12-7-2006

The Effects of Benzo-α-Pyrene on the Insulin-like Growth Factor-I Gene

Brittany Albright Epperson
Yale University

The Effects of Benzo-α-Pyrene on the Insulin-like Growth Factor-I Gene

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Brittiny Albright Epperson

2006

Abstract

THE EFFECTS OF BENZO-α-PYRENE ON THE INSULIN-LIKE GROWTH FACTOR-I GENE.

Brittiny A. Epperson and Ahmed M. Fadiel. Department of Obstetrics and Gynecology, Yale University, School of Medicine, New Haven, CT.

The purpose of this study was to look at the genotoxic and cytotoxic effects of benzo-α-pyrene (BαP), a chemical mutagen that is present in cigarette smoke, on the insulin-like growth factor-I (IGF-I) gene. Women who smoke during pregnancy are more likely to have a growth-restricted baby. We hypothesized that BαP exerts its effects through genotoxic and cytotoxic avenues. The cytotoxicity is manifested by chromosomal abnormalities and a decrease in the rate of cell division. The genotoxicity is manifested by changes in certain genes known to be important in mammalian fetal development such as IGF-I. IGF-I is implicated in intrauterine growth restriction (IUGR), a problem that greatly increases the risk of perinatal morbidity and mortality. To futher understand the mechanism by which BαP influences the normal growth and development of human placental cells, human placental trophoblast cells from an established immortalized cell line were utilized. Cells were cultured in appropriate media, starved (using starvation "Serum Free Medium"), and treated with two doses of BαP, 1μM (dose 1) and 5μM (dose 2). Chromosomes were prepared for cytogenetic analysis and visualized using light microscopy after Giemsa staining. Chromosomal aberrations were identified and the rate of cell division was determined through the analysis of the mitotic index for treated cells compared to a control group. To further understand the influence of BαP on the IGF-I gene expression level, RNA was extracted from control and treated cells, from which cDNA was synthesized and used for further analysis using polymerized chain reaction (PCR). The PCR results were used to better understand the genotoxicity of BαP, while chromosomal aberration analysis was used to determine the cytotoxic effects of BαP on human placental cells. Our results indicate that many

chromosomal abnormalities were present in the treated groups compared to the control group. In addition, there was a significant decrease in the mitotic index of the BαP-treated cells (MI=0.3%) verses the control group (MI=0.93%), *p* value 0.0447. Through the PCR assay, we speculate that there is a dose-related response to BαP of the IGF-I RNA expression level, with low levels in the treated groups compared to the control group. We conclude from these results that BαP influences placental cells at both the gene and chromosome level. It also affects the cell cycle of human placental cells. It is known that smoking is deleterious for fetal development. We believe that the current study brings us closer to understanding the mechanism by which smoking can lead to fetal growth restriction.

Acknowledgements

I would like to thank Ahmed M. Fadiel for his support and mentoring. Because of him I have a better understanding of the scientific method. Thanks to Dr. Frederick Naftolin for welcoming me into his laboratory. Thank you to the Obstetrics and Gynecology department at Yale.

Table of Contents

Introduction

It has been reported that infants born small for gestational age (SGA) as a result of intrauterine growth restriction (IUGR) are at increased risk for, among other disorders, cardiovascular disease as adults. This is termed Fetal Origin of Adult Disease (FOAD) or Fetal Programming and is a widely accepted phenomenon. SGA is defined as infant weight less than the 10^{th} percentile for gestational age. Of note, SGA and IUGR are not interchangeable terms. Infants can be born SGA simply due to genetics without having been growth-restricted *in utero*, and therefore, are not classified with the IUGR SGA infants discussed in this study. IUGR can be defined as the failure of a fetus to achieve its genetically determined growth potential (1). Low birth weight (LBW) is defined as infants weighing less than 2500 grams (5 lbs 8 oz) regardless of gestational age. LBW infants are usually either preterm or intrauterine growth restricted. Thirty-three percent of LBW infants are SGA.

Intrauterine growth restriction occurs in up to 10% of all pregnancies. The perinatal mortality risk of a growth-restricted fetus is up to ten times higher than a healthy fetus. IUGR is the second leading cause of perinatal death after preterm delivery (2). Twenty percent of stillborn fetuses are growth-restricted (3).

FOAD was first described in 1969 in an English study looking at a population born in the early 1900s (4). This study showed a negative correlation between death from cardiovascular disease and weight, head circumference, and ponderal index (weight/length^3) at birth. Another study a few years later showed that death rates were three times higher in those weighing ≤18 lbs at age 1 year compared to those weighing ≥27 lbs (4). This idea piqued the interest of many, and today there is an abundance of

active research in this area (5-13). To prevent cardiovascular disease caused by IUGR, by early diagnosis and intervention of IUGR, would be a great feat. In order to bring scientists closer to accomplishing this, a thorough understanding of IUGR is necessary. A vast amount of research has shown insulin-like growth factor (IGF) to play an essential role in fetal development and has been implicated as a prime target for understanding the causes of IUGR.

Just one of countless causes for IUGR is maternal cigarette smoking during pregnancy (14-23). Cigarettes contain many chemical substances that are proven to be mutagenic and many are found to be carcinogenic. Many of the carcinogens make up a group of compounds called polycyclic aromatic hydrocarbons (PAHs). Of this group, benzo-alpha-pyrene (BαP) is the most widely studied and is an extremely potent carcinogen (24).

This study attempts to look at the effects of BαP on the insulin-like growth factor-I (IGF-I) gene. We also perform cytogenetic analysis and determine the mitotic index on human placental cells exposed to BαP.

Intrauterine growth restriction

Many factors exist that contribute to IUGR. Among those factors are acquired illnesses, such as intrauterine infection, and inherited diseases, like sickle cell anemia (3). Among maternal medical conditions, hypertension, whatever the cause, is most strongly associated with IUGR (25). Placental insufficiency is an important cause, and this can be due to either maternal or fetal factors (26). Table 1 gives a thorough overview of the causes of IUGR. It is estimated that in the U.S. chromosomal aberrations account for ten percent of IUGR cases; and as much as forty percent of IUGR cases may be attributed to environmental factors, such as cigarette smoking or alcohol abuse during pregnancy (3).

Table 1. Causes of intrauterine growth restriction (IUGR). IUGR is when a fetus is small for gestational age, below the 10th percentile for weight, and has not met its genetic potential for growth. It is when the fetus is growth restricted due to pathology. IUGR is a serious problem, increasing the risk of perinatal morbidity and mortality. The causes are often multifactorial and the etiology is often unknown. Among maternal factors, hypertension is the most common cause of IUGR.

Causes of IUGR:

Maternal Demographics

- Age (<16, >35)
- Ethnicity (e.g., African-American)
- Low socioeconomic status
- Single
- Low level of education

Maternal Health

- Hypertension
- Pregnancy-induced hypertension
- Infection (TORCHES)
- Anemia
- Asthma
- Chronic Renal Insufficiency
- Heart Disease
- Substance Abuse
    - Cigarette Smoking
    - Alcohol use
    - Narcotic use
- Under nutrition and Malnutrition (during pregnancy)

Placental

- Hemangioma
- Placental infarct
- Single umbilical artery
- Small placental size

Fetal

- Multiple gestation
- Chromosomal anomaly
    - Trisomy (21, 18, 13)
    - Turner Syndrome
    - X Polysomy
    - Dwarfism
    - Chondrodystrophies
    - Osteogenesis Imperfecta

When a specific illness can be identified, there is no wondering about the cause of IUGR. However, there are instances when fetal growth is insufficient for reasons unknown. Not knowing the cause and how this could have been prevented can be stressful for the clinician and the patient.

