 0.1 ppb BaP and 4.2 ppb total PAHs (Tateno 1990); fruits 0.01 ppb BaP and 0.7 ppb total PAHs (Falco et al. 2003); wheat grain 0.3 ppb BaP and 4 ppb total PAHs (Jones et al. 1989); wheat flour 0.1 ppb BaP and 1.5 ppb total PAHs (Dennis et al. 1991); white bread 0.017 ppb BaP and 3.12 ppb total PAHs (Lodovici et al. 1995) and coffee 0.9 ppb BaP and 25 ppb total PAHs (Klein 1993). Among oils, groundnut oil had higher levels of total PAHs at 750 ppb compared to soybean oil at 220 ppb (Kolarovic and Traitler 1982) and olive oil at 25 ppm (Moret and Conte 2000). In addition, the levels of BaP in groundnut oil (110 ppb) were higher than those in soybean oil (30 ppb) and olive oil (0.1 ppb). In products from animal origin, levels of BaP in fresh fish were at 1.5 ppb and 90 ppb for total PAHs (Baumard et al. 1998b) whereas raw beef meat had BaP levels below detection and total PAHs at 10 ppb (Lodovici et al. 1995).

Cooking methods affect the content of PAHs in a food item. Meats that have been fried or charcoal broiled are especially high in PAH content (Harvey 1997). In cooked foods, the following values were reported (Table 1.5): smoked fish 50 ppb BaP and 800 ppb total PAHs (Akpan et al. 1994); barbecued beef meat 1.5 ppb BaP and 45 ppb total PAHs (Lodovici et al. 1995) and grilled frankfurters 55 ppb BaP and 800 ppb total PAHs (Larsson et al. 1983). The data summarized in Table 1.5 reveal that total PAHs tended to be higher in oils and cooked foods as opposed to vegetables and fruits. The same trend was found in high molecular weight PAHs such as BaP which was detected in grilled frankfurters at 55 ppb (Larsson et al. 1983) as compared to 0.1 ppb in vegetables (Tateno 1990).

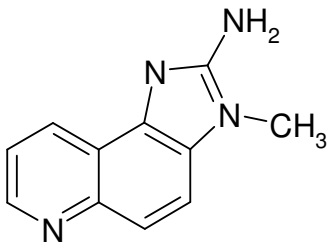
In addition to PAHs, more than 20 derivatives of HCAs were identified in cooked foods, especially meat and fish (Jagerstad and Skog 2005; Jakszyn et al. 2004; Turesky 2007). HCAs consist of two or three rings with an exocylic amino group on one of the rings (Jagerstad and Skog 2005).

Table 1.5. Summary of BaP and total PAH concentrations in parts per billion (ppb) in different food items.

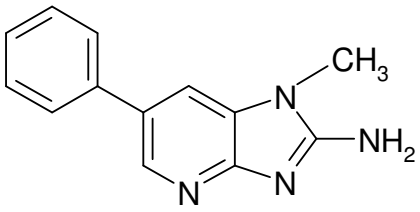
Source	BaP	Total PAH	Reference
Vegetables	0.1	4.2	(Tateno 1990)
Fruits	0.01	0.7	(Falco et al. 2003)
Wheat grain	0.3	4	Jones et al. 1989
Wheat flour	0.1	1.5	(Dennis et al. 1991)
Bread (white)	0.017	3.12	(Lodovici et al. 1995)
Coffee	0.9	25	(Klein 1993)
Olive oil	0.1	25	(Moret and Conte 2000)
Soybean oil	30	220	(Kolarovic and Traitler 1982)
Groundnut oil	110	750	(Kolarovic and Traitler 1982)
Fresh Fish	1.5	90	(Baumard et al. 1998b)
Smoked Fish	50	800	(Akpan et al. 1994)
Raw Beef Meat	nd*	10	(Mottier et al. 2000)
Barbecued Beef Meat	1.5	45	(Lodovici et al. 1995)
Grilled Frankfurters	55	800	(Larsson et al. 1983)

*nd= not detected

They are formed from precursors such as creatine or creatinine, amino acids and sugars. HCAs can be divided into two main classes, the aminoimidazol-quinolines (e.g. IQ) and the aminoimidazol-pyridines (e.g. PhIP) (Jakszyn et al. 2004). The molecular structures of IQ and PhIP are shown in Figure 1.2. HCAs were found to be mutagenic and carcinogenic in *in vitro* and *in vivo* models (Sugimura et al. 2004) however the evidence from epidemiological studies is not sufficient yet to establish a causal link with human cancer cases (Jagerstad and Skog 2005). High quantities of HCAs were associated with prolonged cooking time and high temperature cooking surfaces (Knize et al. 2003). Human exposure to HCAs were estimated to vary between nanograms to micrograms



2-amino-2-methylimidazolo[4,5-f]quinolone (IQ)



2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

Figure 1.2. Structures and nomenclatures of representative heterocyclic aromatic amines.

per day depending on dietary lifestyle and cooking processes (Jagerstad and Skog 2005).

PAHs are a major component of tobacco smoke. About 150 different unsubstituted and methylated PAHs have been detected in tobacco smoke condensate. It has been estimated that approximately 10 to 50 μg of BaP are inhaled into the lungs from one cigarette (Luch 2005; Phillips 1996). According to Harvey (Harvey 1997) more than 150 PAHs occur in gas phase and more than 2,000 were identified in the particulate phase (Harvey 1997). Tobacco smoke is one of the most important sources of PAHs in indoor air.

Background exposures to PAHs are relatively low (air, water and diet) compared to occupational sources. PAH exposure can be significant during industrial processes such as coal tar production, aluminum smelting and also in industries where petroleum and petroleum products are used. Exposure to PAHs in an aluminum production plant was reported at levels ranging between 1488 and 15149 ng/m^3 depending on the job function (Vu Duc and Lafontaine 1996), and as high as 200,000 ng/m^3 in coke ovens (Lewtas et al. 1997). Monitoring of airborne PAHs in the work environment of an iron foundry and two steel plants revealed that the concentrations of PAHs ranged between 321 to 1331 ng/m^3 as compared to 7 ng/m^3 in urban areas of the general environment (Apostoli et al. 2003). Among other occupations, personal PAH monitoring revealed that traffic policemen are exposed to PAH levels ranging from 9 to 140 ng/m^3 in Budapest, Hungary (Szaniszló and Ungváry 2001)and around 1700 ng/m^3 in Beijing, China (Liu et al. 2007). The total PAH exposure levels for toll booth attendants were reported to range between 6300 and 15000 ng/m^3 in Taipei, Taiwan (Tsai et al. 2004).

1.3.2 Source Apportionment

Based on the ratio of various components, PAH mixtures can be classified as originating from petroleum or petrogenic sources, or from pyrolysis or pyrogenic sources. Specific marker compounds may help in the identification of the major sources of PAHs at a given location. In the case of petrogenic sources, low molecular weight compounds (2 and 3 rings) tend to be predominant whereas PAHs emitted by incomplete

combustion of organic materials have a higher proportion of heavier compounds (4 rings or larger). As an illustration, low molecular weight compounds contributed to 98% of total PAHs emitted by coke ovens, 76% and 73% of the emissions from diesel and petroleum engines respectively, and 80% of the PAHs emitted from wood combustion (Khalili et al. 1995). Indeno[1,2,3-cd]pyrene (6 rings) and benzo[ghi]perylene (5 rings) were detected in PAH profiles associated with mobile sources. Benzo[ghi]perylene is known to be a marker of gasoline exhaust emissions (Eiguren-Fernandez et al. 2004). The relative abundance of PAHs present in mixtures can therefore be used to elucidate sources and provide a source fingerprint (Harrison et al. 1996). Molecular indices based on ratios of selected PAH concentrations may differentiate between pyrolytic and petrogenic sources (Edgar et al. 2006). For example, a phenanthrene : anthracene ratio greater than 10 suggests a petrogenic source of PAHs. On the other hand, a fluoranthene: pyrene ratio greater than 1.0 indicates that PAH contamination is most likely due to combustion or pyrolytic processes. Ratios of other PAH compounds such as the ratio of benz[a]anthracene and chrysene, indeno[1,2,3-cd]pyrene i and benzo[ghi]perylene, and benzo[b]fluoranthene and benzo[k]fluoranthene are also commonly used (Morillo et al. 2007).

1.4 Hazardous Effects of Polycyclic Aromatic Hydrocarbons

1.4.1 Ecological Effects

PAH contamination of aquatic environments poses a threat to ecosystems and indirectly to human health. Humans can be exposed to through consumption of fishery products such as mussels, shrimps or other crustaceans and fish. Generally however, this human exposure route is considered to be of lesser significance when compared to PAH exposures from air and other food items such as vegetables.

The effects of PAHs on wildlife can be significant especially through food chain poisoning. However, direct effects of PAHs on aquatic life may also occur. PAHs may persist in sediments and biota due to an inability to efficiently eliminate them. Body burden of PAHs in marine organisms is primarily determined by uptake and elimination

of these contaminants. Uptake is externally controlled by the chemical partitioning characteristics of contaminants between different media such as sediment, water and food. Therefore, occurrence of PAHs in organisms can be an indicator of exposure to contaminated water, sediment and food. Internally, uptake depends on the behavior of the organism and its physiology (Meador et al. 1995).

PAH contamination of coastal harbors, inland rivers and lakes at varying levels has been documented in studies from as early as the 1960s. Laboratory and field studies on feral organisms have attempted to evaluate the ecotoxicological potential of this contamination. Tumors and other diseases have been reported in fish and were most likely linked to PAHs. Field studies of PAH toxicity present the challenge of identifying biological responses caused specifically by PAHs due to the presence of common co-contaminants such as organochlorines and metals. Fish take up PAHs and rapidly metabolize (Lemaire et al. 1990) and excrete them into the bile which is considered a major route of elimination (Varanasi 1989). Thus PAHs tend not to bioconcentrate and might cause adverse effects with little or no chemical trace. Metabolism converts up to 99% of PAH to metabolites within 24 hours of uptake, significantly reducing tissue concentrations of PAH parent compounds (Varanasi 1989). Thus, it is usually very challenging to determine PAH concentrations in fish tissues to assess exposure. Determining the levels of PAH metabolites in bile is usually more significant since it reflects uptake, metabolism and excretion (Meador et al. 1995). The major adverse effects reported in feral fish and linked to PAHs can be categorized into biochemical, histopathologic, immunological, genetic, reproductive, developmental and behavioral effects.

Biochemical effects consist mainly of alteration of Phase I, by induction of mixed function oxidases (MFO) for the most part, and to a lesser extent Phase II enzymes (Stegeman and Hahn 1993). Those effects may also include changes in hormones, energy reserves and serum enzymes. Induction of MFO enzymes was reported in fish around petroleum development sites in a marine environment (Stagg et al. 1995). According to Stegeman and Hahn (1993), MFO induction was associated with

mutagenic and carcinogenic processes and was also linked with effects on reproduction as well as organ and cellular disturbances (Au et al. 1999). Histopathological effects include an effect synonymous with serious chemical injury which is cancer. Several studies suggested that there was a link between polluted water with elevated levels of PAHs and histopathological effects including tumor formation in fish (Baumann and Harshbarger 1995; Myers et al. 1998; Pritchard et al. 1996; Steyermark 1999). Skin and skeletal disorders, liver abnormalities in addition to neoplasms were also among the effects linked to PAH contamination.

Alteration of immune responses in fish can lead to susceptibility to bacterial, viral and parasitic infections and decrease the resistance to carcinogenesis. Pollution in estuaries in the Pacific Northwest of the USA was suggested to be one of the factors contributing to the decline of wild Pacific salmon. Karrow et al. (1999) observed a significant alteration in blood leukocytes in rainbow trout exposed to creosote. Immunotoxicity can result in a variety of adverse outcomes but the most concerning effect is the decreased resistance to infectious diseases. Immunosuppression in juvenile Chinook salmon from Puget Sound sites in Washington, U.S. was documented with total PAH concentrations in stomach contents ranging from 4000 to 15,000 ng/g wet weight (Johnson et al. 2007).

Classical cytogenetic techniques revealed a level of genetic toxicity in larvae of different fish species exposed to PAHs (Carls et al. 1999; Hose and Brown 1998). Higher levels of DNA fragmentation were detected in the blood cells of bullheads from contaminated sites in the US and Canada as compared to reference sites (Pandrangi et al. 1995). Among the PAH effects on reproductive function in fish, impairment of sperm quality and effects on egg hatchability was observed in a specie of fish chronically exposed to PAH contaminated sediment in Canada. PAHs therefore can also present a reproductive risk in fish.

Developmental effects were observed from relatively low PAH concentrations in larval and juvenile fish. Moles and Norcross (1998) reported reduced growth in juvenile flounder with chronic exposure to low levels of PAHs (around 1.6 ppm) in sediment.

Among the behavioral changes that were potential linked with PAH exposure are the alteration of feeding (Gregg et al. 1997; Hinkle-Conn et al. 1998) and swimming behaviors (Carls et al. 1999).

In lab feeding studies during which fish are exposed to higher PAH concentration than detected in the field, physiological changes were detected at PAH concentrations around 25,000 ng/g wet weight in juvenile chinook salmon whereas immunosuppression, CYP1A induction and DNA damage were observed in rainbow trout exposed to PAHs in diet at concentrations around 40,000 ng/g wet weight (Johnson et al. 2007).

Terrestrial vertebrates are mostly exposed to PAHs via their diet. Topical exposure can also be an important route of exposure for incubating birds that become oiled and oil is subsequently transferred to the eggshell from their feathers which can result in an *in ovo* exposure to the embryos. Oiled birds can also ingest oil when preening. Concentrations of PAHs in birds and their eggs are relatively low however due to rapid metabolism and excretion of PAHs (Näf et al. 1992). Many invertebrate-feeding birds and mammals feed on earthworms which were found experimentally to bioaccumulate specific PAHs when exposed to contaminated soils (Ma et al. 1995). BaP-diol epoxide adducts in blood albumin and hemoglobin in woodchucks (*Marmota monax*) from an area near an aluminum electrolysis plant (Blondin and Viau 1992). Induction of liver monooxygenases activity has been demonstrated in wood mice sampled from either a pitch-coke plant or an industrial/oil shipping area (Leupold et al. 1992). Toxic effects of PAH in wild birds and mammals occur mainly through the formation of adducts and the associated risk of carcinogenicity which can be a concern in long-lived species. Immunsuppression, embryotoxicity and general toxic effects of PAHs were reported terrestrial vertebrates such as amphibians and birds.

Effects of acute or chronic exposure to PAHs in wild mammals and reptiles and even birds is not well defined. Therefore, efforts to link PAHs with effects on populations or communities of marine mammals are few. Markedly, PAHs were implicated as possible causal agents for the health problems of beluga whales found dead over a period of 8 years along the shores of St. Lawrence estuary in Canada (Beland

1993) especially due to their high incidence of neoplasms and presence of BaP-DNA adducts in their tissues (Martineau et al. 1994).

PAHs can also have phytotoxic effects. Many plant species are sensitive to PAHs to some extent and are susceptible to impaired growth due to PAH exposures. Plants constitute the point of entry of hazardous compounds into the food chain, and can be used as sentinel species for PAH contamination in the environment. Plants absorb PAHs from soil, water or air and especially in aquatic species assimilation of contaminants is fast even from non-dissolved phases such as sediments. Plants with broad leaves such as lettuce tend to accumulate more airborne particles containing PAHs due to the large surface areas of their leaves (Ramesh et al. 2004). PAHs are usually phytotoxic only at high concentrations if not photoinduced. Sunlight can dramatically increase toxicity of PAHs in plants by phytosensitization and photomodification reactions (Krylov et al. 1997). Photosensitization reactions usually lead to formation of highly damaging reactive oxygen species (ROS) whereas photomodification of PAHs occurs via oxidation and activation of the parents compounds (Mallakin et al. 1999).

1.4.2 Polycyclic Aromatic Hydrocarbons and Human Cancer Risk

Humans are continuously exposed to complex mixtures from a variety of sources. These include air particulate, cooked foods, cigarette smoke, and emissions from industrial or contaminated waste sites. The toxicity of complex environmental mixtures including smoke and soot was recognized as early as the Middle Ages by Paracelsus (Gallo 2001). A primary concern associated with human exposures to complex mixtures of PAHs comes from the fact that many of these compounds have mutagenic and carcinogenic properties (Lijinsky 1991). In addition, a typical PAH mixture includes hundreds of compounds which may act as enzyme inducers, carcinogens, or promoters. Thus, the interactions of the components of a specific PAH mixture are difficult to predict. Historically, human exposures to a variety of PAH complex mixtures have been associated with increased cancer rates (Dipple 1984; Harvey 1991; IARC 1984, 1985; Warshawsky 1992).

As early as 1775, Sir Percival Pott (Pott 2002) linked scrotal cancer with exposure to soot among British chimney sweeps. This provided the first proof of an environmental origin of a form of cancer. This finding was confirmed more than a century later. Early laboratory animal studies in 1915 reported on the production of malignant epithelial tumors by repetitive application of coal tar to the ear skin of rabbits (Yamagiwa 1915). Shortly thereafter, dermal application of complex mixtures to mice was introduced as a method for investigating the carcinogenic potential of coal tars. After successfully producing cancer in rodents under experimental conditions, scientific interest shifted to the identification of the individual chemical(s) responsible for tumor formation as documented by the work of Kennaway (1925). Beginning in 1930 with the help of the British Gas, Light and Coke Company, Hieger (1930) isolated approximately 7 g of a yellow powder from 2 tons of coal tar pitch by repetitive steps of fractional distillation, extraction and crystallization. The pelt showed strong carcinogenic activity in rodents. Further fractionation of the carcinogenic powder produced pure crystalline products with melting point of 176^{o}C and 186^{o}C which were recognized to be isomeric with pentacyclic perylene. Radioactively labeled PAHs which became available in the late 1940s bound to both proteins and DNA fractions in epidermal cells after administration of the compound into the back of mice. DNA was then proposed to be the essential “cellular receptor for carcinogenesis”.

The carcinogenic potencies of a series of PAHs and the extent to which these are bound to DNA *in vivo* were roughly correlated. It was further noted that this process was dependant on a series of additional cellular events and the presence of activating enzymes residing in the endoplasmic reticulum. However, it was not until the early 1970s when Borgen and coworkers (1973) reported that a metabolite of BaP, the 7,8 dihydrodiol, binds to a ten-fold greater extent to DNA *in vitro* than its parent compound. For this to occur, activating microsomal preparations were required. Sims et al. (1974) proposed that a secondary metabolite, the 7,8 dihydrodiol 9,10 epoxide (diol epoxide), derivative of BaP is the chemical agent covalently interacting with DNA. Hence, B[a]P diol epoxide was considered to be the ultimate carcinogen.

In the 1940s it was observed that low doses of PAHs applied to the back of mice did not induce tumors unless croton oil was also applied. Croton oil which had no carcinogenic potencies was considered to act as a promoting agent. Therefore, the initiation stage of cancer is thought to occur when normal cells are irreversibly converted into latent tumor cells that remain dormant until stimulated by promoting agents. Onset of promotional stage would then lead to outgrowth of initiated cells resulting in the proliferation of clones of altered cells which enhances tumor formation. In animal tumor models, this stage is a prolonged period and may be reversible when treatment is terminated. Promoters generally act through receptor-mediated 'epigenetic' mechanisms. Initiating compounds such as PAHs are genotoxic. They covalently bind to DNA and cause lesions. When these lesions are not repaired, the formation of PAH-DNA adducts may result in the generation of apurinic sites and nucleobase-mispairing at these sites which may lead to the induction of mutations (Figure 1.3). The formation of mutations in cancer related genes such as protooncogenes or tumor suppressor genes is therefore generally assumed to be a crucial event in tumor formation. PAHs with initiating and promoting activity are therefore considered as "complete carcinogens". Repeated treatments of animals with high doses of potent PAHs such as BaP and dibenz[a,h]anthracene (DBA) over extended periods of time will produce a significant increase in tumors in the absence of promoters.

The biological activity of PAHs is substantially influenced by the chemical structure of these compounds. Two groups of PAHs have been characterized according to their structural differences, peri-condensed PAHs and cata-condensed PAHs. The peri-condensed PAHs include compounds which form a cycle as their lines connect the ring centers. Furthermore this category can be subdivided into two groups, alternants, formed exclusively of six-membered rings, and non-alternants, that include some five-membered rings. Cata-condensed PAHs do not form cycles, and can be classified as branched, which are usually more thermodynamically stable and less chemically reactive, or non-branched (Ramesh et al. 2004). Figure 1.4. displays the different types of PAH structures.

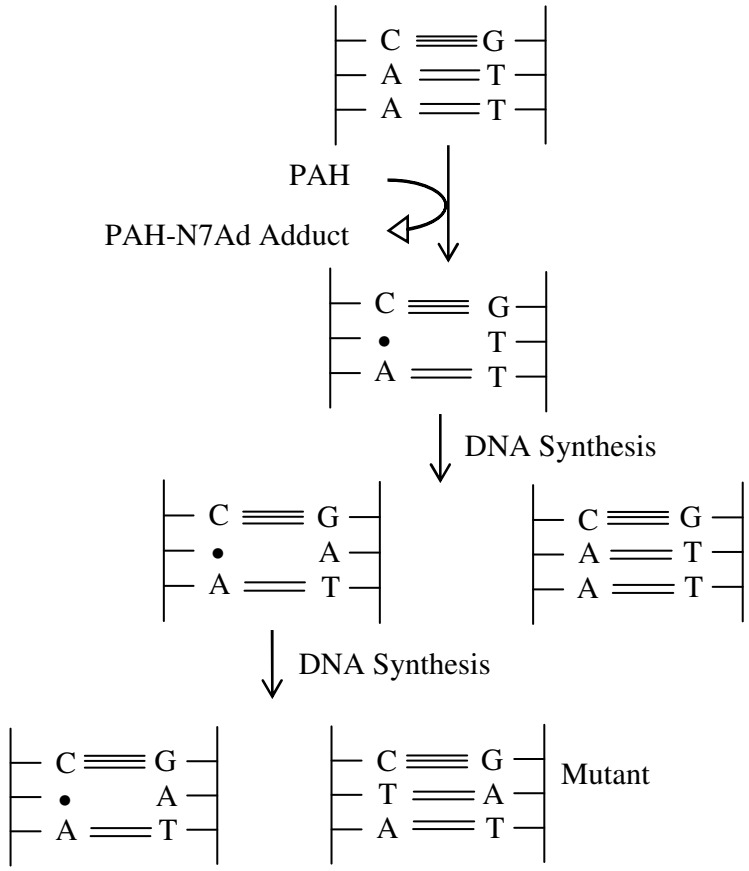


Figure 1.3. Process for inducing A→T transversions from depurinating adenine adducts. Adapted from Skupinska et al. (2004).

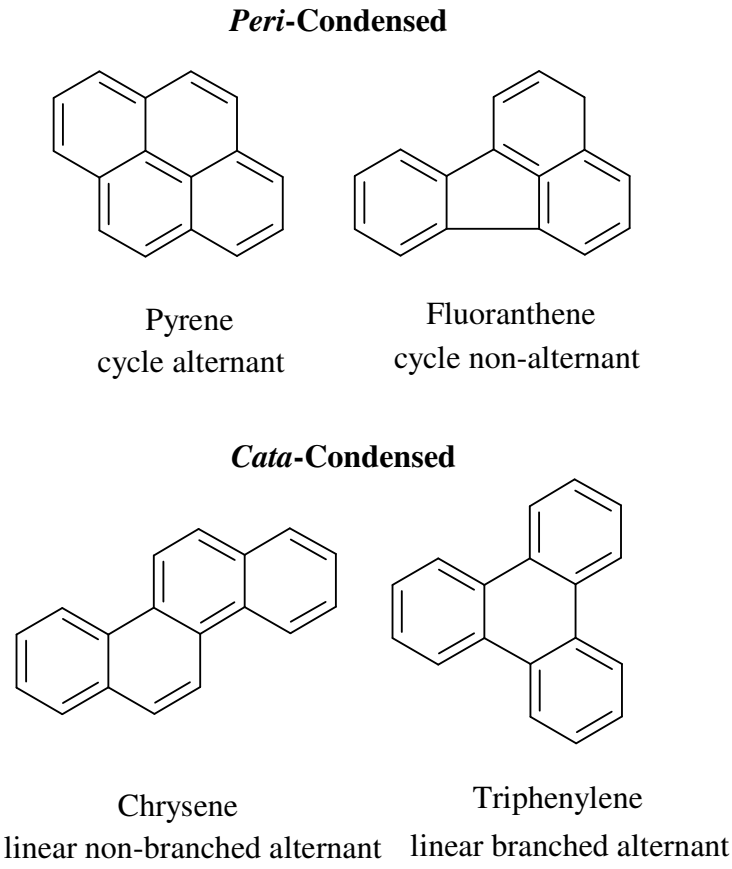
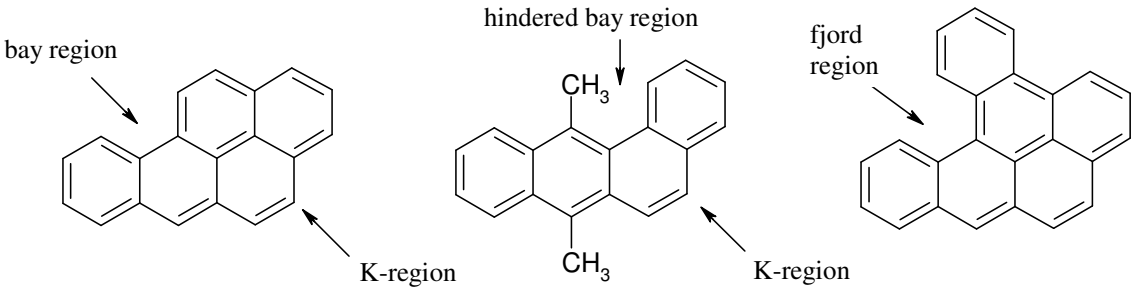


Figure 1.4. Types of PAH structures. Adapted from Ramesh et al. (2004).

PAHs may have varying structural regions; the K region, the L region, the Bay region, the distal bay region, and the peri position (Figure 1.5). The bay region is the most biologically active of the regions in the model carcinogen BaP. It consists of an open inner corner of a phenanthrene moiety. Following metabolism by mammalian enzymes, PAHs can either be excreted from the body, or activated and bound to nucleotides in DNA. Elimination of PAHs usually requires conjugation by Phase II enzymes. The reaction of the benzo[*a*]pyrene-7,8-diol-9,10-epoxide with the N-2 of guanine results in the formation of a DNA adduct.



Benzo[a]pyrene 7,12-Dimethylbenz[a]anthracene Dibenzo[a,l]pyrene

Figure 1.5. Structural features of PAHs that contribute to carcinogenicity. BaP, a known carcinogen, contains a bay region. The diol epoxide metabolite of BaP, which binds to DNA, contains an epoxide group next to the bay region. Methyl groups in DMBA hinder this structural feature further, as does the extra benzene ring in DB[a,l]P. The compounds are arranged in order of their increasing carcinogenic potency, with DB[a,l]P being the most potent. Adapted from Baird et al. (2005).

In most cases, the major source of human exposure to PAHs is through inhalation of vapors or dust. Exposure to PAHs from ingestion of soil, food, and drinking water may also occur. Indoor air is also considered to be an important source of human exposure to PAHs, especially in homes where sources such as environmental tobacco smoke, unvented kerosene space heaters, coal or gas cooking and heating appliances exist.

Epidemiologic data indicate that exposure to PAHs has been associated with human cancers of the skin, lungs, and bladder (Boffetta et al. 1997). Several individual PAHs, including BaP, chrysene, indeno[1,2,3-c,d]pyrene, and benzo[b]fluoranthene have produced carcinogenic, mutagenic, and genotoxic effects in animal studies (Basler et al. 1977; Deutsch-Wenzel et al. 1983; LaVoie et al. 1982; Thyssen et al. 1981). Seven PAHs, including the model carcinogenic PAH, BaP, are classified by the United States Environmental Protection Agency (USEPA) as probable (class B2) human carcinogens (USEPA 2006). PAHs have been observed to induce cancer, hematotoxicity, cardiotoxicity, renal toxicity, neurotoxicity, immunotoxicity, reproductive toxicity, and developmental toxicity in animals and humans (Ramesh et al. 2004). Tobacco smoke, a major source of PAHs has been linked to about 90% of all lung cancer cases and other

smoking-related cancer types and is believed to account for approximately 1.2 million worldwide deaths annually (Luch 2005).

Animal studies have demonstrated that individual PAHs and PAH mixtures may act as potent carcinogens in rodent skin models. These observations are in agreement with results from human studies indicating an increased incidence of skin cancer associated with the use of various coal tar preparations (Grimmer et al. 1982; Lewis et al.1982; Lewis 1983; Mahlum et al. 1984; Mukhtar et al. 1982; Mumtaz et al. 1996; Robinson et al. 1984; Wallcave et al. 1971). One of the first synthetic PAH congeners shown to be carcinogenic in laboratory animal studies was dibenz[a,h]anthracene (Cook 1933). BaP was later identified as an important carcinogenic PAH congener present in many carcinogenic PAH mixtures (Cook 1933). Several PAH congeners have been classified as human carcinogens (either 2A-probable or 2B-possible) by the International Agency for Research on Cancer (IARC) which systematically reviews the carcinogenicity of chemicals such as PAHs (IARC 2004). These compounds include benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, BaP, dibenz[a,h]acridine, dibenz[a,j]acridine, dibenz[a,h]anthracene, dibenzo[a,e]-pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, dibenzo[a,l]pyrene, indeno[1,2,3-c,d]-pyrene, and 5-methylchrysene. All of the carcinogenic PAHs contain 4 or more fused benzo rings. On the other hand, the compounds categorized by the USEPA as probable human carcinogens (B2) include the four ring PAHs; benz[a]anthracene and chrysene, the five ring PAHs; benzo[b]fluoranthene, benzo[k]fluoranthene, BaP, dibenz[a,h]an-thracene, and the six ring PAH; indeno[1,2,3-c,d]pyrene (USEPA 2006).

An excess incidence of lung cancer was reported in occupational studies of subjects exposed to PAH mixtures in emissions from coke ovens and aluminum smelters (Boffetta et al. 1997; Hoshuyama et al. 2006; IARC 1984a, 1984b, 1985; Mastrangelo et al. 1996; Nadon et al. 1995). European chimney sweeps, who were exposed to lower levels of soots and tars have exhibited lower incidence of scrotal cancer. This occurrence has bolstered a causal relationship between PAH exposure and this form of cancer (Butlin 1892). Later studies established the link between increased incidence of cancer

and exposure to other PAH-containing complex mixtures. The mixtures described in those studies included various coal tars, automobile exhaust, creosote, asphalt fumes, cigarette smoke as well as other PAH-containing materials (Cruickshank and Gourevitch 1952; Fisher 1953; Grimmer et al. 1991; Grimmer et al. 1983; IARC 1984a, 1984b, 1985, 1986, 1989; Mumtaz et al. 1996; Pisani et al. 2006; Pittelkow et al. 1981; Roy et al. 1988; Waterhouse 1971; Wynder 1967).

Occupational studies are of particular importance in studying human effects of PAH mixtures. They are usually considered to be the most complete studies in terms of exposure, dose-response and adverse health effects. The International Agency for Research on Cancer (IARC) classified several PAH mixtures common in occupational settings and industrial processes as carcinogenic, probably carcinogenic (2A) or possibly carcinogenic (2B) to humans based on epidemiological and experimental evidence (Boffetta et al. 1997; IARC 1984a, 1984b, 1985, 1989; Mastrangelo et al. 1996). A list of PAH mixtures and exposure circumstances evaluated by IARC is provided in Table 1.6.

Aiming at reviewing studies that report dose-response analysis of PAH levels in relation to cancer risk, Mastrangelo et al. (1996) evaluated a large number of occupational studies published between 1966 and 1996. Ten studies (Armstrong et al. 1994; Bonassi et al. 1989; Clavel et al. 1994; Costantino et al. 1995; Jockel et al. 1992; McLaughlin et al. 1992; Nadon et al. 1995; Spinelli et al. 1991; Tola et al. 1979; Tremblay et al. 1995) that evaluated occupations in aluminum, coke oven, petroleum and foundry industries among others, were found to explicitly mention PAH exposure in qualitative or quantitative forms and relate it to lung or bladder cancer. McLaughlin et al. (1992) measured exposure to BaP, silica, arsenic and radon in 316 lung cancer cases and 1,352 controls from a cohort of Chinese workers in tin mines. Odds ratio of lung cancer in workers exposed to cumulative levels of BaP ranging from 108 to 250 $\mu g/m^3$ was 2.7 however the trend was not significant. From a cohort of aluminum refinery plant

Table 1.6. List of PAH mixtures and exposure circumstances evaluated for carcinogenicity by the International Agency for Research on Cancer (IARC).

PAH Mixture or Process	Epidemiological Evidence	Experimental Evidence	IARC Group	Reference
Bitumens (extracts)	-	Sufficient	2B	(IARC 1985)
Carbon black	Inadequate	Sufficient	2B	(IARC 1984a)
Coal-tars	Sufficient	Sufficient	1	(IARC 1985)
Diesel engine exhaust	Limited	Sufficient	2A	(IARC 1989)
Gasoline engine exhaust	Inadequate	Sufficient	2B	(IARC 1989)
Mineral oils, untreated and mildly-treated	Sufficient	Sufficient	1	(IARC 1984a)
Soots	Sufficient	Inadequate	1	(IARC 1984a)
Shale oils	Sufficient	Sufficient	1	(IARC 1984a)
Aluminum Production	Sufficient	-	1	(IARC 1984b)
Coal gasification	Sufficient	-	1	(IARC 1984b)
Coke production	Sufficient	-	1	(IARC 1984b)
Iron and steel foundry	Sufficient	-	1	(IARC 1984b)

workers, Armstrong et al. (1994) selected 338 lung cancer cases and 1,138 controls to estimate their occupational exposure to BaP. A smoking adjusted lung cancer rate ratio of 2.2 was the highest observed and corresponded to a BaP cumulative exposure level ranging from 100 to 199 $\mu g/m^3$ with a significant dose-response trend. In 138 bladder cancer cases and 414 controls selected from the same cohort of aluminum workers, Tremblay et al. (1995) reported that after adjusting for smoking, the risk of bladder cancer is 6.7 times higher workers exposed to a cumulative level of BaP ranging between 200 and 299.9 $\mu g/m^3$ than in nonexposed workers. However, no study in the review by Mastrangelo et al. (1996) measured internal dose in workers as a biomarker of exposure to PAHs. Workers from different occupational environments were exposed to different chemical mixtures at different intensities and for different durations. Nevertheless, the studies reviewed by Mastrangelo et al. (1996) present an advantage over previous epidemiological studies that base exposure on qualitative job title categories. The reviewed studies, published about two centuries after Pott's report linking scrotal cancer to chimney sweeping, also revealed an excess in lung and bladder cancer incidence with occupational exposure to PAHs.

Boffetta et al. (1997) published a more comprehensive review of cancer risk associated with occupational exposures to PAHs. The industries and occupations evaluated were the following: aluminum production, coal gasification, coke production, iron and steel foundries, and workers exposed to diesel engine exhaust, coal tars and related products, carbon blacks, among other mixtures of PAHs. Lung cancer risk was consistently elevated in most of the reviewed studies, which confirms that lung is the major target organ of the carcinogenic effects of PAHs. In a study on coal gasification workers in Germany, the relative risk of lung cancer was estimated to be 2.9 (Berger and Manz 1992). An increased risk of skin cancer was detected exclusively in settings where dermal exposure occurred. Bladder cancer was less consistent among studies however seemingly connected with PAH exposures from coal tar and pitch in industrial processes such as aluminum production, coal gasification and tar distillation. Laryngeal and renal cancers do not seem to be significantly reported. Exposure misclassification and

controlling for confounders such as tobacco smoking, however remains an issue in these studies.

More recently, a review by Bosetti et al. (2007) presented detailed results from cohort studies linking quantitative occupational PAH exposure to respiratory and urinary tract neoplasms published after the review by Boffetta et al (1997) until the end of year 2005. Epidemiological evidence from the reviewed studies confirms an elevation in lung/respiratory cancer risk among several PAH-related industrial processes, except for aluminum and carbon electrode manufacturers. The pooled relative risk (RR) and 95% confidence interval (CI) by industry was 2.58 (95% CI 2.28-2.92) for coal gasification, 1.58 (95% CI 1.47-1.69) for coke production, 1.40 (95% CI 1.31-1.49) for iron and steel foundries, 1.51 (95% CI 1.28-1.78) for roofers and 1.30 (95% CI 1.06-1.59) for carbon black production. Cancer of the bladder and the urinary system was less evident among the reviewed occupations, with the exception of aluminum production (pooled RR=1.29, 95% CI 1.12-1.49) coal gasification (pooled RR=2.39, 95% CI 1.36-4.21) and iron and steel foundries (pooled RR=1.29, 95% CI 1.06-1.57) which were associated with a modest increase in risk.

In a literature review of recently published studies linking occupational exposures to PAHs and any form of cancer that were not included in the review Bosetti et al. (2007), six major studies were identified. Among those, the study by Audureau et al. (2007) only provided a descriptive analysis of occupational exposures in 106 French subjects who worked in foundries, petrochemical industries and rubber production and were diagnosed with bladder cancer in 2003. PAHs were the main chemicals that 47% of these workers were exposed to during their careers. The remaining five studies provided exposure and risk estimates and are summarized in Table 1.7.

Krishnadasan et al. (2007) selected 362 prostate cancer cases and 1,805 matched controls from a cohort of aerospace and radiation workers in the United States. PAHs were the most common chemicals that workers were exposed to (39%). Crude risk estimates suggested a relation between high levels of PAH exposures estimated by a job exposure matrix and prostate cancer risk. This relation however was not significant after

Table 1.7. Summary of results from occupational studies linking exposure to PAHs and cancer risk.

BaP/PAH Exposure*	Number of Study Subjects	Occupation	Type of Study	Type of Cancer	Risk Estimate† (95% CI)	Reference
Unexposed	392 cases 1,805 controls	Aerospace and Radiation	Nested case-control	Prostate	1.0	(Krishnadasan et al. 2007)
Low/Moderate					1.0 (0.69,1.6)	
High					1.3 (0.73,2.5)	
108.3 μg BaP/m^3-year	6,423	Aluminum Smelting	Retrospective cohort	Bladder	3.0	(Friesen et al. 2007)
119.6 μg BaP/m^3-year				Lung	1.8	
N/A	121,846	Iron-Steel Foundry	Retrospective cohort	Lung	6.54 (1.13-37.8)	(Hoshuyama et al. 2006)
0-0.4 μg BaP/m^3-year	7,298	Asphalt Paving	Cohort	Bladder	1.0	(Burstyn et al. 2007)
0.4-1.0 μg BaP/m^3-year					1.13 (0.44-2.90)	
1.0-1.8 μg BaP/m^3-year					1.67 (0.62-4.48)	
1.8+ μg BaP/m^3-year					1.09 (0.30-3.99)	
N/A	637 cases 244 controls	Petroleum Industry	Case-control	Prostate	0.74-1.48	(Rybicki et al. 2006)

*Cumulative exposure used when available.
†Risk estimate corresponding to lagged exposure used when available.

adjusting for exposure to other chemicals, physical activity and socioeconomic status. For the high exposure group, the relative risk was 1.3 and the 95% CI included the null value.

In a study on coal tar-derived substances and risk of bladder or lung cancer, Friesen et al. (2007) selected a cohort of 6423 men who worked for 3 or more years at an aluminum smelter in Canada for a period extending from 1954 until 1997. The number of cancer cases diagnosed during the study period was 90 bladder and 147 lung cancer cases. Exposure in this study was evaluated using two different measures of PAH exposure, benzene-soluble materials (BSM) and BaP. Those two exposure indices were found to be highly correlated (r=0.94). The median BaP cumulative exposures were 20 and 18 $\mu g/m^3$-year for no lag and 20-year lag, respectively, and the maximum cumulative exposure was 300 $\mu g/m^3$-year. The BaP cumulative exposures and incidence of bladder cancer indicated a strong dose-response relationship with highest relative risk (3.0) corresponding to the highest cumulative exposure. The relative risk of lung cancer was lower (1.8) for the highest cumulative exposure to BaP but the dose-relationship was the same.

Mortality effects of exposure were assessed in a cohort of iron-steel workers in Anshan, China (Hoshuyama et al. 2006). The cohort included in the study consisted of 121,846 male subjects. Standardized mortality ratios (SMRs) and standardized rate ratios (SRRs) were calculated to evaluate mortality risks among male workers potentially exposed to 15 hazardous substances during 14 years of follow-up. Exposure assessment was performed by using a workshop, job title and exposure matrix. Combined exposure to PAHs and two or more dusts increased the risks of lung cancer (SRR=654, 95% CI 113-3,780). Risks of all neoplasms evaluated in the study were also significantly increased with combined PAH exposure (SRR=541, 95% CI 209-1,395).

The association between PAHs and bladder cancer was investigated in 7,298 men employed between 1913 and 1999 in companies applying asphalt in four European countries (Burstyn et al. 2007). To be included in the cohort, each subject had to be employed for at least two seasons of work. Forty eight bladder cancer cases were

detected among which 39 were exposed at least 15 years before diagnosis. Exposure to BaP was used as a marker for 4-6 rings PAHs and was predicted by using information about changes in asphalt paving technology in each company over time, production characteristics, and job histories. Cumulative exposure did not seem to be associated with risk of bladder cancer. Nevertheless, high average exposure levels displayed around 40% increase in bladder cancer risk but no significant dose-response trend. After allowing for 15-year lag, bladder cancer risk increased by two-fold between the highest and lowest PAH exposure categories (RR=1.9, 95% CI 0.66-5.47). Other non-occupational risk factors such as smoking habits, diet and physical activity were lacking in this study.

Occupational exposure to PAHs from wood, petroleum, coal and other sources through inhalation and dermal absorption was evualuated in relation to prostate cancer risk in a worker population in the United States (Rybicki et al. 2006). The subjects recruited for this study included 637 prostate cancer cases and 244 controls. Exposures were predicted by an expert review of job histories. The highest risk estimate adjusted for age, race, prostate-specific antigen (PSA), pack years of smoking and dietary PAH intake, was associated with coal as a source of PAH exposures. The prostate cancer risk estimate associated with PAH exposure from coal was 1.29 (95% CI 0.73-2.3) through inhalation and 1.48 (95% CI 0.68-3.2) through dermal exposure, but associations were not significant. The primary objective of this study was however to determine if variation in the GSTP1 gene modifies the risk of prostate cancer following occupational exposures to PAHs. The *GSTP1 Val*^{105} allele was observed more frequently in cases in the highest quartile of occupational inhalation PAH exposures. In prostate cancer cases, carriage of the *GSTP1 Val*^{105} allele was significantly increased in subjects in the highest quartile of PAH exposure, especially in cases with an earlier onset of disease (under age 60) when the risk was 4.52 (95% CI 1.96-10.41).