The largest concern with a LBW infant is perinatal morbidity and mortality. Neonatal death is 40 times more likely in LBW infants and 200 times more likely in very low birth weight (VLBW) infants, VLBW being defined as less than 1500 grams (27). All LBW infants will spend time in the Neonatal Intensive Care Unit, spending health care dollars even when intervention and/or treatment are not necessary. A fetus that has been growth restricted is at increased risk for premature birth; is less capable of handling the distress of labor and delivery as compared to a normal fetus, increasing the chances of perinatal morbidity and mortality (28); is at greater risk of hypoxia and hypoglycemia pre- and perinatally; is at risk for developing systemic iron deficiency later in infancy and childhood (29); and the growth-restricted fetus may not grow appropriately during infancy, potentially never achieving catch-up growth. It is known that children with LBW have a higher risk of childhood behavioral problems (30).

The growth-restricted fetus is also at increased risk for neurodevelopmental delays. For example, thyroid hormone is essential for fetal development of the central nervous system (CNS). The growth-restricted fetus is at increased risk of having hypothyroxinemia (31), but also infants born to mothers with hypothyroxinemia are at increased risk of being growth restricted (32).

A large population-based study concluded that impaired neurodevelopment during fetal life due to LBW may increase susceptibility to depression later in life (30). In this

study, women who had weighed ≤3 kg at birth had an increased risk of being depressed at age 26 (P<0.001). There was no significant risk increase for men. However, men who had weighed ≤2.5 kg at birth were more likely to report a history of depression at age 26 and to be psychologically distressed at age 16. Mittendorfer-Rutz, *et al.* found LBW, adjusted for gestational age, to be a significant predictor of suicide (33).

Two forms of growth restriction have been described—symmetric and asymmetric. Symmetric growth restriction usually occurs early in gestation and the brain, or head circumference, and the abdomen and soft tissues are equally growth restricted. In asymmetric growth restriction, the brain is able to grow to its appropriate size, while the abdomen and soft tissues are growth restricted. This type of growth restriction usually occurs later in gestation. Asymmetrically growth-restricted fetuses usually have a better outcome than symmetrically growth-restricted fetuses in terms of fetal distress and catch-up growth.

Epidemiology of IUGR

The prevalence of LBW deliveries was more than twice as high among black women (13.3%) as it was among white women (5.7%) in 1990, and has remained relatively consistent. The gap is even wider when VLBW infants are considered. This difference exists even when socioeconomic factors such as income, education, and harmful habits are controlled. LBW rates among Mexican-American, Asians, and Native Americans are not much higher than rates among non-Hispanic whites. The majority of LBW infants are due to IUGR in developing countries, and to pre-term delivery in

developed countries (34). As mentioned above, in the U.S., forty percent of IUGR may be due to environmental factors, most of which may be preventable.

Pathophysiology of IUGR

The mechanisms by which IUGR occurs are not well known. Fetal hypoxia is a major contributor to IUGR. *In utero* oxygen insufficiency can be caused by many things such as: maternal illness, like anemia or congenital heart defects; improper placental implantation, possibly leading to preeclampsia; insufficient umbilical blood flow due to abnormal umbilical vessels; or fetal anemia from Rh isoimmunization. Chronic fetal exposure to hypoxia, rather than acute hypoxic exposure, is implicated in IUGR (35). It has been shown that growth factors are consistently down regulated in response to hypoxia, leading to growth restriction (35). It has been shown that fetal exposure to elevated levels of testosterone in both male and female sheep, rats, and subhuman primates leads to growth restriction and elevated levels of IGFBP-1 and -2 (36).

Smoking and IUGR

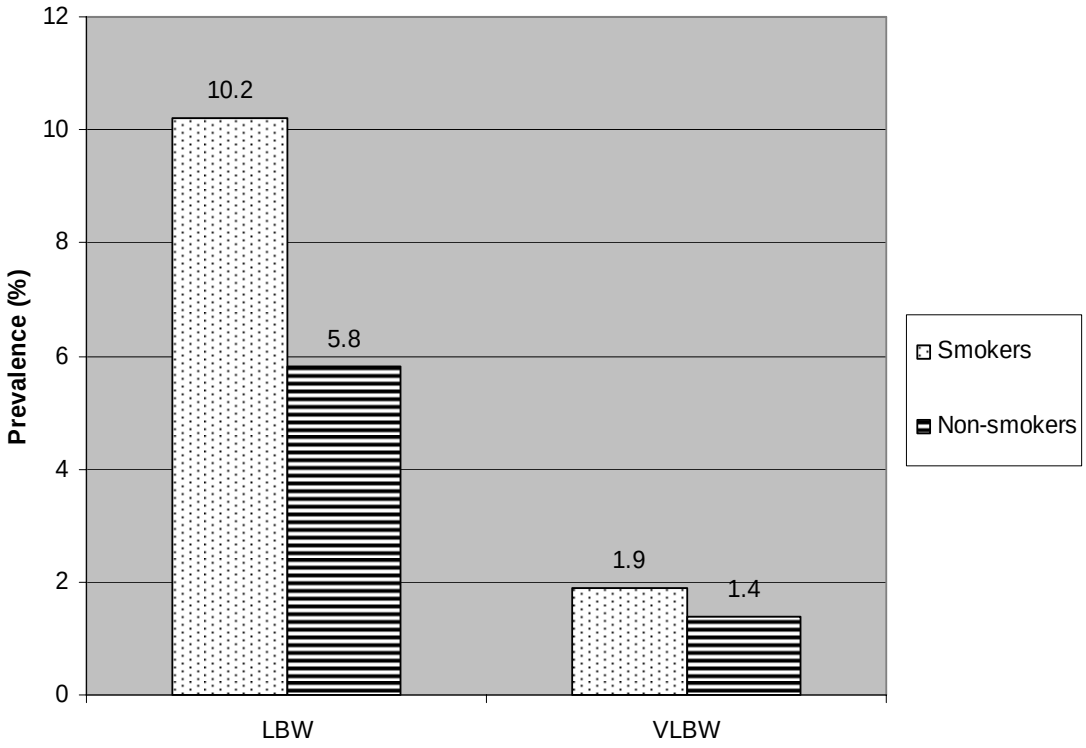
It is well known that certain health-compromising behaviors during pregnancy, such as maternal cigarette smoking, alcohol use, and drug abuse, are associated with SGA infants (21). Also, as the number of health-compromising behaviors increases in an individual, the risk of having a term-low birth weight (term-LBW) infant increases (21). Among cigarette smoking, alcohol use, and drug use, cigarette smoking is the biggest predictor of term-LBW (21). It is important to note that women who engage in the aforementioned health-compromising behaviors are more likely to have other risk factors

for having an SGA infant, such as low socioeconomic status, undernourishment, and/or lack of prenatal care.

In addition to increasing the risk of IUGR, smoking or secondary smoke exposure during pregnancy increases the likelihood of infertility, spontaneous abortion, perinatal death, preterm labor, placental abruption, sudden infant death syndrome (SIDS), preterm premature rupture of membranes, placenta previa, and ectopic pregnancy (37, 38). It is important to note that ectopic pregnancy is the leading cause of maternal death during the first trimester (39). The national cost for pregnancy-related complications due to smoking is $350 million (40).

It is estimated that 19% of all women smoke cigarettes (41). Twenty-five percent of women of reproductive age smoke cigarettes. Twelve percent of all pregnant women are current cigarette smokers. Smoking may decrease infant birth weight by as much as 250 grams. Smokers are three times more likely to have a growth-restricted fetus than are non-smokers (3). Figure 1 shows the prevalence of LBW and VLBW infants among smokers versus non-smokers.

Figure 1. Prevalence of low birth weight (LBW) and very low birth weight (VLBW) among smokers versus non-smokers. LBW is defined as 1500-2499 grams; VLBW <1500 grams. 39 (40).



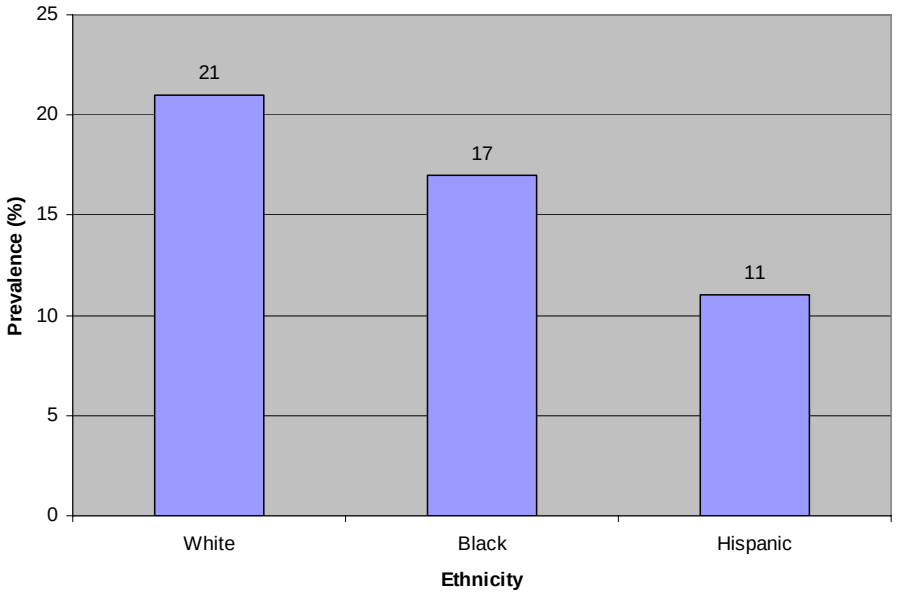
The mechanisms by which smoking leads to IUGR is not completely understood, however, several mechanisms have been proposed: inadequate oxygen delivery to the fetus via insufficient placental gas exchange, chromosomal abnormalities, direct toxic effects of nicotine, and direct toxicity of the more than 4,000 chemicals found in cigarettes (3, 42). Certain maternal genotypes may increase the risk of having a LBW infant in cigarette smokers (43).

Ethnic Breakdown of Women who Smoke during Pregnancy

Smoking in women is highest among American Indian and Alaska Native, intermediate among white and black women, and lowest among Asian or Pacific Islander and Hispanic women. Smoking is 3 times more prevalent in women with 9 to 11 years of

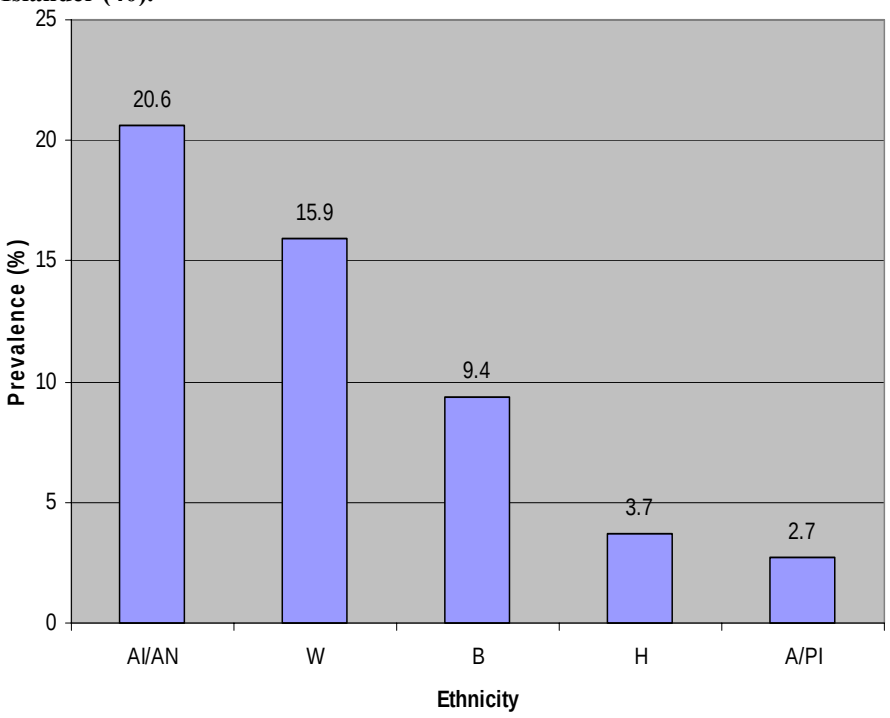
education compared to women with 16 or more years of education (44). As reported in the 2004 National Health Interview Survey, 21% of white women are current smokers, compared with 17% of black women and 11% of Hispanic women (41). See Figure 2 for an overview of smoking in women by race.

Figure 2. Prevalence of smoking among women in the U.S. by ethnicity (44).



The prevalence of smoking among pregnant women is 20.6% in American Indians/Alaska Natives, 15.9% in whites, 9.4% in blacks, 3.7% in Hispanics, and 2.7% in Asian/Pacific Islanders (see Figure 3). The younger the pregnant woman, the more likely she is to smoke during pregnancy (18% among pregnant woman younger than 20 versus 9.8% among pregnant women 35 years or older).

Figure 3. Prevalence of smoking among pregnant women in the U.S. by ethnicity. In general, about one-fourth of the U.S. population smoke cigarettes. This serious problem is compounded when a pregnant woman smokes. Smoking has deleterious effects on the fetus—spontaneous abortion, preterm labor, intrauterine growth restriction, and placental abruption are only a few of the many examples of problems that can occur when a developing fetus is exposed to the harmful toxins of cigarette smoke. This chart shows the ethnic breakdown of smoking among expecting mothers in the U.S. AI/AN=American Indian/Alaska Native; W=white; B=Black; H=Hispanic; A/PI=Asian/Pacific Islander (40).



Benzo-alpha-pyrene

BαP is a polycyclic aromatic hydrocarbon (PAH) and is a well-documented, well-researched mutagen that is present in cigarette smoke (45-48). It was first isolated from coal tar in 1930, and its carcinogenicity was discovered by repeated painting of the substance onto mouse skin (49). Aside from being found in smoking tobacco, it is also found where combustion reactions take place (it is a byproduct of incomplete combustion). These include industrial processes, transportation, energy production and use, food preparation, and open trash burning. It is also found in materials like tar and asphalt. Natural sources include forest fires and grass fires (50). BαP has been implicated in many cancers, particularly lung cancer (51).

Cigarettes contain many different carcinogens--PAHs, *N*-nitrosamines, inorganic compounds, aza-arenes, aromatic amines, aldehydes, heterocyclic aromatic amines, and other organic compounds (46). BαP is the most studied of all cigarette mutagens and is highly potent. Like other cigarette carcinogens, BαP requires metabolic activation prior to causing any damage. The BαP metabolite, (±)B[a]P-*r*7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BPDE), is highly carcinogenic (52). Upon formation of BPDE, adducts are formed by the covalent binding of BPDE to macromolecules such as DNA, RNA, and protein (53). The majority of DNA adducts in healthy individuals are repaired. However, with chronic exposure, as in an individual who smokes, DNA adducts may persist, greatly increasing the risk of mutation and tumorogenesis (46).

Insulin-like Growth Factor

IGF Family

The IGF family consists of insulin, IGF-I, and IGF-II. All are similar in structure and important in cell proliferation and somatic growth. IGF-binding proteins (IGFBPs) are important in regulating the activity of IGF-I and –II. IGF-I and –II act mainly through the IGF-I receptor (IGF-IR). In binding IGF, IGFBPs modulate the interaction of IGF with its receptor. The binding proteins can inhibit this interaction, or they can act by slowly releasing the IGF, prolonging the interaction with the receptor, and enhancing the response (54). Giudice *et al* found elevated levels of fetal cord serum IGFBP-1 and -2 in IUGR fetuses, suggesting that these binding proteins decrease IGF-I transportation to fetal tissues, inhibiting fetal growth (55).