These studies confirm the increased incidence of different types of cancer, especially lung and bladder, following exposures to PAHs in occupational settings. A major flaw however in these studies is exposure assessment. No attempt was made in

any of these studies to measure internal dose of chemicals which would help avoid exposure misclassification and would account for different exposure routes. However, it can be argued that exposure misclassification occurred non-differentially leading to underestimation of risks. In addition, non-occupation risks were seldom adjusted for and could have confounded results. Nonetheless, occupational studies of PAHs and cancer risk present many advantages of which are large sample sizes, and relatively long follow-up periods needed in evaluation of diseases with long latency such as cancer. In addition, assessment of dose-response trends enabled by these studies is of particular importance in determining "safe" doses of PAH exposure over long periods of time.

A number of factors can influence the carcinogenic potency of individual PAHs and PAH mixtures in animal studies. Comparisons among the different studies that evaluated carcinogenicity of PAHs can lead to misleading results. Factors such as species, age, sex and strain of animal, route of administration, vehicle or solvent for the PAH(s) and presence or absence of exogenous and endogenous tumor promoters or inhibitors may significantly modify organ/tissue-specific genotoxic responses to PAHs. Differences in PAH sensitivity may result from differences in ease of penetration to target tissues, basal and/or inducible levels of drug-metabolizing enzyme activities in both target and non-target tissue, and levels of various DNA repair enzyme systems. The difference in aryl hydrocarbon receptor (AhR) affinity among inbred mouse strains leads to difference in CYP enzymes inducibility which was shown to be associated with differences in risk of cancer caused by PAHs (Nebert et al. 2004). Differences in expression, regulation and catalytic activities of metabolic enzymes between rats, mice and humans has also been previously documented (Guengerich 1997). Unidentified components in a mixture may contribute to its carcinogenic potential (Weyand et al. 1995). The carcinogenicity of BaP and other noncarcinogenic PAHs as well as their mixtures was investigated in male C3H/HeJ mouse skin (Warshawsky et al. 1993). A mixture of a noncarcinogenic dose of BaP in combination with 5 noncarcinogenic PAHs resulted in enhanced carcinogenic potency. Similar synergistic responses were observed with coal tar that contained low levels (0.0006%) of BaP. In a different study using

substituted methylbenz[a]anthracenes, no skin tumors were observed when compounds were applied in toluene, whereas the congeners induced tumor formation after application in n-dodecane, as the solvent vehicle. Tissue-specific activation of PAH mixtures was reported by Culp and Beland (1994). Adduct levels detected in lung tissue of coal tar fed B6C3F1 mice was significantly higher than that of the liver and forestomach (Culp and Beland 1994). Furthermore, ingestion of manufactured gas plant residue by female A/J mice induced lung tumors exclusively, whereas feeding or single intraperitoneal (i.p.) injection of BaP to the same mouse strain induced lung and forestomach tumors (Weyand and Wu 1995). Route of administration of the chemical may therefore have implications on the tumor formation process.

The juvenile mouse model for PAH-induced carcinogenicity presents another important example of *in vivo* assay variability. The carcinogenicity of BaP in mice has been thoroughly investigated in many studies (Vesselinovitch 1990; Vesselinovitch et al. 1975a, 1975b). In one of the studies (Vesselinovitch et al. 1975a), administration of a single dose of BaP (75 μg/g) to male B6C3F1 mice induced a significant increase in liver tumors on days 1 (infant) or 15 (juvenile), whereas 42-day-old males exhibited only minimal tumor development when treated with BaP. On the other hand, liver tumor incidence was low in 1-, 15- and 42-day-old female mice treated with a single dose of BaP. Results from these studies suggest that age and sex were important for development of liver tumors in the B6C3F1 mouse model, whereas neither of these two variables affected the frequency of tumor formation in lungs. Perinatal age is considered a sensitive period regarding liver tumor induction largely due to high DNA and cell replication. On the other hand, the sex hormonal environment of the animals may modulate preneoplastic and neoplastic cell growth. More recently, 3-methylcholanthrene (3-MC), a substituted PAH, was demonstrated to activate estrogen receptor-α (ER-α) by direct interactions with this receptor independent of the AhR complex (Abdelrahim et al. 2006).

1.4.3 Non-genotoxic Effects of Polycyclic Aromatic Hydrocarbons

With the extensive wealth of evidence that exist on the carcinogenicity of PAHs to experimental animals and humans, the significance of other physiological effects induced by PAHs becomes overshadowed. Numerous hazardous chemicals or complex mixtures of chemicals found in the environment may disrupt endocrine functions in exposed individuals. These disruptions may potentially lead to deleterious reproductive effects and increased risk for breast cancer as well as cancers of the reproductive system. A number of PAHs have been identified as having chemical structures similar to steroid hormones (Santodonato 1997), which suggests that PAHs are potentially capable of inducing estrogenic and anti-estrogenic responses in humans. Estrogenic responses are largely mediated by the activation of the estrogen receptor (ER) which is a transcription factor that subsequently interacts with responsive genes in hormone-sensitive tissues. Therefore, the ER-mediated mechanism of PAH toxicity is due to their ability to displace natural estrogens and bind to ER. However, PAHs can also exert anti-estrogenic activity. These types of responses are induced by PAHs binding to AhR and upregulating CYP-dependant monooxygenases leading to faster metabolism of steroid compounds (Santodonato 1997).

Recent studies provided further experimental proof that PAHs, more specifically B[a]P and 3-MC in addition to being AhR agonists can also bind and activate ERα independently of AhR (Abdelrahim et al. 2006; Liu et al. 2006). Organic extracts primarily consisting of polycyclic aromatic compounds, isolated from road dust and diesel exhaust particulates were found to contain contaminants that induced significant ER ligand activity (Misaki et al. 2008). Nevertheless, further research is in need to better understand these less studied mechanisms of PAH toxicity and their effects on living organisms.

1.4.4 Congenital Malformations

Birth defects have major worldwide public health implications. They are the leading cause of infant mortality. Birth defects can be categorized as structural,

functional, metabolic, behavioral or hereditary (Jones 1997). Exposure to genotoxic compounds, such as PAHs, is thought to be a contributing factor to the risk of birth defects. One of the most common types of birth defects are neural tube defects (NTDs). NTDs occur when the embryonic neural tube which ultimately forms the brain and spinal cord fails to properly close during the first few weeks of fetal development. Affected individuals suffer from both increased morbidity and mortality compared to their normal contemporaries. Undeniably, some forms of NTDs are uniformly lethal. Despite the fact that there are several known causes of NTDs, a specific etiologic agent(s) has not been identified in the majority of cases. Maternal proximity to hazardous waste sites and exposure to pesticides have both been suggested as risk factors, but are not considered to be established ones for NTDs in offspring. The rarity of NTDs occurrence (<1/1000 births in the United States), variability among exposure assessment methods between studies as well as overly simplistic etiological models has hindered efforts for establishing the degree of association between chemical exposures and NTD risk.

The process of neural tube formation is referred to as neurulation. This process is initiated at approximately 10 days, and is completed by approximately 27 days post-conception (Sadler 2005). Neurulation is a complex process involving the formation of the neural plate, as well as neural fold elevation, bending, adhesion and fusion. Due to the complexity of the events required for neural tube formation, it is likely that NTDs may result from disruptions in more than one developmental pathway. In mice, there is evidence suggesting that NTDs can arise due to genetic mutations affecting convergent extension, elevation/apposition of the neural folds, or fusion (Copp et al. 2003).

Different types of NTDs exist depending on the region where the neural tube failed to close. Defects of neurulation that are restricted to the caudal region of the neural tube are referred to as spina bifida. Spina bifida is characterized by the incomplete development of the posterior neural tube with a protrusion of neural tissue through an opening in the vertebral arches. Approximately 13% of individuals with spina bifida die before their first birthday and 25% die prior to 18 years of age (Wong and Paulozzi

2001). Moreover, some studies suggest that affected individuals are subject to excess mortality throughout their adult years (Bowman et al. 2001; Hunt 1999; McDonnell and McCann 2000; Singhal and Mathew 1999). Individuals with spina bifida who survive are at risk for lower extremity weakness and paralysis, sensory loss, bowel and bladder dysfunction, orthopedic abnormalities, associated malformations of the nervous system (e.g. hydrocephalus), and specific learning disabilities (Northrup and Volcik 2000). Defects of neurulation that are restricted to the cranial region are referred to as anencephaly, and those involving both the caudal and cranial regions are referred to as craniorachischisis. Anencephaly is characterized by failure of closure of the anterior neural tube which leads to absence of brain. Both anencephaly and craniorachischisis are fatal to the offspring.

Prevalence of NTDs exhibit regional and temporal variations. The estimated NTD prevalence, based on United States birth certificate data for 1995, was 40/100,000 (spina bifida: 28/100,000; anencephaly: 12/100,000) (Mathews et al. 2002). Variables such as race, ethnicity and gender influence the prevalence of NTDs. In the United States, the prevalence of NTDs is highest among the offspring of Hispanic women, intermediate in the offspring of non-Hispanic white women and lowest in the offspring of Asian and African-American women (Feuchtbaum et al. 1999). In addition, female offsprings are affected more frequently than males (Shaw et al. 2003).

NTDs are known to be the most common form of all human birth defects, yet the etiologic basis and embryology are poorly understood. The development of these kinds of birth defects is governed by both genetic and environmental components. This may explain why varying rates of NTDs exist in different geographical locations, and among different races and ethnicities. The efficacy of folic acid supplementation for prevention of NTDs appears to be influenced by the genetic capability of the mother to transport and metabolize folic acid (Gelineau-van Waes and Finnell 2001).

Surveillance data indicate overall NTD rates have been falling in the most developed countries in the past three decades. Prenatal diagnosis programs became increasingly important determinants of NTD prevalence. However they are not

considered to be the only factor responsible for the decline of NTD prevalence at birth. More research studies should be undertaken to prevent all folic acid preventable NTDs (Olney and Mulinare 2002). Approximately 400,000 infants with spina bifida are born worldwide every year. Even after adjusting for prenatal diagnosis and elective termination, the general trend of the rates of spina bifida and anencephaly has been on the decline. Around the 23rd day of gestation, fusion of the neural tube starts and proceeds both cranially and caudally. This process is influenced by genetic and environmental factors. An example of a single gene mutation leading to birth defects is the Meckel-Gruber syndrome. On the other hand, trisomy 13 and trisomy 18 are examples of chromosomal abnormalities causing congenital anomalies. The genetic component of birth defects however consists most likely of a polygenic interaction and not a single gene. Other known risk factors of NTDs include maternal use of anti-epileptic drugs, maternal diabetes, hyperthermia and obesity. Periconceptional folate administration reduces the incidence of NTDs but the precise mechanism is still uncertain (Pulikkunnel and Thomas 2005).

Congenital malformations are the leading cause of infant mortality in the United States. The estimated lifetime cost for children born each year in the state of California with spina bifida exceeds $58,375,000. The public health impact of birth defects cannot be overstated. An estimated 5 to 10% of all birth defects are due to *in utero* exposure to known teratogenic agents or maternal factors. As an example, maternal cigarette smoking during early pregnancy was linked to a two-fold increase in risk for both isolated cleft lip with or without cleft palate and isolated cleft palate (Shaw et al. 1996). Several thousand of new compounds synthesized each year reach our environment, from which 10% persist in appreciable amounts as potential environmental toxicants. Many of these compounds have teratogenic potential. Among 2,500 teratogenic agents listed by Shepard (1995), around half were found to induce congenital defects in laboratory animals but only 40 were considered to produce teratogenic effects in humans. Teratogenic agents include infectious organisms, physical factors, maternal metabolic imbalances, drugs, and environmental chemicals. Schardein (1993) observed that out of

3,300 chemicals that have been evaluated for their teratogenic potential, approximately 37% exhibited some evidence of teratogenicity. The studies by Shepard (1995) and Shardein (1993) confirm the existence of a wide gap between animal and human teratogenicity data and that more research efforts are needed to explore the human teratogenic potential of environmental chemicals. However, it is still universally accepted that at a certain dose level, any chemical can be teratogenic. Disruption of normal embryonic development can be produced by both teratogenesis and mutagenesis. The former induces noninherited malformations by altering fundamental embryological processes whereas mutagenesis causes heritable changes in genetic material. The most critical morphogenic processes usually take place in the first two months post conception. At this time, embryos are especially vulnerable to any teratogenic insults.

Maternal nutrition clearly has an impact on the development of the fetus. Supplemental folic acid may reduce incidence of NTDs by 70% but the molecular mechanism is unknown (Finnell et al. 2002). The daily recommended dose for women of childbearing age is 0.4 mg of folic acid. However, the recommended dose for a woman who had previously conceived a child with a NTD is ten fold higher. Intake of folic acid is recommended at one month prior to pregnancy and through three months of gestation. High levels of the amino acid homocysteine, usually indicating a defect in folate metabolism, are considered to be a risk factor for NTDs. Folate is involved in synthesis of nucleic acids and proteins. Reports from China suggest that folate deficiency is prevalent especially in the Northern provinces such as Shanxi (Hao et al. 2003; Hasenau and Covington 2002; Zhang and Smith 2007).

The list of variables that have been implicated as risk factors for NTDs in humans is long and varied. Nonetheless, most of the reported associations are weak and have been inconsistently replicated in subsequent studies. Established NTD risk factors include inadequate maternal intake of folate/folic acid (Wald 1993), maternal pre-gestational diabetes (McLeod and Ray 2002) and maternal use of anticonvulsants valproic acid and/or carbamazepine (Hernandez-Diaz et al. 2001; Lammer et al. 1987; Matalon et al. 2002). There is also a relatively strong evidence that maternal obesity

(Hendricks et al. 2001; Shaw et al. 2002; Shaw et al. 1996; Waller et al. 1994; Watkins et al. 2003; Watkins et al. 1996; Werler et al. 1996) and maternal hyperthermia (Chambers et al. 1998; Lynberg et al. 1994; Milunsky et al. 1992; Shaw et al. 1998) are associated with an increased risk of NTDs in offspring. When considering environmental exposures, living in close proximity to hazardous waste sites has been identified as a potential NTD risk factor in studies conducted by Dolk et al. (1998) and Orr et al. (2002). However based on such studies, it is not feasible to identify the compound or mixture responsible for the increased risk of NTDs since no attempt was made to measure exposure in affected individuals. In addition, these studies were based on a surveillance system that included multiple hazardous waste sites, which vary with respect to their contaminants and the contaminated environmental media. When conducted on individual waste sites, studies might offer a more focused assessment of specific exposures. Nevertheless, even at single sites, multiple contaminants and environmental media are still likely to be present. Due to the relative infrequency of NTDs, epidemiologic studies are often limited by access to a small number of cases. The research described in this dissertation was focused on evaluating the utility of various biomarkers for assessing NTD risk factors.

Disease risk is a complex concept and may be influenced by intricate interactions between genes and environmental factors. In recent years, the opportunity to investigate such interactions has been explored in the context of large-scale epidemiological studies. Initial investigations indicated that the risk of NTDs may be influenced by gene-nutrient interactions (Shaw et al. 2002; Volcik et al. 2003), but such observations remain inconclusive.

Around the early 1990s, an outbreak of anencephaly was reported in the south Texas county of Cameron on the United States-Mexico border (Hendricks 1999). Further investigations indicated that along the entire Texas-Mexico border region, the rates of NTDs were up to 27 per 10,000 live births which is higher than the nationwide average estimated to be around 6 per 10,000 live births (Hendricks et al. 1999). The affected area in south Texas has a predominantly Hispanic population which is known to have higher

rates of NTDs in comparison to any other races (Canfield et al. 1996). However, the detected NTD rates were alarmingly high and warranted attention from state and national experts. For instance, The Centers for Disease Control and Prevention (CDC) together with Texas Department of Health Services initiated a program of NTD surveillance and risk reduction in 14 counties in south Texas (2000). The primary objective of this program was to provide folic acid to women of child-bearing age, particularly to ones who have had an NTD-affected pregnancy to prevent recurrence of these defects. Prevention of birth defects by using folate as a supplement or in diet did not produce the anticipated results in this population (Suarez et al. 2000). Numerous risk factors for NTDs have been evaluated in the study population. Among those are exposure to amine-containing drugs and dietary nitrites and nitrates (Brender et al. 2004), polychlorinated biphenyls (PCBs) (Suarez et al. 2005), heavy metals (Brender et al. 2006) and fumonisins (Missmer et al. 2006) in addition to folate pathway gene polymorphisms (Barber et al. 2000). More recently, researchers investigated the hypothesis that *Helicobacter pylori* seropositivity could potentially lead to compromising bioavailability of nutrients such as folate, vitamin B_{12} and iron to the fetus which increase the NTD risk (Felkner et al. 2007). To date, it appears that the highest risk factor may be maternal exposure to fumonisins, a class of mycotoxins commonly contaminating corn. Cornmeal samples collected from the affected area of Texas during the time of the NTD outbreak had unusually high levels of fumonisins. A particular characteristic of the diet of the Hispanic population residing in south Texas is the heavy consumption of corn in the form of tortillas. It was estimated that Mexican-American women living on the Texas-Mexico border consumed around 90 grams of corn per day as compared to 17 grams of corn-based food per day consumed by Canadian adults (Hendricks 1999). Just before the identification of the NTD cluster in south Texas, there were outbreaks of animal diseases such as equine leukoencephalomalacia which was strongly linked to fumonisin contaminated corn feed (Hendricks 1999). Moreover, a number of *in vitro* and animal studies have indicated that exposure to fumonisins may lead to NTDs (Flynn et al. 1997; Gelineau-van Waes et al.

2005). A study measuring biomakers of exposure to fumonisins in blood of mothers of NTD cases and controls from the affected area found that the risk of NTD increased proportionally with fumonisin exposure up to a threshold level independent of other known risk factors (Missmer et al. 2006).

The relationship between indoor use of coal and the resulting exposure to PAHs has not been thoroughly investigated as a risk factor for birth defects. Most studies however, indicate that air pollution and subsequent exposure to elevated levels of PAHs is associated with adverse birth outcomes in humans. A potential link between coal combustion byproducts, and the risk of congenital malformation was investigated by a study conducted in Nova Scotia, Canada. In this study, the risk of congenital malformations (overall and specific categories) was increased in the offspring of residents of a coking operation, compared to the rest of Nova Scotia (Dodds and Seviour 2001). Airborne PAHs have been implicated in human reproductive effects, notably, DNA adducts and hypoxanthine-guanine phosphoribosyltransferase mutations in newborns as well as preterm birth and intrauterine growth restriction (Dejmek et al. 2000; Perera et al. 2002; Sram et al. 1999). Cigarette smoking during pregnancy has been associated with several adverse birth outcomes including spontaneous abortions, delayed conception, and low birth weight (Preston 1991). Findings from a more recent study suggest that high levels of PAHs as measured by DNA adducts in cord blood, from the World Trade Center fires in New York City in 2001, may have contributed to reduced fetal growth in exposed women (Perera et al. 2005). In a review of the literature on ambient pollution and pregnancy outcomes by Sram and coworkers (2005), low birth weight, premature birth and intrauterine growth retardation seemed to be the reproductive effects most often associated with air pollutants. The study observed that intrauterine growth retardation was specifically linked with PAHs. Detection of DNA adducts in human placenta and cord blood confirms that PAHs may be transferred to the fetus and could predispose it for developing disease later in life (Hansen et al. 1993). Limited evidence is available from animal studies. However a study by Wang and Yu (Wang and Yu 2004) demonstrated a relationship between exposures to PAH mixtures in

cigarette smoke and neural tube defects in hamsters. On the molecular level, activity of placental metabolic enzymes, such as CYP1A1, has been documented to be induced by PAHs as a result of maternal exposures to these compounds, which was reported to be linked with human placental calcifications and Threatened Preterm Delivery (TPD) (Huel et al. 1993). As discussed previously, 3-MC was reported to be capable of directly activating ER-α (Abdelrahim et al. 2006), which could potentially lead to various adverse reproductive effects.

1.5 Human Health Risk Assessment

Humans are continuously exposed to complex chemical mixtures present in their environment. Exposure to higher concentrations of chemicals can potentially occur around hazardous waste sites. As mentioned previously, a number of reports have identified increased rates of adverse health effects ranging from skin rashes to congenital malformations, among residents in proximity to toxic waste sites (Brender et al. 2003; Brender et al. 2006; Comba et al. 2006; Kuehn et al. 2007). These epidemiological studies give insight into effects complex chemicals mixtures including PAHs have on humans. However, without toxicity testing, there is no absolute data on how chemicals react in biological organisms, and the severity of effects they may cause.

The USEPA Risk Assessment Guidance for Superfund (RAGS) (USEPA 1989) has established a 4-step approach that can be used to estimate the human health risk associated with exposure to contaminated media at Superfund sites (Figure 1.6). The initial step in this approach consists of identifying the hazard specific to the site. Hazard Identification involves developing a qualitative assessment of risk at the site. Existing background data on contaminants and contaminated media is gathered. Concentrations of each contaminant are then compared to Risk Based Concentrations (USEPA 2007) to determine which chemicals represent the greatest health threat. Hazard identification typically results in developing a list of Contaminants of Potential Concern, or the 10-15 chemicals which are anticipated to represent the greatest threat to human health (USEPA 2007).

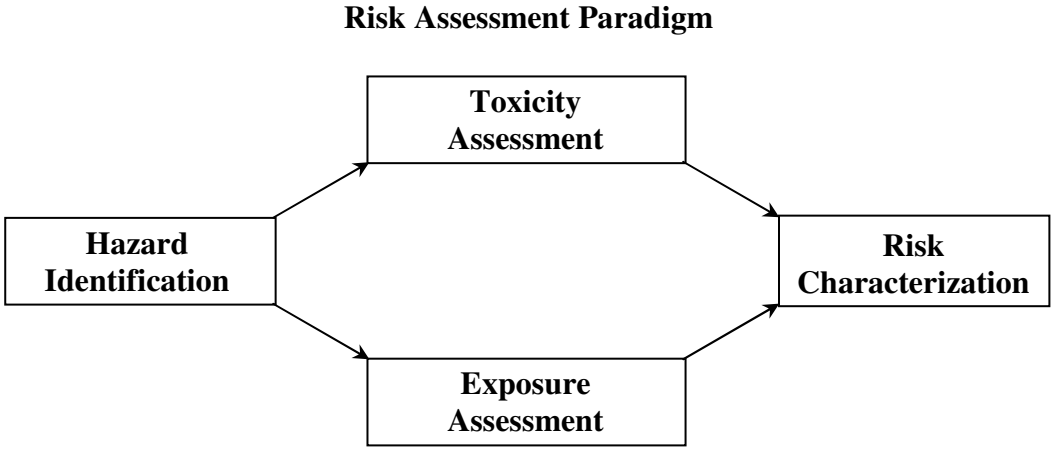


Figure 1.6. The four major steps included in the quantitative evaluation of human health risk for chemically contaminated sites (modified from Asante-Duah (2002)).

The second step in human health risk assessment is the toxicity or dose-response assessment. The Integrated Risk Information System (IRIS) developed by USEPA (USEPA 2006) is most commonly used to identify a Reference Dose (RfD) for non-cancer effects, and/or a Cancer Slope Factor (CSF) for cancer effects. A great deal of uncertainty still exists in many of these toxicity values, although for a large number of chemicals, toxicity values have been established. Sources of uncertainty include species extrapolation, and extrapolation from high doses used in toxicity studies to the relatively low doses that generally occur in environmental exposures. As there is no widely accepted protocol for interpreting the potential interactions of chemical mixtures yet, most risk assessments are usually conservative and assume additive effects. Component interactions in a mixture may affect risk by affecting the pharmacokinetics of other chemicals. Thus, low molecular weight PAHs that are capable of inducing Phase I metabolizing enzymes in the liver may enhance the toxicity of high molecular weight PAHs. Competition for catalytic sites of metabolizing enzymes also occurs as documented by Falahatpisheh et al. (2004) who observed an inhibition of toxicity of BaP by chrysene, another class B2 carcinogenic PAH.

The third step in risk assessment is the exposure assessment which alternatively can be conducted before toxicity assessment. All completed exposure routes at a site for both on-site workers and off-site residents must be determined. This includes exposure to contaminants released onsite that may be present in soil, air, groundwater, surface water, sediment, or food products. For each completed exposure pathway, the concentration of every contaminant of concern in a specific medium is estimated. This estimation may use a mean value, an upper 90^{th} percentile value or more conservatively a maximum value depending on the quantity and quality of existing data. These values are used to estimate a Cumulative Daily Intake (CDI) for each exposure pathway and each contaminant of concern. The most common exposure routes in humans include ingestion, inhalation, or dermal contact with a contaminant of concern. Humans can also be indirectly exposed through ingestion of contaminated plants or animals. In this step, sources of uncertainty include assumptions regarding intake variables for contaminated media, estimate of chemical concentrations in the media, and rates of absorption from various exposure pathways (USEPA 2007).

The last step of risk assessment generally results with the generation of a quantitative site-specific characterization of both cancer and non-cancer risk. The non-cancer risk is a sum of the Hazard Quotient, or the value obtained by dividing the Cumulative Daily Intake by the Reference Dose. Contaminant concentrations are considered acceptable as long as the estimated daily intake is below the No Observable Adverse Effect Level (NOAEL) (or the Reference Dose). Hazard Quotients for each chemical and each exposure pathway are usually summed to obtain a Hazard Index that characterizes the non-cancer risk associated with the site-specific contaminants of concern. The cancer risk is expressed by the Lifetime Cancer Risk (LCR) that is computed as the product of the Cancer Slope Factor and the Cumulative Daily Intake. Cancer risk in residential settings is considered acceptable as long as the sum of Lifetime Cancer Risk for all chemicals and all exposure pathways does not exceed one in one million. These risk estimations help in efforts of ranking sites and assessing acceptable levels for clean-up.

However, as previously discussed, significant sources of uncertainty exist in each step of the risk assessment process, the major one being the potential additive, synergistic, antagonistic or potentiating interactions of complex chemical mixtures (USEPA 1986). Animal studies emphasize the uncertainty associated with basing human risk assessments for environmental complex mixtures on the concentration of a single, albeit prototypic, carcinogenic chemical, due to the potential presence of unidentified components that may have toxicological significance in most environmental mixtures (Rodriguez et al. 1997). PAH interactions in a mixture may affect the inducibility of Phase I, Phase II and DNA repair enzymes which leads to differences in DNA adduct frequencies and persistence. Alterations in these enzymes are also potentially influenced by circadian rhythms (Lavery et al. 1999; Noshiro et al. 1990; Ohno et al. 2000). Mixture interactions may also alter the rates of absorption of certain chemical components, making cells more or less permeable to high molecular weight PAHs which are potentially more toxic.

Pentachlorophenol (PCP) is a chlorinated hydrocarbon present in certain environmental PAH mixtures such as wood preserving waste (Donnelly et al. 1987). PCP was found to be a potent inhibitor of glutathione S-transferase (GST) (Moorthy and Randerath 1996; Mulder and Scholtens 1977). Moorthy and Randerath (1996) demonstrated that administration of 9-hydroxyBaP in a binary mixture with PCP resulted in an almost two-fold increase in DNA adduct intensity. Using microbial cells *in vitro*, an enhanced mutagenic response that was highly dose dependent was observed with mixtures of BaP and PCP (Markiewicz et al. 1996). These data suggest that *in vivo* exposure to BaP:PCP mixtures could produce increased levels of benzo[*a*]pyrene 7,8 diol-9,10 epoxide (BPDE)-N2G adducts. Recent studies with infant and adult B6C3F1 mice, exposed by i.p. injection to BaP alone or BaP plus PCP, found that PCP potentiated BPDE-N2G adduct formation in the liver and lung of adult but not infant mice (Bordelon et al. 2001). In wild-type C57BL/6 mice, exposure to the BaP/PCP mixture produced significantly fewer BPDE-N2G adducts in both tissues than BaP alone (Ress et al. 2002). In the C57BL/6 p53-null mice, however, the level of BPDE-N2G

adducts produced by the BaP/PCP mixture and the BaP were not appreciably different in the liver (Ress et al. 2002). These experiments reveal that PCP may enhance or reduce BPDE-N2G adduct formation, depending on the age, strain and genetic characteristics of the animal model used.

The research conducted as part of this dissertation has investigated the ability of complex PAH mixtures present in residential environments to form DNA adducts in mice as well as human populations.

1.6 Toxicity Test Methods

Chemical analysis provides essential information regarding the major components of a complex mixture. However, chemical analysis is unable to measure the genotoxicity of a mixture or potential mixture interactions. In some cases, chemical analysis may not be capable of detecting all the components of a mixture, mainly because of their similarity in chemical structure. *In vitro* and *in vivo* bioassays therefore are more adequate for evaluating the toxicity of chemicals and mixtures. *In vitro* bioassays whether acellular or consisting of microbial or mammalian cells in culture are useful for screening complex mixtures and investigating mixture interactions. Nevertheless, such assays lack the ability to replicate complex pharmacokinetic processes such as absorption, distribution, metabolism, and excretion. Absorption and distribution may partition mixture components such that the composition of compounds that reach the target organ is very different from the composition of the mixture. Metabolism and excretion are also likely to alter the composition and concentration of mixture components in tissues. In general, it is recommended to use a battery of assays when evaluating the potency of a substance with the idea of selecting tests that detect a broad range of endpoints. This testing method provides a broader understanding of the toxic effects of a substance especially when dealing with complex environmental mixtures. *In vitro*, i*n vivo*, and ultimately epidemiological studies are together substantial tools in recognizing environmental mixtures that potentially could produce deleterious health effects in exposed populations. Combining data from multiple measurements and assays

at different levels of cellular organization provides a more comprehensive dataset, as well as information to better understand the biological plausibility of disease.

1.6.1 In Vitro *Bioassays*

In vitro bioassays are usually the most rapid and inexpensive screening tests to evaluate the toxic potential of complex environmental mixtures. They usually are able to screen a large number of chemicals or chemical mixtures. This characteristic makes them adequate for prioritizing chemical mixtures for further testing in more complex systems. Short-term *in vitro* bioassays were mainly used to identify potential carcinogens (Adams et al. 1996; McCann et al. 1975) or the genotoxic potency of complex environmental mixtures (Donnelly et al. 1998). Numerous *in vitro* bioassays have been described in the literature. Among these, *Salmonella*/microsome and *E. coli* prophage induction are the microbial assays that appear to have the greatest selectivity and sensitivity for complex mixtures of PAHs and PCAs (DeMarini et al. 1990; Tennant et al. 1987). In addition, mammalian cell culture assays have been developed and had a predominant role in bioassay protocols to identify carcinogens since the early 1980s (Brusick 1988). The *in vitro* bioassays discussed in the following text have been selected for testing complex mixtures of PAHs and were used in the research experiments described in this dissertation.

1.6.1.1 *Salmonella*/Microsome Assay

The *Salmonella*/microsome assay is the most widely studied *in vitro* mutagenicity bioassay. The assay was developed by Dr. Bruce N. Ames and hence is commonly known as the Ames assay. The *Salmonella*/microsome assay was first validated in a study including 300 chemicals. The study reported that 90% of the known carcinogens produced a positive mutagenic response (McCann et al. 1975). In addition, the study found a correlation of 83% between carcinogenicity and mutagenicity. The chemical classes of compounds tested in the study included the following: aromatic amines, alkyl halides, polycyclic aromatics, esters, epoxides and carbamates, nitro

aromatics and heterocycles, aliphatics, nitrosamines, fungal toxins and antibiotics, cigarette smoke condensate, azo dyes and diazo compounds. However, other studies that examined the qualitative relationship between mutagenic and carcinogenic potency by testing a large number of chemicals using the *Salmonella*/microsome assay, found low correlations ranging between 0.24 and 0.44 (Fetterman et al. 1997).The protocol of this assay has been revised multiple times but has been described in its current use in Maron and Ames (Maron and Ames 1983).

Various chemicals or mixtures of chemicals have been successfully screened for mutagenic potential using this assay. Among the substances and mixtures tested using this assay are complex environmental mixtures such as wood preserving wastes (Barbee et al. 1996; Brooks et al. 1998; Cizmas et al. 2004; Donnelly et al. 1987; Donnelly et al. 1995; Hughes et al. 1998); house dust (Roberts 1987), manufactured gas plant residues (Cizmas et al. 2004; Randerath et al. 1999) sewage sludges (Donnelly et al. 1990; Perez et al. 2003), cigarette smoke (Roemer et al. 2004), surface waters (Ohe et al. 2004), diesel exhaust (Seagrave et al. 2005), soils (Watanabe et al. 2005) cooked foods (Knize et al. 2003; Shishu and Kaur 2003; Sugimura et al. 2004) as well as binary mixtures of chemicals such as BaP and pentachlorophenol (Donnelly et al. 1990; Markiewicz et al. 1996).

A review by the U.S. National Toxicology Program (NTP) comparing the sensitivity and specificity of short-term bioassays such as the *Salmonella*/microsome assay found that 83% of the compounds that induce a positive response in Ames test were also rodent carcinogens, which was the highest positive predictivity among the tested bioassays (Tennant et al. 1987a). The study selected 73 chemicals, well characterized for carcinogenicity in both sexes of two rodent species, to be assessed in four short-term *in vitro* tests. The short-term bioassays evaluated were the Ames *Salmonella*/microsome mutagenesis assay (SAL), the assays for chromosome aberration (ABS) and sister chromatid exchange (SCE) induction in Chinese hamster ovary cells, and the mouse lymphoma L5178Y (MOLY) cell mutagenesis assay. SAL and ABS were reasonably specific but relatively not sensitive to rodent carcinogens whereas the reverse

was true for SCE and MOLY. The *Salmonella*/microsome assay exhibited the highest specificity (86%) but was the least sensitive (45%) among the tested bioassays. Another report by the same group found that the *Salmonella*/microsome assay is most sensitive to trans-sex/trans-species carcinogens (Tennant et al. 1987b). The main advantages of the *Salmonella*/microsome assay over other short-term tests include its low cost, broad availability, extensive literature and simplicity (Tennant et al. 1987b). The assay has been found to be sensitive to PAHs, nitroaromatic hydrocarbons, mycotoxins, and a various other carcinogens. A high percentage (95%) of PAHs that induced a positive response in *Salmonella* was also found to be rodent carcinogens (Purchase et al. 1976). It has historically been insensitive to chlorinated hydrocarbons, hormones, heavy metals, and several additional classes of chemicals (Ames 1984).

The *Salmonella*/microsome assay is based on bacterial reverse mutation and uses histidine dependent strains of *Salmonella typhimurium*. Researchers discovered that *Salmonella* strains that depended on histidine as nutrition source, had mutations caused by either base-pair substitutions or deletions of one or more bases known as frameshift mutations. Such bacterial strains were found to have the ability to revert back to wild-type (histidine independence) if exposed to mutagens and could be used to recognize and detect chemicals with mutagenic potential (Mortelmans and Zeiger 2000). The inclusion of a mammalian metabolic activation system enabled this assay to be very useful in detecting indirect-acting compounds such as PAHs (Ames et al. 1973). Bacterial strains used in this assay do not have the capability of metabolizing and activating xenobiotic substrates of cytochrome P450. The mammalian metabolic activation system typically used in this *in vitro* assay is the S9 microsomal fraction of rodent liver in the presence of an NADP generating system and other cofactors (Maron and Ames 1983). In most cases, rodents used to make S9 are pre-treated with a metabolizing enzyme inducer such as Aroclor 1254, 3-MC, Phenobarbital (PB) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to increase the level of mixed function oxidases in the microsomal fraction of the rodent liver homogenate used in this assay. The type of induction was found to have a significant effect on the results seen in the *Salmonella*/microsome assay. The

mutagenic potential of BaP whether administered as a single compound, or in a reconstituted mixture, appeared to increase with TCDD-induced enzymes. On the other hand, the range of CYP isozymes induced by Aroclor was best for complex mixtures from environmental extracts (Markiewicz et al. 1996).

Many tester strains of *Salmonella* have been developed, however the standard strains recommended for mutagenesis testing include TA97, TA98, TA100 and TA102. These strains have been engineered to enhance their sensitivity to different classes of genotoxic compounds. The *Salmonella typhimurium* strain used for the research described in this dissertation was strain TA98 which has been shown to be sensitive to frameshift mutagens. This strain has several specific characteristics to enhance its sensitivity (Ames et al. 1973). TA98 contains a primary deletion in the histidine operon referred to as the *his*D3052 mutation. This mutation causes the bacterial strain to be unable to grow in media lacking supplemented histidine. This is due to the fact that *hisG* gene codes for the histidinol dehydrogenase enzyme needed in the process of histidine biosynthesis. This mutation is considered a -1 frameshift mutation which affects the reading frame of a nearby repetitive –C-G-C-G-C-G-C-G- sequence (Isono and Yourno 1974). Chemicals capable of causing frameshift mutations can restore the correct reading frame. Thus, chemicals that have the ability to react with DNA and induce a frameshift mutation will allow bacteria of this strain to revert back to histidine independence.

Another mutation incorported in the TA98 *Salmonella* tester strain is referred to as deep rough or *rfa* mutation. This mutation causes a loss of the lipopolysaccharide membrane which increases its permeability and hence sensitivity to high molecular weight compounds. This is a critical manipulation for the sensitivity of this bioassay since it facilitates the passage of bulky compounds such as carcinogenic PAHs into bacterial cells. The *uvrB* mutation also exists in the TA98 tester strain. This mutation was found to increase the sensitivity of bacterial cells by compromising the DNA excision repair system of these cells. Such cells will significantly lose their ability to repair DNA damage caused by mutagenic chemicals. Deletion of the gene causing this mutation however also eliminated coding for biotin synthesis enzymes. Therefore,

strains with such deletion are no longer biotin independent and will require low levels of biotin in the agar for growth.

The sensitivity of the microbial strains was greatly increased by the addition of the plasmid pKM101. This plasmid increased error prone repair and hence enhanced chemical and UV-induced mutagenesis. Inclusion of this plasmid in bacterial cells has also inferred ampicillin-resistance which is a characteristic measured during strain checks to ensure the presence of this plasmid. This mutation is probably the least stable of all mutations in this tester strain. Although the plasmid pKM101 greatly enhances the sensitivity of the tester strain, it also slightly reduces the selectivity of the strain towards frameshift mutagens.

In addition to these characteristics, the bacteria strain also has a normal background level of spontaneous revertants. These are bacteria cells that revert back to histidine independence without requiring exposure to any mutagenic agents and form visible colonies. In most laboratories, strain TA98 produces a characteristic number of spontaneous revertant colonies ranging from 15 to 50 colonies. With addition of a dose of 25 μg of 2-nitrofluorene (2-NF), a direct acting mutagen, the number of TA98 revertant colonies is expected to be more than 1,000 colonies, whereas, a dose of 10 μg of BaP with metabolic activation is expected to result in the formation of around 300 revertant colonies. Thus, both of these chemicals are routinely used as positive controls in this assay.

As discussed previously, the *Salmonella*/microsome assay was found to be a sensitive system for detecting carcinogenic PAHs, but not carcinogenic PCAs. Also, this assay is relatively insensitive to chemicals that may cause cytotoxic effects at low doses (Maron and Ames 1983). These limitations need to be considered during testing of a substance or mixture. The research described in this dissertation relied on multiple *in vitro* bioassays in conjunction with *in vivo* testing to measure the genotoxic potential of complex mixtures collected from residential environments.

1.6.1.2 Acellular Assay

Positive results reported in the *in vitro* genotoxicity tests may be due to secondary mechanisms such as cytoxicity, cell cycle perturbation and not necessarily related to direct interaction of the chemical with the DNA molecule (Adams et al. 1996).The major advantage of the acellular system is the ability to examine direct reaction of chemical agents with DNA. Generally coupled with ^{32}P-postlabeling to measure DNA adducts, acellular assays can be used to determine whether a single compound, metabolite or chemical mixture directly damages DNA. The cell-free assay is simple and relatively easy to conduct. The cell-free assay is designed to detect DNA adducts or DNA damage induced by chemically-reactive compounds. The assay usually requires only DNA and the test agent, and may be conducted with or without the addition of a metabolic activation system such as the rat liver microsomal fraction (Segerback 1990; Shah and Bhattacharya 1986). Since this assay does not use cells, it provides an opportunity to use the maximum soluble concentration of the test compound, increasing the sensitivity of the assay due to the fact that DNA damage cannot be repaired (Adams et al. 1996). Thus, acellular tests can provide an early assessment of the mutagenic/carcinogenic potential of compounds as well as important insight for the mechanisms of positive responses in cell-based genotoxicity studies (Adams et al. 1996).

Numerous studies have used acellular systems to study and distinguish between the direct or indirect genotoxic effects of different chemicals. The formation of reaction products in hemoglobin and DNA after treatment with ethylene oxide and N-(2-hydroxyethyl)-N-nitrosourea in an acellular test was reported (Segerback 1990). ^{32}P-postlabeling was coupled with an acellular assay using rat live S9 metabolizing system to test factors suspected to affect recoveries of PAH-DNA adducts (Segerback and Vodicka 1993).

Various types of compounds and mixtures have been tested in similar systems. These include petroleum oils and oil coal tar mixtures (Reddy et al. 1997), particulate and semivolatile fractions of vehicle exhausts (Pohjola et al. 2003), cigarette smoke condensate (Randerath et al. 1992), wood preserving waste (Randerath et al. 1994), PAH

mixtures recovered from contaminated lake sediments (Randerath et al. 1999) and organic mixtures from urban air particles (Binkova et al. 2007). Acellular assays have also been used in mechanistic and metabolism studies (Moorthy 2002; Moorthy et al. 1996; Sanyal and Li 2007). Acellular systems have also been very useful in studies to test the capacity of certain agents to decrease or inhibit the effect of known carcinogens (Le Bon et al. 1992; Salgo et al. 1999; Shah and Bhattacharya 1986; Smith et al. 1998).

1.6.2 In Vivo *Bioassays*

As discussed previously, *in vitro* bioassays while useful in measuring the ability of chemicals to react with DNA and cause genotoxic damage, cannot replicate complex pharmacokinetic processes. Biological processes such as absorption, distribution, bioactivation, detoxication, and DNA repair can modify the potential of environmental mixtures to induce toxic effects in animals or humans. Thus, in addition to *in vitro* testing, it is important to evaluate the toxicity of environmental mixtures using *in vivo* systems as well as epidemiological studies. Animal studies allow genotoxicity measurements to be obtained under controlled conditions, but do account for pharmacokinetic interactions. Epidemiologic studies provide information regarding the adverse impact of occupational or environmental exposures, but often lack quantitative information regarding exposures.