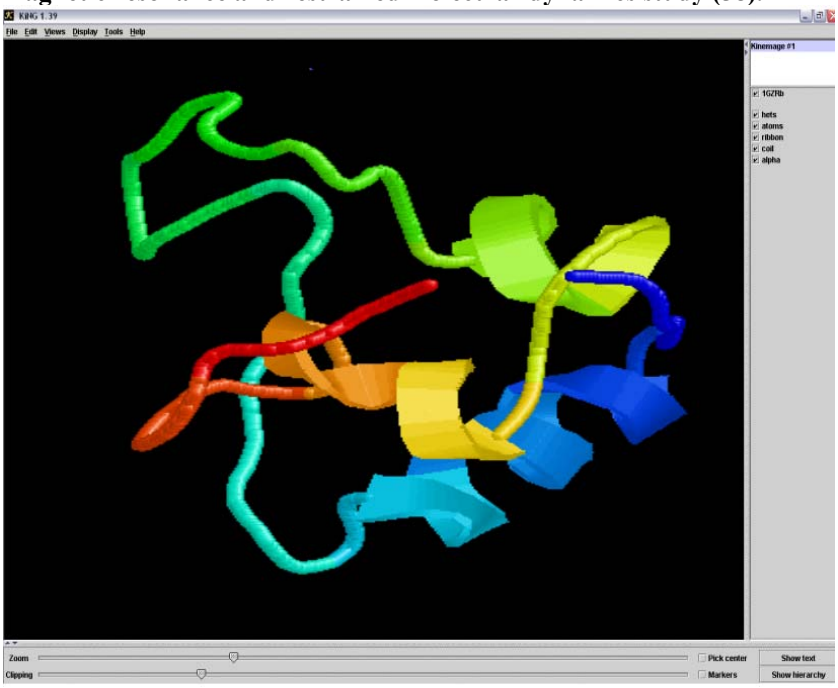
IGFBP-1 has been strongly implicated in IUGR. Studying zebrafish, Kajimura *et al* found that over expression of IGFBP-1 even under normoxic conditions resulted in growth reduction. Also, knockout IGFBP-1 embryos exposed to hypoxic conditions had a significant reduction in growth restriction. Furthermore, growth and development were unaffected in IGFBP-1 knockout embryos under normoxic conditions. It has been described that other stressful conditions besides hypoxia lead to an increase in IGFBP-1, such as malnourishment, stress, and chronic disease (56).

IGF-I Genomics

The IGF-I gene is located on chromosome 12 and has 6 exons. Two promoter regions have been identified. IGF-IA and IGF-IB are two cDNAs of IGF-I, produced by

alternative RNA splicing (56). We have utilized computational genomics and proteomics to further understand the stature of the IGF-I gene/protein (see Figure 4).

Figure 4. A screen snapshot for the IGF-I protein in three dimension view (3-D) visualized using KiG Java software obtained from the PDB (57). The protein is Solution structure of hIGF-I, a nuclear magnetic resonance and restrained molecular dynamics study (58).



As described by Brzozowski *et al* IGF-I has an extension at the C-terminus known as the D-domain (57). The helical core of the IGF-I protein, consisting of three helices, is similar to its equivalent in insulin (57). However, there is an IGF-I-specific C-loop that extends ~20 Å away from the core. There is also the presence of a peptide-bond cleavage between Ser35 and Arg36 resulting in an apparent gap between residues 35 and 39 (57). These structural specificities set IGF-I apart from insulin, despite having high sequence similarity.

Several studies have shown that a defect in the IGF-I gene results in severe growth restriction overall and in numerous organ systems. This occurs even in the

presence of increased GH levels. For example, IGF-I gene or IGF-I receptor gene knockout mice resulted in a 40-45% reduction in the size of the mice (59). To show that IGF-I plays a role in growth, Laron's group, along with four other groups, administered biosynthetic IGF-I long-term to children with primary IGF-I deficiency (Laron Syndrome). This resulted in an increase in yearly mean growth velocity. Exogenous GH was also administered to patients with isolated growth hormone deficiency (IGHD), also showing an increase in growth velocity. This shows that both IGF-I and GH promote linear growth (59).

IGF-IR mutations have also been described in persons with unexplained intrauterine growth restriction and severe short stature. Abuzzahab *et al* showed two single-base-pair substitutions in exon 2 of the IGF-IR gene in a 14-year-old who had poor fetal and postnatal growth, persistent short stature, and an abnormal psychiatric evaluation (nonverbal learning disorder, obsessive tendencies, excessive fantasy role playing, and social phobias). Fibroblasts from this patient showed reduced binding of IGF-I to the IGF-IR and the IGF-IR phosphorylation studies were abnormal. This study also describes a boy with severe short stature and various dysmorphic features who was found to be heterozygous for a point mutation in exon 2 of the IGF-IR gene. In fibroblasts from this patient, the number of IGF-IRs was lower than in control subjects (60).

IGF-I Physiology

IGF-I, also known as somatomedin C, is essential for mammalian growth and development. It is a 70-amino acid polypeptide containing three α-helices. It is produced mainly by the liver (this production is stimulated by growth hormone), through which it exerts its endocrine effects. IGF-I is produced by other nonhepatic tissues as well, having autocrine and paracrine functions. Yakar *et al* found that mice with a liver IGF-I gene deletion had an 80% reduction in circulating IGF-I. This suggests that the majority of circulating IGF-I is produced in the liver under the regulation of growth hormone (GH). The study, however, showed that postnatal and peripubertal growth were normal despite this vast reduction in liver IGF-I (61). GH induces the production of IGF-I, but it is not the only regulator. Nutritional status and liver blood insulin levels also induce the production of IGF-I. IGF-I expression in the reproductive organs is affected by sex steroids, and expression in bone is affected by estrogen and PTH.

Functions of IGF-I include bone growth and metabolism. One study showed that in women, absence of the wild-type (192-bp) allele in the promoter region of the IGF-I gene is associated with lower bone mineral density levels and higher rates of bone loss (62). The absence of this 192-bp allele is also associated with lower levels of circulating IGF-I (63). Another study showed a decrease in the rate of long bone ossification in IGF-I gene knockout mice (64). The same study also showed both sexes of these mutant mice to have a reduction in size of reproductive organs and to be infertile. IGF-I has been shown to be important in CNS development. D'Ercole *et al* showed that transgenic mice containing ectopic IGFBP-1 in the brain had retarded brain growth [IGFBP-1 binds IGF-I, preventing its action (65)]. IGF-I is important for proliferation of the vasculature. Du *et*

al showed that vascular smooth muscle cells transfected with antisense IGF-IR cDNA had a decreased number of IGF-IRs and subsequently smooth muscle cell growth was inhibited (66). See table 2 for a complete overview of the function of IGF-I.

Table 2. Functions of Insulin-like Growth Factor-I (IGF-I) gene and protein. It is similar in structure to proinsulin. IGF-I is a ubiquitous growth factor and plays numerous roles in normal physiology as well as in pathology, such as cancer. IGF-I works in conjunction with growth hormone and also independently. It is produced mainly by the liver, where it acts in an endocrine fashion. It is also produced by other body tissues, where it acts in a paracrine and autocrine manner.

GROWTH
IUGR Stimulates smooth muscle cell migration and proliferation. Survival factor for fibroblasts, vascular smooth muscle cells, neurons, cardiomyocytes, and tumor cells (suppression of IGF-I signaling induces massive apoptosis *in vivo* and *in vitro*). Acromegaly (through overproduction of GH) Osteoblast differentiation (IGF-I regulates Osterix, a vital transcription factor for bone growth) IGF-I may promote erythropoiesis. IGF-I may enhance keratinocyte viability and contribute to a return to epidermal homeostasis, following UVB exposure. Inhibits apoptosis via PI-3-kinase and Bcl-X pathways. IGF-I accelerates regeneration of nerve and skeletal muscle following nerve injury (IGF-I causes satellite cell proliferation, marked by increases in cyclin-D1, required for G1 phase of cell cycle)
REPRODUCTION
Elevate LH levels in women with PCOS causing anovulation. Regulate ovarian cells through leptin.
HEART DISEASE
Increased circulating IGF-I and IGFBP-3 may be stimulators of atherosclerosis. IGF-I may play a role in the pathogenesis of Idiopathic Hypertrophic Cardiomyopathy (*in vitro* studies demonstrate an overexpression of IGF-I in cardiomyocytes of HCM tissue)

CANCER Increased breast cancer risk in women with promoter polymorphisms in IGF-I and IGFBP-3 genes. IGF-I promotes prostate carcinogenesis. IGF-I inhibits apoptosis and stimulates cell proliferation, promoting tumor development. IGF-I/IGFBP-3 imbalance may have implications in hepatocarcinogenesis and liver tumor development in patients with hepatic cirrhosis. Promotes cell division in breast cancer cells. Colorectal carcinogenesis.
CNS Neuron differentiation. Neurotransmitter release. Stimulation of dendritic growth.
ENDOCRINE Serum glucose regulation via alterations in intestinal glucose transporter gene expression.

IGF-I and Fetal Development

During gestation, IGF-I and -II play the major role in fetal growth, and after birth, GH, working through the actions of IGF-I, has the major role. Several studies reviewed in Baker *et al* showed that the embryonic growth effects of IGF-I are GH-independent, since mutant animals with no GH or no pituitary gland showed no impairment in prenatal growth (64). Several studies have found a positive correlation between serum IGF-I levels in the neonate and gestational age at birth (55, 67-69). This suggests that a higher amount of IGF-I is needed in a fetus undergoing a more rapid rate of growth during the later stages of gestation. In a study to determine the effects of null mutations of IGF-I in mice, Baker *et al* found that after embryonic day 13.5, IGF-I null mice embryos grew at a

slower rate and were 60% smaller at the end of gestation than the wild-type controls. The IGF-I null mice also grew at a slower rate postnatally, and after 8 weeks of age, were 30% smaller than the wild-type controls (64).

IGF-I levels have been shown to be significantly lower in IUGR fetuses compared to fetuses with weights above the mean for gestational age (67, 69). However, other studies have found this not to be the case (55). The causes for IGF-I deficiency are not known, but several studies have been done reporting the presence of an IGF-I gene mutation or polymorphism in persons with IGF-I deficiency, IUGR, or both. Mutations have also been described in the IGF-I receptor (IGF-IR) gene. Woods *et al* reports a 15-year-old boy with severe intrauterine growth restriction that persisted after birth, profound sensorineural deafness, and mental retardation that had IGF-I deficiency and a homozygous deletion of exons 4 and 5 of the IGF-I gene (70). Another study reports a boy with IGF-I deficiency and pre- and postnatal growth failure who was found to have a homozygous T→A transversion in the polyadenylation signal sequence in exon 6 of the IGF-I gene, resulting in similar phenotypic characteristics of the aforementioned study growth failure, sensorineural deafness, and delayed psychomotor development (71).

Other factors that influence fetal growth and lead to growth restriction include, but are not limited to, maternal malnutrition, substance abuse, anemia, prescription drug use, infection, and young age (3, 72), as well as a reduction in uteroplacental circulation. It is unclear if all causes of IUGR are related to alterations in IGF-I, or members of the IGF family. However, malnutrition, leading to hypoglycemia, causes up- and down regulation of insulin, IGF-I, and IGF-II (72). Many other growth factors exist that have

been implicated in IUGR, such as leptin, neuropeptide Y, and vasoactive intestinal peptide (72).

Fetal Origin of Adult Disease

Environmental insults, such as malnourishment and decreased oxygen supply, may cause the fetus to undergo some type of adaptation in order to protect vital organ systems, like the CNS. When nutrients are sparse, the fetus directs blood flow to the brain, preserving normal brain development, while causing other vital organs to be growth restricted. This adaptation early in fetal development may result in permanent alterations in anatomical structure, such as decreased cell number, that cause the fetus to be born SGA and puts the infant at high risk for disease later on in life. For example, one study showed that mice exposed to a low protein diet prenatally had smaller hearts and fewer cardiomyocytes than mice exposed to a normal protein diet prenatally (73). Some environmental insults, such as cigarette smoke, may cause mutations in the IGF-I gene, resulting in decreased levels of circulating IGF-I, and producing an SGA infant. One study has shown that there is an increase in the number of DNA adducts in the term placentas of smokers (74). DNA adducts are insults to the DNA and are considered to be the first step in causing a genetic mutation.

Acquired single nucleotide polymorphisms (SNPs) are alterations in the sequence of the genetic material of an individual. To be considered a SNP, the single nucleotide (A, C, T, or G) alteration must occur in at least 1% of the population (75). Research has shown that SNPs can cause a change in cell function, can increase or decrease an individual's risk of acquiring certain diseases, and may even alter the response an individual has to a drug (75). SNPs may occur due to an environmental insult. Acquired SNPs can cause permanent alterations in the DNA sequence and can be passed on to offspring.

A study looking at a polymorphism in the promoter region of the IGF-I gene found that individuals who were noncarriers of the 192-bp allele, compared to homozygous and heterozygous carriers, had significantly lower circulating IGF-I levels. To show that low levels of IGF-I are associated with CV and cerebrovascular disease, two early markers of atherosclerosis were assessed: carotid intima-media thickness and aortic pulse wave velocity (76). Both of these parameters were significantly increased in hypertensive noncarriers compared with hypertensive carriers. This study suggested a hypothesis as to why this difference was observed only in hypertensive subjects: those with a higher hemodynamic load needed more vascular protection, which is provided by the effects of IGF-I. Noncarriers of the 192-bp allele were unable to produce sufficient amounts of IGF-I that would protect the vasculature from damage caused by hypertension.

Some of the adult diseases that have been described to be associated with low birth weight are Syndrome X (central obesity, hypertension, dyslipidemia, impaired glucose tolerance), type II diabetes mellitus, and atherosclerosis (77-81). Animal studies show that even brief exposure to malnourishment *in utero* leads to permanent changes in blood pressure (BP), cholesterol metabolism, and insulin responses to glucose (82). IUGR individuals have defects in the action of insulin and its secretion, as well as fewer pancreatic ß-cells (83). It has even been speculated that IUGR may predispose an individual to a sedentary lifestyle, which in turn would increase the risks of developing cardiovascular (CV) disease (84). Interestingly, those at highest risk for CV disease as adults are those who were small at birth and obese as adults. One study found the inverse relationship between birth weight and adult blood pressure had more to do with thinness

at birth rather than birth weight alone (85). This same study found that among the heaviest boys, those who were thin at birth had a predicted probability of high BP almost twice that of those who were relatively heavy at birth.

The devastation of heart disease is well known. The cost of CV disease in the United States is projected to be $394 billion in 2005, including both health care expenditures and lost productivity from death and disability (86). Globally, the number of deaths due to CV disease exceeds 12 million annually. It is important to fully understand the pathophysiology of IUGR in order to obtain early detection and intervention methods, theoretically decreasing the incidence of CV disease.