1.6.2.1 DNA ^{32}P-postlabeling Assay

The ^{32}P-Postlabeling assay is widely used in epidemiological and toxicological studies designed to measure DNA adducts. The name of this assay is derived from the fact that radioactive orthophosphate (^{32}P) is incorporated into DNA. DNA addition products or adducts are formed when electrophilic compounds covalently bind to DNA which contains nucleophilic centers. Such chemicals are usually exogenous to the body. Endogenous compounds form adducts also known as I-compounds and can be functionally important however most likely do not reflect DNA damage (Zhou et al. 2005). Those age-dependent endogenous DNA modifications were discovered in tissues

of control animals by ^{32}P-postlabeling (Randerath et al. 1986). I-compounds are believed to represent markers of oxidative stress or DNA repair. Exogenous DNA adducts are commonly used as biomarkers of exposure or early effect reflecting the biologically effective dose of a genotoxicant exposure. The formation of exogenous DNA adducts is generally considered the initiation step in the process of carcinogenesis (Farber and Sarma 1987).

The detection limit of the ^{32}P-postlabeling assay is 1 adduct in 10^9 normal nucleotides, which is equivalent to one DNA base modification per cell (Brandt and Watson 2003). Other methods of identifying and quantifying DNA adducts include fluorescence immunoassays and gas chromatography/mass spectrometry (GC/MS). However, the ^{32}P-postlabeling assay is considered as the most sensitive method which makes it the assay of choice for detecting low levels of DNA adducts such as in human exposure assessment studies (Hemminki et al. 2000). In a study to determine the levels of DNA adducts in lung cancer patients by ^{32}P-Postlabeling and the enzyme-linked immunosorbent assay (ELISA), DNA adduct levels measured by ^{32}P-postlabeling were relatively higher in all study subjects (Cheng et al. 2001). In addition to the high sensitivity of this assay, another advantage of ^{32}P-postlabeling as an assay is the fact that it requires low amounts of DNA ranging between 1 to 10 μg of DNA (Hwang and Bowen 2007). Also, the ^{32}P-postlabeling assay does not require prior knowledge of adduct structures and hence is adequate for measuring DNA lesions formed with unidentified mutagens/carcinogens and mixtures in experimental animals and humans (Randerath and Randerath 1994). The quantitative results generated by this assay however, should always be interpreted with caution since the efficiency of adduct labeling is often undetermined or uncontrolled (Poirier and Weston 1996). Although the procedure is quantifiable, often times, uncertainty regarding the efficiency of adduct labeling renders this a qualitative indication of exposure (Phillips 1997). Another major drawback of this assay is the inability to identify chemical structures of adducts. Nevertheless, additional steps such as co- and re-chromatography can be performed to aid with structural identification (Lu et al. 1986).

Functionally, DNA adducts can lead to mutations. Scenarios of how a mutation can occur after base adduction include nucleotide misincorporation, slippage by DNA polymerase, and misrepair. Mutations can be repaired, or they may lead to cell death. Mutations that occur in critical genes such as oncogenes and tumor suppressor genes, may lead to a variety of unwanted effects, among which is tumor formation (Figure 1.7). Tumor formation is a multi-stage process that may involve multiple chemical exposures over many years. In order to evaluate the carcinogenic potential of an environmental chemical mixture, it is important to understand the mechanism through which the mixture induces carcinogenesis. Around two decades ago, Farber and Sarma (1987) developed a model for tumor formation in the liver. The steps described in their model were largely identified through the use of the resistant hepatocyte model created by Solt and Farber in 1976 (Solt and Farber 1976). According to this model three major steps occur between exposure to a chemical carcinogen and development of a malignant tumor. The process begins when the chemical is absorbed and distributed into systemic circulation. Once in circulation, the chemical may be activated through metabolizing enzymes into its ultimate chemically-reactive form. Initiation is considered to be the first major step to occur during the tumor formation process. This step consists of the carcinogen binding with DNA to form a mutation. This mutation might potentially lead to the formation of abnormal, initiated cells. The growth of the initiated cells is promoted through subsequent mutations, to critical genes such as tumor suppressor genes or oncogenes that alter normal cellular functions. Promoted cells have a variety of altered biochemical characteristics and possibly form small foci. Continued exposures may result in the progression of these foci into neoplastic cells. The nodules formed by neoplastic cells may be observable, but may also grow unnoticed with minimal effect on the affected organism. In the case of a malignant tumor, the neoplastic cells have the capability to outgrow the normal structure of the tissue in which they grow, and may break off from a primary tumor and migrate to another location in the host forming a secondary tumor (Solt and Farber 1976).

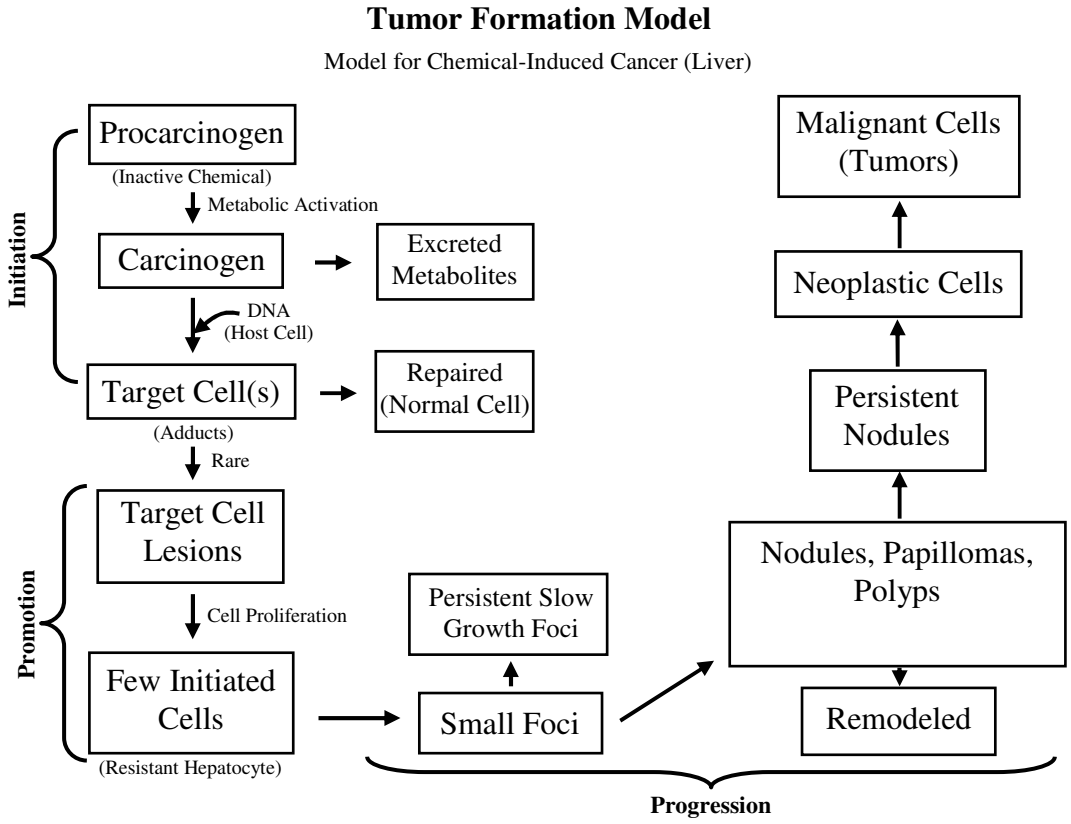


Figure 1.7. Diagram of the tumor pathway model for chemical-induced liver cancer. Adapted from Farber and Sarma (1987) and Phillips (2006).

The active metabolite of BaP, BPDE shown to bind to the N-2 of guanine, was found to cause a GC → TA mutation (Jelinsky et al. 1995). Tumorigenesis and levels of DNA adducts were correlated (Poirier and Beland 1994; Poirier et al. 1995) and tumor formation was reduced when chemopreventative agents were used to reduce DNA adduct formation (Breinholt et al. 1995; Dashwood et al. 1998; Egner et al. 2003). Therefore, measuring DNA adducts is substantial in identifying potential carcinogens present in the environment, studying mechanisms of carcinogenesis and DNA repair, in addition to biomonitoring and human health risk assessment.

According to K. Randerath and E. Randerath (1994) the ^{32}P-postlabeling assay has been applied in more than 100 laboratories to measure adducts formed in DNA

preparations treated with hundreds of test chemicals. Chemicals evaluated in the ^{32}P-postlabeling assay include arylamines, nitroaromatics, nitrosamines, azo compounds, dye-stuffs, PAHs, heterocyclic polycyclic aromatics, epoxides, quinones, methylating and other alkylating agents, mycotoxins, alkenylbenzenes, and antibiotics, in addition to chemicals generating oxygen free radicals. Moreover, DNA lesions induced by complex mixtures such as tobacco smoke, iron foundry and coke oven carcinogens in occupational settings, as well as unidentified sources has also been assessed by ^{32}P-postlabeling.

The ^{32}P-postlabeling technique has been used in several studies to detect DNA adducts formed by BaP (Booth et al. 1999; Boysen and Hecht 2003; Godschalk et al. 1998; Gupta et al. 1982; Lu et al. 1986; Reddy et al. 1984; Reddy and Randerath 1986). Adduct analysis by ^{32}P-postlabeling has been performed on DNA isolated from various tissues including white blood cells or peripheral blood lymphocytes which are readily accessible in humans. Other tissues that have been used to detect BaP adducts include skin, placenta, kidney, liver, lung, breast, and pancreas (Hemminki et al. 2000). The ^{32}P-postlabeling test has been used in biomonitoring human populations exposed to environmental or occupational levels of genotoxic chemicals (McClean et al. 2007; Tang et al. 2006), as well as cell-based or cell-free *in vitro* studies (Binkova et al. 2007; Sevastyanova et al. 2007) evaluating the genotoxic potential of complex mixtures. Animal studies conducted to investigate adduct formation after treatment with environmental complex mixtures has also commonly used ^{32}P-postlabeling as part of their methods (Cizmas et al. 2004; Randerath et al. 1999). The research presented in this dissertation has used ^{32}P-postlabeling to detect DNA adducts in a cell-free system, as well as in animal studies and biological tissues collected from human study subjects.

1.7 Metabolism

Exposure to PAHs may occur through dermal absorption, inhalation of particulate or ingestion. PAH absorption through the skin is relatively slow, whereas absorption through the respiratory or gastrointestinal tracts are more rapid. Once

absorbed into systemic circulation, PAHs usually undergo Phase I (oxidation) and Phase II (conjugation) metabolism to increase solubility and the rate of elimination. The process of mammalian metabolism of xenobiotics such as PAHs typically is divided into two distinct phases, Phase I and Phase II (Brandt and Watson 2003). The major reaction in Phase I is usually hydroxylation catalyzed by a class of enzymes referred to as monooxygenases or cytochrome P450 (CYP). These enzymes called mixed function oxidases (MFOs) are ubiquitously present in multicellular organisms and expressed or induced in mammalian tissues, mainly in the endoplasmic reticulum (microsomal fractions). Other reaction types that occur in Phase I include reduction and hydrolysis. Phase II metabolic transformations consist mainly of converting the hydroxylated and other metabolites produced in Phase I to various polar metabolites by conjugation to, for example, glucuronic acid, sulfate, glutathione or certain amino acids. The overall purpose of those two phases is to increase water solubility of the metabolized compounds by the addition of a polar entity, thus facilitating their excretion from the body. Without this process, water insoluble compounds such as PAHs could remain in tissues for extended periods. The rate and extent of PAH metabolism generally depends on structure of the specific compound. Due to their similar chemical structure consisting of multiple fused benzene rings, PAHs as a group, undergo similar biotransformations. The multiple pathways for Phase I and Phase II metabolic pathways of PAHs are diagrammed in Figure 1.8.

The first step in Phase I metabolism of PAHs is typically oxidation (epoxidation) and is catalyzed by CYPs. CYPs are hemoprotein that associate with another membrane-bound enzyme that NADPH-dependant flavoprotein cytochrome P450 reductase. Monooxygenation of xenobiotics in mammalian species including humans are mainly catalyzed by CYP enzyme families 1 to 3 such as CYP1A1 and CYP1B1 (both in extrahepatic tissues) and to a lower extent CYP1A2 and CYP3A4 (mostly in the liver). CYP1B1 is present in almost all organs except liver and lungs. CYP1A1 expression is usually low in mammalian tissues. CYP3A4 is most abundant in tissues followed by CYP1A2.

PAHs can induce their own biotransformation. They can significantly increase expression of CYP1A1 (BaP, 7,12-dimethlybenz[*a*]anthracene, 3-methylcholanthrene) and CYP1B1 in liver, lung and most extrahepatic tissues by binding to cytosolic AhR. The AhR-ligand forms a complex with the aryl hydrocarbon nuclear translocator (ARNT). In the nucleus, ligand-activated AhR-ARNT heterodimer becomes competent to bind to aryl hydrocarbon response element (AhRE) or xenobiotic response element (XRE). CYP1 protein is induced following this mechanisms, as well as GST and UDP-glucuronosyltransferase (UGT) enzymes considered to be Phase II enzymes.

PAHs are generally chemically inert molecules. In certain cases however, Phase I metabolism converts unreactive compounds to intermediate chemically and biologically highly reactive species. Studies of the *in vivo* biological effects of PAHs have established that the most potent carcinogenic members contain 4 to 6 benzo rings and often possess certain structural features such as sterically crowded bay or fjord regions. A multistep enzymatic activation pathway with a sequence of non-K-region epoxidation, hydrolysis of the primary epoxide to a dihydrodiol, and further epoxidation at the adjacent double bond produces vicinal diol-epoxides as the carcinogenic metabolites of PAHs. Formation of diol-epoxide at bay- or fjord- region is considered as one of the major if not the predominant pathway of metabolic activation of most mutagenic and carcinogenic PAHs. The activation pathway leading to bay or fjord region diol-epoxides requires two additional enzymatic steps after initial monooxygenation (epoxidase) at a non-K-region double-bond for example, position 7,8 in BaP. Firstly, arene oxides initially produced undergo enzymatic hydrolysis to trans-dihydrodiols. Microsomal epoxide hydrolase (mEH) is involved in metabolism of arene oxides originating from PAHs. CYP enzymes are again responsible for subsequent epoxidation of trans-dihydrodiols to vicinal bay- diol-epoxides mainly CYP1A1 and 1B1. Additional

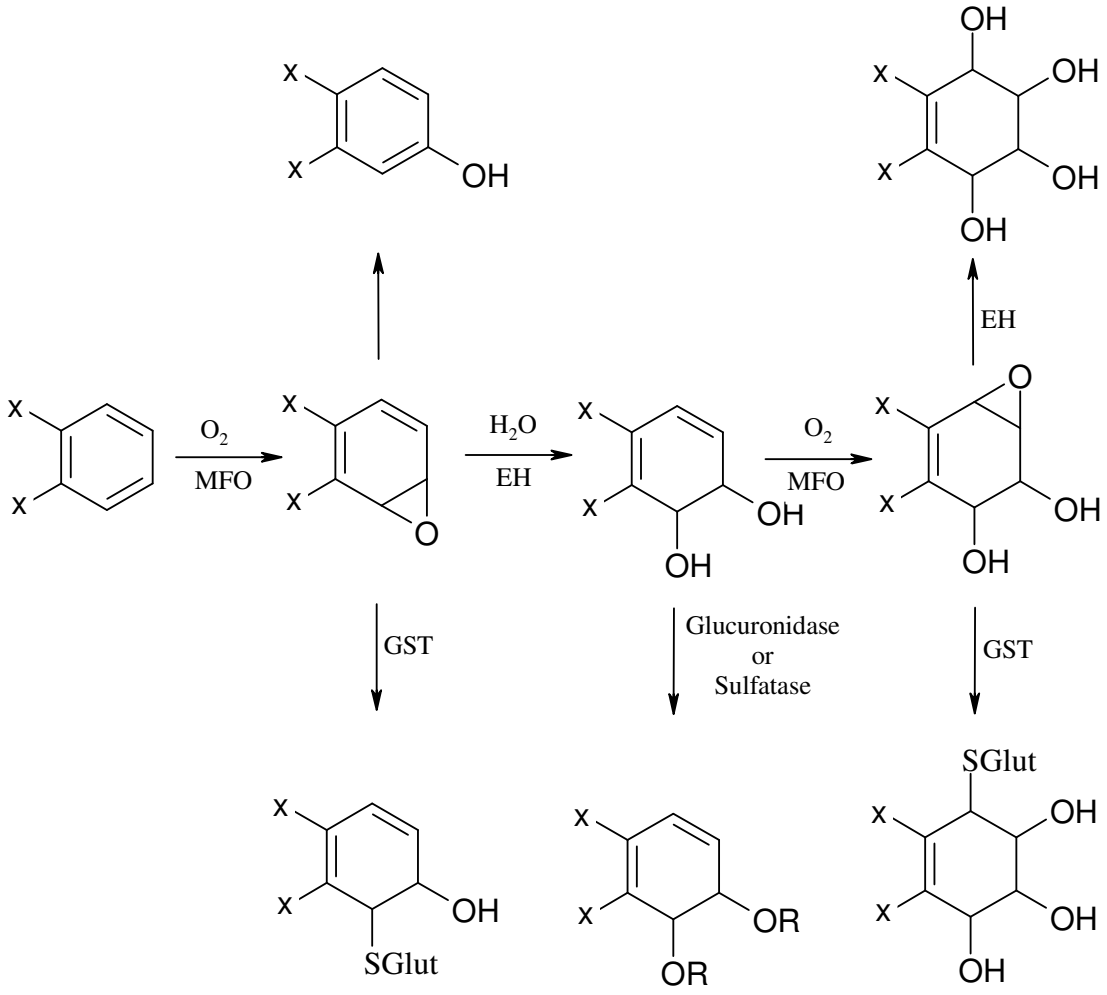


Figure 1.8. Metabolic pathways of PAH metabolism (Harvey 1991). MFO is mixed function oxidase, EH is epoxide hyrolase, GST is glutathione-S-transferase, R = glucuronate or sulfate.

metabolic activation pathways of PAHs-diols into ultimate carcinogens occur via the radical cation pathway catalyzed by peroxidases as well as the *ortho*-quinone pathway catalyzed by aldo-keto reductase (AKR) (Shimada 2006; Xue and Warshawsky 2005). Thus, BaP as a model PAH can be converted by Phase I enzymes (CYP, mEH, AKR or peroxidases) to a large number of metabolites: arene oxide, phenols, trans-dihydrodiols, quinones and diol-epoxide (Figure 1.9).

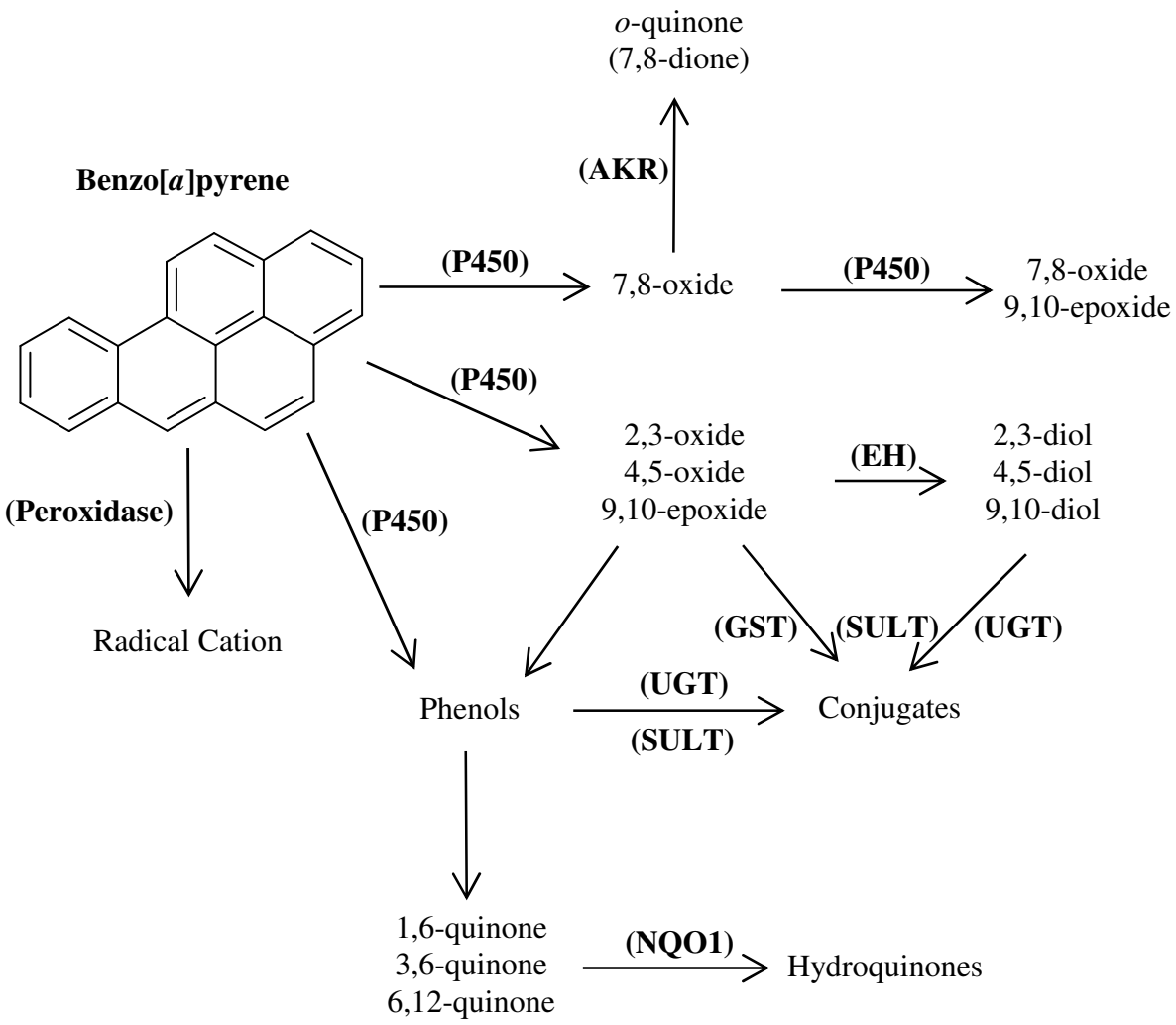


Figure 1.9. Scheme depicting the several different pathways for metabolic activation of benzo[a]pyrene. These pathways are assumed to apply generally to all PAHs, due to the structural similarities of these compounds. Scheme was adapted from Shimada (2006). P450 is cytochrome P450, EH is Epoxide hydrolase, GST is Glutathione transferase, UGT is UDP-glucuronosyltransferase, SULT is sulfotransferase, NQO1is NADPH-quinone oxidoreductase 1 and AKR is aldo-keto reductase.

Oxidative metabolism of PAHs therefore yields highly reactive diol-epoxide derivatives that may bind covalently to cellular macromolecules, including DNA, to form addition products referred to as adducts. These metabolites mostly react with the purine bases to form covalently linked bulky adducts. This genotoxic reaction is mediated through either

trans or less frequently *cis* opening at the exocyclic positions N-2 of guanine and N-6 of adenine. This might perturb the normal Watson-Crick double helix compromising transcription and replication. This process may lead to errors such as nucleotide misincorporation during the next round of DNA replication and potentially induce irreversible changes in the sequence of key genetic targets. This can also lead to suppression of transcriptional activity and disruption of gene expression (Johnson et al. 1997) which may reduce cell survival. Mutations that activate protooncogenes and de-activate tumor suppressor genes may occur and cause disruption of regulatory processes which might lead to the initiation the tumor formation process (Phillips and Grover 1994). Elevated levels of DNA adducts and P53 mutations have been associated with PAH exposures in human studies (Alexandrov et al. 2002; Gaspari et al. 2003; Hainaut and Pfeifer 2001). Thus, PAHs can become tumorigenic after metabolic activation, inducing DNA damage through DNA adduct formation and causing mutations in growth-controlling genes such as tumor suppressor or oncogenes. Cellular defense mechanisms exist especially through apoptosis and the global genome nucleotide excision repair pathway which provide an essential line of defense against the mutagenic and carcinogenic activity of PAH diol-epoxide metabolites.

Benzo[a]pyrene, the most extensively studied PAH congener was used as a model compound for the metabolism of this class of chemicals. The major pathway for the oxidative Phase I metabolism of BaP occurs through cytochrome P-450 enzymes. CYP1A1 was demonstrated to have an essential role not only for PAH-mediated toxicity, but also for detoxification of orally administered BaP (Uno et al. 2004; Uno et al. 2001). Mice models with knocked out CYP1A1 gene were found to be protected against liver toxicity and death (Uno et al. 2001). Conclusions drawn from this study indicated that the reported resistance of mice was due to a decrease in production of the normally large amounts of toxic metabolites. Nevertheless, three years later, a study by the same group detected higher levels of DNA adducts in the *Cyp1a1*(-/-) knockout mice as compared to the levels induced in *Cyp1a1*(+/+) wild-type mice. According to these results, CYP1A1 was shown to be necessary for the detoxification of orally administered

BaP. More recently, mice data showed that a balance between tissue-specific expression of CYP1A1 and CYP1B1 influences the susceptibility to toxic and possibly carcinogenic effects of orally dosed BaP (Uno et al. 2006).

Phase I oxidation reactions generally produce arene oxides. Arene oxides may then be transformed into compounds such as phenols by spontaneous reaction or trans-dihydrodiols by a hydration reaction catalyzed by microsomal epoxide hydrolase. Arene oxides may also covalently bind to glutathione by spontaneous reaction catalyzed by GST. At this stage, 6-hydroxybenzo[*a*]pyrene has been formed, and is then oxidized to 1,6-, 3,6-, or 6,12-quinones via spontaneous or metabolic reaction. Two further phenols may be oxidized; 3-hydroxybenzo[*a*]pyrene to 3,6-quinone and 9-hydroxybenzo[*a*]pyrene to the K-region 4,5-oxide. The 4,5-oxide can then be hydrated to 4,5-dihydrodiol (4,5,9-triol). Glucuronides and sulfate esters may then be conjugated from the phenols, quinones, and dihydrodiols, while glutathione conjugates can also be formed from the quinones. In addition to conjugation, dihydrodiols may undergo further metabolism through additional oxidation reactions. Such reactions may also modify the structure of the dihydrodiols via cytochrome P-450 enzymatic pathways. This reaction generally results in the formation of the 7,8-dihydrodiol-9,10-epoxide. Conjugation of diol epoxides can occur spontaneously or by a GST catalyzed reaction. Alternatively, the diol epoxides may form tetrols via spontaneous hydrolization. The 7,8-dihydrodiol-9,10-epoxide is generally considered as the ultimate carcinogenic metabolite of BaP (ATSDR 1995).

As mentioned previously, USEPA classifies seven PAHs as probable human carcinogens. BaP is listed as a class B2 or probable human carcinogen. In its pure form, this compound appears as pale yellow needles or plates in the solid form (Harvey 1997; USEPA 2006). Numerous animal studies using several different routes of administration and numerous genotoxic assays established the carcinogenic potential of BaP (Culp et al. 1998; Gaylor et al. 2000; Ramesh and Knuckles 2006; Rodriguez et al. 1997; USEPA 2006). Species of animals demonstrating positive carcinogenic responses include rats, mice, hamsters, and guinea pigs. Routes of exposure indicated to be carcinogenic in

animals include dietary, gavage, inhalation, intratracheal instillation, dermal application, intraperitoneal injection, subcutaneous injection, intravenous, transplacental, implantation in the stomach wall, lung, renal parenchyma and brain, injection into the renal pelvis, and vaginal painting. Sites of tumors formation seen after oral administration of BaP include forestomach, squamous cell papillomas and carcinomas.

Often considered a model carcinogen, BaP has multiple structural areas for metabolic activation, including a bay-region. The pathway for binding to DNA is shown in Figure 1.10.

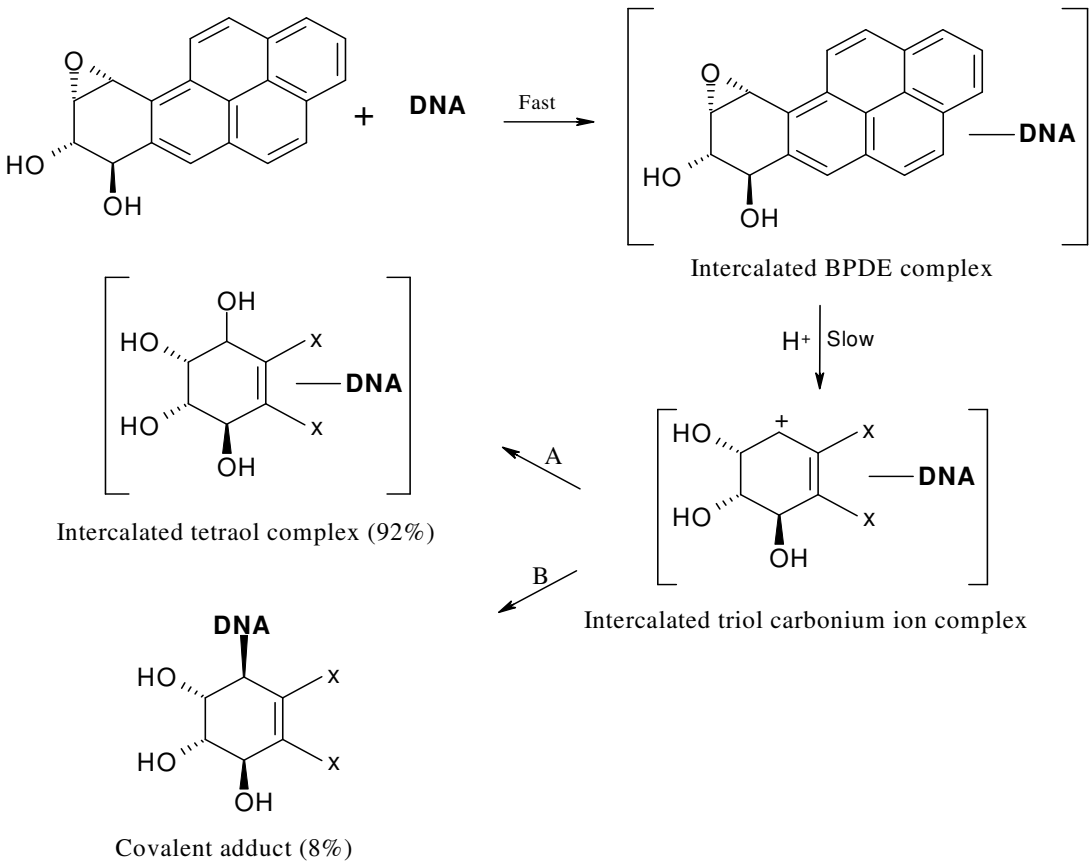
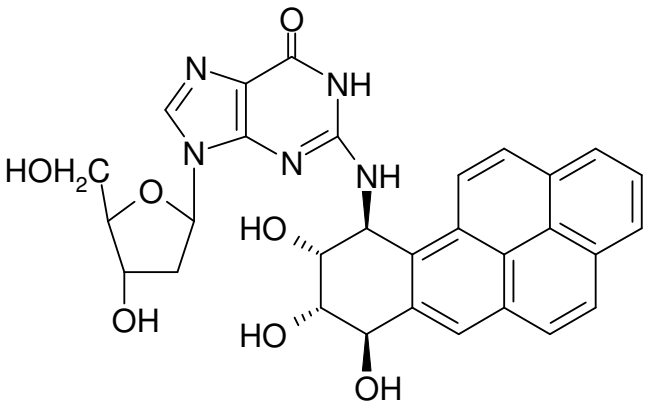


Figure 1.10. Mechanism by which the *anti* configuration of benzo[a]pyrene diol epoxide binds to DNA covalently (Harvey 1991).

The structure of BPDE, the most reactive metabolite of BaP, bound to the N-2 of the deoxyguanosine molecule is shown in Figure 1.11. BaP was administered at 0, 1, 10, 20, 30, 40, 45, 50, 100 and 250 ppm in the diet of male and female CFW-Swiss mice in a past study by Neal and Rigdon (1967). Forestomach tumors were detected in the 20 ppm or higher dose ranges. The incidence of tumors was also found to increase with dose. In a study by Brune and coworkers (1981) Sprague-Dawley rats were fed BaP at 0.15 mg/kg. Dosing of the laboratory rats occurred every nineth day or 5 times a week until death, yielding an average yearly dose of 6 or 39 mg/kg respectively. Tumors were observed in the forestomach, esophagus and larynx. The incidence of tumor followed a linear trend based on dose.



anti - BPDE-N-2-dG

Figure 1.11. Benzo[a]pyrene diol epoxide, *anti* configuration, adduct attached at the N-2 position of deoxyguanosine (Harvey 1991).

The formation of bulky DNA adducts is generally considered the first phase (initiation) in the steps that occur during transformation of a normal cell into a malignant cancer cell (Figure 1.7). These steps are generally referred to as initiation, promotion and progression (Ramesh et al. 2004). The current study integrates results from *in vitro* and *in vivo* studies with data obtained from the analysis of biological samples collected from human populations. Cell culture and animal studies were used to investigate the

genotoxicity of complex PAH mixtures collected from residential environments. These data were then compared with PAH composition and concentrations, as well as DNA adduct levels in placenta, serum and lymphocytes of human subjects. The results of this research will be used to investigate genetic factors affecting sensitivity towards genotoxic PAH mixtures.

1.7.1 Genetic Polymorphisms

The potential for xenobiotic chemicals to react with DNA and induce genotoxic damage is largely defined by the activity of Phase I and Phase II metabolizing as well as DNA repair enzymes. Variations in genotype, or single nucleotide polymorphisms (SNPs), have been shown to exert a significant influence on the sensitivity of individuals towards carcinogenic PAHs. Included in this dissertation are experiments to investigate the impact of SNPs on the ability of PAHs to form DNA adducts in animals as well as humans.

Levels of PAH-DNA adducts have been found to vary considerably among individuals with similar exposure to PAHs. This implies that inherited differences exist in formation of these adducts. Viau et al. (1995) reported that 57% of a given amount of pyrene, usually abundant in PAH mixtures, is excreted via urine and 18% via feces within 24 hr in rats. The rate of elimination of PAH mixtures, as determined by the urinary concentration of 1-hydroxypyrene (1-OHP), is affected by genetic polymorphisms of Phase I (CYP1A1) and Phase II metabolic enzymes (GST) (Luch 2005). Increased risk of lung cancer has been associated with polymorphisms in drug metabolizing enzymes namely CYP1A1 and GST according to several studies listed by Hecht and coworkers (2006). Hecht et al. (2006) investigated 11 polymorphisms in a group of 346 smokers. High ratios of phenanthrene to phenanthrene metabolites were significantly correlated with the presence of the CYP1A1I462V polymorphism. Female subjects and subjects with the GSTM1 null genotype exhibited a stronger correlation effect. It was also noted that the highest 10% of the parent PAH compound to metabolite ratios could not be predicted by any single polymorphism or by certain combinations.

Occupational studies observed that the formation of DNA adducts in lymphocytes from asphalt paving workers was found to increase during each day of the workweek. Mean adduct levels were lowest on Mondays (0.3 adducts per 10^9 nucleotides) and highest on Fridays (5 adducts per 10^9 nucleotides). Furthermore, a 3-fold increase in DNA adduct formation was observed in white blood cells of workers in the task associated with the highest PAH exposures. The lowest adduct level was found among roller operators (0.7 adducts per 10^9 nucleotides) whereas the highest was among screedmen (2.3 adducts per 10^9 nucleotides) (McClean et al. 2007). Coke oven workers were also observed to have elevated BaP-DNA adduct levels in lymphocytes. In the study of coke oven workers, the GSTM1 null genotype was found to be associated with significantly higher levels of adducts among workers (60 adducts per 10^9 nucleotides) as compared to the GSTM1 active genotype (33 per 10^9 nucleotides) at the same exposure level (Pavanello et al. 2004). 'At risk' genotypes identified by Rojas et al. (1998) correlated with increased DNA adduct levels (174 per 10^9 nucleotides).

Genetic polymorphisms of metabolic enzymes therefore have been shown to affect an individual's capacity to either activate or detoxify PAHs and their metabolites. Newly developed technologies allowing determinations of single nucleotide polymorphisms have opened possibilities for studies focusing on individual susceptibility to PAH-induced carcinogenesis. The CYP subfamily of enzymes is generally assumed to provide the majority of catalytic activity towards the initial oxidation of most xenobiotic chemicals, including the PAHs. In most cases, the Phase I enzymes increase the polarity and reactivity of xenobiotics, whereas the Phase II enzymes react with the polar end of the molecule resulting in detoxification. The main CYPs in humans that participate in PAH metabolism are 1A1, 1A2, 1B1, 2C9, 3A4 and 3A5. Exposure to PAHs has been shown to induce expression of 1A1, 1A2, and 1B1 (Iwanari et al. 2002). The induction mechanism is through ARNT binding to XRE such as located upstream of CYP1A1 gene. Three genetic polymorphisms were detected within the CYP1A1 gene. CYP1A1 MspI (CYP1A1*2A) and Ile/Val (CYP1A1*2B or *2C) more prevalent in Asians than Caucasians (Pavanello 2006). The mEH enzyme

plays a dual role in detoxification and activation of carcinogens. GSTM1 and GSTP1 are major Phase II enzymes that catalyze the conjugation reaction of BPDE. GSTT1 has both detoxification and activation properties and hence is difficult to predict the biological consequences of a null genotype (Pavanello 2006). Individuals with the GSTM1 null genotype have a slight increase in lung cancer risk and similarly modest increase in the risk of bladder cancer. Studies on the GSTM1 gene deletion are widely conducted to investigate the effects GSTM1 enzyme deficiency which has been linked to lung cancers among cigarette smokers (Brockmoller et al. 1996; Butkiewicz et al. 2000; McWilliams et al. 1995; Strange et al. 1991).

The assessment of a single polymorphic genotype is not likely to provide a reliable estimate of individual susceptibility to PAH-induced cancers. Gene-gene interaction exists, for example GSTM1 may regulate the induction of other metabolizing enzymes such as CYP1A1 and CYP1A2 (Butkiewicz et al. 2000; Vaury et al. 1995). GSTM1 deficiency not only leads to an increase in hepatic CYP1A2 activity in active smokers, but also to significant increased levels of bulky PAH-DNA adduct in lung tissues of smokers and ex-smokers as compared to individuals carrying wild-type GSTM1 (Rojas et al. 1998; Stucker et al. 2002). A deficiency in GSTM1 might also lead to saturation of the GSTP1 enzyme pathway, and lower its detoxification capability. A commonly accepted concept is that individuals with a combination of a genetically determined increased capacity to activate pro-carcinogenic cigarette smoke constituents such as PAHs, and the concurrent impaired capacity to detoxify genotoxic metabolites, would be at particular risk of developing PAH-induced cancers. However, data are not yet available from molecular epidemiology studies to confirm this assumption.

In addition to polymorphisms in xenobiotic metabolizing enzymes, biomonitoring of genotoxic risk is also investigating newly discovered polymorphisms in DNA repair genes. The ability of normal individuals was found to differ significantly in their ability to repair DNA damage by exogenous substances such as tobacco smoke or endogenous agents such as oxidation products. DNA repair is specific for a class of damage. Double-strand breaks are repaired by homologous recombination-dependant

repair or in an end-joining reaction. Most small base modifications are repaired by the Base Excision Repair pathway. Bulky adducts and helix-distorting adducts are removed primarily by a major, versatile cellular pathway the Nucleotide Excision Repair pathway. The genes that are mostly commonly studied include ERCC1 (excision repair cross-complementing 1), ERCC2 or XPD (excision repair cross-complementing 2), XRCC1 and XRCC3 (X-ray repair cross-complementing groups 1 and 3) as well as hOGG1 (human 8-OH-guanine glycosidase). ERCC2 or XPD participates in the Nucleotide Excision Repair pathway (Friedberg 2003) whereas ERCC1 is involved in the incision step of Nucleotide Excision Repair. XRCC1 plays a role in the Base Excision Repair pathway and XRCC3 contributes in DNA double-strand break recombination repair. The human OGG1 gene is part of the Base Excision Repair gene family and encodes for a DNA glycosylase/AP-lyase specifically involved in the excision of 8-OH-dG:dC but not 8-OH-dG:dA (Boiteux and Radicella 2000).

DNA repair polymorphisms may affect the levels of DNA adducts in an exposed population since they repair genetic damage. Levels of DNA adducts in populations exposed to genotoxic compounds was related to DNA repair genotype differences. A significant increase in DNA damage in populations was observed with the presence of only one of the mutated alleles of DNA repair genes (XRCC1 399 Gln and/or XPD 751 Gln). Never-smoking XRCC1 399 Gln homozygote individuals exhibited a significantly higher levels of DNA adducts in their white blood cells on average with a level of 16 per 10^9 nucleotides compared with 6 per 10^9 nucleotides in Gln/Arg heterozygotes and 7 per 10^9 nucleotides in Arg/Arg homozygotes (Matullo et al. 2001). Presence of a least one variant allele in XPD exon 23 was associated with a significant three-fold times increase in risk for lung cancer among never-smokers younger individuals (<70 years) after adjusting for age, gender and environmental tobacco smoke (Hou et al. 2002). Additionally, polymorphisms of XPD repair gene in exon 23 were found to be significant predictors for total DNA adduct levels whereas polymorphisms of XPD repair gene in exon 6 were related to formation of B[a]P-"like" DNA adducts (Binkova et al. 2007). A recent study demonstrated a negative influence of exposure to PAHs from

traffic emissions on DNA repair efficiency, and also suggested that smoking might be a factor influencing that process. Monitoring was performed in a group consisting of policemen and bus drivers occupationally exposed to PAHs and a group of matched controls. DNA damage and repair in lymphocytes.of study subjects were evaluated using a modified version of the single cell gel electrophoresis (SCGE) assay, known as Comet assay. A significant decrease in repair efficiency due to exposure to PAHs was observed in the exposed individuals. A negative influence of tobacco smoking on the efficiency of DNA repair was observed (Cebulska-Wasilewska et al. 2007).