A large, systematic review found an inverse relationship between birth weight and systolic blood pressure (SBP) in adulthood. On average, a 1-kg decrease in birth weight corresponded with a 2 to 4 mmHg increase in SBP. The review also found a positive correlation between postnatal catch-up growth of SGA infants and adult SBP (87).

Another proposed cause of adult heart disease in terms of fetal growth restriction is congenital oligonephropathy. The premise of this theory is that IUGR causes impaired renal growth, resulting in fewer nephrons and a decrease in glomerular filtration surface area. This may result in systemic and glomerular hypertension as an adult. Silver *et al* found that the renal volume in growth-restricted fetuses was significantly smaller than in healthy fetuses (2).

Some studies are beginning to propose prophylactic treatment options for IUGR infants. IUGR rats are shown to have features of human type 2 diabetes—β-cell secretory defects, insulin resistance, and a reduction in β-cell mass. IUGR rats treated short-term in the neonatal period with exendin-4 (Ex-4), a glucagon-like peptide-1 analog that

promotes β-cell proliferation, had improved glucose tolerance. These rats eventually became normoglycemic, and later had no signs of diabetes. The effects of Ex-4 on glucose homeostasis in these rats were permanent (83).

A study by Vickers *et al* showed that treatment with GH in intrauterine growth-restricted rats improved systolic blood pressure and overall fat mass (88). N-3 fatty acid intake through the consumption of dietary fish may be associated with an increase in birth weight and a decrease in the risk of IUGR and preterm labor. An English study looked at the correlation between n-3 fatty acids and gestation duration and birth weight. In the univariate analysis of this study, fish consumption was positively associated with birth weight, and negatively associated with the frequency of IUGR (89).

Hypothesis

The purpose of this study was to look at the genotoxic and cytotoxic effects of benzo-α-pyrene (BαP), a chemical mutagen that is present in cigarette smoke, on the insulin-like growth factor-I (IGF-I) gene.

Materials and Methods

Cell Culture

Cells from an HTR V8 EVCT cell-line of human placental cells were cultured in culture flasks (Falcon™, 75cm^2 canted neck, vented cap). Cells were allowed to grow in bovine serum for 72 hours. Cells were grown to subconfluence. After this time the cells were starved for 48 hours. Then cells were either treated or untreated. The untreated flask was the control. Cells were treated at two doses: dose 1 = 1μM BαP and dose 2 = 5μM BαP. BαP has been reported to induce DNA adducts at a concentration of 2.5 μM (90). In the mouse hepatoma cell line TAOc1B(a)Prc1, only 40 nM of BαP was required to induce a 2-fold increase in sister chromatid exchange (SCE) frequency (91). Screening cell cultures for SCE's is a frequent method of testing chemicals for potential DNA damaging (and thus probably mutagenic) effects (92). Cells incubated for 72 hours, after which time the treatment was removed and flasks were immediately frozen at -20 C until use.

Chromosomal Preparation and Light Microscopy Analysis

Cells from the HTR human placental cell line were prepared for chromosomal analysis. The cells were cultured and allowed to enter metaphase. The mitotic inhibitor Colcemid was added to arrest cells at metaphase. Chromosomes were then extracted by exposing cells to a hypotonic solution followed by a series of fixative solutions. The cells then expanded and the chromosomes were allowed to spread out. For staining, the slides were placed in Giemsa stain solution (1mL Giemsa stain to 50mL H_20) for 40 minutes. The slides were then rinsed with distilled water and allowed to air-dry. Cells from the

control and dose 1 were prepared and analyzed.

For the analysis of chromosomal aberrations, the entire area of each slide was viewed under a light microscope (Carl Zeiss, Inc., Thornwood, NY) and all chromosomes were analyzed. Chromosomes were visualized at 1000x. Chromosomes were considered abnormal if there were numerical aberrations (aneuploidy) or structural aberrations (condensed, contracted, clumped, sticky, or ringed). Photographs of selected chromosomes from each group (control or treated) were taken.

Mitotic index (MI) is the fraction of cells in a microscope field that contain condensed chromosomes. MI is a method of quantifying cellular proliferation. For mitotic index analysis, the slides were viewed under light microscopy. Ten random fields were viewed and the number of metaphasic cells per 100 cells were counted for each field. A student t-test was performed in order to determine the significance.

RNA Extraction and Gel Electrophoresis

The TRIzol® Reagent Method was used (see protocol sheet from Invitrogen Life Technologies™, form no. 18057N). Cells were grown to subconfluence in culture flasks (Falcon™, 75cm^2 canted neck, vented cap). *Homogenization:* The cells (control, dose 1, and dose 2) were lysed directly in the culture dish by adding 1mL TRIzol® Reagent per 10cm^2 area of culture dish, passing the cell lysate several times through a pipette in order to disrupt the cell membrane and homogenize the solution. The samples were transferred to Eppendorf (E) tubes. *Phase Separation:* The homogenized samples were incubated for 5 minutes at 15 to 30° C. to permit the complete dissociation of nucleoprotein complexes. 0.2mL of chloroform per 1mL of TRIzol® Reagent was added. Each sample tube was

shaken vigorously by hand for 15 seconds and incubated at 15 to 30° C. for 2 to 3 minutes. The samples were centrifuged at 12,000 x *g* at 4° C. for 15 minutes. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol® Reagent used for homogenization. *RNA Precipitation:* The colorless upper aqueous phase was transferred to a new E tube and the organic phase was saved for subsequent DNA isolation. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5mL of isopropyl alcohol per 1mL of TRIzol® Reagent was used. Samples were incubated at 15 to 30° C. for 10 minutes and centrifuged at 12,000 x *g* at 4° C. for 10 minutes. The RNA precipitate formed a visible gel-like pellet on the side and bottom of the tube. *RNA Wash:* The supernatant was removed and the RNA pellet was washed once with 75% ethanol, using 1mL per 1mL TRIzol® Reagent used. Samples were then vortexed and centrifuged at 7,500 x *g* at 4° C. for 5 minutes. *Redissolving the RNA:* The RNA pellet was allowed to dry under a fume hood for 10 minutes. The RNA pellet was dissolved in 50µL RNAse-free water and incubated for 10 minutes at 55 to 60° C. The RNA solution was frozen at 4º C. until use.

For the purpose of confirming the purity of the RNA extracted, agarose gel electrophoresis was run. The gel was prepared by adding 0.5gm agarose to 37mL ddH_20 in a 250mL Erlenmeyer flask and microwaving for 1 minute 10 seconds until agarose dissolved completely. Care was taken so mixture did not boil over. Mixture was cooled for 3 minutes over ice. 5mL of 10x MOPS solution (0.2M MOPS, 50mM sodium acetate, 5mM EDTA, pH 7.0) and 8.75mL 37% formaldehyde was added; swirled flask to mix.

Mixture was poured into gel tray and comb was placed; gel was allowed to cool over ice for about 20 minutes. RNA samples were prepared by adding 5µL RNA solution, 10µL RNA denaturation buffer (10mL 100% deionized formamide, 3.5mL 37% formaldehyde, and 1.5mL 10x MOPS), 1µL ethidium bromide (500µg/mL), and 5µL RNA Loading Buffer (Sigma cat. No. R-1386). Prior to placing in gel, RNA samples were incubated in 65 C. water bath for 10 minutes and placed on ice for 2 minutes. Samples were run in buffer solution (40mL 10x MOPS, 360mL ddH20, and 70mL 37% formaldehyde) at 50 volts for about 2 hours, or until dark blue dye had migrated about 3/4 across length of gel. Gel was then viewed under UV light transilluminator and picture taken using a Polaroid camera with photographic hood.