1.8 Biomarkers of Polycyclic Aromatic Hydrocarbons

Traditionally, toxic potencies of chemicals were assessed based on overt symptoms manifested among exposed individuals. More recent studies have focused on identifying appropriate markers in biological organisms that can be used to quantify exposure or as an early indicator of effect. These markers are generally observed in an exposed population prior to overt signs or symptoms of disease. Such indicators are referred to as biomarkers (Skupinska et al. 2004). Concisely defined, biomarkers are biological particles that undergo detectable change when the individual is exposed to hazardous substances (Kleiner et al. 2003; Niyogi et al. 2001). Ultimately, biomarkers should help quantify exposures and detect disease in the early stages (Bentsen-Farmen et al. 1999). More importantly, improved methods for quantifying sources of exposure are useful for managing risk by reducing exposure. An essential component of primary prevention of diseases induced by environmental contaminants is to rank various sources of exposure and identify methods that would effectively reduce these exposures.

Biomarkers are usually divided into three broad categories including biomarkers of exposure, effect and susceptibility. An overview of the endpoints that are typically used to measure exposure, susceptibility and effects is provided by the “biomarker paradigm” represented in Figure 1.12. The paradigm originally proposed by the National Research Council (1987) indicates that a series of biological and molecular events occurs between the initial exposure to a xenobiotic compound and the onset of disease.

Furthermore, the paradigm shows that each step might be ruled by additional factors that could modify the individual susceptibility to exogenous toxicants.

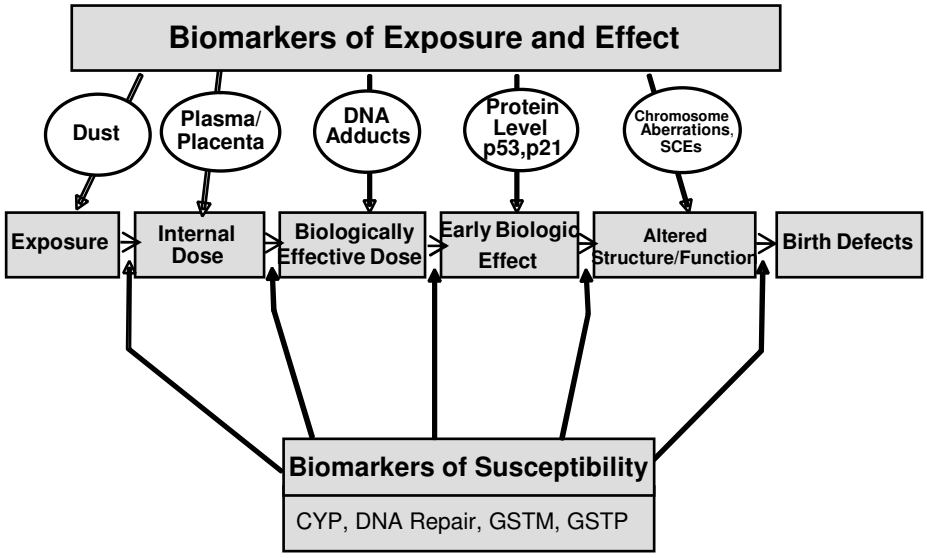


Figure 1.12. Sequence of events from exposure to overt adverse health effect. This scheme was modified from NRC, 1987.

The research conducted for this dissertation has focused on investigations to correlate external dose or exposure with internal dose and biologically effective dose. External dose was estimated by measurements of PAHs in household dust; and, a questionnaire was used to investigate other major sources of maternal exposure such as smoking status.

Biomarkers of internal dose were obtained through measurement of DNA adducts in venous blood from parents or placenta from children. In addition, genotyping of selected genes was performed to measure genetic sensitivities. When combined, these biomarkers data provided important insight into the sources of exposure and mechanisms of diseases with environmental etiologic factors.

Biomarkers of exposure usually consist of the unchanged compound or its metabolite measured in biological material such as blood, tissue, urine, feces, exfoliated

cells, sweat, nails (Brandt and Watson 2003). BaP, dibenz[a,c]anthracene and chrysene were detected in placenta, maternal blood, umbilical cord blood and breast milk in non-smoking women in India. Levels of BaP were highest in breast milk at 0.3 ppm compared to 0.04 ppm in venous blood (Madhavan and Naidu 1995). The method of choice for biomonitoring recent PAH exposures in human populations, especially when different routes of exposure are combined, is the determination of PAH metabolites in urine. Concentrations of the PAH unchanged parent compound in urine are generally low, and instead one or more metabolites of the predominant hydrocarbon are usually present in high enough concentrations to be determined (Grimmer 1994; Jongeneelen et al. 1988). In mammals, 1-OHP represents the main metabolite of pyrene, a four ringed PAH abundant in complex mixtures of PAHs (Keimig et al. 1983). PAH profiles may vary significantly from an exposure source to another, however pyrene is a dominant compound in mostly all PAH mixtures. The half-life for 1-OHP in urine is relatively long, lasting up to 48 hours. Several studies have demonstrated that 1-hydroxypyrene is a good indicator of PAH exposure (Alexandrie et al. 2000; Pan et al. 1998; Siwinska et al. 2004). A review by Dor et al. (1999) concluded that 1-OHP was the most relevant biomarker for assessing exposure to PAHs. However, Sram and Binkova (2000) concluded based on their review of results from molecular epidemiology studies published between 1997 and 1999 that 1-OHP is a more effective biomarker in measuring occupational rather than environmental PAH exposures due to its reduced sensitivity to ambient PAH exposure levels in air. In addition, 1-OHP might not accurately reflect the internal body burden of carcinogenic PAHs mainly because these are mostly high molecular weight congeners predominantly excreted in feces (Grimmer 1994).

Humans exposed to PAHs in the environment or in their occupation have exhibited elevated excretion levels of 1-OHP in their urine. Urinary excretion of 1-OHP was correlated to lung cancer risk in occupational exposures to PAHs. A level of 2.3 μmol/mol creatinine was associated with a relative lung cancer risk of 1.3 in coke oven

workers (Jongeneelen 1992). Selected data on urinary 1-hydroxypyrene levels are summarized in Table 1.8 and reported values are given as μmol/mol creatinine.

Table 1.8. Summary of urinary 1-hydroxypyrene (1-OHP) concentrations in μmol/mol creatinine in selected human populations.

Type of Exposure	Number of Individuals	1-OHP	Reference
General Population (China)	70	0.5-1.6	(Zhao et al. 1990)
General Population Non-smoker	19	0.08	(Granella and Clonfero 1993)
General Population Smoker	22	0.2	(Granella and Clonfero 1993)
Children (living near a steel mill)	350	0.05	(Lee et al. 2007)
Cooking (Females)	108	0.5	(Chen et al. 2007)
Highway toll station (pre-shift)	32	1	(Tsai et al. 2004)
Highway toll station (post-shift)		3	
Traffic Police	89	0.14	(Merlo et al. 1998)
Coal-electrodes production (pre-shift)	17	4	(Bentsen-Farmen et al. 1999)
Coal-electrodes production (post-shift)		10	
Coal tar distillation	4	4-12	(Jongeneelen et al. 1986)
Aluminum Production	5	1.2-8.8	(Vu Duc 1996)
Coking plant	447	4.2-5.2	(Chen et al. 2007)

Urinary 1-hydroxypyrene was measured in 70 residents from three cities in North China; Beijing, Shenyang and Taiyuan (Zhao et al. 1990). These cities are believed to have severe air pollution due to the heavy dependence of China on coal as a source of energy. The level of 1-OHP in urine was different for residents of the three cities and ranged between 0.5 and 1.6 μmol/mol creatinine. Residents of Shenyang exhibited the highest 1-OHP levels and those of Beijing the lowest levels. PAHs were also measured in city air, and the results show that pyrene was a major component and ranged between 11 to 18% of total PAHs in air. No difference in the levels of 1-OHP was detected between smokers and non-smokers. Smoking of an average of 10 cigarettes per day was found to have a greater influence on urinary 1-OHP levels in automotive repair workers than occupational exposures (Granella and Clonfero 1993). Urine of smokers had 0.2 μmol 1-OHP/mol creatinine which was more than two-fold of the levels found in non-smokers. Children, age 7-15 years, living near a large steel mill in Korea excreted 1.3 times higher levels of 1-OHP in their urine when compared to another group residing much farther from the factory. However, potential confounders such as the ambient air PAH concentrations and the dietary and indoor sources of PAHs were not directly measured (Lee et al. 2007). Mean urinary 1-OHP concentrations in 108 Chinese females exposed to cooking oil fumes were found to be 0.5 μmol/mol creatinine (Chen et al. 2007). Levels of 1-OHP in the urine of the study subjects were associated with cooking frequency in the kitchen. Subjects were mostly non-smokers and did not have heating systems except electric air conditioners. The concentration of PAHs in kitchen air as well as dietary sources of PAHs were not investigated in this study. Pre-shift and post-shift urinary 1-OHP levels were quantified in 32 female highway toll booth attendants (Tsai et al. 2004). Mean post-shift 1-OHP levels in urine of study subjects was three-fold (3 μmol/mol creatinine) that in pre-shift samples. Total PAH exposure level in air from the breathing zone of booth attendants was at an average of 11,400 ng/m^3; pyrene in particular was at an average of 105 ng/m^3. Among the factors that might affect urinary levels of 1-OHP such as smoking habit, only the total PAH exposure level was significant. A study on traffic police officers in Italy did not find significant difference in

the level of their urinary 1-OHP as compared to a control group working indoors (Merlo et al. 1998). Mean concentrations of 1-OHP excreted in the urine of traffic police officers was 0.14 μmol/mol creatinine as compared to a level of 0.09 μmol/mol creatinine in control subjects. BaP air level was used as a surrogate of exposure to pyrene. The geometric mean of BaP air concentrations detected in traffic police officers was 3.67 ng/m^3, which was 70 times higher than that detected in control subjects (0.05 ng/m^3). Mean 1-OHP concentrations were significantly higher in the urine of smokers compared to non-smokers in both groups. In addition, the number of cigarettes smoked per day was found to well predict the level of 1-OHP excreted in urine. Urinary 1-OHP excretions were two-fold higher or more in subjects who smoked 15 cigarettes or less (0.176 μmol/mol creatinine) and subjects who smoked more than 15 cigarettes (0.226 μmol/mol creatinine) compared to non-smokers (0.089 μmol/mol creatinine). Exposure to environmental tobacco smoke (ETS) was also associated with high 1-OHP levels in urine, unlike consumption of broiled/grilled meat and fresh fruit and vegetables. Despite the fact that seasonal variation of airborne PAH concentrations was not detected, levels of urinary 1-OHP in traffic police officers varied among season and were highest between the months of January and March (0.238 μmol/mol creatinine).

In a study conducted at a coal-electrode production plant, pre and post-shift levels of urinary 1-OHP were measured in a group of workers occupationally exposed to high levels of PAHs (Bentsen-Farmen et al. 1999). The mean urinary 1-OHP was 4 μmol/mol creatinine in pre-shift samples and 10 in post-shift samples. Workers were equipped with personal samplers to measure the concentration of PAHs in the workplace air. Mean air PAH level in the particulate phase were 38,000 ng/m^3 but pyrene was more abundant in the gaseous phase where it formed 72% of total PAHs. No significant correlation was found between 1-OHP and workplace pyrene exposure in this study. This finding suggested that skin exposure to PAHs might be a major exposure route in this plant which confirmed that estimation of total PAH exposure is best accomplished by biological markers such as quantitation of PAHs in body fluids. Urine samples from workers at a coal tar distillation plant were analyzed for 1-hydroxypyrene (Jongeneelen

et al. 1986). The time weight average (TWA) exposure over 8 hours for these workers ranged between less than 2000 to 280,000 ng/m^3 for total PAHs and less than 2000 to 96,000 ng/m^3 for pyrene. Levels of 1-OHP in the urine of workers ranged between 4 and 12 μmol/mol creatinine depending on the job function. The concentration of 1-OHP in the urine of workers exceeded the upper 95th percentile of a control group. In this study, smokers did not have a significant increase in their 1-OHP urinary levels. In workers at an aluminum production plant, levels of urinary 1-OHP appeared to range between 1.2 and 8.8 μmol/mol creatinine depending on the performed task (Vu Duc 1996). Personal air sampling revealed that pyrene was predominant among the detected PAHs and ranged between 789 and 9477 ng/m^3. Levels of 1-OHP in urine of workers and the concentrations of pyrene in their breathing zone correlated fairly well. However, correlation between 1-OHP and BaP was poor in this study which indicates that 1-OHP might not be a sensitive biomarker to carcinogenic PAH exposures. Chinese coke oven workers at two different coking plants had 1-OHP levels in their urine ranging from 4.2 to 5.2 μmol/mol creatinine (Chen et al. 2007). The highest 1-OHP levels were detected among topside workers with concentrations ranging from 5.5 to 15.5 μmol/mol creatinine. Workers who used respirators had lower 1-OHP levels in their urine that ranged from 2 to 4 μmol/mol creatinine. Only in one plant, workers who smoked more than 10 cigarettes per day had significantly higher urinary levels of 1-OHP than non-smokers. Levels of 1-OHP were 6 μmol/mol creatinine in urine of heavy smokers as compared to 4 μmol/mol creatinine in urine from non-smokers. Occupational exposure in this study was mostly predominant which might minimize the effect of cigarette smoking on urinary 1-OHP levels.

Large inter-individual variations in urinary 1-OHP excretion exist. Studies have shown that the level of 1-OHP and other PAH biomarkers in urine can be affected by selected genetic polymorphisms of drug metabolizing enzymes. Genetic variants of CYP and GST enzymes that contribute to PAH metabolism were found to alter the rate of 1-OHP excretion in urine. Higher concentration of 1-OHP was found in the urine of traffic police officers carrying the heterozygous variant of the CYP1A1 MspI genotype and

consuming less than 15 cigarettes per day, as compared to individuals homozygous for the wild-type allele. On the other hand, non-smoking policemen or individuals smoking more than 15 cigarettes per day revealed no significant genetic influence over levels of 1-OHP in their urine (Merlo et al. 1998). A two-fold higher levels of 1-OHP were detected in smokers with heterozygous CYP1A1 MspI genotypes compared to smokers with the wild-type genotype, in a non-occupationally exposed group of Japanese, Hawaiian and Caucasian subjects (Nerurkar et al. 2000). Among coke oven workers in Italy, GSTM1 null genotype was associated with increased levels of 1-OHP excretion (Brescia et al. 1999). Similar results were reported by Alexandrie et al. (2000) in a study on aluminum smelter workers. Other studies however have found reduced levels of 1-OHP excretion associated with the inactive GSTM1 genotype (Schoket et al. 2001) or no significant effect (Merlo et al. 1998) in occupationally exposed individuals. Therefore, it appears that the influence of polymorphisms especially in Phase II metabolic enzymes such as GST is not clear yet.

A biomarker of effect is a biological measurement that indicates that the organism is responding to an exposure at some level. DNA adducts are one example of a biomarker of effect that can be used to measure early indicators of genotoxic effects that precede the onset of health effects such as adverse pregnancy outcomes or cancer. Since bulky DNA adducts reflect persistent genetic damage at a target site, they may not exhibit the same degree of variability as biomarkers that only reflect recent exposures. DNA adducts may also account for multiple routes of exposure and differences in toxicokinetics and repair amongst exposed subjects (Godschalk et al. 2003). Phillips (Phillips 2005) recognizes that monitoring the formation of DNA adducts in lymphocytes as a surrogate tissue provide a valuable tool for investigating environmental exposure in healthy individuals. Other biomarkers of effects include protein adducts, levels of functionally critical protein such as p53 and p21, chromosome aberrations and sister chromatid exchange (Angerer et al. 1997; Shaham 1996).

The formation of DNA adducts is generally considered to be the earliest critical event that can be detected in the complex multi-stage process of chemically induced

carcinogenesis caused by compounds such as PAHs (Boyd and Barrett 1990; Eriksson et al. 2004; Kondraganti et al. 2003). Monitoring human exposure to carcinogens by means of DNA adduct formation provides an integrated measurement of carcinogen intake, metabolic activation and delivery to the target macromolecule. Cell culture, animal, and human studies have demonstrated that PAH-DNA adducts play an important role in the transformation of normal cells into malignant cells (Godschalk et al. 2003). In a cell culture study by Dennisenko and Pao (1996), it was found that BPDE-DNA adduct formation in the *p53* tumor suppressor gene *in vitro* corresponded with mutational hotspots on the same gene in human lung cancer tissue. Data from animal studies also suggests that levels of DNA adducts in target organs are related to overall cancer risk. Poirier and Beland (1994) reported an overall linear relationship between levels of DNA adducts and the dose of a carcinogen administered to rodents. DNA adducts were also generally correlated with tumorigenesis. In human studies, a direct link was reported between bulky DNA adducts in white blood cells (WBCs) and lung cancer risk in a prospective study within the Physician's Health Study (Tang et al. 2001). In this study, 'healthy' smokers at the time of sampling and who had elevated levels of DNA adducts in their WBCs were three times more likely to develop lung cancer than smokers with low adduct levels. In a review article, Godschalk et al. (2003) lists several human studies that report correlations between DNA adducts and cancer risk in exposed populations. Among those are a study by Ryberg et al. (1994) that found higher levels of DNA adducts in female smokers (13.55 per 10^8 nucleotides) when compared to male smokers (9.75 per 10^8 nucleotides). These results correspond well with epidemiological reports on a greater risk of tobacco-induced lung cancer among women. Studies with lung cancer patients found that PAH-DNA adduct levels in their lung tissue and WBCs were higher when compared to healthy controls (Perera et al. 1989; Tang et al. 1995). Overall, *in vitro*, *in vivo* and human studies indicate that as biomarkers of exposure, DNA adduct levels provide important information for predicting human cancer risk.

DNA adduct levels are usually reported as number of adducts per normal nucleotides. Levels of 1 adduct/10^5 nucleotides correspond to around 10,000 DNA base

modifications per cell, since each cell contains 10^9 DNA bases. At such levels, there is reasonable statistical probability that a mutation might be formed (Brandt and Watson 2003). At low levels such as the detection limit of ^{32}P-poslabeling (1 adduct/10^9 normal nucleotides) the chance of a mutation occurring is low and thus it is less probable that such level of adduct formation can be significant in terms of cancer risk. DNA adduct levels ranging from 1 adduct/10^9 normal nucleotides to 1 adduct/10^{10} normal nucleotides are common for ambient exposure to PAHs (Phillips 1997). Thus, although qualitative detection of low levels of adducts is usually viewed as a positive indicator of exposure, the relevance of low adduct levels to human cancer is less clear. However, predisposing polymorphic genes exist such as those involved in PAH metabolism and DNA repair. Certain genotypes may increase the degree of DNA adduct formation and thus cancer risk, even when low level exposure occurred (Brandt and Watson 2003). In addition, even low levels of DNA adducts in a sensitive receptor (such as the developing fetus) is likely to represent appreciable risk. Previous studies detected B[a]PDE-DNA adducts in human placenta *in vivo* (Manchester et al. 1988; Manchester et al. 1990; Whyatt et al. 1998) and cord blood (Arnould et al. 1997; Tang et al. 2006). The levels of PAH-DNA adducts detected in placental tissue were 85 adducts per 10^9 nucleotides (Whyatt et al. 1998) and 3 adducts per 10^9 nucleotides in umbilical cord blood (Tang et al. 2006).

Among biomarkers of structural or functional alterations in humans are cytogenetic endpoints such as chromosome aberrations (CAs), micronuclei (MN) and sister chromatid exchanges (SCEs). These cytogenetic modifications are mainly due to errors of DNA replication, which can be caused by mutagens (Wilson and Thompson 2007). The frequency of chromosomal aberrations in peripheral blood lymphocytes was found to be a predictor of cancer risk in several human cohorts (Norppa et al. 2006). Previous studies have also confirmed the relationship between PAH exposure and p53 expression or chromosome damage (Nakatsuru et al. 2004; Siwinska et al. 2004; Wilding et al. 2005). With regards to the biomarkers of effect, data from previous studies have established the utility of measurements of protein levels and chromosome damage as an indicator of genotoxic effects (Lodovici et al. 2004; Pavanello and

Clonfero 2004; Salazar et al. 2004; Whyatt et al. 2000; Wilding et al. 2005). A study population in an environmentally polluted part of Poland exhibited increased levels of various biomarkers such as DNA adducts, CA, SCE and ras oncogene expression (Perera et al. 1992).

Chromosomal aberrations were shown to be an intermediate step in tumor formation pathway and to be indicators of exposure as well as susceptibility (Bonassi et al. 2000; Hagmar et al. 1994). Coke oven workers exhibited an increased level of chromosomal aberrations and SCE as compared to control subjects. The exposure among coke oven workers ranged from 0.6 to 550 $\mu g/m^3$ and 0.002 to 50 $\mu g/m^3$, for carcinogenic PAHs and BaP, respectively. The respective values in controls were 0.1 to 1.5 $\mu g/m^3$ and from 0.002 to 0.01 $\mu g/m^3$. The frequency of CA and SCE was found to be related to exposure to carcinogenic PAHs (Kalina et al. 1998). Occupational exposure to PAHs in airport personnel resulted in higher mean value of SCE frequency and CA as compared to controls. The exposed group showed a higher mean value of SCE frequency compared to controls (4.6 versus 3.8) and an increase (1.3-fold) of total structural CA in exposed as compared to control subjects (Cavallo et al. 2006). However no difference in the level of urinary 1-OHP or MN was detected between the exposed and control groups in the same study. In Turkey, exposure to urban air pollution significantly increased the levels of CAs in traffic policeman and taxi drivers (Burgaz et al. 2002).

The tumor suppressor gene p53 has been reported to play a critical role in cell responses to genotoxic chemicals such as cell cycle arrest, DNA repair and apoptosis (Park et al. 2006). The p53 gene, very frequently altered in human cancer cells, is found to be mutated in around 50% of all human tumors (Cariello et al. 1994). Cells exposed to genotoxic agents exhibit increased levels of the p53 protein which in turn lead to an up-regulation of the Cyclin-dependant kinase (Cdk) inhibitor, $p21^{WAF1/CIP1}$ protein (Park et al. 2006). The expression of both p53 and $p21^{WAF1/CIP1}$ proteins were found to be induced by PAHs *in vitro* (Binkova et al. 2000; Mahadevan et al. 2001). Thus, presence of both proteins in blood serum has the potential to be used as a molecular marker of exposure to specific carcinogens in environmental monitoring and risk assessment studies. Previous

studies have used p53 in blood serum as a marker of cancer (Brandt-Rauf and Pincus 1998; Charuruks et al. 2001) and PAHs (Krajewska et al. 1998; Pan et al. 1998) among other genotoxic agents. A more recent study by Rossner et al. (2003) however have found no correlation between p53 and $p21^{WAF1/CIP1}$ plasma levels, as well as a negative correlation between p53 levels and PAHs exposure. In addition, smoking was found to have no effect on the levels of either protein. The contradictory results presented in the study by Rossner et al. (2003) suggest that the use of p53 and $p21^{WAF1/CIP1}$ plasma levels as biomakers of carcinogenic PAH effect might require further examination.

Finally, biomarkers of susceptibility are related to the genotype of an individual. The genetic make-up does not usually establish a disease condition but most likely identifies a certain sensitivity that makes a person at higher risk for disease. SNPs in genes coding for drug metabolizing or DNA repair enzymes could result in a faster or slower metabolism and DNA repair efficiency in human tissues. Therefore, SNPs can modify the levels of biomarkers detected in tissues by altering retention and/or elimination of hazardous chemicals from the body.

1.9 Environmental Health in China

As a consequence of the major economic developments in the last decade, air pollution has become one of the most serious environmental concerns in The People's Republic of China. Burning of solid fuels such as coal for power generation and industrial production as well as residential heating and cooking is considered to be a major source of atmospheric pollution in Chinese cities (Zhang et al. 2007). Indoor air pollution affects a large share of the population in China, especially in rural areas, small cities, and in less developed peri-urban areas of large cities (Zhao et al. 2006). Coal, wood and other biomass fuels remain the primary heating and cooking fuels for the great majority of the Chinese population (Alford et al. 2002).

The northern province of Shanxi is referred to as the "Coal Warehouse of China". This region has extensive coal fields, some regulated by the government and some owned and operated by families. Shanxi provides at least one quarter of China's

coal. The coal mined from this region is commonly used for power generation. Coal is used in food preparation and for heating purposes in homes or stores. The thermal decomposition of the solid coal fuel produces PAHs, which are gaseous at high temperatures, but condense onto the surface of soot particles during cooling. Consequently, residents can be exposed to PAHs and other byproducts either by inhalation of the airborne soot particles or ingestion of soot particles that deposit on food. In Shanxi, many women spend up to four hours per day cooking in an unventilated kitchen.

Given the importance of coal combustion as an energy supply, the main pollutants affecting air quality in China include particulate matter (PM) and sulfur dioxide (SO_2). Emission of 16 PAH congeners (Figure 1.13) on the USEPA priority pollutants list from major sources in China have increased substantially from around 18,000 tons in 1980 to more than 25,000 tons in 2003 (Xu et al. 2006). The USEPA priority pollutant PAHs include acenaphthene, acenaphthylene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene (OFR 1982). Major sources of PAH emissions in China according to Zhang et al. (2007) are biomass (59%), domestic coal combustion (23%) and coke production (15%). However when considering major cities only (with population larger than 1 million) the source profile is different with coke production (49%), domestic coal combustion (34%), vehicular fuel (8%), aluminum production (6%) being the major sources. The concentration and composition of PAHs from each of these sources is likely to be appreciably different. The province of Shanxi where many large coal mines are located, has several major sources of PAH emissions (Zhang et al. 2007). Domestic use of coal can present serious health problems because the coals usually are mined locally and burned in poorly vented or unvented stoves.

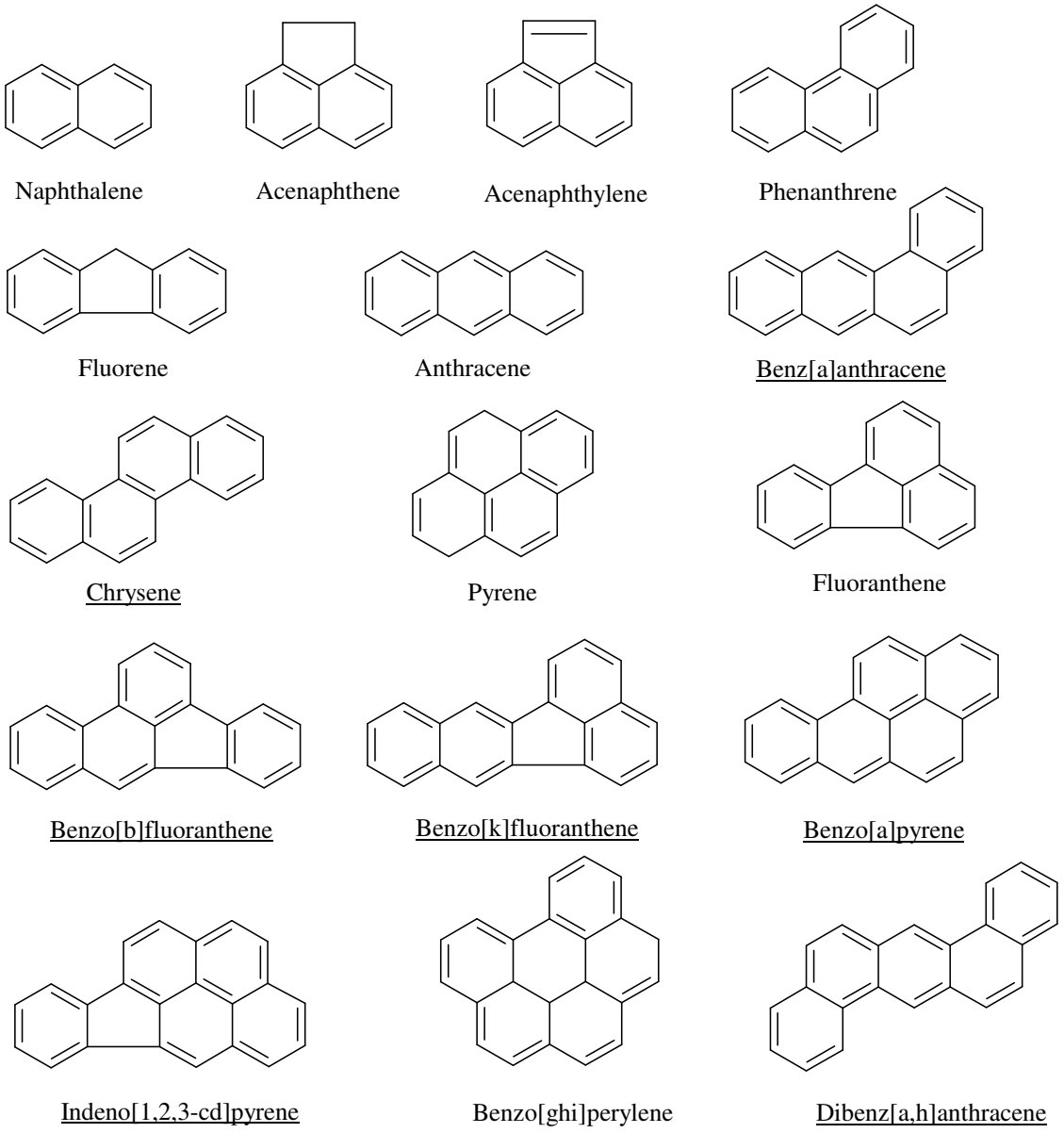


Figure 1.13. Structures and nomenclatures of the 16 PAH congeners on the USEPA priority pollutant list. The seven USEPA class B2 carcinogens are underlined. Figure adapted from Yan et al. (2004).

In addition, many of the smaller mines produce a lower grade coal which burns at a lower temperature and may produce higher PAH emissions than a higher grade coal. China remains the world's largest coal producer and consumer. A substantial portion is

used for domestic energy needs. More than 75% of China's primary energy needs are supplied by domestic coal (Finkelman et al. 1999). Domestic coal combustion for heating and cooking as mentioned before is still very frequent in Chinese homes, especially those in poor rural areas due to lack of electric power. Indoor use of coal for heating and cooking is a major source of PAH exposure for rural women in China. Qian and coworkers (2001) found that across several districts in four Chinese cities, approximately 51% to 71% of households use coal for heating and cooking. Solid fuels are typically burned in open stoves and numerous studies indicated that the resulting levels of indoor air pollution are very serious compared with outdoors. Since most people, especially women, spend a large percentage of time indoors, indoor air pollution has a disproportionate impact on human health (Zhao et al. 2006).

Indoor air pollution has been shown to pose a major health risk leading to serious respiratory diseases such in children as well as adults. In a study of several Chinese cities, Qin et al (1991) found that the concentrations of PM_{10} in kitchens that rely on coal ranged from 291 to 665 $\mu g/m^3$. Exposure to PAHs, from the inhalation of particulates or ingestion of soot particles has been implicated as a potential explanation for the high rates of esophageal and lung cancer found in some regions of China. The rates of esophageal cancer in parts of Henan province in China are among the highest in the world. Ingestion of PAH-coated soot produced during unvented coal combustion was reported to be a possible cause or contributing factor for the increase in esophageal cancer risk in Henan (Wornat et al. 2001). However, other studies have suggested a link between the consumption of fermented cabbage and esophageal cancer in Chinese populations (Cheng et al. 1992; Li and Yu 2003). PAHs produced during residential smoky coal combustion have also been cited as the primary cause of high levels of lung cancer in China (Mumford et al. 1987). Chinese women using smoky coal domestically for heating and cooking were found to be highly exposed to chemicals implicated as causative factors for lung cancer (Lan et al. 2000; Mumford et al. 1987). In several European countries, the risk of developing lung cancer in whole-life users of solid cooking fuel was twice more than that of non users (Lissowska et al. 2005). The

combustion of smoky coal produces emissions that are composed mainly of organic matter, of which 40% are PAHs. It is estimated that the daily dose of BaP inhaled by a non-smoker exposed to household smoky coal is 30 times more than that of a smoker that is not exposed to PAHs in coal (Mumford et al. 1995). Urinary excretion of depurinated BaP-DNA adducts was shown to be 600 fold higher in non-smoking Chinese women exposed to smoky coal as compared with a control group of smokers (Casale et al. 2001).

A meta-analysis of Chinese epidemiological studies that evaluated the risk of lung cancer from indoor air pollution, was conducted by Zhao and coworkers (2006). Domestic coal use and indoor exposure to coal dust were among the criteria used as indicators of indoor air pollution. The pooled odds ratios were 2.66 (95% CI: 1.39-5.07) for domestic coal use whereas the pooled odds ratios for exposure to coal dust was 2.42 (95%CI: 1.62-3.63). These results indicate that there is an association between indoor air pollution due to coal use and lung cancer risk.

Other health impacts found to be more frequent in solid fuel users include respiratory illnesses in children and chronic obstructive pulmonary disease in adults (Zhang and Smith 2007). Also associated with indoor air pollution are conditions such as asthma, adverse pregnancy outcomes, loss of eye sight and cardiovascular diseases, adding to population morbidity and mortality (Smith and Maeusezahl-Feuz 2005). Estimates from the World Health Organization (WHO) indicate that indoor air pollution is responsible for more than 1.6 million premature deaths each year in the developing world (WHO 2002). In China alone, those estimates indicate that about 420,000 die each year from the effects of indoor air pollution (Zhang and Smith 2007).

1.9.1 Birth Defects in China

The birth prevalence of NTDs in China is approximately 12/10,000 which is among the highest in the world. The prevalence of NTDs in China is higher in rural (25/10,000) than in urban (8/10,000) areas, higher in the north (20/10,000) than in the south (6/10,000), and higher in females (16/10,000) than in males (10/10,000) (Dai et al.

2002). In addition, relative to the United States, the distribution of NTD types in China tends to be shifted toward the most severe forms (e.g. spina bifida with anencephaly, craniorachischisis) (Moore et al. 1997). In rural areas of Shanxi, the prevalence of NTDs was reported to be as high as 140/10,000 in 2002 (Zhang et al. 2006). Birth prevalence of NTDs in rural areas of the Northern provinces of China is considered to be among the highest in the world. This rate is ten times greater than that in the Western world (Moore et al. 1997). NTDs account for one third of stillbirths and one fourth to one third of neonatal deaths in China (Li et al. 2003).

The mean incidence of NTDs in North China is five fold higher than that of South China (Pei et al. 2003). This large difference is mainly considered in the context of different climate and diet between these two regions. South China regarded as generally wealthier area, has an average yearly temperature 22% greater than North China (60^{o}F vs. 49^{o}F) and 43% more rainfall. Plant and animal foodstuffs in South China are scarcer than in North China. Diets in South China contain a large variety of foods including meat, fish, shellfish, green vegetables, and rice. Diets in South China generally also include much less corn, Chinese sorghum, and potato in contrast to North China. Dietary sources of folate and vitamin A are therefore more abundant and varied in South China. In addition, the consumption of increased quantities of corn in Northern China increases the potential for exposure to mycotoxins, another risk factor for NTDs. As mentioned previously etiologic factors for NTDs are varied and only some of which may be modified by folate supplementation (Melnick and Marazita 1998). Consumption of folic acid before and during the first 28 days of pregnancy lowered rates of NTDs in a region in the North of China with elevated NTDs incidence and one with low incidence in Southern China. Differences in background rates were hypothesized to be at least in part due to dietary intake of folate (Berry et al. 1999).

1.10 Objectives and Specific Aims

Exposure to toxic chemicals at any stage of human life may result in a variety of adverse health outcomes. Maternal exposures in particular may lead to preterm or low

birth weight children as well as offspring born with birth defects. In virtually all cases, human diseases are the result of complex interactions between genetic and environmental factors, broadly defined to include nutritional factors and lifestyle, in addition to exposure to chemicals or other toxins. A diagnostic matrix that could be used to track contaminants from the environment to receptors, and to estimate the dose of exposure necessary to cause a disease, would represent a valuable tool for reducing the burden of environmentally induced disease. Improved knowledge of the major sources of exposure would enhance opportunities to reduce exposure; while, improvements in the development of biomarkers of effect and sensitivity would help identify high-risk individuals. The primary goal of this research was to evaluate tools for monitoring genotoxic PAHs in the environment, as well as in biological receptors. The study focused on environmental and biological samples from Shanxi, China because this area is known to have elevated levels of PAH contamination. Subjects recruited to participate in the research included children born with NTDs and their parents because it was assumed that this group represents receptors that would be sensitive to genotoxic effects associated with PAH exposure. One advantage of monitoring biomarkers in children born with birth defects is that the exposure precedes the effect by less than one year. The various experiments conducted for this research were designed to employ cell culture, animal and human studies to test the hypothesis that the genotoxicity of complex PAH mixtures was correlated with the concentration of BaP.

The overall focus of this study was to evaluate a series of biomarkers for use in monitoring populations exposed to combustion by-products. The data used in this dissertation describe a study conducted in a human population with high PAH exposures in rural regions of China. This study was largely a preliminary study for a subsequent case-control study in a much larger population. However, it is emphasized that the current study was intended as an exposure assessment. Biomarkers of exposure were compared in cases and controls without attempting to develop epidemiological analyses, primarily because of sample size limitations. As noted by Smith (2008), high exposures usually occur where studies are difficult to conduct. While such studies may not provide

data for detailed epidemiologic evaluations, they are still quite valuable because they can provide unique evidence of risk associated with increased levels of chemical exposures.

The specific aims of this research include:

1. Compare the *in vitro* and *in vivo* genotoxicity of polycyclic aromatic hydrocarbon mixtures extracted from residential dust.

2. Measure biomarkers of exposure, effect and susceptibility to polycyclic aromatic hydrocarbons in children born with a neural tube defect and matched controls.

3. Measure biomarkers of exposure, effect and susceptibility to polycyclic aromatic hydrocarbons in venous blood of parents of children born with a neural tube defect and matched controls.

In order to accomplish the goals of this research, a series of experiments have been completed. First, residential dust was collected from houses in the Shanxi province in China. The dust was extracted and tested in a battery of *in vitro* and *in vivo* assays. These data were used to investigate the dose-response relationship for B[a]P in PAH mixtures under controlled laboratory conditions. Subsequently, study subjects were recruited from county hospitals in Shanxi, China and informed consent was obtained. Biological tissues were collected from recruited NTD cases and matched controls and when available from their parents. Tissues were extracted and analyzed at Texas A&M University. These analyses were performed to measure biomarkers of exposure, effect and susceptibility to PAHs and investigate any difference in level of biomarkers that may be produced by genetic polymorphisms.

CHAPTER II
GENOTOXICITY OF COMPLEX CHEMICAL MIXTURES IN RESIDENTIAL FLOOR DUST*

2.1 Introduction

Until recent decades, air pollution was largely considered an outdoor phenomenon. Most research studies have focused on sources of pollutants and exposures that occur in the outdoor environment. Fewer studies describe contaminant concentrations in the home environment. According to various reports from the United States and Europe, most people spend more than 90% of their time indoors (Butte and Heinzow 2002; Graham and McCurdy 2004). This estimate could be higher for sensitive groups such as infants, elderly and chronically ill. Thus, the duration of human exposure to air pollutants is likely appreciably longer indoors (Naufal et al. 2007).

According to the WHO (2002), the largest source of indoor air pollution is cooking and heating with solid fuels such as wood and coal. Poorly vented cooking and heating is common in nearly half of the world, and is especially prevalent in rural China. Components of solid fuel smoke consist of thousands of chemicals many of which can have harmful effects on human health (Peabody et al. 2005). Byproducts of solid fuel combustion which include toxic gases such as carbon monoxide, formaldehyde and sulfur dioxide are also the chief sources of respirable particles that can carry other pollutants frequently detected indoors (Cooke 1991). Indoor concentrations of many of these hazardous substances might reach levels that are multiple times higher than those outdoors (USEPA 1987). Indoor contaminants are subject to different environmental factors as compared to outdoor contaminants and thus may persist longer (Paustenbach et al. 1997). Particles of soot formed after the combustion of organic fuels may be of

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respirable size and were found to contain carcinogenic polycyclic aromatic hydrocarbons (PAHs) (USEPA 1987).

PAHs are usually found as complex mixtures of hundreds to thousands of compounds with different number of aromatic rings, arrangements, and substituents. Seven PAHs have been classified by the U.S. Environmental Protection Agency (EPA) as probable or class B2 human carcinogens (USEPA 2006). Due in part to their relatively low vapor pressures and low water solubility (Skupinska et al. 2004), PAHs will not remain suspended in indoor air and are likely to accumulate in the dust fraction. Levels of PAHs in house dust were found to exceed their levels in surrounding soil (Roberts and Dickey 1995). Dust can therefore act as a reservoir for household pollutants (Butte and Heinzow 2002) and potentially increase indoor exposure levels. Adverse human health effects documented after exposure to house dust include respiratory and reproductive effects as well as cancer (Maroni 1995). Routes of exposure to house dust consist of mainly inhalation, non-dietary ingestion, and to a lesser extent dermal absorption. Inhalation is particularly significant after certain activities that might re-suspend dust such as sweeping or cleaning (Thatcher and Layton 1995). Non-dietary ingestion of PAHs in dust is not a significant concern for adults; however, due to both behavior and pharmacokinetic differences it can be of major importance in children. In fact, house dust is believed to be a major source of childhood exposure to PAHs and other toxic substances (Roberts and Dickey 1995). Due to behavioral characteristics such as mouthing and food-handling, non-dietary ingestion of contaminants is potentially the main route of exposure in children. Dermal and inhalation exposures may also be important in children and are related to behaviors such as crawling on the floor and contact with dirt and grass (Black et al. 2005). In a review article on the mutagenic hazards of settled house dust, Maertens et al. (2004) listed numerous published studies that document the detection of PAHs in house dust. However, only a single study (Roberts 1987) has examined the mutagenic potential of house dust.

In rural areas of China, coal constitutes the main energy source. Rodents captured from a coal mining area showed evidence of significantly higher extent of DNA

damage compared to animals from a control area. DNA damage was assessed by DNA migration, damage index and percentage of damage. Activities such as stripping and crushing coal produce PAHs into the environment and DNA adducts formation was thought to present an important contribution to the high level of DNA damage found the the blood cells of exposed rodents (Leon et al. 2007). Household use of coal was found to cause more health problems in adults and children than any other fuel (Peabody et al. 2005). Previous studies (Chuang et al. 1992; Mumford et al. 1995) from an area affected with a high incidence of lung cancer suggested an association with indoor use of coal for heating and cooking under unvented conditions. The lung cancer rate was believed to be associated with high concentrations of PAHs present in coal smoke. Exposure to coal smoke was also associated with increased K-*ras* mutation frequency in nonsmoking female lung cancer patients which is believed to be induced by PAHs (Keohavong et al. 2003). Levels of PAHs recorded in indoor air from eight Chinese homes generally exceeded those in outdoor air (Liu et al. 2001). Due to different activities and ventilation conditions, PAH levels were highest in the bedroom, followed by the kitchen, living room and balcony. Different cooking methods and temperatures were found to affect the composition and concentrations of PAHs in air of domestic and commercial kitchens in China (Zhu and Wang 2003).