DNA Extraction and Gel Electrophoresis

During RNA extraction using the TRI Reagent method, the cells from the culture flasks were homogenized with TRI Reagent and after several steps as described above for RNA extraction, an E tube was spun and the upper aqueous phase containing the RNA was extracted. For DNA extraction, the E tubes were centrifuged at 12,000 x *g* at 4 C. for 5 minutes. 0.5mL of back extraction buffer (BEB: 4M guanidine thiocyanate, 50mM sodium citrate, 1M Tris, water) was added to each E tube. Tubes were mixed intensively for 3 minutes and then spun at 10,000 x *g* at 4 C. for 30 minutes. The upper aqueous phase containing the DNA was transferred to new E tubes. 0.4 mL of isopropanol was added to each tube. Tubes were allowed to incubate at room temperature for 5 minutes. Samples were centrifuged at 10,000 x *g* at 4 C. for 15 minutes. The supernatant was removed and the DNA pellet was washed with 0.5mL of 70% ethanol and spun at 12,000

x *g* at 4 C. for 15 minutes. The ethanol was poured off and the DNA pellet was dissolved in TE buffer (0.01 M Tris, pH 7.3, 0.001M Na2EDTA) and stored at 4 C. until use.

For the purpose of confirming the purity of the DNA extracted, agarose gel electrophoresis was run. The gel was prepared by adding 0.5 gram agarose to 50 mL TE buffer solution (10mL 1M Tris, pH 8.0, 200µL 0.5M EDTA; added ddH_20 to 1000mL) in a 250mL Erlenmeyer flask and microwaving for 1 minute until agarose dissolved completely. Care was taken so mixture did not boil over. Mixture was cooled for 3 minutes over ice. 1µL ethidium bromide (500µg/mL) was added; swirled flask to mix. Mixture was poured into gel tray and comb was placed; gel was allowed to cool over ice for about 20 minutes. DNA samples were prepared by adding 50µL DNA solution and 10µL loading buffer (25mg bromophenol blue, 4g sucrose, and H_20 to 10mL). Samples were run in TE buffer at 50 volts for about 1 hour, or until dye had migrated about 3/4 across length of gel. Gel was then viewed under UV light transilluminator and picture taken using a Polaroid camera with photographic hood.

cDNA Synthesis

(Sigma Enhanced Avian RT First Strand Synthesis Kit, Product no. STR-1). The following was added to a PCR tube: 5µg RNA template (extracted from human placental cell line, see above *RNA Extraction and Gel Electrophoresis*), 1µL deoxynucleotide mix, 1µL 3' antisense specific primer, ddH_20 quantity sufficient to bring total volume to 10µL. Samples were gently mixed and briefly centrifuged. Sample tubes were placed in thermal cycler at 70° C. for 10 minutes. The tubes were then placed on ice, centrifuged, and the following components added: 2µL 10x buffer for eAMV-RT, 1µL enhanced avian RT,

1µL RNase inhibitor, and 6µL ddH_2O. The reaction tubes were incubated at 25° C. for 15 minutes. The tubes were placed in thermal cycler at 50° C. for 50 minutes. The samples were then used for PCR (see next section).

PCR of DNA and cDNA and Electrophoresis

(SuperArray BioScience Corporation user manual part #1016A version 1.2) The following was added to a sterile PCR tube for each sample (control, dose 1, and dose 2): 12.5µL ReactionReady™ HotStart Sweet PCR master mix (PA-007), 9.5µL ddH_20, 1µL cDNA template, and 1µL primer. See Table 3 for more information on the primers. The samples were placed in the thermal cycler and the program from Table 4 was run.

Table 3. IGF-I primer sequences.

IGF-I Exon	Strand	Primer Sequence
1	Forward	GCTAAATCTCACTGTCACTGCTAAATT
	Reverse	GAATTCCCCAATGACAACAAAGAG
2	Forward	CCTGATTAATGACAGTCGTGG
	Reverse	CCAGATACGGGCACTCATTC
3	Forward	GCACCCTAACATGAGGCGACTCTG
	Reverse	GGATCCCACCCAGGTGGGCTTAC
4	Forward	GCTCATTCAAAGGGACAACATGGG
	Reverse	TGCTCCTCTCTCATCATCCTTGCC

Table 4. Thermal cycler program for PCR.

Cycles	Duration	Temperature
1	15 minutes	95º C
35	15 seconds	95º C
	30 seconds	55º C
	30 seconds	72º C

An agarose gel was prepared by adding 1 gram agarose to 50mL TAE (40mM Tris-acetate, 2mM EDTA, pH 8.0) in a 250mL Erlenmeyer flask and microwaving for 1 minute 10 seconds until agarose dissolved completely. Care was taken so mixture did not boil over. Mixture was cooled for 3 minutes over ice. 20μL ethidium bromide was added; swirled flask to mix. Mixture was poured into gel tray and comb was placed; gel was allowed to cool over ice for about 20 minutes. Samples were run in TAE buffer at 90 volts for about 1 hour, or until dye had migrated about 2/3 across length of gel. Gel was then viewed under UV light transilluminator and picture taken using a Polaroid camera with photographic hood. The gel photo was converted to digital form using a standard scanner and the gel photo was analyzed using the software NIH *Image*, Scion Image for Windows.

I conducted all laboratory procedures described above except chromosomal preparation of the HTR placental cells. This procedure was performed by the research supervisor, Dr. Ahmed Fadiel.

Results

The cytogenetic analysis was done using the Giemsa stain method under light microscopy. This analysis showed marked differences between the chromosomes from the control cells and the chromosomes from the BαP-treated cells. Furthermore, the chromosomal aberrations differed amongst the two different doses of BαP. Chromosomes from the control group are shown in Figures 5-7. Chromosomes in Figure 5 are euploidic, all sister chromatids have normal centromeres, and the chromatid length is within normal limits. Figures 6 and 7 show chromosomes that are euploidic and structurally normal.

Dose 1 showed chromosomal clumping, as reported in Trier *et al* (93) and Grant (94) and also contraction, stickiness, and fragmentation (94). Chromosomes treated with dose 1 are shown in Figures 8-11. Figure 8 reveals hypoploidy and chromosomal contraction. Figure 9 shows hypoploidy and chromosomal contraction and clumping. Figure 10 shows sticky and clumped chromosomes. Figure 11 demonstrates hypoploidy, chromosomal contraction, and also a ringed chromosome.

Dose 2 showed polyploidy with clumping and stickiness (Figures 12 and 13). Figure 12 shows polyploidy. In addition to polyploidy, Figure 13 also reveals clumped and sticky chromosomes.

Figure 5. Control group (no treatment). Normal chromosomes. Chromosomes prepared from human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. The number of chromosomes is 46 (human diploid). All sister chromatids have normal centromeres and the chromatid length is within normal limits.

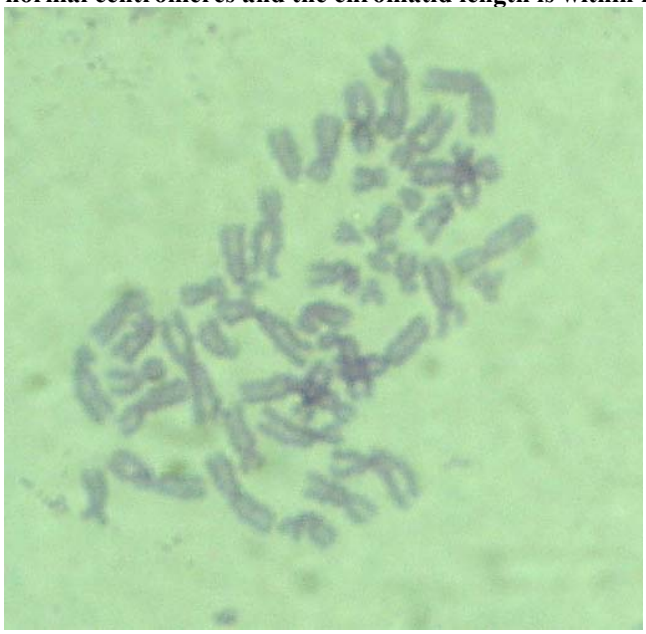


Figure 6. Control group (no treatment). Normal chromosomes. Chromosomes prepared from human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. The centromeres appear intact and the chromatid length appears to be within normal limits.