In the current study, dust was collected from the floor of homes in a typical rural community that burn coal fuel indoors. These homes were located in a region of China affected by a high frequency of neural tube defects (NTDs) among other health problems. After solvent extraction, the chemical composition of each dust sample was quantified and its genotoxicity assessed in a battery of *in vitro* and *in vivo* bioassays. The main objective of this study was to evaluate the genotoxic potential of house dust extracts; and to determine if there was a correlation between genotoxicity and the concentration of benzo[a]pyrene (B[a]P) or carcinogenic PAHs.

2.2 Materials and Methods

2.2.1 Site

Dust samples were collected from houses in Taigu County in the province of Shanxi in North China. Shanxi is the leading province in coal production in China and provides as much as the third of China's coal. The coal mined from this region is commonly used indoors for cooking and heating. The major products of thermal decomposition of coal include PAH as well as related nitrogen and sulfur containing polycylic aromatic compounds (Chen et al. 2005; Mumford et al. 1995; Wornat et al. 2001). Residents may be exposed to PAHs and other byproducts either by inhalation of the airborne soot particles or ingestion of soot particles that deposit on food. Dust samples were collected from the surface of floors in houses. A sample of uncombusted coal dust was also collected from a stockpile near houses.

2.2.2 Collection of Dust Samples

A total of four floor dust samples (E1, E2, E3, E4) were collected from four different houses in Taigu County in April 2005. The houses were comparable in terms of the presence of dirt in the sampling areas. All of the floors from which dust was collected were made of brick. The materials used to collect residential dust samples included a pre-ashed and pre-weighed glass fiber filter cloth (type A/E, 20.3 cm x 25.4 cm, Gellman Sciences, Ann Arbor, MI), a measuring tape, an aluminum foil pouch, a plastic bag and nanograde isopropyl alcohol. Dust samples were collected from an area in the kitchen. Approximately eight samples from each house were collected and composited. Dust samples were collected from adjacent areas delineated on the floor surface of each house. The precise dimensions of the sampling areas were recorded. A glass fiber filter cloth was saturated with isopropyl alcohol. The collection of the dust sample was accomplished by wiping the cloth across the delineated floor area from the near end to the far end and back until the entire area to be sampled was wiped. The cloth was checked periodically for dust accumulation. If the cloth appears to be saturated with dust, sampling was stopped and the area that had been sampled was measured and

recorded. Upon completion of the floor swipe sample collection, each glass fiber filter cloth was wrapped in an aluminum foil pouch and transferred to a ziploc bag. All the dust samples were shipped on ice packs to the analytical laboratory in the United States.

2.2.3 Sample Extraction and Chemical Analysis

Dust samples were extracted with a methylene chloride:acetone (95:5 v/v) mixture in a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE). After the extraction was complete, the sample extracts were combined, dried under a stream of nitrogen and weighed. An aliquot of the combined residue was then transferred to pre-weighed sterile glass culture tubes with teflon-lined caps, dried under a stream of nitrogen, reweighed and stored at 4°C.

Dust extracts were analyzed for PAHs and their alkylated homologues using USEPA method 8270C (USEPA 1997). This method was developed for PAH quantitation and has been described previously (Cizmas et al. 2003). Analysis was performed using an Agilent 5975 gas chromatograph with a mass selective detector in selected ion monitoring mode. A 60 m x 0.25 mm ID x 0.25 mm film thickness column (Agilent Technologies, Palo Alto, CA) was used. The injection port was maintained at 300°C and the transfer line at 280°C. The temperature program was as follows: 60°C for 6 min, increased at 12°C/min to 180°C and then increased at 6°C/min to 310°C and held for 11 min for a total run time of 47 min.

2.2.4 Microbial Mutagenicity Assay

The *Salmonella*/microsome assay was used to evaluate the mutagenic potential of the complex mixture extracted from dust. The *Salmonella* tester strain TA98 was kindly supplied by Dr. Bruce Ames (University of California, Berkeley, CA). The procedures used were the same as those described by Ames et al. (1975) with modifications as suggested by Maron and Ames (1983). *Salmonella* cultures were prepared by incubating 50 µl of a frozen permanent *Salmonella* stock in 10 ml sterile oxoid broth (Oxoid #2,

Unipath Ltd., Basingstoke, Hampshire, England) in a rotary shaker incubator for 10 hr. at 37°C.

Extracts were tested in the presence of metabolic activation (S9) in the standard plate incorporation assay (Maron and Ames 1983). The S9 supernatant of homogenized Aroclor 1254 induced Sprague-Dawley rat liver was obtained from Molecular Toxicology, Inc. (Boone, NC). Positive indirect-acting (B[a]P: 10 µg/plate) and negative solvent (dimethyl sulfoxide (DMSO)) controls were included in each test to ensure consistency in TA98 sensitivity and S9 mix activity. The S9 mix contained 30% rat liver S9 fraction per ml and 70% cofactor supplement per ml (11.4 mM $MgCl_2$, 47 mM KCl, 7.1 mM glucose-6-phosphate, 5.7 mM NADP, and 140 mM potassium phosphate buffer, pH 7.4). The amount of S9 mixture applied per plate was 500 µl.

Dust extracts were resuspended in DMSO and tested on duplicate plates in two independent experiments at five dose levels (1.0, 0.5, 0.25, 0.1, 0.05 mg/plate). To a 2.5 mL volume of top agar was added approximately 1-2 x 10^8 cells, 50 µL of sample extract, and 0.5 mL of S9 mix. The top agar was then vortexed and poured onto a minimal glucose agar plate. Plates were incubated at 37°C for 72 hr. Revertant colonies were counted on an Artek Model 880 automated colony counter (Dynatech Laboratories, Chantilly, VA). A response was considered positive if the average number of revertants at a minimum of two dose levels was greater than twice the average response for the corresponding negative solvent control (Chu et al. 1981).

2.2.5 Treatment of DNA In Vitro

A modification of the procedure of Randerath et al. (1992) was used to measure the *in vitro* formation of DNA adducts. Placental DNA was isolated from two cases (children born with a neural tube defect) and two matched controls (with no visible abnormality). DNA samples were treated with residential dust and coal extracts suspended in DMSO. All families that provided biological samples for this study were informed of the nature of the study and signed consent forms approved by the Texas A&M University Institutional Review Board (IRB no. 2003-0430).

In this experiment, 150 µg of DNA was added to a solution with 3-methylcholanthrene induced Fischer-344 rat liver microsomes, NADPH (100 mM) and DNA binding buffer (150 mM Tris-HCl, 150 mM KCl and 5 mM $MgCl_2$, pH 7.6). Dust or coal extract in DMSO was added last. The dose of dust and coal extracts was determined based on the contents of B[a]P. The reaction concentration of B[a]P was adjusted to 1 µM for each extract. In addition to the solvent control (DMSO), an aliquot of E1 was added to a solution including all reaction reagents except microsomes to serve as another negative control. The solution was incubated at 37°C for 2 h. The reaction was stopped enzymatically to start the process of DNA extraction as described previously (Moorthy 2002).

2.2.6 In Vivo *Genotoxicity*

Female ICR mice (21-24 g) were purchased from Harlan (Houston, TX). Mice were fed Laboratory Rodent Diet 5001 and provided with tap water ad libitum. Institutional guidelines on animal care and use were followed in all experiments. Mice were divided into eleven groups with four mice each. A patch of hair (approximately $4cm^2$) was shaved on the back of each mouse three days prior to treatment. Animals were treated topically with 150 µL extract on the shaved area using a glass capillary micropipette. Methylene chloride (150 µL) and BaP (100 nmol) were applied as the negative and positive controls, respectively. Treatment groups of dust extracts included: E1 at 3 mg/mouse, 1.2 mg/mouse and 0.48 mg/mouse; E2 at 3 mg/mouse, 1.2 mg/mouse and 0.48 mg/mouse; E3 at 3 mg/mouse and E4 at 3 mg/mouse. The coal extract was also tested at a dose of 3 mg/mouse. Twenty-four hours after treatment, animals were sacrificed by suffocation using CO_2. Skin and lungs were harvested and stored at -80ºC until DNA isolation. DNA isolation was conducted as previously reported (Gupta 1984). DNA concentration and purity was measured spectrophotometrically by absorbances at 260 and 280 nm. A_{260}/A_{280} ratio for all samples was between 1.6 and 1.8.

2.2.7 ^{32}P-Postlabeling

The nuclease P1-enhanced bisphosphate version of the ^{32}P-postlabeling assay was performed as described by Reddy and Randerath (1986). Briefly, DNA (6 to10 µg) was enzymatically degraded to normal (Np) and modified (Xp) deoxyribonucleoside 3'-monophosphates. After 3'-dephosphorylation of normal nucleotides with nuclease P1 the enriched nuclease P1-resistant modified 3'-nucleotides were converted to 5'-^{32}P-labeled deoxyribonucleoside 3', 5'-bisphosphate derivatives by incubation with carrier-free [γ-^{32}P] ATP and T4 polynucleotide kinase. Adducted radioactive nucleotides were separated by multidirectional anion-exchange thin-layer chromatography (TLC) using polyethyleneimine (PEI)-cellulose sheets. Labeled products were purified and partially resolved by one-dimensional development with 2.3M NaH_2PO_4 pH 5.75 overnight (D1). Bulky labeled adducts retained in the lower (2.8 x 1.0 cm) part of the D1 chromatogram were contact-transferred to fresh thin-layer sheets and resolved by two-dimensional TLC. The first dimension employed 3.82M lithium formate + 6.75M urea, pH 3.35. The second dimension was developed with 0.72M NaH_2PO_4 + 0.4M TRIS + 7.65M urea, pH 8.2. Radioactivity of each TLC map was determined by using an Instant Imager (v.2.04; Packard Instrument, Downers Grove, IL). DNA adduct levels were quantified as mean relative adduct labeling (RAL) values $\pm$ SD using the following equation:

RAL = sample count rate/ (DNA-P x specific activity $_{ATP}$),

where the sample count rate is measured in cpm, DNA-P represents the pmol of DNA monomer units assayed per replicate, and the specific activity of the ATP is in units of cpm/pmol. Equality of means was tested at the 95% confidence level using the unpaired Student's t-test completed with the Stata v. 8.0 software (StataCorp 2003). The criterion for significance was set at ($P<0.05$).

2.3 Results

2.3.1 Chemical Analysis

The concentrations by area and mass of the US EPA priority PAHs and total PAHs detected in all five extracts are presented in Table 2.1. Total carcinogenic PAHs

Table 2.1: Concentration of US EPA priority PAHs detected in organic extracts of four house dust samples (E1-E4) and coal.

Chemical	Concentration by Area				Concentration by Mass				
	E1 (µg/m^2)	E2 (µg/m^2)	E3 (µg/m^2)	E4 (µg/m^2)	E1 (µg/g)	E2 (µg/g)	E3 (µg/g)	E4 (µg/g)	Coal (µg/g)
Naphthalene	0.8	0.7	0.1	0.2	n/a[f]	1.2	0.02	0.03	0.02
Acenaphthylene	18	5	1	4	n/a	8	0.2	0.5	0.01
Acenaphthene	0.7	0.3	0.1	0.1	n/a	0.5	0.01	0.02	0.02
Fluorene	4	1	0.3	0.3	n/a	1.8	0.1	0.03	0.2
Phenanthrene	600	134	51	88	n/a	240	10	10.3	2.1
Anthracene	57	13	5	12	n/a	20	0.9	1.4	0.1
Fluoranthene	772	298	87	190	n/a	530	16	22.2	0.3
Pyrene	667	200	75	160	n/a	355	14	18.5	0.4
Benz[a]anthracene	460	110	48	110	n/a	195	9	13	0.3
Chrysene	632	234	125	230	n/a	410	24	27	0.6
Benzo[b]fluoranthene	486	183	108	193	n/a	325	20	23	0.7
Benzo[k]fluoranthene	112	33	16	17	n/a	60	3	1.9	0.1
Benzo[a]pyrene	181	49	27	46	n/a	85	5	5.4	0.2
Indeno[1,2,3-c,d]pyrene	144	44	23	30	n/a	80	4	3.5	0.1
Dibenz[a,h]anthracene	43	14	7	10	n/a	25	1.3	1.2	0.1
Benzo[g,h,i]perylene	112	39	20	26	n/a	70	4	3	0.3
Total PAHs[a]	**10100**	**3240**	**1350**	**2750**	**n/a**	**5760**	**255**	**320**	**32**
cPAHs[b]	**2060**	**667**	**353**	**636**	**n/a**	**1180**	**67**	**75**	**2**
cPAHs(%)[c]	**20**	**21**	**26**	**23**	**20**	**21**	**26**	**23**	**6**
B[a]P(%)[d]	**1.8**	**1.5**	**2**	**1.7**	**1.8**	**1.5**	**2**	**1.7**	**0.6**
Fluoranthene/Pyrene[e]	**1.2**	**1.5**	**1.2**	**1.2**	**1.2**	**1.5**	**1.2**	**1.2**	**0.8**

[a]Total PAHs = Total polycyclic aromatic hydrocarbons quantified.
[b]cPAHs = Total probable or class B2 human carcinogenic PAHs quantified based on USEPA, 2006.
[c]cPAHs(%) = Percent of total PAHs constituted by carcinogenic PAHs.
[d]B[a]P(%) = Percent of total PAHs constituted by benzo[a]pyrene.
[e]Fluoranthene/Pyrene = Ratio of fluoranthene to pyrene.
[f]n/a= not available.

(cPAHs) are also reported for each sample extract. For dust sample E1 the mass of PAHs per dust weight could not be determined due to the abrading, and partial loss of filter mass, of the pre-weighed filters by the floor surface. Total PAHs (by area) in the four dust extracts ranged from 1350 μg/m^2 for sample E3 to 10100 μg/m^2 for sample E1 (Table 1). By mass, PAH concentrations were lowest in the extract of the coal sample and highest (5760 μg/g) in sample E2. Carcinogenic PAH concentrations by area ranged from 353 μg/m^2 for sample E3 to 2060 μg/m^2 for sample E1. Carcinogenic PAHs composed approximately 20% of the mass of total PAHs for all four dust samples, and less than 10% the mass of the coal extract. B[a]P concentrations in the extracts were between 1.5 and 2.0% for dust and 0.6% for the coal. Because of the extraction process, concentrations of low molecular weight hydrocarbons are not likely to be accurate. Extracts collected for the current study consistently exhibited low concentrations of these compounds. The predominant PAH in dust extracts E1 and E2 was fluoranthene, while chrysene was the predominant carcinogen in all four dusts and the predominant PAH in extracts E3 and E4. The PAH detected at the highest concentration in the coal extract was phenanthrene, and benzo[b]fluoranthene was the predominant carcinogen. Overall, these data indicate that the PAH composition of the samples was variable with E3 having the highest carcinogenic PAH fraction whereas E1 had the highest concentration of PAHs by area but a relatively low carcinogenic PAH fraction. Levels of PAHs in the uncombusted coal extract were much lower than those detected in dust extracts.

2.3.2 Bacterial Mutagenicity

The microbial mutagenicity of the five PAH mixtures extracted from the dust and coal samples is presented in Table 2.2. Due to the limited mass of material available, and because PAHs are generally indirect-acting mutagens, the sample extracts were tested only in the presence of metabolic activation. All of the samples induced a doubling of revertants as compared to the solvent control background. Samples E3 and E4 induced approximately 200 more revertants than the solvent control at the highest dose tested.

Table 2.2: Bacterial Mutagenicity, as measured with *S. typhimurium* TA98 plus S9, of organic extracts of four house dust samples (E1-E4) and coal from Shanxi, China.

Sample	Dose (mg/plate)	Total TA98 his+ revertants Mean(±SD)	Response*
E1	0	38±9	
	0.05	39±6	
	0.1	53±6	±
	0.25	50±5	
	0.5	59±16	
	1	83±9	
E2	0	38±9	
	0.05	39±6	
	0.1	55±2	±
	0.25	44±14	
	0.5	74±19	
	1	83±24	
E3	0	38±9	
	0.05	51±20	
	0.1	56±10	++
	0.25	74±13	
	0.5	118±6	
	1	238±11	
E4	0	38±9	
	0.05	36±1	
	0.1	54±1	++
	0.25	71±20	
	0.5	110±12	
	1	231±23	
Coal	0	38±9	
	0.05	54±9	
	0.1	58±9	+
	0.25	75±7	
	0.5	73±10	
	1	101±8	

* ± = Two-fold increase in revertants at one dose.
+ = Doubling of revertants at two doses.
++= Doubling of revertants at two doses and four-fold increase at one dose.

The coal extract exhibited a positive response, whereas E1 and E2 showed a weak positive response since they only induced a doubling of revertants at a single dose.

2.3.3 DNA Adducts Induced in Vitro

Representative profiles of DNA adducts from human placenta DNA treated with dust and coal extracts *in vitro* are shown in Figure 2.1. Dust extracts from four houses elicited qualitatively identical patterns overall. However, the coal extract displayed a unique pattern (Figure 2.1). The quantitative analysis of DNA adducts was reported as total DNA adducts which consisted of the addition of the levels of individual DNA adduct spots. Levels of total DNA adducts among the different extracts were compared between the placenta DNA from NTD cases and controls. However, no statistically significant differences were observed between the latter two groups. Therefore, the values of DNA adducts from NTD cases and controls were combined when data from different extracts were compared (Figure 2.2). Mean Relative Adduct Labeling (RAL) per 10^9 nucleotides for total adducts were 123.4, 249.2, 512.1 and 206.5 for E1 to E4, respectively. Coal extract produced very low levels of DNA adducts (17.7 per 10^9 nucleotides). All five extracts displayed a statistically significant increase in adduct levels over the solvent control. Coal extract induced significantly lower levels of DNA adducts compared to all dust extracts. In contrast, E1 generated significantly higher levels of DNA adducts than any other dust extract. E1 did not cause any DNA adduct formation without the addition of microsomes.

2.3.4 DNA Adducts Induced in Vivo

Topical treatment of female ICR mice with extracts of house dust and coal, followed by ^{32}P-postlabeling and two-dimensional mapping in the previously described solvents resulted in representative skin DNA-adduct profiles as shown in Figure 2.3. Similar patterns of DNA adducts were observed in all four dust extracts. Coal, however, exhibited a slightly different pattern of DNA adducts. The pattern of B[a]P-DNA adducts was similar to the one previously reported in Talaska et al. (1996). Mean levels

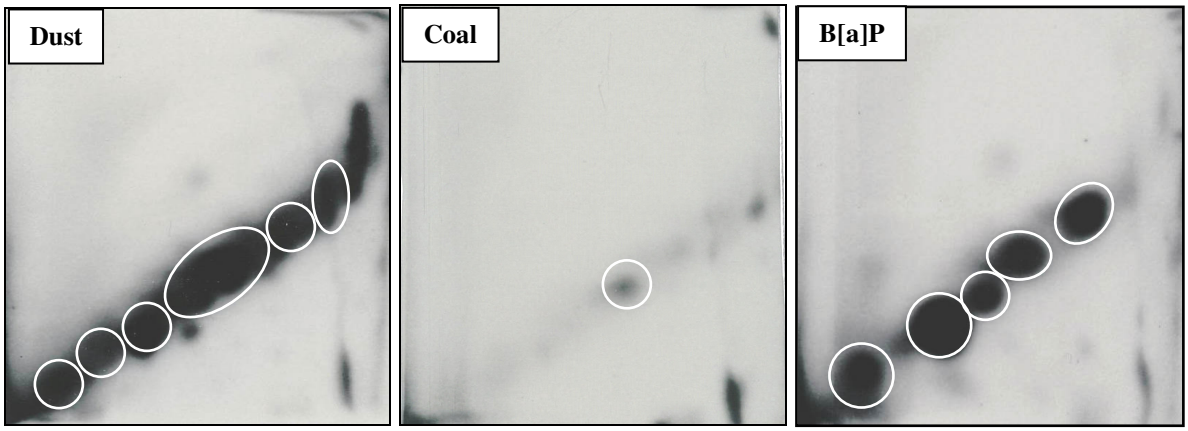

Figure 2.1. Autoradiograms of ^{32}P-postlabeled placenta DNA adducts of induced *in vitro* by organic extracts of house dust, coal and B[a]P. Autoradiography for 16 hr. at -80^{o}C using Kodak XAR-5 film (Dust and B[a]P maps were exposed for 6 hr. only).

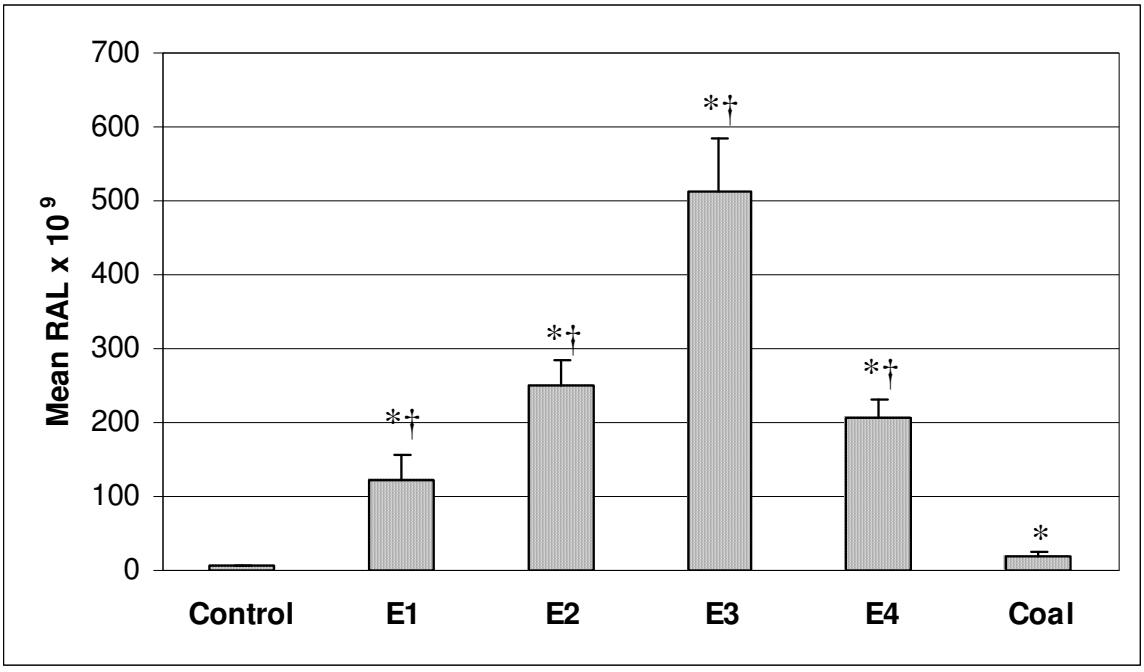

Figure 2.2. Levels of total DNA adducts induced in placenta DNA from neural tube defect cases and controls treated by house dust and coal extracts expressed as Relative Adduct Labeling (RAL) per 10^9 nucleotides (mean± SD) values.
*Significant from control (P<0.05).
†Significant from coal (P<0.05).

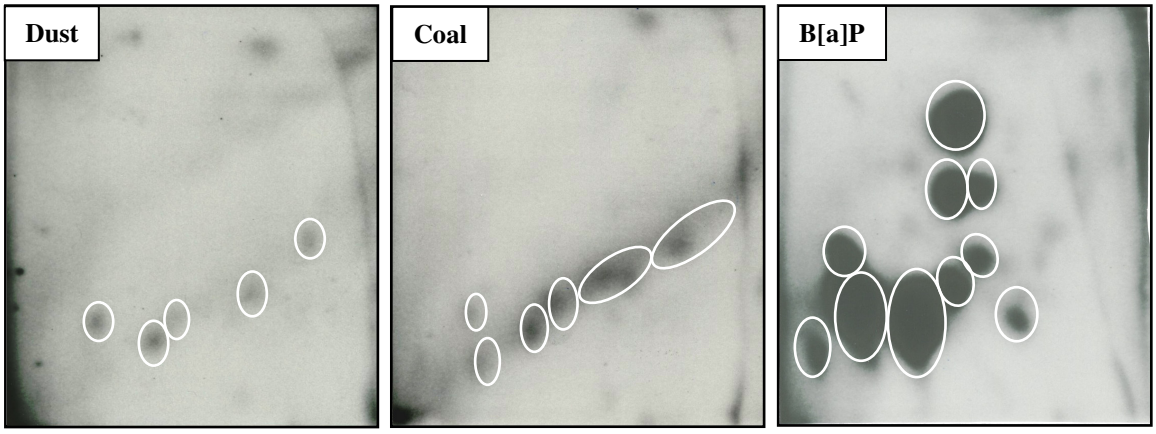

Figure 2.3. Autoradiograms of ^{32}P-postlabeled skin DNA adducts of female ICR mice treated dermally with organic extracts of house dust, coal and B[a]P. Autoradiography for 24 hr. at -80^{o}C using Kodak XAR-5 film.

of total DNA adducts in skin were quantified and compared across the five different extracts, as well as B[a]P. Data is presented in Table 2.3. Overall, dust and coal extracts displayed very low genotoxicity in mice. No dose-responses were observed in E1 and E2 extracts. In contrast to *in vitro* data, the coal extract induced relatively higher levels of mice skin DNA adducts compared to dust extracts (Table 2.3). Figure 2.4 demonstrates a comparison of the levels of total DNA adducts of mice skin treated with 3 mg of dust and coal extracts. Except for E2, all dust extracts at the 3 mg dose as well as coal induced a statistically significant increase in total adduct levels over the solvent (methylene chloride) control. E1 (1.2 mg) induced significantly more adducts than the solvent control, but not at the lowest dose (0.48 mg). Dose-response patterns were not observed for E1 or E2. Interestingly, coal extract yielded significantly higher levels of adducts at the 3 mg dose than any dust extracts at the same dose. DNA adduct levels were not significantly different between dust extracts. Total lung adduct values were lower when compared to skin, the organ of treatment application (data not shown).

Table 2.3. Mean Relative Adduct Labeling (RAL) values (± SD) of total DNAadducts induced in skin DNA of ICR female mice dermally exposed to house dust and coal extracts.

Sample	Dose (/mouse)	Mean±SD
Control (Methylene Chloride)	150 µL	1.07±0.34
E1	3 mg	1.88±0.51[a]
	1.2 mg	2.37±0.48[a]
	0.48 mg	1.50±0.36[a]
E2	3 mg	1.44±0.39
	1.2 mg	1.41±0.38
	0.48 mg	1.52±0.27
E3	3 mg	2.54±0.92[a]
E4	3 mg	2.20±0.48[a]
Coal	3 mg	9.81±0.65[b]
B[a]P	100 nmol	220±82

[a] Extract induced significantly more adducts than the control ($P<0.05$).
[b] Extract produced significantly the highest adduct levels among all extracts ($P<0.05$).

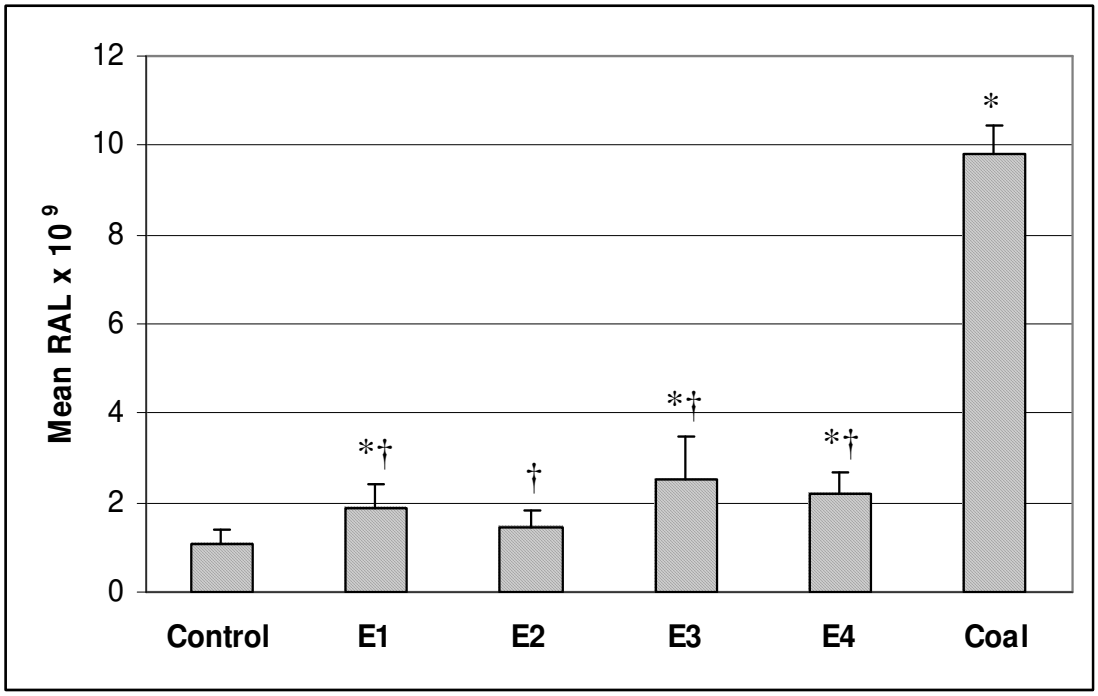

Figure 2.4. Comparison of levels of total skin DNA adducts expressed as Relative Adduct Labeling (RAL) per 10^9 nucleotides (mean± SD) values of female ICR mice treated dermally with organic extracts (3 mg) of dust and coal. Coal produced significantly more adducts than any dust extract ($P<0.05$).
*Significant from control ($P<0.05$).
†Significant from coal ($P<0.05$).

2.4 Discussion

This study used chemical analysis and short-term bioassays to compare the PAH composition and genotoxic potency of complex PAH mixtures from four residential dust samples and coal. Overall, the PAH components and their concentrations in the house dust samples were in accordance with the results of the studies reviewed by Maertens et al. (2004). The reported concentrations of PAH were 10 to 100 fold higher than those detected by our lab during similar studies conducted in Sumgayit, Azerbaijan and Rio Bravo, Texas (unpublished data). Both of these communities do not burn coal indoors. Acenaphthene and acenaphthylene were found in lower concentrations whereas pyrene and fluoranthene were found to be among the most abundant PAHs in the house dust samples collected in Shanxi, China. In addition, the ratio of fluoranthene to pyrene in all four dust samples was greater than 1 (Table 2.1) suggesting that PAHs in dust were generated by pyrolytic processes (Baumard et al. 1998a). Based on chemical analysis, sample E1 had the highest concentration by area of total PAHs and B[a]P, followed by E2, E3 and E4. However, the biological response did not correlate well with genotoxicity predicted from chemical analysis. Data from chemical analyses did not accurately predict the toxicity of PAH mixtures as noted in previous studies (Cizmas et al. 2004; Randerath et al. 1999). Due to the complexity of such mixtures, genotoxic responses appear to be affected by chemical interactions.

In the mutagenicity assay, E1 and E2 induced a weak positive response whereas E3 and E4 which had lower levels of total PAHs and B[a]P induced a strong positive response. The lack of correlation between B[a]P levels and mutagenic response has been previously reported (Donnelly et al. 1993). These results suggest a potential chemical interaction existing among the hundreds of chemicals in the complex mixtures that altered genotoxicity. Using DNA isolated from placental tissue, sample E3 exhibited the greatest *in vitro* binding affinity when compared to the rest of the dust extracts. In the animal study adduct levels were lower, although the overall trend was similar. E3 and E4 induced significantly higher levels of DNA adducts than E1 or E2 *in vivo*. Overall, dose-response relationships were not linear for either sample E1 or E2. Coal, which gave a

positive response in the mutagenicity assay, induced the lowest levels of DNA adducts among all samples *in vitro*. However, coal induced the highest levels of total DNA adducts formation in mice skin when compared to the dust samples.

In mice, the extracts with the higher concentrations of chemicals may have saturated metabolic activation enzymes. This effect has been previously noted in the literature by Ramesh and Knuckles (2006) who found non-linear dose-DNA adduct relationship in tissues of rats exposed to high doses of B[a]P in their diet. It is also possible that high concentrations of PAHs could have induced phase II enzymes in the adult mice skin tissues that partially detoxified genotoxic chemicals. Furthermore, cytotoxicity at the site of contact and increased DNA repair and/or cell turnover could have attenuated DNA adduct formation *in vivo* (Randerath et al. 1999). Such effects are not likely to be encountered in *in vitro* systems where enzymatic systems are typically pre-induced and generally not limiting. Results from a previous study (Courter et al. 2006) suggested that urban dust particulate matter inhibited CYP metabolic capacity, thus altering PAH-DNA adduct formation and tumor initiation. Additionally, Godschalk et al. (2000) found that the levels of DNA adducts in skin peaked at 2 days after acute exposure by dermal application of B[a]P on male rats. Mice in our study were only exposed for 24 hr.

It is important to note however that overall, the biological potency observed in the genotoxicity testing was well predicted by the chemical analysis of carcinogenics in the extracts. E3 had the highest percentage of carcinogenic PAHs (Table 2.1) and induced the maximum genotoxic response *in vitro* with placenta DNA or bacteria, as well as in whole animals. The percentage of the PAH fraction composed of carcinogenic PAHs appears to be a good qualitative indicator of genotoxicity.

Data from this study confirmed the presence of genotoxic compounds at relatively high levels in the residential environment in Shanxi, China. Results from this study could not however determine the major source of those compounds, whether it was from coal burning or tobacco smoking or a combination of both. In addition, the study results indicate that DNA adducts are correlated with carcinogenic PAH concentrations

in dust. The results suggest that compounds with carcinogenic potency are present in floor dust of homes at high enough concentration to potentially cause adverse human health effects. Future research would benefit from information regarding concentration of dust borne PAHs in human serum and frequencies of DNA adducts in lymphocytes.

CHAPTER III
BIOLOGICAL INDICATORS OF PAHS IN VENOUS BLOOD FROM A HUMAN POPULATION

3.1 Introduction

Humans are continuously exposed to hazardous chemicals. These chemicals usually exist as complex mixtures. Complex mixtures often contain hundreds of different chemical components. These include components that are proven to be toxic to human or ecological receptors and others with a less characterized toxicity. An example of such complex mixtures are PAHs, a class of chemicals produced from the incomplete combustion of organic substances such as coal, gas, wood, or tobacco. PAHs are widespread environmental contaminants existing in air, water, soil and sediment. Certain PAHs have been classified as carcinogenic compounds by the USEPA (2006) and the IARC (2004). Epidemiological studies on populations exposed to PAH mixtures have demonstrated a link between these exposures and cancer of the lung, respiratory system and stomach (Bertrand et al. 1987; Krewski and Thomas 1992; Puisieux et al. 1991; Vyskocil et al. 2004). Smoking and exposure to environmental tobacco smoke (ETS) has been associated with cancer in the lung, bronchus, larynx, bladder, cervix and oral mucosa (Lee et al. 2006; Phillips 1997; Yach and Wipfli 2006).

In an effort to prevent long-term effects caused by such chemical exposures, biological indicators of PAH exposure and effect such as DNA adducts have been developed. These biological indicators serve as a diagnostic matrix to estimate exposure and identify potential early indicators of adverse health effects. PAH exposure levels are classically measured by air sampling. Internal dose is detected by the determination of a parent chemical or metabolite in body fluids (blood or urine). Biological effects as a measure of the internally effective dose are considered more relevant for the assessment of the ultimate health risks such as cancer. These effects are monitored by biochemical markers including covalent binding products to DNA or proteins in addition to DNA stand breaks or cytogenetic markers such as micronuclei (MN), chromosome aberrations

(CA) and sister chromatid exchange (SCE) (van Delft et al. 2001). DNA adducts are considered as a marker for potential risk of genotoxic effects such as cancer or birth defects. DNA adducts may also be used to reflect individual variations in exposure, absorption, metabolic activation and DNA repair. The estimated half life of DNA adducts ranges between three to four months (Mooney et al. 1995).

More recently, markers of genetic predisposition have also been characterized to help identify individuals with a sensitive or resistant genotype. More recently, genetic predisposition was found to affect individual susceptibility to disease and biomarkers of exposure and effect. Identification of individuals with sensitive or resistant genotypes is essential in human health risk assessment. Genetic polymorphisms include variations in genes for Phase I and Phase II drug metabolizing enzymes such as CYP450 and GST as well as genes for repair of DNA adducts. As a major Phase II enzyme, GSTM1 deletion is prevalent in humans. In the US, GSTM1 null occurs in around 51% Caucasians, 46% among Hispanics, 59% among Asians, and 29% among African-Americans (Engel et al. 2002). GSTT1 is also polymorphic in humans. Frequency of GSTT1 null genotypes in US studies range from 15% to 27% for Caucasians, 22% to 29% for African-Americans, and 10 to 12% for Hispanics (Cotton et al. 2000).

Absence of GSTM1 enzyme activity reportedly increases susceptibility to cancer. GSTT1 on the other hand detoxifies reactive alkylating compounds and 10 to 20% of individuals have genetic deficiency for GSTT1 (Nelson et al. 1995). Populations exposed to environmental pollution showed increased levels of several markers of genotoxicity including PAH-DNA adducts, ras oncogene overexpression, chromosome aberrations, and sister chromatid exchange (Perera et al. 1992; Perera et al. 1999).

Most studies on environmental exposures to PAHs in human populations focus on areas where air pollution is a concern. Epidemiological studies show that exposures to PAHs (or air pollution) is associated with increases in mortality and/or morbidity from respiratory illnesses, cardiovascular diseases and cancer (Taioli et al. 2007). A number of epidemiological studies have revealed that urban communities are at an increased risk for developing cancer especially lung cancer, with a relative risk of 1.5 (Nielsen et al.

1996).The first report considered as an evidence that environmental pollution might elicit DNA adduct formation in humans was by Hemminki et al. (1990). The main objective of the study was to evaluate occupational PAH exposures in coke oven workers in Poland. Two control groups were recruited for the study, one composed of local residents and another included rural residents. Unexpectedly, DNA adduct levels in local control subjects were nearly as high as the levels detected in the coke oven workers. Seasonal variations in adduct levels measured in white blood cells of the residents of Upper Silesia in Poland were consistent with air pollution levels and found to be more than two-fold higher in winter compared to summer (Perera et al. 1992). DNA adducts measured by ^{32}P-postlabeling in white blood cells of a non-smoking women group with outdoor occupations in an air polluted city in the Czech Republic was associated with their personal PAH exposure (Binkova et al. 1995). In a recent study on the genotoxic effects of air pollutant exposures on a human population living downwind to an industrial complex in Thailand, Peluso et al. (2008) found that the level of bulky DNA adducts in the leukocytes of residents was 0.85 ± 0.07 per 10^8 nucleotides which was significantly elevated compared to residents living in a control district which had DNA adduct levels of 0.53 ± 0.05 per 10^8 nucleotides. Smoking habits did not seem to have had any effect on the DNA adduct levels in this study.

Non-smoking healthy male subjects from a rural and urban areas of Denmark and Athens, Greece were recruited in a PAH biomonitoring study. DNA adducts were found to increase going from rural to small urban and large urban residential areas (Athens, Greece). No influence of the GSTM1 genotype on DNA adduct levels was found in this study (Nielsen et al. 1996). No difference in lymphocyte PAH-DNA adduct levels between GSTM1 deficient and proficient persons was observed in other studies (Binkova et al. 1998; Hou et al. 1995; Ryberg et al. 1994). However, findings reported by Georgiadis et al. (2004) indicate that non-smoking students exposed to urban air pollution and ETS with GSTM1 deletion had higher levels of DNA adducts in their lymphocytes compared with GSTM1 “wild-type” subjects. Never smoking women with GSTM1 null had statistically significant greater risk of developing lung cancer from

exposure to ETS (Bennett et al. 1999). Lung cancer risk in GSTM1 null individuals exposed to indoor coal combustion emissions was elevated compared to individuals with an active copy of GSTM1. GSTT1 polymorphisms did not seem to be associated with lung cancer risk (Lan et al. 2000). However, individuals with null genotypes of GSTT1 were found to seemingly have an increased risk for developing colorectal cancers (Deakin et al. 1996). Inactive GSTT1 was also associated with increased risk for some forms of brain tumors (Hand et al. 1996; Kelsey et al. 1997).

The biological effects of GSTT1 deletion are still considered difficult to predict because it has both activating and detoxifying properties which affect many environmental pollutants (Pavanello 2006). Previous studies have found that the GSTT1 null variant to be associated with increased DNA damage and adduct levels (Georgiadis et al. 2005; Perera et al. 2002). However in a report of BPDE-DNA adduct levels in the leukocytes of smokers, while the GSTM1 deletion variant led to an increase in adduct levels, the GSTT1 variant led to a decrease (Lodovici et al. 2004). In addition, a recent report by Garte et al. (2007) suggested that GSTT1 deletion had protective effects on DNA oxidation over a group of 8 different individual PAH compounds.

Polymorphisms in DNA repair genes are also being developed as genetic predisposition indicators. As an example, excision repair cross-complementing group 2 (ERCC2) is involved in the nucleotide excision repair pathway (NER) by recognizing and repairing many structurally unrelated lesions such as bulky adducts and thymidine dimers (Manuguerra et al. 2006). DNA adducts levels in leukocytes of coke oven workers (16.6 ± 2.1 per 10^9 nucleotides) were significantly higher than those in metropolitan residents (5.2 ± 1.4 per 10^9 nucleotides) and suburban gardeners (6.5 ± 1.0 per 10^9 nucleotides). DNA adducts in subjects with ERCC2 Lys751Gln wild genotype was significantly higher than in those who have either heterozygous or homozygous variant alleles. No significant association between DNA adducts and polymorphisms of metabolic enzymes (GSTM1, GSTT1, CYP1A1 and mEH) was detected (Hu et al. 2007).

The study described in this chapter was directed at the analysis of biomakers of exposure, effect and susceptibility in parents of children with birth defects and controls in the Chinese province of Shanxi. As a measure of the internal dose of PAHs, PAH concentration in blood plasma was measured. The frequency of aromatic DNA adducts was quantified by ^{32}P-postlabeling technique in white blood cells from recruited subjects as a measure of internal effective dose. Polymorphims of two major Phase II metabolic enzymes (GSTM1, GSTT1) were also evaluated to detect genetic senstivities.
The biomarkers of exposure, effect, and sensitivity evaluated for this diissertation (Chapters III and IV) are summarized in Table 3.1.

Table 3.1. Summary of the PAH biomarkers tested in the study.

Biomarker	Sample	Method	Reference
Exposure			
PAH Concentrations	Venous Blood (Plasma) Placenta	SW-846 Methods 3545 and 8270C	(USEPA 1997)
Effect			
Aromatic DNA Adducts	White Blood Cells Placenta	^{32}P-postlabeling	(Reddy and Randerath 1986)
Susceptibility			
GSTM1	White Blood Cells Placenta	Polymerase Chain Reaction (PCR)	(Bailey et al. 1998)
GSTT1	White Blood Cells Placenta	Polymerase Chain Reaction (PCR)	
XRCC1	Placenta	SNPlex, TaqMan Assay	
OGG1	Placenta		
MGMT	Placenta		
ERCC2	Placenta		
APEX1	Placenta		
XRCC3	Placenta		

3.2 Materials and Methods

3.2.1 Study Site

Shanxi province in China was selected as a site for this research since it was anticipated that elevated concentrations of PAHs were present in the environment. Shanxi is located in north China approximately 200 miles northwest of the capital, Beijing. Shanxi – known as the 'Coal Warehouse of China'– is the leading province in coal production in China and provides as much as one quarter of China's coal. The coal mined from this region is commonly used indoors for cooking and heating. Residents in Shanxi may be exposed to PAHs and other byproducts either by inhalation of the airborne soot particles or ingestion of soot particles that deposit on food. The incidence of NTDs in Shanxi is one of the highest in the world. The overall prevalence rate for neural tube defects in China is 12.95 per 10,000 live-births; while in the northern part of the country (where this study was conducted), the rate is 19.90 per 10,000 births (Dai et al. 2002). In comparison, the estimated NTD prevalence in the United States, based on birth certificate data for 1995, was 4/10,000 (Mathews et al. 2002). Subjects were recruited for participation in the study through a collaboration with Dr. Li Zhu and the Institute for Children and Reproductive Health located in Beijing, China. Staff at county hospitals in Taigu, Pingding, Xiyang and Zezhou recruited participants for the study.

3.2.2 Subject Recruitment

Children and parents of children born with NTDs as well as children and parents of children from a matched control population were recruited for the study from four county birth hospitals. The types of congenital malformations that were studied were selected based on evidence suggesting an environmental component in their etiology. Neural tube defects, including anencephaly and spina bifida, are readily recognizable at birth based on a routine newborn physical examination. All spontaneous abortions, late fetal deaths (stillbirths), and live births occurring in four hospitals in the Shanxi region (including Tai Yuan) in northern China were recruited for the study. Subject recruitment was facilitated by the presence of a birth defects surveillance system in China since 1992

as described by Li et al. (2003). The consent form, questionnaire and all study protocols were reviewed and approved by the Texas A&M University Institutional Review Board (IRB) prior to initiation of the study. A copy of the Texas A&M University IRB approved protocols (No. 2003-0430) for this research is attached to this document. Each hospital received training from project staff, as well as a protocol book that was translated into Chinese.

3.2.2.1 Case Ascertainment

Babies with NTDs were identified at the time of birth by the attending physician. Birth attendants were trained to perform a standardized assessment of the infant immediately after delivery. Procedures were established at each of the participating hospitals for a standardized examination of all babies, both live births and late fetal deaths. Information on malformations of any type was entered on the reporting form. When a baby was born with an NTD a special case reporting form was completed and a study coordinator was contacted and informed of the birth of a potential "case." In cases of fetal or neonatal deaths, procedures were established with the hospital to retain the remains in the pathology department until the diagnosis can be confirmed. If the diagnosis was confirmed the birth was entered into the registry and the mother contacted to participate in the case-control study.

3.2.2.2 Case Mother Enrollment

When the physician verified the diagnosis of the appropriate congenital malformation, they confirmed with the hospital staff that it is acceptable to contact the mother. Whenever possible, the mother was contacted while in the hospital and the study was described to her. The details of the study were presented and, if it is considered appropriate by the physician, informed consent for participation was obtained and the questionnaire was administered. The mother was asked to sign an informed consent form (in Chinese) and to complete the study interview at that time or another

time before discharge. If this was not possible, information on how to contact her after discharge was obtained and a possible timeframe for the interview was determined.

3.2.2.3 Control Selection

One control was selected for each case from among the births at the same hospital where the case was born. Each month of the study, one additional control was recruited from each birth hospital. Controls were identified from the hospital's birth registry records, selecting births immediately following the case, or when case subjects were born preterm a case was selected from children born at the approximate date for a full term delivery. The babies' records were reviewed for the presence of congenital malformations. If there was any record of the baby having a congenital malformation, of any type, the next birth from the birth records was selected and reviewed.

3.2.2.4 Study Questionnaire

Case and control mothers who provided informed consent to enroll in the study were interviewed in-person using a standardized questionnaire that was translated to Chinese. The questionnaire was designed to ask about potential risk factors and confounders that are relevant to the circumstances in China. The questionnaire was based on the one developed by the Centers for Disease Control and Prevention and the California State Birth Defects Surveillance programs. This questionnaire is currently being used in a national collaborative case-control study of congenital malformations in the United States. It included questions on topics such as: occupation, chronic and acute illnesses, smoking habits, nutrition and alcohol use, prescription and non-prescription drug use, socio-demographic information, a complete reproductive history along with a family history of birth defects or genetic diseases, and a maternal residential history relative to the study pregnancy. The questions were time specific, asking about these factors for the period three months prior to pregnancy and during pregnancy, by trimester. Questions about the father of the subject included inquiries about occupation, race and ethnicity, age, smoking habits, and alcohol and drug use. Questions related to

history of congenital malformations and genetic diseases in either the mother's or the father's family and about consanguinity were also included. The questionnaire was developed in English with modifications appropriate for Chinese culture. The questionnaire was reviewed by an individual outside of the project for consistency with the English version and was approved by the Texas A&M University Institutional Review Board. A copy of the questionnaire is attached as appendix I.

3.2.3 Sample Collection and Shipping

3.2.3.1 Environmental Samples

Floor or window dust was collected from selected households in the study province of Shanxi. The households were comparable in terms of the presence of dirt in the sampling areas. All of the floors from which dust was collected were made of brick. The materials used to collect residential dust samples included a pre-ashed and pre-weighed glass fiber filter cloth (type A/E, 20.3 cm x 25.4 cm, Gellman Sciences, Ann Arbor, MI), a measuring tape, an aluminum foil pouch, a plastic bag and nanograde isopropyl alcohol. Whenever possible, dust samples were collected from an area in the kitchen. Dust samples were collected from adjacent areas delineated on the floor, window or wall surface of each house. The precise dimensions of the sampling areas were recorded. A glass fiber filter cloth was saturated with isopropyl alcohol. The collection of the dust sample was accomplished by wiping the cloth across the delineated floor area from the near end to the far end and back until the entire area to be sampled was wiped. The cloth was checked periodically for dust accumulation. If the cloth appeared to be saturated with dust, sampling was stopped and the area that had been sampled was measured and recorded. Upon completion of the floor swipe sample collection, each glass fiber filter cloth was wrapped in an aluminum foil pouch and transferred to a ziploc bag. All the dust samples were shipped on ice packs to the analytical laboratory in the United States.

3.2.3.2 Venous Blood Samples

Biological samples were collected from all study subjects. These included the case and control children, as well as their mothers and fathers. These samples were collected in the field by hospital personnel and processed for transport to the Peking University Health Science Center in Beijing, China. A volume of 10-15 mL of venous blood was collected in sodium heparin BD Vacutainer tubes (VWR, catalogue # VT6480) from the parents of all case and control subjects. Venous blood samples were divided into plasma for organics analysis and white blood cells (WBCs) for DNA isolation. Samples were stored at appropriate temperatures to be shipped on refrigerant gel packs to Texas A&M University for processing and analysis. Shipment of samples was carried out according to the regulations of the US Department of Transportation and International Air Transport Association (IATA). Samples were packaged according to IATA packing instruction 650 and classified under the category of "Diagnostic Specimens" UN 3373.

3.2.4 Sample Extraction and Chemical Analysis

3.2.4.1 Extraction of Dust Samples.

Extraction of dust filters was performed as described in Chapter II.

3.2.4.2 Extraction of Venous Blood

Venous blood samples were centrifuged in the heparinized collection tubes at 3000 RPM for 10 minutes. This procedure served to separate blood into an upper lighter plasma layer and a lower denser cell layer. The plasma layer was transferred by pipette to borosilicate glass vials to store at -80^{o}C for PAH extraction and analysis.

The method of plasma liquid-liquid extraction is modified from the USEPA Manual of Analytical Methods for the Analysis of Pesticides in Human and Environmental Samples (Watts1980). Five ml of plasma and 10 ml of methanol were mixed together to denature plasma proteins. Samples were then sonicated for 1 minute intervals for 3 times. A break during sonication is needed to prevent heating of the

samples. Afterwards, samples were decanted into seperatory funnels and 10 ml of dichloromethane was added to begin the extraction process and to rinse each sample vial. Following the first extraction, plasma samples were extracted with 3 x 20 ml dichloromethane. Each extraction was shaken for 3 minutes with a 20 minute settling period. The combined extracts were filtered on a solid phase extraction (SPE) column (Restek) that contains a layer of combusted sodium sulfate which aids in removing aqueous sample residues. The volume of the extract was reduced to 2 to 3 ml and submitted for determination of PAH analytes in a gas chromatography-mass spectrometry (GC/MS) tandem.

3.2.4.3 Chemical Analysis

A modified USEPA SW-846 Method 8270C (USEPA 1997) was used for the quantitative determination of polycyclic aromatic hydrocarbons (PAHs) and their alkylated homologues in extracts of dust and biological tissue. This method was developed for PAH quantitation and was described previously (Cizmas et al. 2003). Analysis was conducted on a Hewlett-Packard 5890 Series II gas chromatograph with a 5972 mass selective detector in selected ion monitoring mode. A 60m x 0.25mm ID x 0.25mm film thickness column (Agilent Technologies, Palo Alto, CA) was used. The injection port is maintained at 300°C and the transfer line at 280°C. The temperature program is as follows: 60°C for 6 minutes, increased at 12°C/min to 180°C and then increased at 6°C/minute to 310°C and held for 11 minutes for a total run time of 47 minutes.

3.2.5 DNA Isolation and ^{32}P-Postlabeling

DNA was extracted from human tissues, digested and labeled with γ^{32}P[ATP] following methods described previously by Reddy and Randerath (1986). A 0.2 to 0.5 g weight of minced mouse tissue was weighed into a 15 mL glass tube. A 3 mL volume of 1% SDS/1mM EDTA was added, and the tissue was homogenized with an Ultra Turrax (Ultra Turrax T25, Fisher Scientific, Pittsburgh, PA) for 30 to 60 sec at 15,000 to 20,000

RPM. Next, 100 to 150 µL proteinase K (15 mg/mL) was added to the homogenate, and the sample was then vortexed and incubated for 40 min at 38°C. The next step involves deproteinization using three solvent extractions. A 170 µL volume of 1M Tris-HCL, pH 8.0, and 35 µL 100 mM EDTA were added, and then vortexed. For the first extraction, a 3 mL volume of phenol saturated with 50 mM Tris-HCL, pH 8.0, 1 mM EDTA was added, and shaken for a minimum of 3 minutes. The sample was then spun for 10 min at 10000 rpm. The aqueous phase was transferred to a fresh tube. For the second extraction, a 3 mL volume of 1:1 mixture of saturated phenol and SEVAG (24 volumes Chloroform + 1 volume isoamyl alcohol) was added, and shaken for at least 3 minutes. The sample was then spun as described above. The aqueous phase was then transferred to a fresh tube. For the third extraction, a 3 mL volume of SEVAG was added, and shaken as described above. The sample was then processed as previously described, and the aqueous phase was transferred to a fresh tube.

For precipitation of DNA (+RNA), 0.3 mL (=0.1 volume) 5 M NaCl + 3.3 mL ice-cold absolute ethanol are added, and vortexed. The sample was placed in -20°C freezer for 30 min. The sample was then spun for 10 min at 10000 rpm, and supernatant discarded. Precipitant was washed 2 times with 3 mL 70% ice-cold ethanol, and supernatant decanted. The sample was semi-dried for 4-5 min. The DNA (+RNA) was dissolved in 1 mL 0.01 SSC (150 mM NaCl, 15 mM Na citrate) + 10 µL 100 mM EDTA. Next, 50 µL 1 M Tris-HCL, pH 8.0, + 15 µL Rnase A (10 mg/mL) + 16.5 µL Rnase T1 (5,000 units/mL) are added to the sample. The sample was then vortexed, and incubated for 40 min at 38°C. After incubation, 500 µL 0.01 x SSC was added to increase volume. Deproteinization was again carried out by solvent extraction. A 1.5 mL volume of SEVAG was added, and shaken for at least 3 minutes. The sample was processed as stated above, and the aqueous phase transferred to a fresh tube. DNA was precipitated by adding 150 µL 5 M NaCl + 1.5 mL ice-cold absolute ethanol. The sample was put in -20°C freezer for 30 minutes. The DNA was spun, washed, and semi-dried as stated above. DNA was redissolved in 0.3 to 0.6 mL 0.01 x SSC to reach the

desired concentration of 2 µg/µL. The concentration of DNA was checked via UV-Vis Spectrophotometer, the A_{260} (DNA)/A_{280} (RNA) ratio should range from 1.6 to 1.8.

DNA digestion and labeling were preformed as follows: 6 to10 µg of DNA in 5 µL of SSC and/or water were digested using 3 µL of 0.2 U micrococcal nuclease and 2.4 µg spleen phosphodiesterase per µL (MN/SPD) and 2.4 µL of IS-buffer mix (10 µg DNA, 100 mM $CaCl_2$ and 300 mM Na succinate) for 3.5 hr. at 37°C. MN/SPD digestion cleaves the 5'-nucleotide-phosphate bonds, leaving the 3'-monophosphates of the normal and adducted deoxyribonucleosides. The DNA was then digested with 4.75 µL of nuclease P1 digestion mix (4 µg/µL nuclease P1, 1 M NaOAc and 1 mM $ZnCl_2$) for 40 min at 37°C. Nuclease P1 digestion cleaves the 3'-nucleotide-phosphate bonds on normal nucleotides only. It was reported that adducted nucleotides were mostly or partially resistant to nuclease P1 3'-dephosphorylation (Reddy and Randerath 1986). The sample was then labeled using 3.86 µL of polynucleotide kinase (PNK) labeling mix (kinase buffer, 100 µC*i*/µL ATP and 30 U/µL PNK) and incubated for 40 min at 37°C. PNK labeling attaches the radioactive phosphate ([γ-^{32}P]ATP) at the 5'-hydroxyl group end of the adducted nucleotides through [^{32}P]phosphate transfer from ([γ-^{32}P]ATP). The normal nucleotides lost their 3'-phosphate during nuclease P1 digestion, so PNK will not phosphorylate them with the ([γ-^{32}P]ATP). Once samples were labeled, two specific activity (SA) tubes (2 pmol/µL dAP and 50 mM CHES, pH 9.5) were then labeled with 2.5 µL PNK labeling mix, and incubated the same as the samples.

The samples, with the exception of the SA tubes, were then digested with 1.5 µL of 40 mU/µL potato apyrase for 30 min at 37°C. Apyrase digestion destroys the excess ATP by removing [^{32}P] from the ATP ([^{32}P]-ATP $\longrightarrow$ ADP + [^{32}P]). Once apyrase incubation was completed, normals and SA tubes were diluted. Normals are a qualitative check to make sure that each sample was digested and labeled well. Tubes contain 250 µL of 20 mM CHES, pH 9.5. 1 µL of sample was added to the corresponding tube, and then 5 µL per sample were spotted on PEI-cellulose sheets. SA dilution tubes contain 996 µL of 20 mM CHES, pH 9.5. 4 µL of labeled SA tubes (d*pAp mix) were added to each corresponding SA tube, and then 5 µL were spotted on

PEI-cellulose sheets. Normals and SA PEI-cellulose sheets were run in 0.28 M $NH_4(SO_4)_2$ + 50 mM NaH_2PO_4, pH 6.7 to 13 cm past the origin line, approximately 1 to 2 hr. While normals and SA sheets were running, labeled samples are spotted onto a PEI cellulose sheet (D1 development). D1 sheets were run in 80 mL of 2.3 M NaH_2PO_4, pH 5.75, for 16 hr. D1 development removes traces of normal nucleotides after the nuclease P1 treatment, as well as residual orthophosphate by pushing them to the wick at the top of the sheet, leaving the [^{32}P] adducted nucleotides behind. D1 sheets were developed on autoradiographic film, and locations of the spots were then drawn on the back of the PEI-cellulose sheets. Spots were then cutout from the D1 PEI-cellulose sheet and transferred to single PEI-cellulose sheets (2D maps) using a strong magnet. The 2D maps were run vertically in 65 mL of 95% LFU, pH 3.35 + 5% dH_2O (D3 development) to top marked line after being pre-developed in 25 mL of dH_2O to the origin. The 2D maps were then checked for transfer, cut at the second line from the top, washed twice in 250 mL of dH_2O for 7 minutes, dried, and a wick attached to the right side in preparation for the final development (D4 development). For the D4 development, the 2D maps were run horizontally in 65 mL of 90% PTU, pH 8.20 + 10% dH_2O to the top of the wick after being pre-developed in 25 mL of 50% 0.8 M NaH_2PO_4, pH 8.2 + 50% dH_2O to the second line marked from the left side. The 2D maps were then checked for separation, cut just below the wick, washed twice in 250 mL of dH_2O for 5 min, dried, and cut for autoradiographic development and imager reading. An instant imager was used to calculate counts per minute (CPM) per spot. DNA adduct levels were quantified as mean relative adduct labeling (RAL) values $\pm$ SD using the following equation:

> RAL = sample count rate/ (DNA-P x specific activity $_{ATP}$), where the sample count rate is measured in CPM, DNA-P represents the pmol of DNA monomer units assayed per replicate, and the specific activity of the ATP is in units of cpm/pmol.

3.2.6 Genotyping

DNA from whole blood cell samples were genotyped for null deletions in GSTM1 and GSTT1 using the methods described by Bailey et al. (1998). PCR was used to amplify and assay the GSTM1 and GSTT1 alleles. The products were digested with the restriction enzymes *HinfI* and *NcoI*, electrophoresed, and visualized using ethidium bromide staining. The genotyping call rate was about 99.6%.

3.2.7 Statistical Analyses

Statistical analyses were performed using SigmaStat (Systat 2004). Data was available from environmental and human measurements (DNA adducts, genetic polymorphisms, and PAH levels in blood). Values, including PAHs in environmental or biological samples, were normalized by log transformation as needed and arithmetic values used for other variables. Descriptive statistics was performed first. Second, multivariate analyses were performed by using t-test or One Way Analysis of Variance (ANOVA) unless the normality test failed ($P<0.05$). When the normality failed, Mann-Whitney Rank Sum Test or Kruskal-Wallis One Way Analysis of Variance on Ranks was carried out. For bivariate correlation, Pearson's correlation coefficient was determined. The criterion for statistical significance was a P-value < 0.05.

3.3 Results

Dust samples collected from houses located in the study site were chemically characterized for sixty different PAH compounds. These compounds are listed in Table 3.2. They include low and high molecular weight PAHs, in addition to their alkylated homologues. Low molecular weight PAH compounds typically consist of 2- or 3- ring PAHs such as naphthalene (2 benzene rings) and anthracene (3 benzene rings). High molecular weight PAHs generally include compounds with 4 or more rings such as benz[a]anthracene (4 rings) and the model carcinogenic PAH, BaP (5 rings). The chemicals analyzed in this study include carcinogenic PAH congeners (USEPA Class B2) as well as those listed as priority pollutants by the USEPA.

The dust samples collected from residential environments in Shanxi province, included 13 floor dust samples, 8 window dust samples, as well as 2 wall dust samples and 2 samples of light bulb dust. Table 3.3. summarizes the concentrations of PAHs detected in house dust. The data is reported by sample type and divided by carcinogenic, priority and total PAHs, in mass per area sampled. The data indicate the presence of PAHs in residential dust. The total PAH concentration however seems to vary between sample types and was found to range from 19 to 10,093 ng/m^2.

Table 3.2. List of the 60 PAHs quantified in environmental and biological samples. Chemicals listed in **bold** are class B2 carcinogens, whereas those listed in *italics* are USEPA priority PAHs.

List of PAHs Quantified in Environmental and Biological Samples by Number of Rings.

2-Ring PAHs:	C2-Benzothiophene	*Phenanthrene*	C2-Fluoranthenes/Pyrenes	***Benzo(b)fluoranthene***
Decalin	C3-Benzothiophene	C1-Phenanthrene/ Anthracene	C3-Fluoranthenes/Pyrenes	***Benzo(k)fluoranthene***
C1-Decalin	Biphenyl	C2-Phenanthrene/ Anthracene	Naphthobenzothiophene	Benzo(e)pyrene
C2-Decalin	**3-Ring PAHs:**	C3-Phenanthrene/ Anthracene	C1-Naphthobenzothiophene	***Benzo(a)pyrene***
C3-Decalin	*Acenaphthylene*	C4-Phenanthrene/ Anthracene	C2-Naphthobenzothiophene	Perylene
C4-Decalin	*Acenaphthene*	Dibenzothiophene	C3-Naphthobenzothiophene	***Dibenz(a,h)anthracene***
Naphthalene	Dibenzofuran	C1-Dibenzothiophene	***Benz(a)anthracene***	C1-Dibenz(a,h)anthracene
C1-Naphthalenes	*Fluorene*	C2-Dibenzothiophene	***Chrysene***	C2-Dibenz(a,h)anthracene
C2-Naphthalenes	C1-Fluorenes	C3-Dibenzothiophene	C1-Chrysenes	C3-Dibenz(a,h)anthracene
C3-Naphthalenes	C2-Fluorenes	**4-Ring PAHs:**	C2-Chrysenes	**6-Ring PAHs:**
C4-Naphthalenes	C3-Fluorenes	*Fluoranthene*	C3-Chrysenes	***Indeno(1,2,3 c,d)pyrene***
Benzothiophene	Carbazole	*Pyrene*	C4-Chrysenes	*Benzo(g,h,i)perylene*
C1-Benzothiophene	*Anthracene*	C1-Fluoranthenes/Pyrenes	**5-Ring PAHs:**	

Table 3.3. Summary of Concentrations of Total and Carcinogenic PAHs (μg/m^2), as well as USEPA Priority PAHs Detected in Various Dust Samples Collected from Homes in Shanxi, China.

Floor (N=13)		Window (N=8)		Wall (N=2)		Light Bulb (N=2)	
USEPA Priority PAHs		**USEPA Priority PAHs**		**USEPA Priority PAHs**		**USEPA Priority PAHs**	
Mean	632	**Mean**	110	**Mean**	29	**Mean**	162
Median	133	**Median**	36	**Median**	29	**Median**	162
Min	26	**Min**	12	**Min**	17	**Min**	25
Max	4288	**Max**	426	**Max**	42	**Max**	299
Std. Dev.	1181	**Std. Dev.**	144	**Std. Dev.**	17	**Std. Dev.**	193
SEM	328	**SEM**	51	**SEM**	12	**SEM**	137
Carcinogenic PAHs		**Carcinogenic PAHs**		**Carcinogenic PAHs**		**Carcinogenic PAHs**	
Mean	313	**Mean**	38	**Mean**	22	**Mean**	110
Median	53	**Median**	25	**Median**	22	**Median**	110
Min	12	**Min**	5	**Min**	9	**Min**	15
Max	2058	**Max**	107	**Max**	35	**Max**	205
Std. Dev.	574	**Std. Dev.**	35	**Std. Dev.**	18	**Std. Dev.**	134
SEM	159	**SEM**	12	**SEM**	13	**SEM**	95
Total PAHs		**Total PAHs**		**Total PAHs**		**Total PAHs**	
Mean	1482	**Mean**	200	**Mean**	84	**Mean**	412
Median	307	**Median**	84	**Median**	84	**Median**	412
Min	50	**Min**	19	**Min**	45	**Min**	65
Max	10093	**Max**	823	**Max**	123	**Max**	759
Std. Dev.	2794	**Std. Dev.**	271	**Std. Dev.**	55	**Std. Dev.**	491
SEM	775	**SEM**	96	**SEM**	39	**SEM**	347

Carcinogenic PAHs (cPAHs) ranged from 5 to 2,000 ng/m^2. Figure 3.1 represents bar graphs of priority, carcinogenic and total PAHs compared across sampling surfaces. According to the graph, wall dust seems to contain the least amount of PAHs whereas floor is clearly the most abundant in PAHs. Hence, when breaking up PAHs by benzene ring number, results from floor dust was compared to all other sampling surfaces combined (window, wall and light bulb dust). Four-ring PAHs were the most abundant in house dust. The mean concentration of 4-ring PAHs detected in floor dust was 376 ng/m^2, whereas in other surfaces it was 64 ng/m^2. In floor dust, 5-ring PAH were the second most abundant class with a mean concentration of 128 ng/m^2 followed by 3-ring PAHs at 91 ng/m^2. Table 3.4 summarizes the PAH levels in house dust by ring number. Figure 3.2 consists of a graphical representation of the levels of PAH in floor and other surface dust. These data indicate the presence of PAHs in the residential environment of the study population.

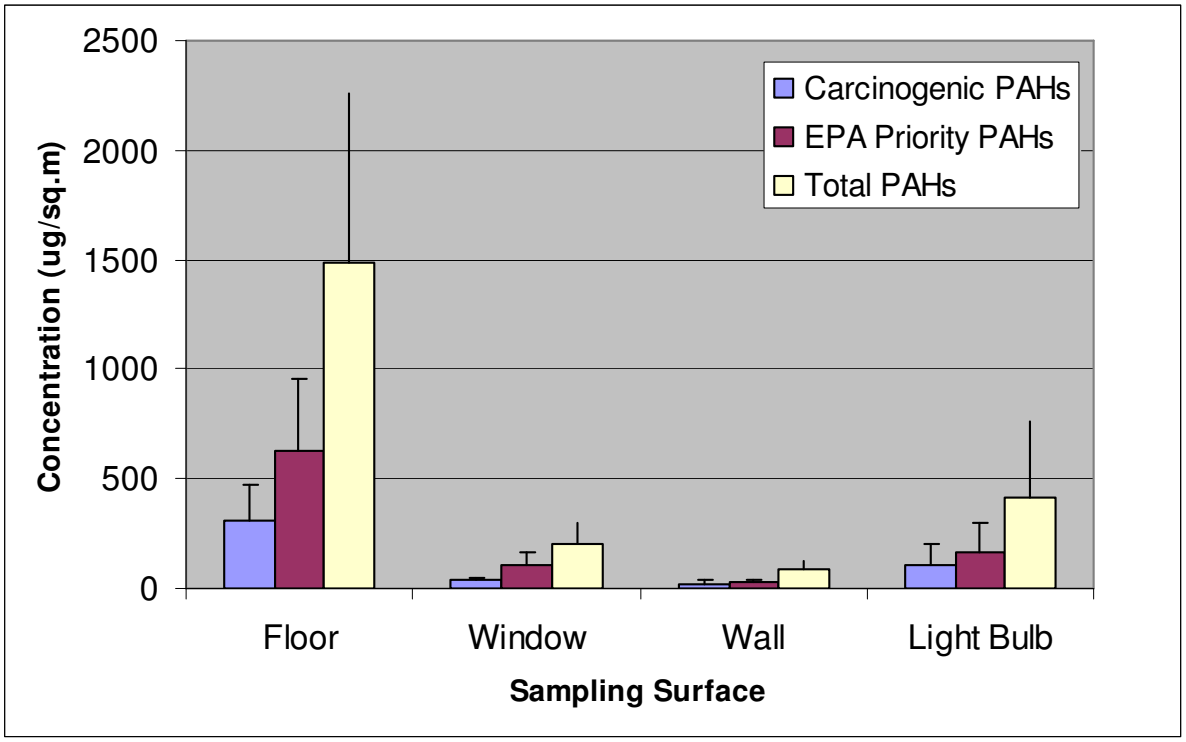

Figure 3.1. Graphical representation of total and carcinogenic PAHs, as well as USEPA priority PAHs detected in various dust samples collected from homes in Shanxi, China.

Table 3.4. Summary of PAHs detected in floor and other dust samples (μg/m^2) collected from homes in Shanxi, China by number of rings.

	Floor			Other		
PAHs	Mean	Std. Dev.	SEM	Mean	Std. Dev.	SEM
2 rings	1	1	0	0	1	0
3 rings	91	182	50	21	42	12
4 rings	376	701	194	64	80	23
5 rings	128	231	64	18	23	7
6 rings	20	40	11	1	2	1

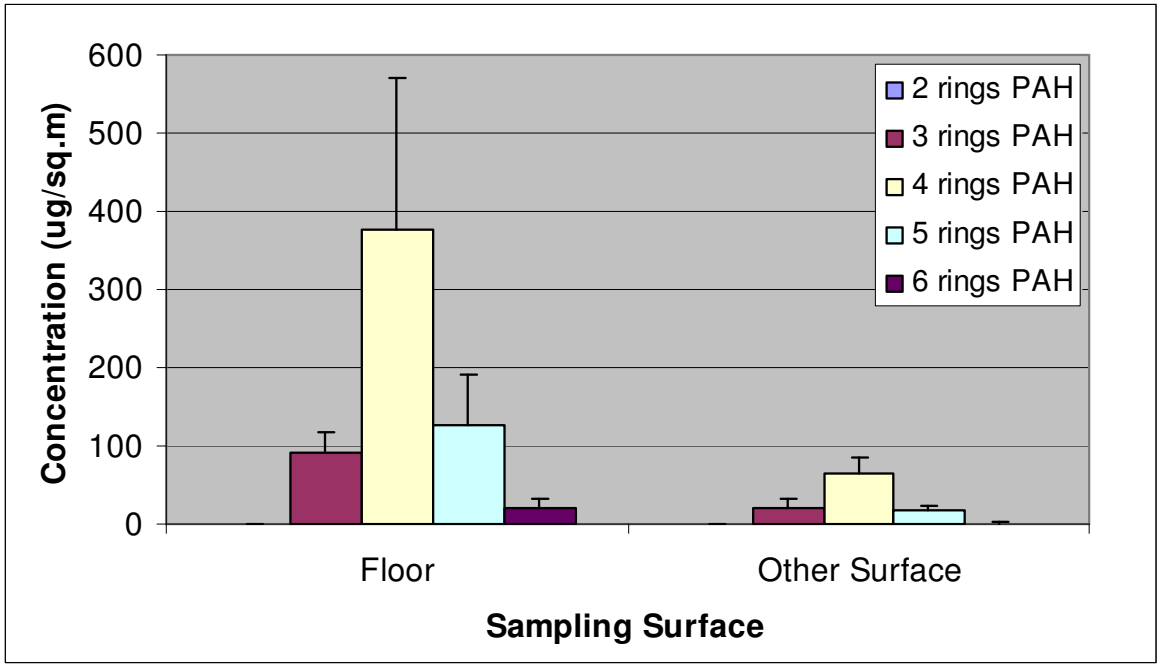

Figure 3.2. Graphical representation of PAHs detected in floor and other dust samples collected from homes in Shanxi, China by number of rings.

Venous blood was collected from 53 mothers that delivered a baby in a participating birth hospital during the time of the study. In addition, 51 of their husbands provided venous blood samples for analysis in this study. Among the mothers, 35 delivered a baby with a neural tube defect and 18 delivered visibly normal babies and were recruited as controls. As for the fathers, 32 were the parents of a baby with a neural tube defect and 19 were fathers of visibly normal children. More characteristics of the adult population that provided venous blood samples are summarized in Table 3.5. As a measure of internal dose, venous blood samples from mothers and fathers were extracted by liquid-liquid extraction and analyzed for the same list of PAHs described previously in Table 3.2. Concentrations of PAHs in mothers and fathers by case status are summarized in Table 3.6. The median level of PAHs detected in venous blood from mothers of cases (232 ng/mL) was significantly higher than that detected in venous blood from control mothers (99 ng/mL). Similar results were found for fathers where case fathers had a median PAH level of 170 ng/mL whereas control father has median PAH level of 130 ng/mL, however the difference was not statistically significant. Figure 3.3 illustrates the difference in total PAH concentration in venous blood of mothers and fathers by case status.

Table 3.5. Characteristics of the adult population that provided venous blood for analysis.

Characteristic	N(Valid Percent)
Sex	
Male	51(49%)
Female	53(51%)
Mothers	
Case	35(66%)
Controls	18(34%)
Fathers	
Case	32(63%)
Controls	19(37%)
Smoking status among fathers	
Yes	38(75%)
No	13(25%)
Passive smoking among mothers	
Exposed	27(51%)
Not Exposed	26(49%)
Mother's occupation	
Farmer and fishery	52(79%)
Other	14(21%)
Mother's highest level of education	
Primary school or lower	15(22%)
Junior high	47(70%)
High school or higher	5(8%)
Father's highest level of education	
Primary school or lower	8(12%)
Junior high	52(78%)
High school or technical secondary school	7(10%)
Mothers cooking in the kitchen	
Almost none	11(16%)
Sometimes	18(27%)
Every day	38(57%)
Major fuel for cooking	
Black coal, Firewood or Natural Gas	17(26%)
Hard coal	48(72%)

Table 3.6. Concentrations of total PAHs (ng/mL) in venous blood of mothers and fathers by case status.

	Mother		Father	
	Case	Control	Case	Control
Min	29	33	52	25
Median	232	99	170	130
Max	762	234	523	345
Mean	258	120	217	159
Std dev	156	69	152	102
SEM	26	16	27	23
Sample size	35	18	32	19
P-value	<0.001		0.25	

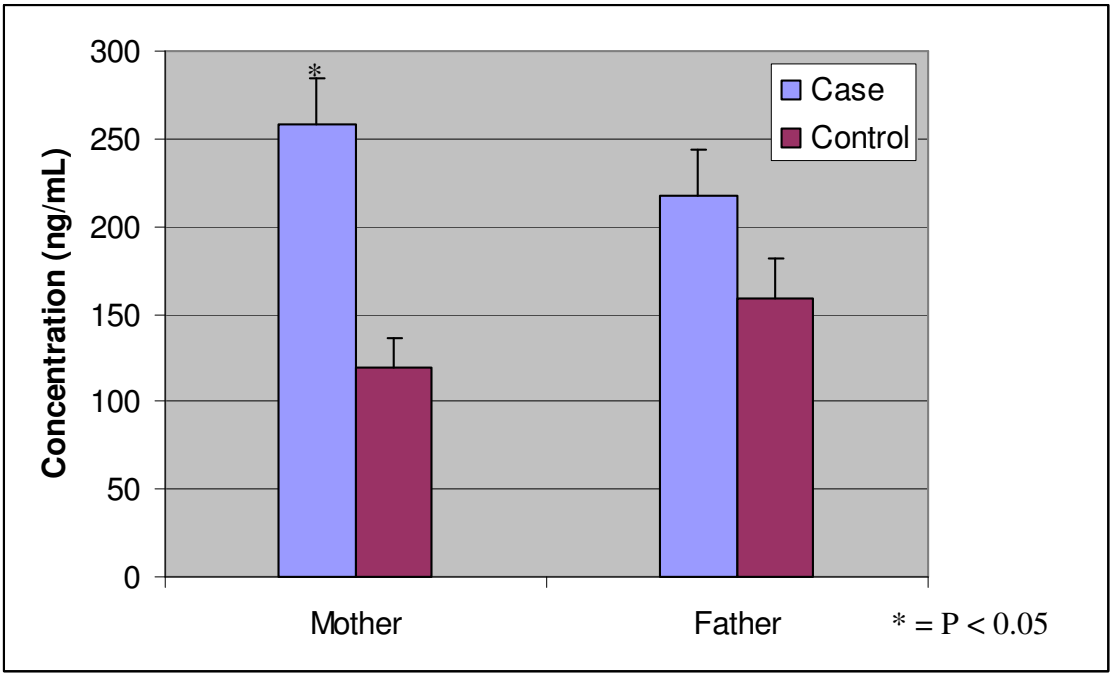

Figure 3.3. Bar graph representing the concentrations of total PAHs detected in venous blood collected from mothers and fathers of neural tube defect cases and controls.

More importantly, cPAHs were detected in venous blood samples from both parents of cases and controls. In addition, cPAHs were significantly more elevated in case mothers (14 ng/mL) when compared to controls mothers (6 ng/mL). Among fathers, those with case children had a median cPAHs level of 10 ng/mL; whereas those with control children had a median cPAHs level of 5 ng/mL. These differences in cPAH levels among case and control fathers were also statistically significant. Table 3.7 and Figure 3.4 summarize the data on carcinogenic PAHs among case and control mothers, as well as case and control fathers.

Table 3.7. Concentrations of carcinogenic PAHs (ng/mL) in venous blood of mothers and fathers by case status.

	Mother		Father	
	Case	Control	Case	Control
Min	0.2	1	1	1
Median	14	6	10	5
Max	65	49	50	48
Mean	19	9	15	10
Std dev	16	11	13	14
SEM	3	3	2	3
Sample size	35	16	31	18
P-value	0.045		0.014	

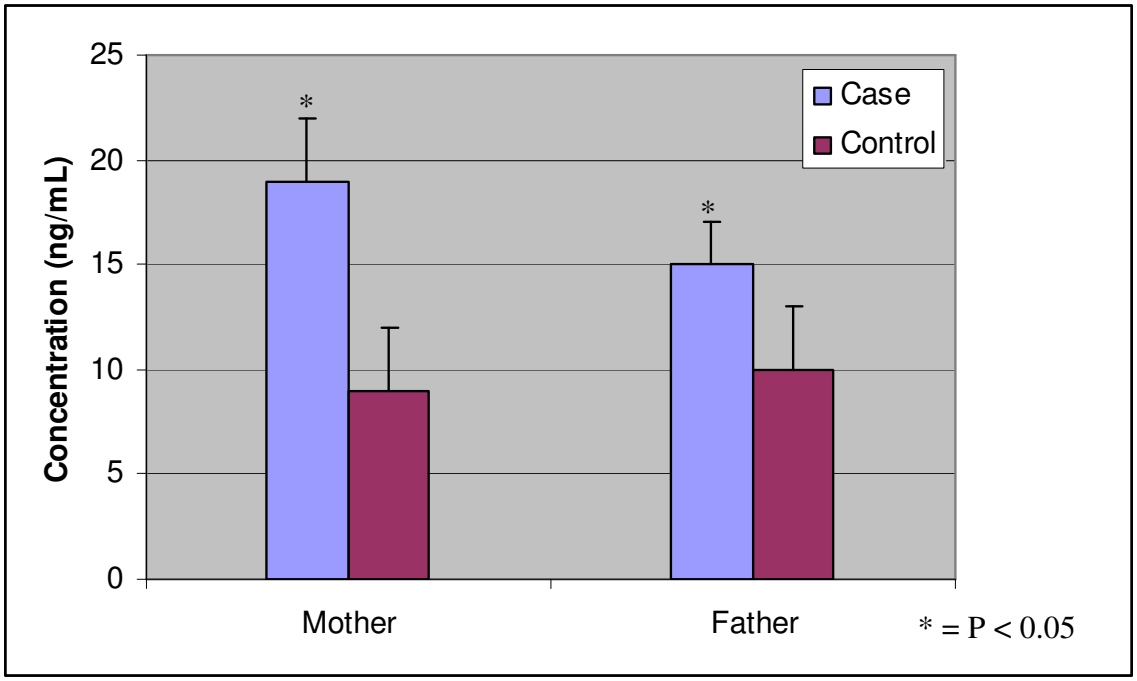

Figure 3.4. Bar graph representing the concentrations of carcinogenic PAHs detected in venous blood collected from mothers and fathers of neural tube defect cases and controls.

Passive smoking was self-reported by mother subjects through the study questionnaire. Table 3.8 reviews the concentration of carcinogenic and total PAHs in mothers exposed to passive smoking (occasionally or everyday) and those who were not exposed. Mothers exposed to passive smoking had median levels of total and cPAHs in their blood of 219 ng/mL and 9 ng/mL respectively. Mothers who were not exposed to passive smoking had median levels of 187 ng/mL total PAHs and 7 ng/mL cPAHs. However, these differences in PAH concentrations were not statistically significant.

Smoking among fathers was very common with seventy-five percent reporting to be smokers. Smokers included subjects who occasionally smoked to those who smoked more than 20 cigarettes per day. Total and carcinogenic PAH were broken up by the smoking status of the fathers to detect any difference in their levels among smokers and non-smokers. Non-smoking fathers had a median level of 272 ng/mL total PAHs in their venous blood compared to 120 ng/mL for smokers, but the difference were not statistically significant. Table 3.9 sums up these results.

Table 3.8. Concentrations of total and carcinogenic PAHs (ng/mL) in venous blood of mothers by exposure to passive smoking.

	Total PAHs		Carcinogenic PAHs	
	Exposed	Not Exposed	Exposed	Not Exposed
Min	33	29	1	0.2
Median	219	187	9	7
Max	762	533	65	51
Mean	233	188	16	15
Std dev	176	110	16	15
SEM	34	22	3	3
Sample size	27	26	25	25
P-value	0.493		0.831	

Table 3.9. Concentrations of total and carcinogenic PAHs (ng/mL) in venous blood of father by smoking status.

	Total PAHs		Carcinogenic PAHs	
	Smoker	Non-Smoker	Smoker	Non-Smoker
Min	25	52	1	2
Median	120	272	8	9
Max	523	441	50	41
Mean	182	236	12	13
Std dev	135	143	13	12
SEM	22	40	2	4
Sample size	38	13	38	11
P-value	0.430		0.606	

In addition to measuring levels of PAHs in venous blood, white blood cells (WBCs) were isolated from blood to be used for DNA extraction. Once isolated, bulky DNA adducts were analyzed by ^{32}P-postlabeling. Control mothers and control fathers exhibited a significantly higher level of DNA adducts as compared to case mothers and case fathers. Table 3.10 summarizes the DNA adduct levels detected in WBCs of mothers and fathers by case status. Median level of DNA adducts in case mothers was 11 per 10^9 normal nucleotides as compared to 16 per 10^9 nucleotides in controls. Similarly, median level of DNA adducts in case fathers was 10 per 10^9 nucleotides which was significantly lower than 19 per 10^9 nucleotides, the median levels of bulky DNA adducts in control fathers. Smoking status did not seem to be associated with DNA adduct levels in this study. Table 3.11 reviews the levels of DNA adducts by passive smoking status in mothers and smoking status in fathers. Figures 3.5 and 3.6 demonstrate representative autoradiograms of smoker and non-smoker as well as case and control subjects.

Table 3.10. Bulky DNA adduct levels (per 10^9 nucleotides) in venous blood of mothers and fathers by case status.

	Mother		Father	
	Case	Control	Case	Control
Min	4	8	4	6
Median	11	16	10	19
Max	22	32	23	55
Mean	12	17	11	21
Std dev	4	7	5	12
SEM	1	2	1	3
Sample size	33	16	28	16
P-value	0.013		0.001	

Table 3.11. Bulky DNA adduct levels (per 10^9 nucleotides) in venous blood of mothers and fathers by smoking status.

	Mother		Father	
	Passive Smoker	Not Passive Smoker	Smoker	Non-Smoker
Min	8	4	4	4
Median	12	13	12	15
Max	26	32	27	55
Mean	13	14	13	17
Std dev	5	7	6	14
SEM	1	1	1	4
Sample size	24	25	31	13
P-value	0.565		0.681	

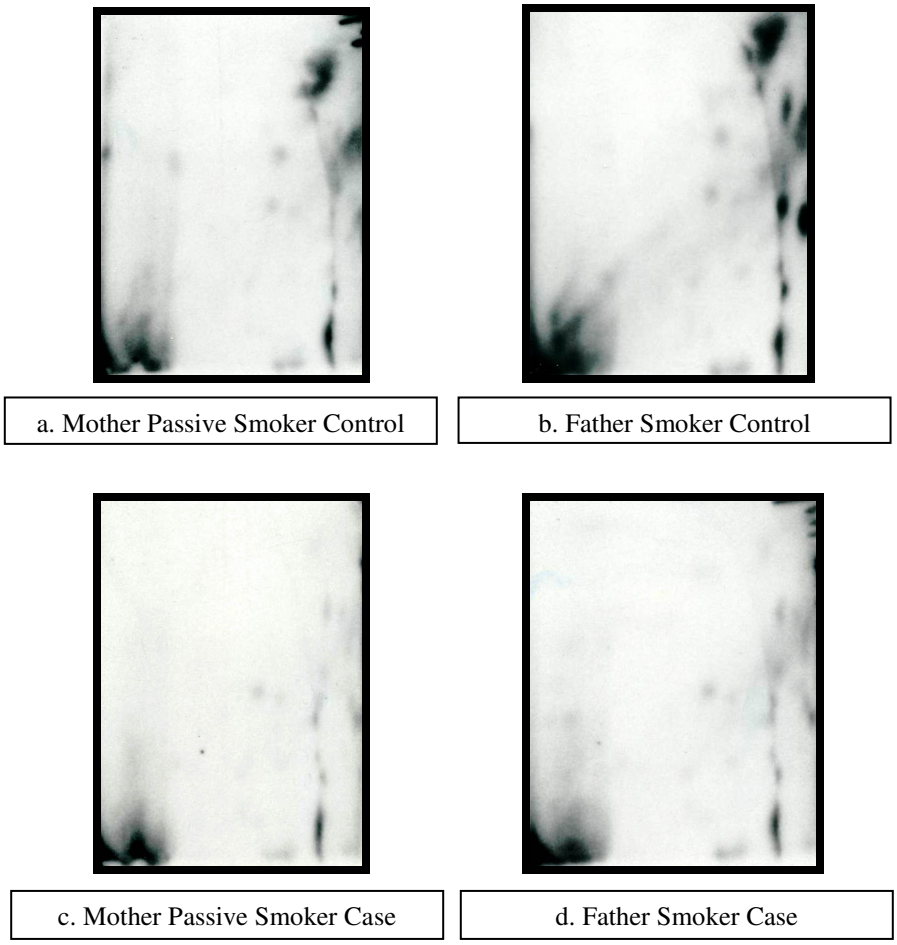

Figure 3.5. Autoradiograms of ^{32}P-postlabeled WBC bulky DNA adducts from smoker subjects. Autoradiography for 24 hr. at -80^{o}C using Kodak XAR-5 film.

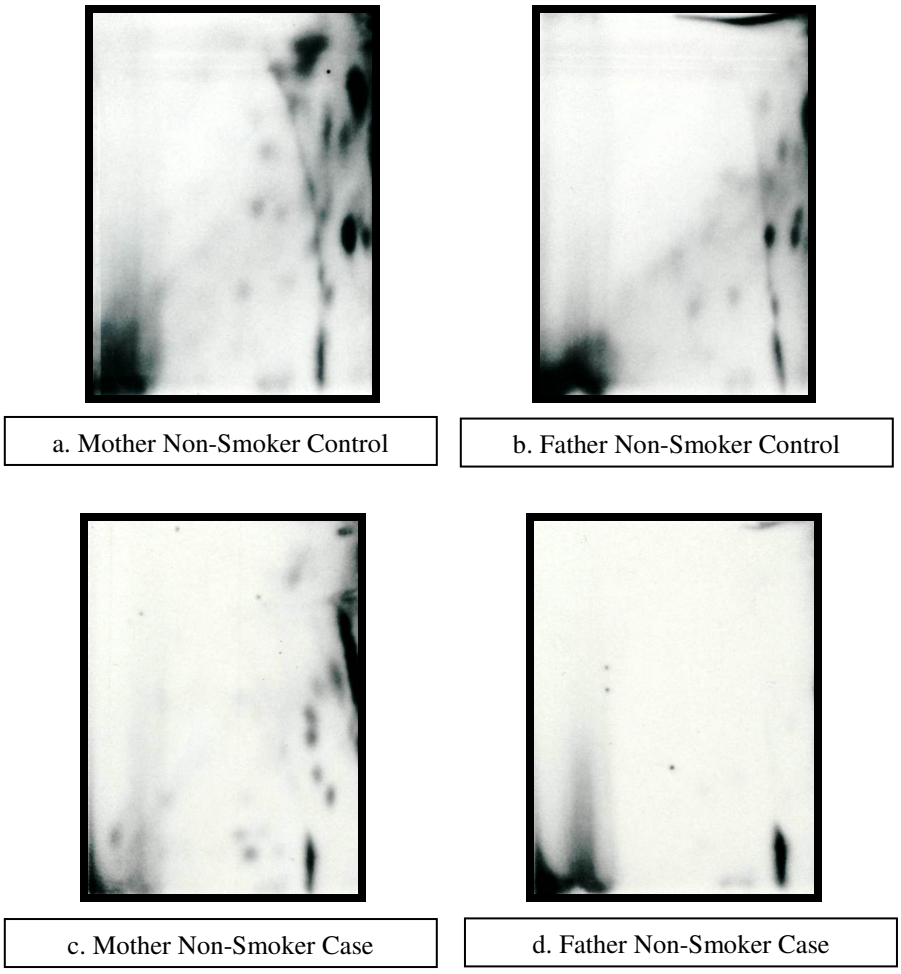

Figure 3.6. Autoradiograms of ^{32}P-postlabeled WBC bulky DNA adducts from non-smoker subjects. Autoradiography for 24 hr. at -80^{o}C using Kodak XAR-5 film.

Interestingly, among the variables that significantly affected the concentrations of DNA adducts was the type of fuel used for cooking. Mothers reported using different types of cooking fuel including hard coal, black coal, firewood and natural gas. Hard coal seems to be the most abundantly used cooking fuel. When DNA adduct levels in WBCs of mothers who used hard coal as a cooking fuel were compared to those who use another type of cooking fuel, they seemed significantly elevated. In fact, median levels of DNA adducts in WBCs of mothers using hard coal were 14 per 10^9 nucleotides which was significantly elevated compared to 10 adducts per 10^9 nucleotides, the median level found in WBCs of mothers using other types of cooking fuel. These data are summarized in Table 3.12 and graphically presented in Figure 3.7.

Table 3.12. Bulky DNA Adduct Levels (per 10^9 nucleotides) in Venous Blood of Mothers by Cooking Fuel Type.

	Hard Coal	Other types
Min	7	4
Median	14	10
Max	32	22
Mean	15	11
Std dev	6	4
SEM	1	1
Sample size	34	14
P-value	0.022	

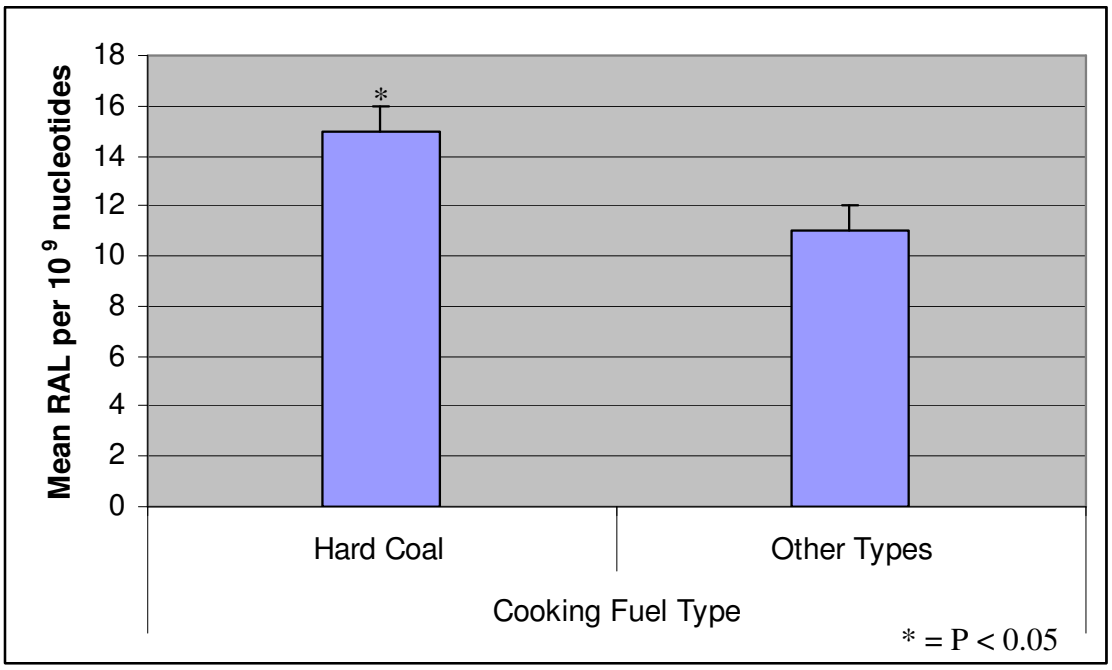

Figure 3.7. Bar graph representing bulky DNA adduct levels in venous blood collected from mothers by cooking fuel type.

Genotyping of two major Phase II metabolic enzymes, GSTM1 and GSTT1, revealed that the GSTM1 deletion occurred in 26% of mothers and 19% of fathers; whereas GSTT1 null genotype was found in 19% of the mothers and 19% of the fathers (Table 3.13). GSTM1 null mothers had a median level of DNA adducts of 11 per 10^9 nucleotides compared to 13 per 10^9 nucleotides in wild-type mothers. Fathers on the other hand had median levels of 14 adducts per 10^9 nucleotides in null individuals and 12 adducts per 10^9 nucleotides in individuals with an active copy of GSTM1. However, the difference in median levels of DNA adducts were not statistically significant (Table 3.14). Mothers with an inactive copy of GSTT1 exhibited a median level of 11 adducts per 10^9 nucleotides, compared to 13 adducts per 10^9 nucleotides in wild-type individuals. Fathers with an inactive copy of GSTT1 were found to exhibit a median level of 15 adducts per 10^9 nucleotides in their WBCs, as compared to a median level of 11 adducts per 10^9 nucleotides for those with a functional GSTT1 enzyme. Similarly to GSTM1

results, difference in DNA adduct levels among different GSTT1 genotypes were not statistically significant (Table 3.15).

Table 3.13. Prevalence of GSTT1 and GSTM1 Deletion in Recruited Subjects.

Genotype	N(Valid Percent)
GSTT1 - Mother	
Wildtype	27(59%)
Null	19(41%)
GSTT1 - Father	
Wildtype	25(57%)
Null	19(43%)
GSTM1 - Mother	
Wildtype	20(44%)
Null	26(56%)
GSTM1 - Father	
Wildtype	25(57%)
Null	19(43%)

Table 3.14. Bulky DNA adduct levels in venous blood of mothers and fathers by GSTM1 genotype.

	Mother		Father	
	GSTM1		GSTM1	
	Null	Wild-type	Null	Wild-type
Min	4	8	8	6
Median	11	13	14	12
Max	32	32	55	36
Mean	13	15	16	15
Std dev	6	6	11	8
SEM	1	1	3	2
Sample size	23	19	18	19
P-value	0.266		0.727	

Table 3.15. Bulky DNA adduct levels in venous blood of mothers and fathers by GSTT1 genotype.

	Mother		Father	
	GSTT1		GSTT1	
	Null	Wild-type	Null	Wild-type
Min	4	8	6	6
Median	11	13	15	11
Max	32	32	55	36
Mean	14	14	19	13
Std dev	7	6	12	7
SEM	2	1	3	2
Sample size	16	26	15	22
P-value	0.990		0.080	

In an attempt to find if exposures in mothers and fathers are correlated, a scatter plot was generated with total PAH levels in blood from fathers versus those from mothers (Figure 3.8). The computed correlation coefficient (R) was 0.4 and the correlation was statistically significant (P=0.01). Additionally, the levels of blood PAHs in non-smoking fathers were plotted against the levels of total PAH in mothers. The correlation seemed to be stronger (R=0.7) and still statistically significant (P<0.01) (Figure 3.9).

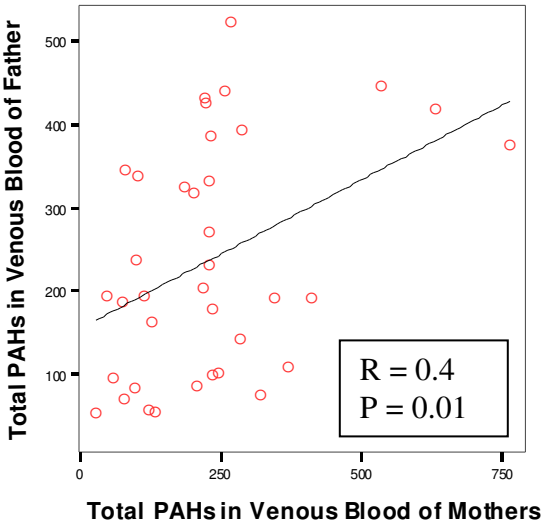

Figure 3.8. Scatter plot, total PAHs in venous blood from fathers versus total PAHs in venous blood from mothers.

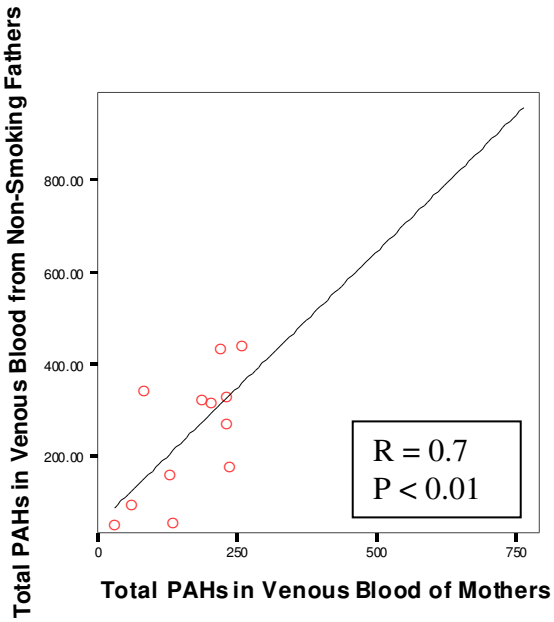

Figure 3.9. Scatter plot, total PAHs in venous blood from non-smoking fathers versus total PAHs in venous blood from mothers.

3.4 Discussion

This research followed the biomarkers paradigm linking chemical exposures to adverse health outcomes (NRC, 1987). This paradigm describes the intermediate stages following chemical exposures, where the chemical or its metabolite can be monitored by measuring biochemical changes that occur in the exposed organism. This paradigm typically consists of four major phases leading to the onset of the undesirable effect. These consist of external exposure, internal dose, biologically effective dose and early biological effects. The human study portion of this dissertation described in chapters III and IV measured external exposure, internal dose as well as biologically effective dose in an attempt to understand the potential link between PAHs and birth defects.

External exposure consisted of measuring chemical levels in homes located in the study area of Shanxi, China. House dust was analyzed for PAHs to assess their abundance inside living places in the study province. Results indicated that the concentration of PAHs was elevated. The detected levels of PAHs were 10-fold higher than levels detected in the homes of other environmentally devastated study populations such as for example Sumgayit, Azerbaijan (data not shown). Floor dust exhibited the highest levels of PAHs compared to other sampling surfaces such as window, wall or light bulbs. In addition, the PAHs detected in floor dust were mostly high molecular weight compounds such as BaP which are typically more potent than their lower molecular weight congeners. Floor dust therefore presents a potential threat to the health of individuals living in this area, especially children who have behavioral and pharmacokinetic characteristics that might lead to higher exposures than adults.

As a measure of internal dose, venous blood samples from parents of NTD cases and control were analyzed for PAHs. Levels of PAHs in venous blood typically reflect more recent exposures. Interestingly, total PAH levels in parents of cases were higher than those of controls. The differences were striking in venous blood samples collected from mothers.

DNA adducts were measured as biomarkers of early biological effects and surprisingly were significantly higher in controls when compared to NTD cases.

Smoking in fathers and passive smoking in mothers did not seem to have a significant effect on the levels of PAHs or DNA adducts in blood.

Deletions in two major Phase II metabolic enzymes GSTM1 and GSTT1 were evaluated as a measure of genetic sensitivity in this population. However, individuals with inactive copies of GSTM1 or GSTT1 did not seem to exhibit significantly higher levels of DNA adducts.

Among the variables that significantly affected DNA adduct levels in venous blood of mothers was the type of fuel used for cooking. Mothers who used hard coal seem to have higher levels of DNA adducts in their blood when compared to those using a different type of cooking fuel. Solid fuel for cooking was previously associated with an increased risk of lung cancer in Europe (Lissowska et al. 2005). In China, cooking frequency was associated with higher levels of urinary 1-hydroxypyrene in females as well as males (Chen et al. 2007). These findings suggest a common environmental source of exposure in males and females. The strong correlation between total PAHs in blood of non-smoking fathers and mothers could be another indication of a common source of PAH exposures.

Overall, these data indicate an elevated PAH exposure in the residential environment of the study population. Presence of genotoxic PAH compounds was confirmed in the environment as well as biological tissues from the study participants. Major limitations to this study include sample size as well as genetic polymorphism data. Polymorphisms in additional metabolic genes (such as Phase I metabolic enzymes) as well as DNA repair genes needed to be evaluated in the future to help identify genetic sensitivities that might help explain the relationship between internal dose of PAHs and bulky DNA adducts.

CHAPTER IV
BIOLOGICAL INDICATORS OF EXPOSURE TO COMBUSTION BY-PRODUCTS IN PLACENTAL TISSUE

4.1 Introduction

The placenta is considered an important source of materials for molecular epidemiologic studies because it is readily available and responsive following maternal exposure to environmental pollutants (Marafie et al. 2000). Through placental transfer, all lipid-soluble xenobiotics potentially enter the conceptus. The factors influencing the rate of the xenobiotic transfer include placental membrane permeability, placental blood flow, and pH differences between the maternal and fetal circulations as well as molecular size, lipid solubility and protein binding properties of the compound itself. Exposure of the embryo or fetus to xenobiotic compounds might lead to deleterious effects including *in utero* death, initiation of birth defects, and production of functional abnormalities. The fetus is potentially very sensitive to environmental air pollution exposures due to high rates of cell proliferation, greater number of cells at risk, lower immunologic competence and decreased capacity to metabolize carcinogens and repair DNA.

Experimental evidence indicates that the placenta contains a mixed function oxidase (MFO) system induced by maternal smoking or exposure to other xenobiotics (Everson et al. 1986; Juchau et al. 1978; Manchester et al. 1988; Vaught et al. 1979). Drug metabolizing enzymes such as CYP1A1, 1B1, 2C8, 2D6, 2E1, 3A4, 3A5, 3A7 are also present in the fetal liver after the embryonic phase (8 to 9 weeks of gestation) (Hakkola et al. 1998). A growing number of studies demonstrate relationships between DNA adduct levels in placenta, exposure to environmental PAHs or tobacco smoke and the activity of cytochrome P450s (Everson et al. 1987; Everson et al. 1988; Everson et al. 1986; Hansen et al. 1993; Lagueux et al. 1999; Topinka et al. 1997; Whyatt et al. 1998).

Maternal exposure to chemicals could not only compromise placental development and exchange but also the hormonal signaling necessary for the continued growth of the fetus *in utero* (Miller et al. 2004). The timing of the exposure to hazardous chemicals is critical in determining the effects on the fetus. Sensitive periods of development exist during gestation due to higher rates of cell proliferation or changing metabolic capabilities (Moore and Persaud 2003). For neural tube defects (NTDs) this window is the first 28 days of gestation. NTDs especially spina bifida has a strong environmental component in its etiology coupled with a possible genetic predisposition in some cases (Thorogood 1997). While less investigated, paternal exposures to chemicals can possibly induce direct genetic damage to male germ cells transmitted to the offspring (Chia and Shi 2002; Selevan et al. 2000; Sram et al. 1999). A recent study suggest that semen quality indicators such as sperms total motility, forward progression, functional test, kinetics were lower in men employed at motorway toll gates compared to controls (De Rosa et al. 2003). Table 4.1 summarizes the established and suspected risk factors for NTDs.

Epidemiological studies revealed that high levels of ambient air pollution increase the risk of morbidity and mortality and cause higher incidence of respiratory and cardiovascular diseases and lung cancer. Emerging evidence indicates that air pollution is also associated with elevated risk of adverse pregnancy outcomes (Glinianaia et al. 2004; Maisonet et al. 2004). Potential teratogens in tobacco smoking include PAHs, metals, formaldehyde and others. Air pollution was linked to childhood mortality, low birth weight (LBW), premature birth, intra uterine growth retardation (IUGR), and birth defects (Sram et al. 2005). The association of ambient air pollution and birth defects was described in one report only. Ritz et al. (2002) evaluated the effects air pollutants such as carbon monoxide (CO), nitrogen dioxide (NO_2), ozone (O_3), and particulate matter less than 10 μm in diameter (PM_{10}) on occurrence of birth defects in Southern California between 1987 and 1993. The average monthly exposure for each pregnancy was estimated from ambient monitoring stations. The study was inconclusive for PM_{10}. Certain fetal heart phenotypes were susceptible to the adverse effects of two

ambient pollutants CO and O_3. Carbon monoxide was associated (during the second month of gestation) with ventricular septal defects and O_3 exposure with aortic artery and valve defects.

Biological indicators of exposure, effect and susceptibility provide a useful tool to evaluate chemical exposures on the placenta and determine their effects on the fetus. Reports of DNA adduct detection in human placenta of smoking women were published as early as the 1980s (Everson et al. 1986). Earlier studies demonstrated that DNA adducts can be detected in cord blood and other fetal tissues (Hatch et al. 1990). Studies have suggested that PAH-DNA adducts are positively associated to the risk of IUGR (Dejmek et al. 2000; Sram et al. 1999), decreased birth weight and length and head circumference (Perera et al. 1999; Perera et al. 1998). Exposure to particulate matter early in the pregnancy was associated with IUGR or fetal growth retardation leading to low birth weight (Dejmek et al. 1999). After evaluating a larger population, it was found that carcinogenic PAHs are responsible for the fetal growth retardation. First gestational month was the critical exposure window for fetal growth retardation (Dejmek et al. 1999). IUGR positively related to the level of DNA-PAH adducts in placenta and DNA adduct levels depended on metabolic genotypes (Sram et al. 1999; Topinka et al. 1997; Topinka et al. 1997). Low birth weight was associated with increased fetal, neonatal, and infant mortality (Arias et al. 2003; Rees et al. 1996; Seeds and Peng 2000), subsequent poorer health and delayed physical and cognitive development (Barker 1996; Dietz 1994; Matte et al. 2001; Rice and Barone 2000; Richards et al. 2002), and increased susceptibility to stress in adulthood (Nilsson et al. 2001) as well as an increase in risk of type II diabetes, hypertension, and coronary heart disease during adulthood (Barker et al. 2002).

In animal studies, BaP impacted fertility (Kristensen et al. 1995), body weight (Bui et al. 1986), incidence of malformation (Legraverend et al. 1984), immunologic effects (Rodriguez et al. 1999), sexual behavior (Csaba et al. 1993) and transplacental carcinogenesis (Anderson et al. 1995). Prenatal exposure of rats to 25-100 $\mu g/m^3$ BaP

Table 4.1. Established and suspected environmental risk factors for NTDs.

Variable	Relative Risk	Selected References
Established Risk Factors		
Inadequate maternal intake of folic acid	2 - 8	(Wald 1993)
Pre-gestational maternal diabetes	2-10	(McLeod and Ray 2002)
Anticonvulsants (Valproic Acid and carbamazepine)	3-4	(Lammer et al. 1987; Matalon et al. 2002)
Suspected Risk Factors		
Maternal Obesity	1.5-3.5	(Waller et al. 1994; Watkins et al. 2003)
Maternal Hyperthermia	2	(Chambers et al. 1998; Shaw et al. 1998)
Maternal Vitamin B12 Status	3	(Ray and Blom 2003; Suarez et al. 2003)
Maternal Diarrhea	3-4	(Felkner et al. 2003)
Isotretinoin	Unknown	(Rothman et al. 1995)
Gestational Diabetes	Unknown	(Janssen et al. 1996; Sheffield et al. 2002)
Fumonisins	Unknown	(Hendricks 1999; Sadler et al. 2002)
Agent Orange (paternal exposure)	Unknown	(IOM 1994)
Chlorination Disinfection by-products	Unknown	(Dodds and King 2001; Klotz and Pyrch 1999)
Electromagnetic Fields	Unknown	(Blaasaas et al. 2002)
Pesticides	Unknown	(Shaw et al. 1999)
Proximity to Hazardous Waste Sites	Unknown	(Dolk et al. 1998; Orr et al. 2002)

through maternal inhalation significantly decreased the fetal survival rate and birth weight in a dose-dependant manner (Archibong et al. 2002).

Reported mechanisms by which PAHs can affect *in utero* fetal development include binding of PAHs to AhR receptors causes anti-estrogenic activity through increased metabolism and depletion of endogenous estrogens (Carpenter et al. 2002) thus disrupting the endocrine system by altering steroid function. In addition, DNA damage resulting in activation of apoptotic pathways (Nicol et al. 1995) or binding to receptors for placental growth factors resulting in a decreased exchange of oxygen and nutrients available (Dejmek et al. 2000) may induce fetal toxicity or interference with transcription, DNA replication, or protein synthesis (Bostrom et al. 2002).

Total bulky DNA adduct levels in placenta samples collected from mothers living in two regions of the Czech Republic with different pollution levels was significantly higher in the most polluted area (2.12 ± 1.46 per 10^8 nucleotides compared to 1.48 ± 1.09 per 10^8 nucleotides). Elevated DNA adduct levels were also found in smoking mothers (10 or more cigarettes per day) by comparison with non-smoking mothers (3.21 ± 1.39 versus 1.32 ± 0.88 adducts per 10^8 nucleotides). Higher DNA adduct levels were detected in the individuals with GSTM1 null genotype by comparison with GSTM1 wild-type genotype (2.05 ± 1.30 versus 1.66 ± 1.39 adducts per 10^8 nucleotides). Seasonal variation in DNA adduct levels was noted only in GSTM1 null genotype (Topinka et al. 1997).

Epidemiological case-control studies in Southern California showed that increased risk of low birth weight and premature birth associated with increases in ambient air pollution related to traffic and the resulting petroleum combustion products emitted from vehicles residential proximity to heavy traffic roadways influenced the occurrence of low birth weight and/or pre-term birth in infants in Los Angeles County between 1994 and 1996. Exposure to traffic-related air pollution was estimated using a distance-weighted traffic density measure that includes residential proximity and traffic levels on roadways surrounding homes. Relative risk for pre-term birth was reported to be 1.08 (95%CI= 1.01-1.15) for infants in the highest distance-weighted traffic density

quintile (Ritz et al. 2002; Wilhelm and Ritz 2003). Mean adduct levels in human placenta and umbilical cord vessels were two-fold higher in smokers (43 ± 11 per 10^9 nucleotides) as compared to non-smokers (20 ± 4 per 10^9 nucleotides) (Hansen et al. 1992).

This text reports on a study to evaluate biomarkers of exposure and early genetic damage by PAHs in placenta samples collected from NTD cases and controls in the Chinese province of Shanxi. Polymorphims of two major Phase II metabolic enzymes (GSTM1, GSTT1) and six DNA repair genes (XRCC1, OGG1, MGMT, ERCC2, APEX1, XRCC3) were also measured to evaluated genetic sensitivities.

4.2 Materials and Methods

4.2.1 Subject Recruitment

Children and parents of children born with NTDs as well as children and parents of children from a matched control population were recruited for the study from four county birth hospitals. The types of congenital malformations that were studied were selected based on evidence suggesting an environmental component in their etiology. Neural tube defects, including anencephaly and spina bifida, are readily recognizable at birth based on a routine newborn physical examination. All spontaneous abortions, late fetal deaths (stillbirths), and live births occurring in four hospitals in the Shanxi region (including Tai Yuan) in northern China were recruited for the study. Subject recruitment was facilitated by the presence of a birth defects surveillance system in China since 1992 as described by Li et al. (2003). The consent form, questionnaire and all study protocols were reviewed and approved by the Texas A&M University Institutional Review Board (IRB) prior to initiation of the study. A copy of the Texas A&M University IRB approved protocols (No. 2003-0430) for this research is attached to this document. Each hospital received training from project staff, as well as a protocol book that was translated into Chinese.

4.2.2.1 Case Ascertainment

Babies with NTDs were identified at the time of birth by the attending physician. Birth attendants were trained to perform a standardized assessment of the infant immediately after delivery. Procedures were established at each of the participating hospitals for a standardized examination of all babies, both live births and late fetal deaths. Information on malformations of any type was entered on the reporting form. When a baby was born with an NTD a special case reporting form was completed and a study coordinator was contacted and informed of the birth of a potential "case." In cases of fetal or neonatal deaths, procedures were established with the hospital to retain the remains in the pathology department until the diagnosis can be confirmed. If the diagnosis was confirmed the birth was entered into the registry and the mother contacted to participate in the case-control study.

4.2.2.2 Case Mother Enrollment

When the physician verified the diagnosis of the appropriate congenital malformation, they confirmed with the hospital staff that it is acceptable to contact the mother. Whenever possible, the mother was contacted while in the hospital and the study was described to her. The details of the study were presented and, if it is considered appropriate by the physician, informed consent for participation was obtained and the questionnaire was administered. The mother was asked to sign an informed consent form (in Chinese) and to complete the study interview at that time or another time before discharge. If this was not possible, information on how to contact her after discharge was obtained and a possible timeframe for the interview was determined.

4.2.2.3 Control Selection

One control was selected for each case from among the births at the same hospital where the case was born. Each month of the study, one additional control was recruited from each birth hospital. Controls were identified from the hospital's birth registry records, selecting births immediately following the case, or when case subjects

were born preterm a case was selected from children born at the approximate date for a full term delivery. The babies' records were reviewed for the presence of congenital malformations. If there was any record of the baby having a congenital malformation, of any type, the next birth from the birth records was selected and reviewed.

4.2.2.4 Study Questionnaire

Case and control mothers who provided informed consent to enroll in the study were interviewed in-person using a standardized questionnaire that was translated to Chinese. The questionnaire was designed to ask about potential risk factors and confounders that are relevant to the circumstances in China. The questionnaire was based on the one developed by the Centers for Disease Control and Prevention and the California State Birth Defects Surveillance programs. This questionnaire is currently being used in a national collaborative case-control study of congenital malformations in the United States. It included questions on topics such as: occupation, chronic and acute illnesses, smoking habits, nutrition and alcohol use, prescription and non-prescription drug use, socio-demographic information, a complete reproductive history along with a family history of birth defects or genetic diseases, and a maternal residential history relative to the study pregnancy. The questions were time specific, asking about these factors for the period three months prior to pregnancy and during pregnancy, by trimester. Questions about the father of the subject included inquiries about occupation, race and ethnicity, age, smoking habits, and alcohol and drug use. Questions related to history of congenital malformations and genetic diseases in either the mother's or the father's family and about consanguinity were also included. The questionnaire was developed in English with modifications appropriate for Chinese culture. The questionnaire was reviewed by an individual outside of the project for consistency with the English version and was approved by the Texas A&M University Institutional Review Board. A copy of the questionnaire is attached as appendix I.

4.2.2 Placenta Samples Collection

Placental tissue samples corresponding to a wet weight of 20 g each were collected postpartum from the fetal side. Tissue sections were taken from all parts of the placenta to be representative of the whole placenta. Excess blood was drained and the tissue sample was rinsed with 30 ml of sterile saline solution. Tissue samples were placed in sterile 50 ml polypropylene centrifuge tubes (BD Falcon, BD Biosciences, San Jose, CA) on dry ice.

4.2.3 Extraction of Solid Tissue Samples

Prior to extraction, pre-weighed aliquots of placenta samples were lyophilized in a FreeZone 12 Liter Console Freeze Dry System (Labconco, Kansas City, MO) at -50^{o}C and 0.012 mBar which is the setting specific for human tissues. Three days later, dried placenta samples were ground using pre-cleaned glass mortars and pestles pre-rinsed in ethanol. Dried placenta samples are weighed and submitted for extraction.

This standard operating procedure is based on EPA SW-846 Method 3545, and provides an accurate and precise method for extraction, isolation, and concentration of selected organic compounds from solid samples. Final extracts were used in the quantitative determination of aromatic hydrocarbons by chromatographic procedures. An automated extraction apparatus (Dionex ASE200 Accelerated Solvent Extractor) was used to extract various organics from 2 to 15 g of pre-dried samples. The extractions were performed using dichloromethane solvent inside stainless-steel extraction cells held at elevated temperature and solvent pressure. The extracts dissolved in the solvent were then transferred from the heated extraction cells to glass collection vials containing activated copper granules to minimize matrix interference during quantitative determinations. Extracts were then concentrated to a final volume of 1 mL and transferred to pre-weighed sterile glass culture tubes with teflon-lined caps, dried under a stream of nitrogen, reweighed using an evaporative solvent reduction apparatus (Zymark TurboVap II) and stored at 4°C.

4.2.4 Clean-up of Placenta Samples

4.2.4.1 Glassware and Preparation of Sodium Sulfate and Alumina

Glassware was thoroughly cleaned with a detergent solution and rinsed with water. Glassware openings were rinsed with clean aluminum foil and the glassware was combusted at 400°C for a minimum of 4 hr. Glassware not suitable for combustion (such as volumetric glassware) was rinsed 3 times with acetone to remove water, then rinsed 3 times with dichloromethane. Glassware was sealed with solvent-rinsed aluminum foil. All glassware was stored in a clean environment to prevent the accumulation of dust and other contaminants.

Sodium sulfate was purified by combustion in a pre-cleaned shallow aluminum pan at 400°C for at least 4 hours. The sodium sulfate was then stored in an oven at 120°C until use. Immediately prior to use, sodium sulfate was placed in a desiccator to cool.

Alumina was purified by combustion in a pre-cleaned shallow aluminum pan at 400°C for at least 4 hours and stored in an oven at 120°C until use. Immediately prior to use, alumina was placed in a desiccator to cool. Silica gel was purified by placing it in a suitable glass container in an oven at 170°C for at least 16 hr. and stored in an oven at 170°C until use. Immediately prior to use, silica gel was placed in a desiccator to cool.

4.2.4.2 Silica-gel/Alumina Column Cleanup

Extracts were processed through a silica-gel/alumina column cleanup and then an HPLC cleanup to remove interfering lipid compounds. It is important that the laboratory temperature is below 27°C in order to keep the reagents in the columns from separating. Prior to use, the sodium sulfate, alumina and silica gel were removed from the oven, placed in a desiccator and allowed to cool. To deactivate the alumina prior to use, alumina (10 g per chromatography column including one extra) was weighed into a combusted glass jar with a Teflon-lined screw cap and 1% (w/w) reagent water was added to the alumina. The jar was placed on the tumbler and mixed for a minimum of 1 hour. The deactivated alumina was used the same day it was prepared.

To deactivate silica gel prior to use, silica gel (20 g per chromatography column including one extra) was weighed into a combusted glass jar with a Teflon-lined screw cap and 5% (w/w) reagent water was added to the silica gel. The jar was placed on the tumbler and mixed for a minimum of 1 hour. Deactivated silica gel was used the day it was prepared.

A glass wool plug was added into the bottom of a clean solvent rinsed liquid-chromatography column and packed down using a clean solvent rinsed glass or stainless steel rod. A beaker was placed beneath the column opening to catch waste effluent. The column was rinsed three times with dichloromethane. The stopcock was closed and the column filled the column with 20 cm of dichloromethane. One cm of combusted sodium sulfate was added to the column and rinsed down with dichloromethane. In order to evenly distribute the sodium sulfate the column glass was gently tapped.

Ten grams of alumina was weighed into a beaker. The alumina was poured into the column and the remaining alumina was rinsed from the beaker into the column using dichloromethane. Gently, the column glass was tapped to evenly distribute the alumina. The alumina was allowed to settle and the stopcock was opened for 15 to 20 seconds in order to pack the alumina tightly and remove air bubbles, then closed.

Approximately 20.0 g of silica gel was weighed into a beaker and dichloromethane added to form a slurry. The slurry was poured into the column and the remaining silica gel was rinsed from the beaker into the column using dichloromethane. Gently the column glass was tapped to evenly distribute the silica gel. The silica gel was allowed to settle and as performed with alumina, the stopcock was opened for 15 to 20 seconds to pack the silica gel and remove air bubbles, and then closed.

Another 1 cm of combusted sodium sulfate was added to the top of the column and rinsed down with dichloromethane. The column glass was tapped to evenly distribute the sodium sulfate. The sodium sulfate was rinsed down with dichloromethane. The stopcock was opened and the solvent drained to the top of the sodium sulfate.

A 50 mL volume of pentane was added to the column through a glass funnel,

slowly not to disturb the top of the column. The column was drained to the top of sodium sulfate layer. The tip of the column was rinsed with dichloromethane. The waste beaker is then replaced with a clean 250-mL flask.

The sample extract was transferred to the top of the column with a combusted disposable glass pipette. The column was drained to just below the top of the sodium sulfate layer. The extract vial was rinsed three times with 1-mL portions of 50:50 pentane/dichloromethane, transferring the rinses to the top of the column with the same disposable glass pipette tip used to transfer the extract to the column. The solvent was drained to just below the top of the sodium sulfate layer.

A volume of 200 mL of the 50:50 pentane/dichloromethane solution was added to the column through a glass funnel, slowly not to disturb the top of the column. The stopcock was partially opened to totally drain the solvent at a flow rate of 1 mL/min. The collected fraction contains aromatic and chlorinated hydrocarbons.

4.2.4.3 Water Bath Extract Concentration

Three to five Teflon boiling chips were added to the collection 250-mL flask and a 3 ball Synder column was inserted. Each boiling flask was placed into a 55 - 60°C water bath to reduce the solvent volume to approximately 5 mL. The reduced volume extract was transferred into a Kurderna-Danish (KD) concentrator tube.One boiling chip was added to each KD tube and the sample volume was reduced from 1 mL to 5 mL in a water bath maintained at 55 - 60°C. The residual sample is then transferred into a 4 mL clean extract vial using a disposable glass pipette and stored at 4°C ± 2°C until HPLC analysis. The extract vial was rinsed two times with 1-mL portions of dichloromethane, transferring the rinses to the HPLC vial with the same disposable glass pipette. Dichloromethane was added to the HPLC vial to a final volume of 4 mL.

4.2.4.4 HPLC Cleanup

Lipid removal from the aromatic fraction was accomplished using a Waters Model 590 Programmable Solvent Delivery Module high performance liquid

chromatography (HPLC) pump with two size exclusion columns in series (21.2 x 300 mm Phenogel 10μm GPC 100A columns) and a guard column (7.8 x 50mm Phenogel 100A). The columns were operated at 25°C. A volume of 2 mL of the aromatic fraction sample extract was injected onto the HPLC columns using the autosampler, and eluted with 7 mL/min dichloromethane. Samples were introduced into the column using a Waters 717 plus autosampler, capable of introducing a 0.5 mL to 2 mL sample. Elution times for compounds of interest were monitored by the use of a Waters 486 UV absorbance detector and recorded on a Waters 746 Data Module. The appropriate fraction was collected with a Waters Fraction Collector. The following HPLC conditions were used:

Pump:	delivered 7 mL/min dichloromethane
Autosampler:	injected 2 mL/min
Run Time:	35 min
Absorbance Detector:	254 nm

Collection time intervals were based on the retention time of three standards; 4,4'-dibromooctochlorobiphenyl, biphenyl, and perylene. This standard mixture was initially injected in duplicate and re-injected after every 6 samples. The collection window was set to minimize the collection of interfering lipid compounds but maximize the collection of analytes of interest. Analytes of interest were collected at specific time intervals into 40-mL TurboVap II concentrator tubes with 0.5-mL endpoint marks. The following fraction collector conditions were used:

Action Mode: 7(= B window)
Bottle Arrange (1, 2, 3, 4 = microplate): No 1
Waiting Time [bottle mode]:
Wait Time: 0 min 0 sec

Windows:

Window 1:

Start Time: 17 min 10 sec

End Time: 22 min 40 sec

Window 2:

Start Time: 0 min 0 sec

Fraction Capa (bottle mode):

Time Fraction: 5 min 31 sec per tube*

Condition (bottle mode):

Multiple Sample (0 = single, 1 = mult): 1

Peak (0 = nonpeak, 1 = +peak): 0

*The time fraction was set for 1 sec longer than the total window time.

The Waters 746 Data Module was programmed as follows:

CH A

Plot Off

CS 0.5

Attenuation 128

The following standard retention times were used to adjust the collection window:

4,4'-dibromooctachlorobiphenyl: 17.60 min

Biphenyl: 19.07 min

Perylene: 21.66 min

Collected extracts were reduced to a volume of 0.5 mL following the procedures described previously, and transferred to clean 2 mL amber vials. After all processing steps have been completed, each extract was transferred to a 2-mL amber extract vial

using a combusted disposable glass pipette and stored at –20°C ± 4°C until further analysis.

4.2.5 DNA Isolation from Placenta Samples and ^{32}P-postlabeling

DNA was isolated from placenta samples and postlabled following the methods described in Chapter III.

4.2.6 Genotyping

Genotyping assays for variants of selected genes were performed in Dr. Richard Finnell's laboratory at the Institute of Biosciences and Technology. Placenta DNA from a subset of case and control subjects were used in those assays. Based on experience with this population and information from the literature, the study focused on polymorphisms in genes for three Phase I and II metabolizing enzymes (CYP1A1, GSTM1, GSTT1) and six DNA repair genes (XRCC1, OGG1, MGMT, ERCC2, APEX1, XRCC3).

The selected SNPs were submitted to Applied Biosystems (Foster City, CA) to establish our unique SNPlex 48-plex assays on the 6 DNA repair candidate genes that were analyzed for in DNA isolated from biological samples. One limitation of the SNPlex technology is that realistically, anywhere from 5~20% of the SNPs will fail in the assay design, due to factors such as GC content or distance between SNPs. For those SNPs that were identified but could not be included in this SNPlex design, alternative methods such as individual TaqMan assays were used. For insertion/deletions and short tandem repeat (STR) polymorphisms, we performed our analyses on the fragment size, either using gel or capillary electrophoresis (Genescan on ABI 3100 Genetic Analyzer). Direct sequencing using BigDye Terminator (Applied Biosystems, Foster City, CA) continued to serve as the 'gold standard' for validation of genotyping data.

According to the manufacturer, the SNPlex^{TM} genotyping system (Applied Biosystems, Foster City, CA) is considered a high throughput and low cost assay based on universal multiplexed oligonucleotide ligation assay (OLA)/PCR technology and drag

chute mobility modifiers. The SNPlex assay starts with 3 unique ligation probes and a region of target gene sequence called the Genome Equivalent Region (GER). This is a portion of sequence that is complementary to the gDNA target on either side of the SNP site. The Locus Specific Oligo (LSO) requires a phosphate group on the 3' end to enable the ligase enzyme to perform the ligation. There are two allele specific oligos (ASO) each contains a unique "ZipCode" sequence and they play the role in identifying the PCR amplified ligation products prior to detection. After the ligation, the residual LSO and ASO probes will be removed utilizing two different exonucleases. Each of these 3 probe constructs contains universal PCR priming sites, which enable the assay to use the same PCR primers for every multiplexed PCR reaction. The reverse universal PCR primer has a Biotin attached to the 5' end that will be used to isolate or capture the double stranded DNA for detection. This technology allows up to 48 SNPs to be tested in a single reaction, consuming as little as 1ng DNA per SNP. The data was analyzed using GeneMapper™ v 3.5.

In addition to using SNPlex to analyze target SNPs in our DNA repair candidate genes, TaqMan assays were used for CYP1A1 SNPs that were impossible to multiplex. The TaqMan assay used two primers surrounding the SNP to generate short amplicons (about 50 to 100bp). The amplicon was subsequently interrogated with two allele specific probes labeled with different fluorescent dyes. These allele specific probes each had a fluorescent tag (e.g., FAM and VIC) on one end and a quenching dye at the other end. When the probe sequence matched the template sequence, the probe bound tightly to the template and the Taq polymerase cleaved the quencher free using its 5' exonuclease activity and allowed the fluorescence of the reporter to release. The TaqMan allele discrimination assay was performed on ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The Assay was purchased from ABI either as an Assay-on-Demand (off-the-shelf), or Assay-by-Design (made-to-order). The reaction was performed in 384-well plate format and duplicates were used for each sample to ensure accurate genotyping. The results were then exported into EXCEL file for statistical analysis. The TaqMan assay is considered a very efficient and

accurate technique however, this assay cannot run in a multiplex manner and requires relatively large amount of DNA for multiple SNP assays.

4.2.7 Statistical Analyses

Statistical analyses were performed using SigmaStat (Systat 2004). Data was available from environmental and human measurements (DNA adducts, genetic polymorphisms). Values, including PAHs in environmental or biological samples, were normalized by log transformation as needed and arithmetic values used for other variables. Descriptive statistics was performed first. Second, multivariate analyses were performed by using t-test or One Way Analysis of Variance (ANOVA) unless the normality test failed ($P<0.05$). When the normality failed, Mann-Whitney Rank Sum Test or Kruskal-Wallis One Way Analysis of Variance on Ranks was carried out. The criterion for statistical significance was a P-value < 0.05.

4.3 Results

Neural tube defects cases and matched controls were recruited from participating birth hospitals in Shanxi, China. Among the 156 families that signed consent to be included in the study, 72 were identified as cases and 84 were identified as controls (Table 4.2). Among neural tube defect cases, 37% were diagnosed with anencephaly, whereas 44% were diagnosed with spina bifida and 11% had both types of neural tube defect as reported in Table 4.3.

Table 4.2. Case status of study subjects.

Case Status	N(Valid Percent)
Case	72(46%)
Control	84(54%)

Table 4.3. Type of neural tube defect cases.

Type of Neural Tube Defect	N(Valid Percent)
Anencephaly	22(37%)
Spina Bifida	26(44%)
Anencephaly + Spina Bifida	11(19%)

During the initial period of the study, neural tube defects were monitored in four participating study counties including PingDing, Taigu, Xiyang and Zezhou. The results indicate that the frequency of NTDs ranged from 8 to 24 cases per 1,000 births in these counties as displayed in Table 4.4.

Table 4.4. Frequency of neural tube defects in Shanxi province, China from January to June, 2005. (Data from Pingding and Taigu counties is from January to May, 2005. Data from Xiyang and Zezhou counties is from January to June, 2005.)

Area	Live-births	Stillbirths	NTDs cases	NTDs Incidence Rate (per 1,000 live-births)
Pingding	1,363	21	21	15
Taigu	1,145	17	9	8
Xiyang	1,289	5	23	18
Zezhou	1,761	37	42	24

Questionnaire results are summarized in Table 4.5. Lifestyle questions related to PAH exposures were framed. Briefly, 97% of the mothers did not report smoking during pregnancy. However, around 88% reported being exposed to passive smoking. In addition, 88% reported using hard coal as cooking fuel, while the rest used black coal, firewood or natural gas. Only 37% of the fathers did not smoker, whereas the rest smoke at least “occasionally” according to the questionnaire results. Also of importance in the etiology of NTDs is folic acid supplementation. Only 10% of the mothers participating in this study reported using folic acid supplementation.

Table 4.5. Summary of questionnaire responses by mothers that provided placenta samples for analysis.

	N(Valid Percent)
Sex of Baby	
Male	77(52%)
Female	70(48%)
Pregnancy Unexpected	
Yes	35(24%)
No	111(76%)
Sequence of Pregnancy	
1st pregnancy	65(44%)
2nd or more pregnancy	84(56%)
Sequence of Delivery	
0 to 1	81(55%)
2 or more	66(45%)
Number of Prenatal Exams during Pregnancy	
0 to 2 exams	70(48%)
3 to 8 exams	76(52%)
Maternal BMI	
Underweight (Below 18.5)	4(2%)
Normal (18.5 - 24.9)	90(68%)
Overweight (25.0 - 29.9)	43(26%)
Obese (30.0 and above)	7(4%)
Maternal Age at Delivery	
Under 25	56(38%)
25 to 35	74(53%)
Over 35	13(9%)
Current Occupation	
Farmer and fishery	115(77%)
All other occupations	34(23%)
Maternal Education Level	
Primary school or lower	24(16%)
Junior high	108(73%)
High school or technical secondary school	11(7%)
Junior college or higher	6(4%)
Previously had babies with birth defects	
Yes	8(5%)
No	141(95%)
Paternal Education Level	
Primary school or lower	17(11%)
Junior high	111(74%)
High school or technical secondary school	18(12%)
Junior college or higher	4(3%)

Table 4.5. (continued)

	N(Valid Percent)
Consanguinity	
Yes	1(1%)
No	148(99%)
Cold or Fever 1 month before to 2 months after conception	
Yes	29(19%)
No	118(79%)
Anemia 1 month before to 2 months after conception	
Yes	9(6%)
No	139(93%)
Hyperemesis gravidarum 1 month before to 2 months after conception	
Yes	29(20%)
No	117 (79%)
Hepatitis 1 month before to 2 months after conception	
Yes	2(1%)
No	144(97%)
Other infectious diseases 1 month before to 2 months after conception	
Yes	3(2%)
No	144(97%)
Prophylactic medication 6 months before to 2 months after conception	
No	150(100%)
Anti-epilepsy or sedatives 1 month before to 2 months after conception	
Yes	2(1%)
No	147(99%)
Antibiotics 1 month before to 2 months after conception	
Yes	8(5%)
No	141(95%)
Analgesic 1 month before to 2 months after conception	
Yes	4(3%)
No	145(97%)
Other medications 1 month before to 2 months after conception	
Yes	13(9%)
No	136(91%)
Folic acid supplementation	
Yes	13(10%)
No	116(90%)

Table 4.5. (continued)

	N(Valid Percent)
Smoking 1 month before to 2 months after conception	
Yes	5(3%)
No	145(97%)
Passive smoking 1 month before to 2 months after conception	
Yes	88(59%)
No	62(41%)
Drinking liquor 1 month before to 2 months after conception	
Yes	22(15%)
No	127(85%)
Drink beer, red or rice wine 1 month before to 2 months after conception	
Yes	28(19%)
No	122(81%)
Cook in kitchen 1 month before to 2 months after conception	
Almost none	22(15%)
Sometimes	47(31%)
Every day	81(54%)
Drink herbal tea 1 month before to 2 months after conception	
Almost none	125(83%)
Sometimes	21(14%)
Every day	4(3%)
Kitchen separate from living room or bedroom	
Yes	129(87%)
No	20(13%)
Major fuel for cooking	
Black coal, Firewood or Natural Gas	60(41%)
Hard coal	88(59%)
Pregnancy during winter	
Yes	53(38%)
No	86(62%)
Stove used for heating	
Yes	38(72%)
No	15(28%)
Stove in living room or bedroom	
Yes	31(82%)
No	7(18%)
Major fuel for heating	
Black coal, Firewood or Natural Gas	13(34%)
Hard coal	25(66%)

Table 4.5. (continued)

	N(Valid Percent)
Ventilation use	
Daily	14(37%)
Sometimes	22(58%)
Almost none	2(5%)
Air quality during heating	
Fume every day	2(5%)
Fume sometimes	21(55%)
Clean air	15(40%)
Contact with toxis materials 1 month before to 2 months after conception	
Yes	6(4%)
No	143(96%)
Long term exposure to noise 1 month before to 2 months after conception	
Yes	2(1%)
No	147(99%)
Ultrasonic examination 1 month before to 2 months after conception	
None	14(9%)
Once	39(26%)
More than once	96(65%)
X-ray examination or treatment 1 month before to 2 months after conception	
None	149(100%)
Meat consumption 1 month before to 2 months after conception	
Less than 1 meal per week	88(59%)
1 to 3 meals per week	41(28%)
4 or more meals per week	19(13%)
Egg or milk consumption 1 month before to 2 months after conception	
Less than 1 meal per week	55(37%)
1 to 3 meals per week	47(32%)
4 or more meals per week	46(31%)
Fresh veggie consumption 1 month before to 2 months after conception	
Less than 1 meal per week	11(7%)
1 to 3 meals per week	29(20%)
4 or more meals per week	108(73%)
Fresh fruit consumption 1 month before to 2 months after conception	
Less than 1 meal per week	16(11%)

Table 4.5. (continued)

	N(Valid Percent)
1 to 3 meals per week	41(27%)
4 or more meals per week	92(62%)
Soy bean consumption 1 month before to 2 months after conception	
Less than 1 meal per week	57(38%)
1 to 3 meals per week	59(40%)
4 or more meals per week	33(22%)
Pickle or preserved food comsumption 1 month before to 2 months after conception	
Less than 1 meal per week	90(60%)
1 to 3 meals per week	37(25%)
4 or more meals per week	22(15%0
Pullulated potato consumption 1 month before to 2 months after conception	
Less than 1 meal per week	117(80%)
1 to 3 meals per week	24(16%)
4 or more meals per week	6(4%)
Vinegar consumption 1 month before to 2 months after conception	
Less than 1 meal per week	17(11%)
1 to 3 meals per week	37(25%)
4 or more meals per week	95(64%)
Cooking habit 1 month before to 2 months after conception	
Non-fried foods (salad or roasted)	23(15%)
Stir-fried	126(85%)
Source of drinking water 1 month before to 2 months after conception	
Tap water	99(66%)
Other water sources (well, river, pond or others)	50(34%)
Major crops consumed 1 month before to 2 months after conception	
Wheat flour	144(97%)
Rice or other	4(3%)
Psychic trauma 1 month before to 2 months after conception	
Yes	2(1%)
No	147(99%)
Husband's smoking behavior	
None	37(25%)
Occasionally	41(27%)
1 to 10 per day	33(22%)
More than 10 per day	38(26%)
Husband drinking liquor	

Table 4.5. (continued)

	N(Valid Percent)
None	48(32%)
Occasionally	89(60%)
Frequently	11(8%)
Husband drinking beer, red or rice wine	
None	47(32%)
Occasionally	92(62%)
Frequently	9(6%)
Husband contact with toxic materials 1 month before to 2 months after conception	
Yes	13(9%)
No	136(91%)

Placental tissue samples were collected from participating mothers after their delivery of a NTD case child or control. Placentas were used as a mean to explore the chemical exposures occurring to the fetus during gestation. After freeze-drying and solvent extraction, placentas were analyzed for PAHs listed in Table 3.2. Median concentration of PAHs in placental tissue sampled obtained from cases was 168 ng/g dry weight as opposed to 158 ng/g dry weight of PAHs detected in placental tissue collected from controls. Carcinogenic PAHs were at median concentrations of 6 ng/g dry weight in cases and 11 ng/g dry weight in controls. However, differences in PAH levels in placenta by case status were not statistically significant. Levels of total and carcinogenic PAHs in case and controls are presented in Table 4.6 and graphically in Figure 4.1.

Table 4.6. Concentrations of total and carcinogenic PAHs (ng/g dry weight) in placental tissue by case status.

	Total PAHs		Carcinogenic PAHs	
	Case	Control	Case	Control
Min	7	44	1	1
Median	168	158	6	11
Max	505	529	108	196
Mean	165	159	15	17
Std dev	116	98	24	37
SEM	19	19	4	7
Sample size	39	26	38	26
P-value	0.810		0.279	

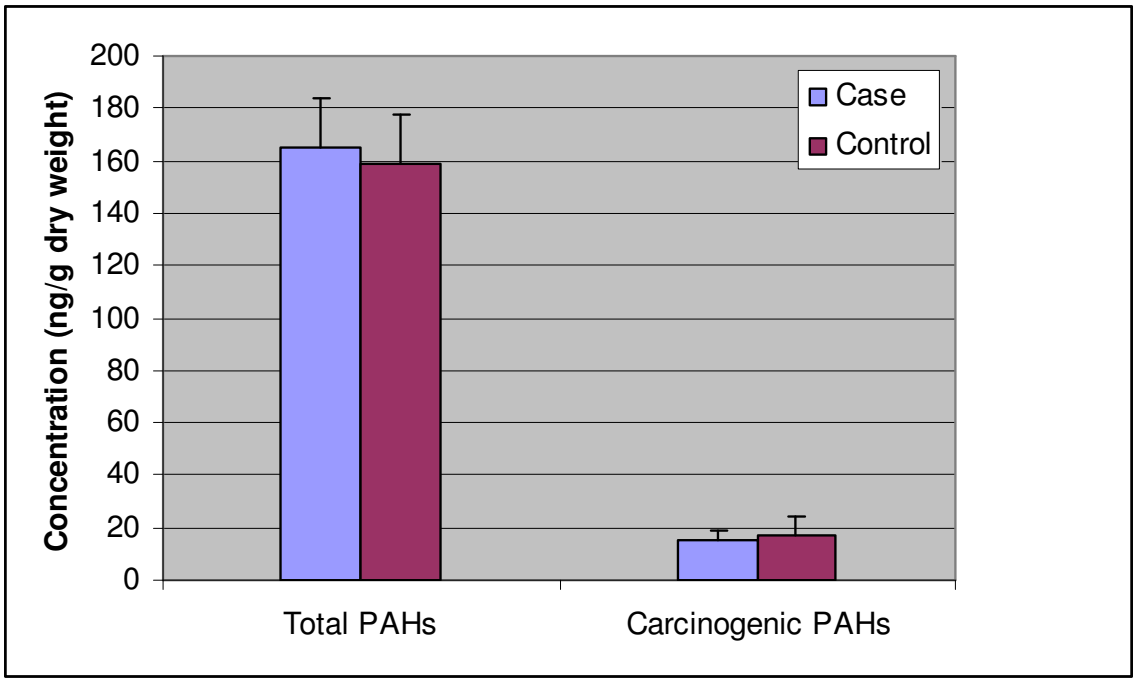

Figure 4.1. Bar graph representing mean total and carcinogenic PAH levels in placental tissue collected from NTD cases and controls.

The effect of passive smoking on placenta levels of total and carcinogenic PAHs was also evaluated. The median concentration of total PAHs in placentas from mothers who reported being exposed to passive smoking was 173 ng/g dry weight which was elevated compared to placentas originating from mothers with no exposure to passive smoking that had a total PAH median concentration of 140 ng/g dry weight.
In addition, carcinogenic PAH in the same two groups showed an elevation in placenta tissues from mothers exposed to passive smoking. These data reviewed in Table 4.7 and presented graphically in Figure 4.2 did not show any statistically significant difference in PAH concentrations.

Table 4.7. Concentrations of total and carcinogenic PAHs (ng/g dry weight) in placental tissue by passive smoker status of the mothers that provided the samples.

	Total PAHs		Carcinogenic PAHs	
	Passive Smoker	Not Passive Smoker	Passive Smoker	Not Passive Smoker
Min	7	12	1	1
Median	173	140	9	7
Max	529	389	196	92
Mean	172	152	19	12
Std dev	127	81	37	17
SEM	21	15	6	3
Sample size	36	29	35	29
P-value	0.787		0.710	

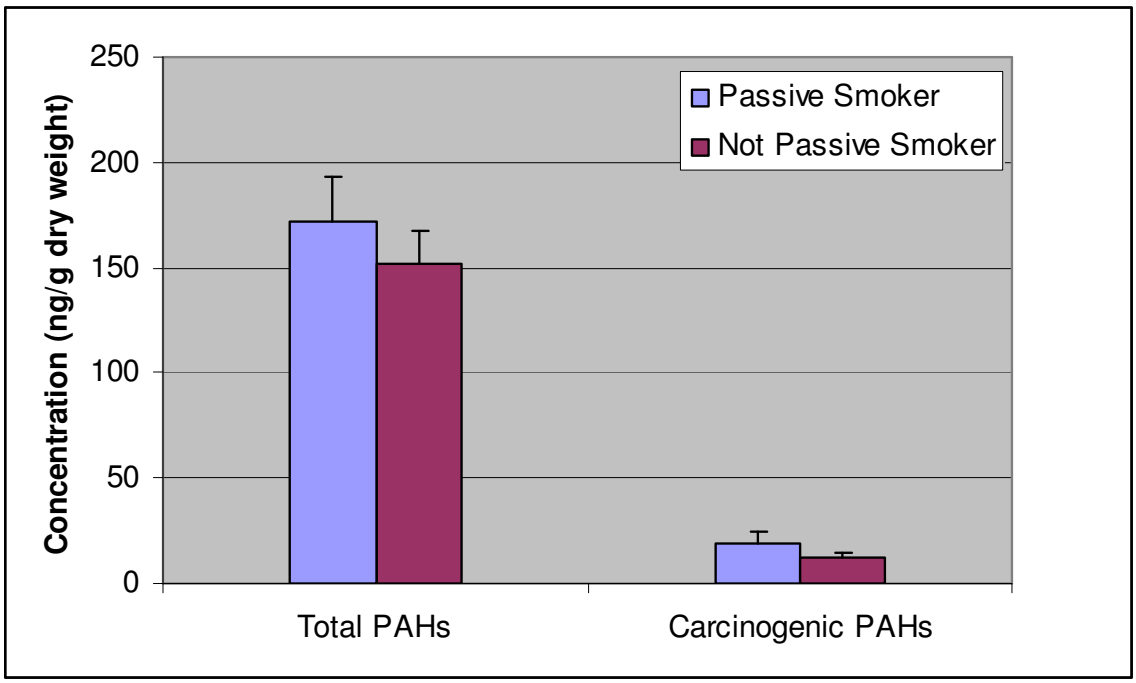

Figure 4.2. Bar graph representing total and carcinogenic PAH levels in placental tissue by passive smoking status of the mothers.

In addition to measuring the internal dose of PAHs in placental tissue, DNA was extracted from placentas and used to measure bulky DNA adducts by ^{32}P-postlabeling. However, as displayed in Table 4.8, levels of DNA adducts in placenta tissue from cases or controls were not to be statistically different. Mean levels of DNA adducts were 9 adducts per 10^9 nucleotides in both groups. DNA adducts levels were seemingly not significantly different between mothers exposed to passive smoking and mothers who are not. Mean levels of DNA adducts were around 9 adducts per 10^9 nucleotides in both groups also (Table 4.9). Figure 4.3 shows scanned autoradiograms of DNA adducts in placentas from a control non-smoker, case non-smoker, control smoker and case smoker.

Table 4.8. Bulky DNA adduct levels (per 10^9 nucleotides) in placental tissue by case status.

	DNA Adducts	
	Case	Control
Min	3	3
Median	8	9
Max	18	17
Mean	9	9
Std dev	3	3
SEM	0.4	1
Sample size	62	54
P-value	0.510	

Table 4.9. Bulky DNA adduct levels (per 10^9 nucleotides) in placental tissue by passive smoking status of the mothers that provided the samples.

	DNA Adducts	
	Passive Smoker	Not Passive Smoker
Min	3	4
Median	8	8
Max	17	18
Mean	9	9
Std dev	4	3
SEM	0.4	0.4
Sample size	67	49
P-value	0.739	

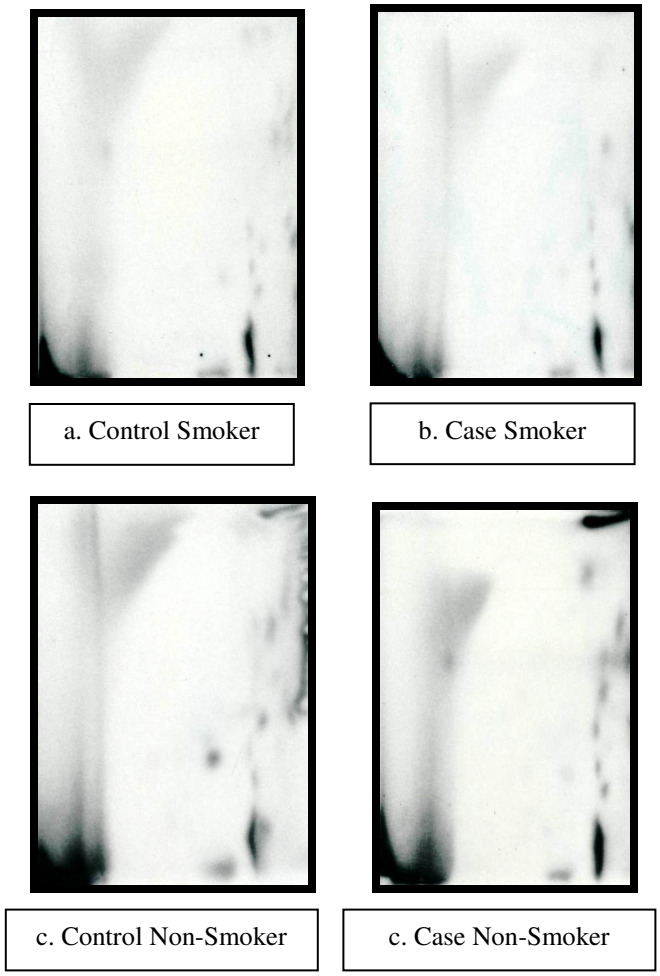

Figure 4.3. Autoradiograms of ^{32}P-postlabeled placenta bulky DNA adducts from non-smoker subjects. Autoradiography for 24 hr. at -80^{o}C using Kodak XAR-5 film.

Cooking fuel type seemed to be associated with DNA adducts in WBCs of fathers and mothers as described in the previous chapter. Concentrations of total and carcinogenic PAHs as well as DNA adducts in placenta samples from mothers who reported using hard coal as cooking fuel were compared to those leves in placentas from mothers who used other cooking fuel types. These results are reported in Table 4.10. Total and carcinogenic PAH median levels did not seem to be elevated in placentas from mother using hard coal when compared to the rest. However, median DNA adduct levels seem to be slightly increased in hard coal users (9 adducts per 10^9 nucleotides) as

compared to the others (7 adducts per 10^9 nucleotides). The difference in DNA adducts levels between these two groups was statistically significant.

Bulky DNA adduct levels were also evaluated by time of delivery, winter or summer (Table 4.11). Winter included births during the months of January through April, whereas summer included births during the months of May through July according the date of delivery provided by the study questionnaires. Around 58% of mothers in this study have delivered in the summer. Interestingly, DNA adducts levels seem to be significantly more elevated in summer deliveries as compared to winter deliveries. Placentas collected in the summer exhibited median DNA levels of 9 adducts per 10^9 nucleotides, whereas as those collected in the winter were found to have median DNA adduct levels of 7 adducts per 10^9 nucleotides.

Table 4.10. Total and carcinogenic PAHs (ng/g dry weight) as well as bulky DNA adduct levels (per 10^9 nucleotides) in placental tissue by cooking fuel type.

	Total PAHs (ng/g dry weight)		Carcinogenic PAHs (ng/g dry weight)		DNA Adducts	
	Hard Coal	Other	Hard Coal	Other	Hard Coal	Other
Min	44	12	1	1	3	4
Median	154	161	7	13	9	7
Max	529	505	196	108	17	18
Mean	166	172	14	24	9	8
Std dev	98	136	29	32	3	3
Std error	14	34	4	8	0.4	0.5
Sample size	47	16	47	15	74	40
P-value	0.788		0.122		0.049	

Table 4.11. Bulky DNA adduct levels (per 10^9 nucleotides) in placental tissue by time of delivery.

	DNA Adducts	
	Winter	Summer
Min	3	3
Median	7	9
Max	16	18
Mean	8	9
Std dev	3	4
Std error	0.4	0.4
Sample size	49	67
P-value	0.007	

In addition to adduct measurement, DNA was used in genotyping two major Phase II metabolism enzymes in addition to DNA repair enzymes of which ERCC2 is most involved in bulky DNA adduct repair. The prevalence of GSTM1 and GSTT1 deletions in placental tissue were 41% and 40% respectively (Table 4.12). Median DNA adducts were 8.2 per 10^9 nucleotides in placentas with an inactive copy of GSTM1 as compared to 7.9 adducts per 10^9 nucleotides in placenta with a functional GSTM1. Wild-type GSTT1 placentas exhibited median levels of 8.4 adducts per 10^9 nucleotides, as compared to 7.6 adducts per 10^9 nucleotides in GSTT1 null placentas. These results summarized in Table 4.13 were not statistically significant.

Table 4.12. Prevalence of GSTM1 and GSTT1 deletion in placentas.

Genotype	N(Valid Percent)
GSTT1	
Wildtype	70(64%)
Null	40(36%)
GSTM1	
Wildtype	69(63%)
Null	41(37%)

Table 4.13. Bulky DNA adduct levels in placental tissue by GSTM1 and GSTT1 genotype.

	DNA Adducts (per 10^9 nucleotides)			
	GSTM1		GSTT1	
	Null	Wild-type	Null	Wild-type
Median	8.2	8.0	7.6	8.4
Sample size	38	64	36	66
P-value	0.592		0.435	

The presence and frequency of heterozygote alleles on the ERCC2 gene at position 312 and 751 are summarized in Table 4.14. At position 312, homozygous allele CT was present in 12% of placentas, whereas on position 751, GT was present in 18% of the placenta samples. Median DNA adduct levels were higher for the heterozygote alleles at both positions of the ERCC2 gene compared to the homozygous alleles. Placentas with the heterozygote CT allele on position 312 of the ERCC2 gene had significantly lower DNA adduct levels (6 adducts per 10^9 nucleotides) compared to the homozygous individuals (9 nucleotides per 10^9 nucleotides). As for position 751 on the ERCC2 gene, placentas of individuals with GT alleles also exhibited lower DNA adduct levels compared to the homozygous alleles, however the difference was not statistically significant (Table 4.15).

Table 4.14. Prevalence of homozygous and heterozygous alleles of ERCC2 DNA repair gene position 751 and 312 in Study Subjects.

Allele	ERCC2 (Asp312Asn)	Allele	ERCC2 (Lys751Gln)
	N(Valid percent)		N(Valid percent)
CC + TT	107(88%)	TT + GG	100(82%)
CT	14(12%)	GT	22(18%)

Table 4.15. Bulky DNA Adduct Levels in Placental Tissue by Single Nucleotide Polymorphism in ERCC2 gene.

	DNA Adducts (per 10^9 nucleotides)			
	ERCC2 (Asp312Asn)		ERCC2 (Lys751Gln)	
	CT	CC+TT	GT	TT+GG
Median	6	9	6	8
Sample size	13	101	20	95
P-value	0.021		0.147	

Finally, Table 4.16 summarizes the total and carcinogenic PAH concentrations in addition to DNA adduct levels across children, mothers and fathers. The levels of PAHs in tissues from the parents or the children did not seem to vary significantly. Nevertheless, DNA adduct levels were significantly lower in children compared to the levels in WBCs from their parents. Interestingly, when cases and controls were divided by high or low total PAH exposures in their mothers, cases were 11 more times (Odds Ratio =11; 95% Confidence Interval= 3-46) likely to be born to a mother with a high PAH exposure (Table 4.17). High PAH exposure was defined as total PAH level above the median in venous blood of mothers (206 ng/mL) and lower exposure was defined as total PAH level below the median.

Table 4.16. Summary of biomarker of exposures in children and their parents. (tPAHs = total PAHs in venous blood in ng/mL or placenta in the case of a child in ng/g dry weight; cPAHs = carcinogenic PAHs in venous blood in ng/mL or placenta in the case of a child in ng/g dry weight; DNA adducts in WBCs or placenta in the case of a child in adducts per 10^9 normal nucleotides).

	Child			Mother			Father		
	tPAHs	cPAHs	DNA Adducts	tPAHs	cPAHs	DNA Adducts	tPAHs	cPAHs	DNA Adducts
Min	7	0.5	3	29	0.2	4	25	1	4
Median	154	8	8	206	8	12	162	8	13
Max	529	196	18	762	65	32	523	50	55
Mean	163	16	9	211	16	14	195	12	14
Std dev	109	30	3	148	16	6	137	12	9
Std error	13	4	0.3	20	2	1	19	2	1
Sample size	65	64	116	53	50	49	51	49	44
P-value	0.170	0.517	<0.001						

Table 4.17. Contingency table categorizing cases and controls by their mother PAH exposure (median of PAH levels in mothers blood = 206 ng/ml, OR=Odds Ratio).

	Total PAHs (ng/mL) in Mothers		
	Case	**Control**	Total
Higher than Median	24	3	27
Lower than Median	11	15	26
Total	35	18	53
			OR = 11 (95% CI = 3 - 46)

4.4 Discussion

The research described in this chapter is the second part of the human exposure assessment study discussed in chapter III. This part of the study measured biomarkers of exposure, early biological effect and susceptibility in placenta samples collected from NTD cases and controls. At the onset of the study, the frequency of NTDs was monitored in four study counties in Shanxi. Compared to the incidence of NTDs in the United States and most of the Western countries, the frequencies of NTDs in Shanxi was alarmingly high. The frequencies of NTDs in the selected counties of Shanxi ranged from 8 to 24 cases per 1,000 live-births. The reported NTD rates in the United States is below one case per 1,000 births (Mathews et al. 2002).

This research proceeded by measuring biological indicators of coal byproducts such as PAHs in placenta tissues from cases and controls. The internal total and carcinogenic PAH dose in NTD cases were elevated compared to controls, but were not statistically significant. In addition, the levels of PAHs were higher in the placentas of mothers who reported being exposed to passive smoking compared to those who did not. However, these differences in PAH levels were not statistically significant.

DNA adducts in placenta were not significantly different among cases and controls. Mothers exposed to passive smoking did not seem to have statistically significant differences in the DNA adduct levels in their placenta compared to those who reported not to be exposed to passive smoking.

Deletions in two major Phase II metabolic enzymes GSTM1 and GSTT1 were evaluated as a measure of genetic susceptibility in placentas. However, differences in DNA adduct levels between placentas with inactive copies of GSTM1 or GSTT1 did not seem to be statistically significant when compared to those with wild-type genotypes.

Single nucleotide polymorphisms (SNPs) on positions 312 and 751 of the ERCC2 gene seem to be the most studied SNPs of this specific DNA repair gene. ERCC2 is involved in bulky DNA adduct repair as part of the Nucleotide Excision Pathway (Manuguerra et al. 2006). Heterozygote alleles on positions 312 and 751 of the ERCC2 gene seem to have a protective effect against DNA adduct formation. This finding needs

to be confirmed with a bigger sample size as it suggests that individuals with the less common SNP may have a more efficicent nucleotide excision repair mechanisms.

Although total PAHs and carcinogenic PAHs did not show statistically significant differences, DNA adducts formed in placentas of mothers who used hard coal as cooking fuel were significantly higher than adducts in placentas of mothers who used other fuel types. This finding seems to be consistent with the data from the mother population discussed in chapter III. Therefore, it might prove to be important to further investigate the coal type that is associated with higher DNA adduct levels in the human population.

It is important to note that while levels of PAHs in biological tissues did not vary significantly between a child and his mother or father, DNA adducts in the parents were significantly higher than those detected in children. This finding suggests that fetuses might be protected to a certain extent against chemicals that their mothers are exposed to. Another interesting finding was the association between maternal PAH exposures and disease. NTD cases were 11 times more likely to be born to mothers with a PAH exposure level higher than the median as compared to mothers with exposure levels lower than the median. This result may be worthy of further investigation in future studies about the potential role of maternal exposures to PAHs in the etiology of human birth defects.

Overall, these results suggest a potential role of PAHs in causing birth defects in the human study population. Genotoxic PAH compounds were present in placenta tissues and were capable of inducing DNA adduct formation in these tissues. Hard coal as cooking fuel seem to be of potential concern due to its association with elevated levels of DNA adducts in the study participants. Limitations to this study include sample size as well as genetic polymorphism data. A larger sample size is required to increase the power of the study and the ability to detect any weak associations between exposure and outcome variables. Polymorphisms of additional metabolic genes (such as Phase I metabolic enzymes) as well as DNA repair genes needed to be analyzed in the future to help identify genetically sensitive groups and help elucidate the relationship between PAHs levels in tissues and bulky DNA adduct formation.

CHAPTER V
SUMMARY

The research in this dissertation was conducted to monitor biomarkers of exposure to complex mixtures of PAHs in a human population exhibiting an unusually high rate of neural tube defects. PAHs are ubiquitous in the environment and may present a threat to the health of exposed human and ecological receptors. The study population was located in Shanxi province, North China where the weather is typically cold and dry most of the year. Individuals particularly in this part of China rely heavily on indoor burning of coal for heating and cooking. Coal mining in Shanxi is very prevalent as the province is considered to be the top coal-producing region in China. Coal combustion is a source of various harmful chemicals of which are PAHs.

This research included identification of the level of PAH contamination in dust collected from selected homes in the study region, as well as their profile and genotoxicity using a battery of tests such as microbial cell cultures, acellular assays and animal models. Floor dust was sampled from several houses in the study area. Dust loading on surfaces such as windows, walls and light bulbs was also sampled to compare the PAH profiles of the different residential surfaces. After pressurized fluid extraction, the dust organic residues were chemically analyzed for PAHs. PAH concentrations from the analyzed dust samples were variable but generally elevated as compared to other environmentally devastated study sites such as Rio Bravo in South Texas and Sumgayit, Azerbaijan. The main sources of PAH seem to be pyrogenic, especially that high molecular weight PAHs (4 rings or more) were more abundant than lower molecular weight ones. This finding was compatible with the fact that the major source of PAHs was thought to be burning coal indoor. A sample of unburnt coal collected from a stockpile in the study location was also extracted and analyzed for PAHs. Levels of PAH in coal were significantly lower than the ones detected in house dust. In addition, the PAH profile in coal was petrogenic, as anticipated.

A series of short-term bioassays were then conducted to investigate the genotoxic potential of the residues isolated from house dust. In the *Salmonella* microsome assay, complex chemical mixtures isolated from house dust generally induced a strong positive mutagenic response, whereas those isolated from coal only exhibited a positive response. In the acellular assay, DNA isolated from the placenta of neural tube defect cases and matched controls was incubated with dust and coal residues in the addition of a metabolic enzyme system. Following incubation, bulky DNA adducts were detected and quantified by ^{32}P-postlabeling. House dust residues exhibited a strong response and elicited significantly higher levels of DNA adducts as compared to coal. The response did not vary by whether DNA was isolated from a NTD case or control. Overall, the patterns of DNA adducts observed after treatment with house dust and coal were similar, however the one induced by complex mixtures isolated from coal was of lower intensity.

Residues from dust and coal were also applied to the skin of CD-1 mice to measure the induction of DNA adducts. Benzo[a]pyrene and methylene chloride were also applied to mice to serve as positive and negative controls, respectively. Following dermal application, dust samples induced the maximum level of genotoxic damage in skin tissue, with a much lower frequency of adduct observed in lungs. Interestingly, the coal extract induced significantly higher levels of DNA adducts in skin tissue as compared to dust. Possible explanations to these findings include a plateau effect caused by saturation of activating enzyme systems and/or cell death triggered by higher levels of adducts induced by dust mixtures. It was also observed that the overall pattern of bulky DNA adducts were slightly different between dust and coal. The autoradiogram for the dust showed multiple distinct spots, whereas the coal which contained lower levels of PAHs exhibited less distinct spots and more of a diagonal radioactive zone typical of complex chemical mixtures such as cigarette smoke. Results from the short-term bioassays and animal study, suggest that dust collected in houses in Shanxi contains complex mixtures of chemicals with a potential to induce genotoxic damage in human receptors. Moreover, the genotoxic response seems to be correlated to the fraction of class B2 carcinogen PAHs.

With the knowledge that potentially genotoxic compounds exist in the residential environment of the study population, parents of neural tube defect cases and those of matched controls were recruited for the biomonitoring part of this research. Indicators of exposure and early effect were measured in biological specimens from parents of cases and controls as well as placenta tissues to assess fetal exposures. A questionnaire was also administered to the mothers of cases and controls to gather data on lifestyles, nutritional status among other risk factors of birth defects. In addition, DNA extracted from biological tissues was genotyped for Phase II metabolic enzymes and DNA repair enzymes.

Venous blood from parents of cases and controls was collected in China and shipped back to Texas A&M University for analysis. Plasma was isolated from whole blood and extracted by liquid-liquid extraction to be used to measure levels of PAHs in blood as an indicator of internal dose. DNA was isolated from blood cells and used for adduct analysis as well as genotyping. Total PAH levels in parents of cases were higher than those of controls. The differences were especially remarkable in venous blood samples collected from mothers of cases and controls. Surprisingly, DNA adducts in WBCs were significantly higher in controls when compared to NTD cases. Smoking (active or passive) did not seem to have a significant on the levels of PAHs or DNA adducts in blood. Phase II metabolic enzymes GSTM1 and GSTT1 polymorphisms did not seem to be significantly associated with DNA adducts induction in this population.

The type of fuel used for cooking seems to have significantly affected DNA adduct levels in venous blood of mothers. Hard coal use induced higher levels of DNA adducts in blood cells of mothers when compared to those using a different type of cooking fuel. Total PAHs in blood of non-smoking fathers and mothers were strongly correlated which could be an indication of a common source of PAH exposures more likely to be environmental.

Placenta samples were collected in participating birth hospitals immediately after the delivery of a recruited case child or matched control. The placenta samples were also shipped back to Texas A&M University for analysis. After homogenization, an aliquot

of placental tissue was freeze-dried and solvent extracted by pressurized fluid extraction to be used for measuring PAH levels as internal dose. Another aliquot of placental tissue was used to isolate DNA for adduct and genotyping analyses. Total and carcinogenic PAH levels in NTD cases were higher compared to controls, but were not statistically significant. Passive smoking did not seem to be associated with significantly higher levels of PAHs in placentas.

Placenta of NTD cases did not exhibit higher levels of DNA adducts when compared to controls. Passive smoking did not seem to be associated with the induction of significantly higher levels of DNA adducts in placentas.

Differences in DNA adduct levels between placentas with null copies of GSTM1 or GSTT1 did not seem to be statistically significant as compared wild-type genotypes. Heterozygote alleles on position 312 of the ERCC2 DNA repair gene were associated with a lower level of DNA adducts and imply that these subjects may possibly have more efficient DNA repair capabilities.

As detected in blood cells from mothers, hard coal induced significantly higher levels of DNA adducts in placentas of mothers who used it as cooking fuel compared to mothers who used other fuel types. It was also found that DNA adducts formed in the parents were significantly higher than those identified in children. Finally, elevated PAH levels in mothers appear to be a potential risk factor for having a child with neural tube defects.

Humans are exposed to complex chemical mixtures such as PAHs at every stage of their life. The dose, frequency, and timing of the exposure determine to a large extent the severity of the adverse health effects exhibited by human receptors. Neural tube defect was a suitable disease to investigate as an adverse health effect of PAH exposures due to its relatively short latency period. The potential link between exposure to PAHs and adverse reproductive outcomes has been thoroughly investigated in recent years. However, the association between PAH exposures and birth defects is still novel and not very well studied yet. The conclusions of this research include:

1. Dust collected from houses in the study province contained elevated levels of PAHs that have proved to have genotoxic potential in *in vitro* and *in vivo* bioassays
2. PAHs detected in venous blood of mothers were significantly higher in cases as compared to controls, but did not correlate well with DNA adduct levels
3. DNA adduct levels detected in placenta tissues did not correlate with disease and did not seem to be associated with GSTM1 or GSTT1 polymorphisms

Future studies are warranted on biomonitoring a larger sample size of the study population to enhance the statistical power of the study. More whole families (father, mother and child) need to be recruited for the study. It also seems substantial to include more genes in the genetic polymorphism analyses, especially polymorphisms of Phase I metabolic enzymes that might be common in Chinese populations. The available literature almost invariably stresses upon the importance of evaluating a combination of genetic polymorphisms with functional compatibility as opposed to single polymorphisms. While the logistics (sample collection, storage conditions and shipping) of studies might tend to be undervalued in scientific contexts, future studies would benefit from improved logistics to ensure the best quality of biological samples shipped back to the United States for processing. In addition, future efforts would profit from indoor air sampling as a measure of external dose to airborne hazardous chemicals, as well as the measurement of nutritional and tobacco smoking markers in study subjects. Identification of other chemicals of concern that might be emitted by coal combustion or during coal mining activities would also be of importance in deciphering any links between environmental contamination and disease.

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