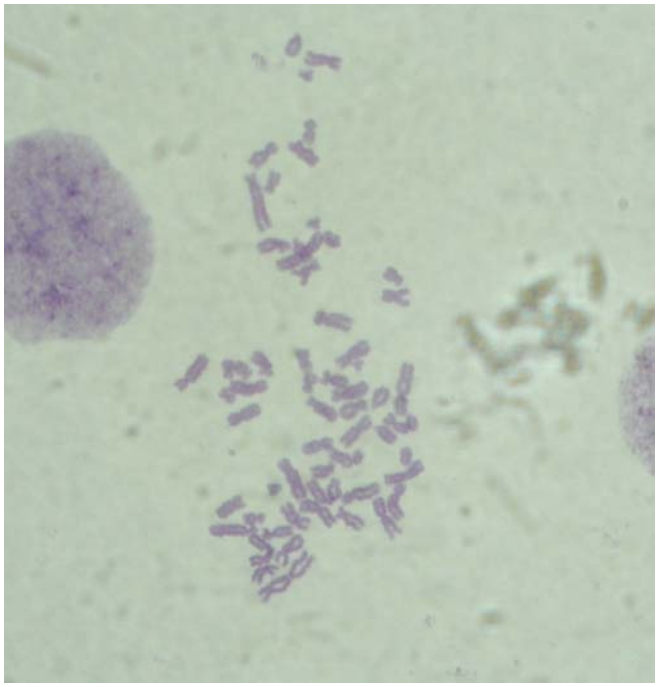


Figure 7. Control group (no treatment). Normal chromosomes. Chromosomes prepared from human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. The number of chromosomes is 46 (human diploid).

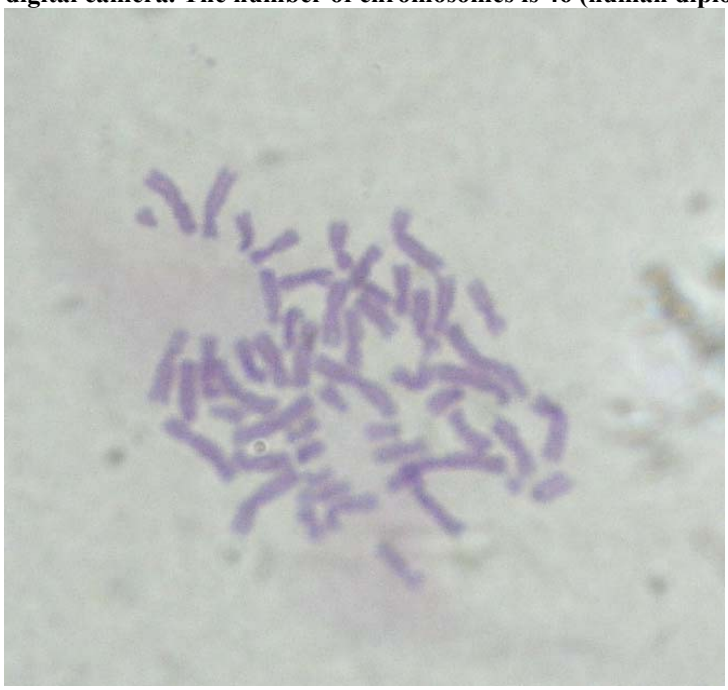


Figure 8. Treated group, dose 1 (cells treated with 1µM B(α)P). Chromosomes prepared from B(α)P-treated human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. Aberrant chromosomes are hypoploidic and appear condensed and contracted.

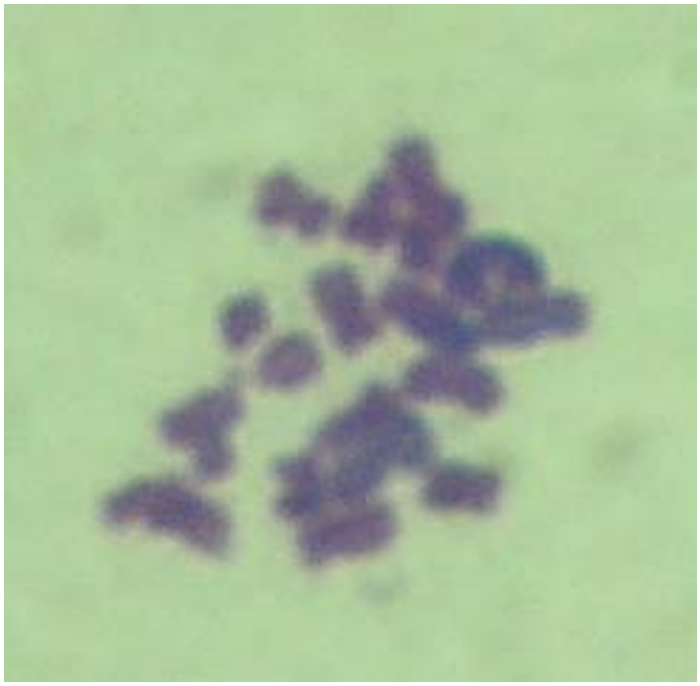
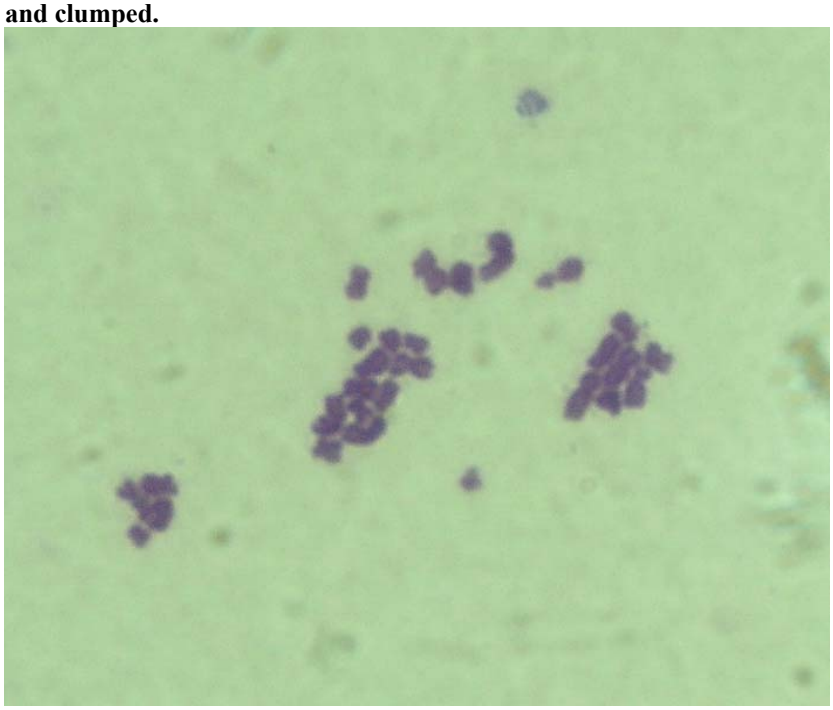


Figure 9. Treated group, dose 1 (cells treated with 1µM B(α)P). Chromosomes prepared from B(α)P-treated human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. Aberrant chromosomes are hypoploidic and appear contracted and clumped.



.

Figure 10. Treated group, dose 1 (cells treated with 1µM B(α)P). Chromosomes prepared from B(α)P-treated human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. Aberrant chromosomes appear sticky and clumped.

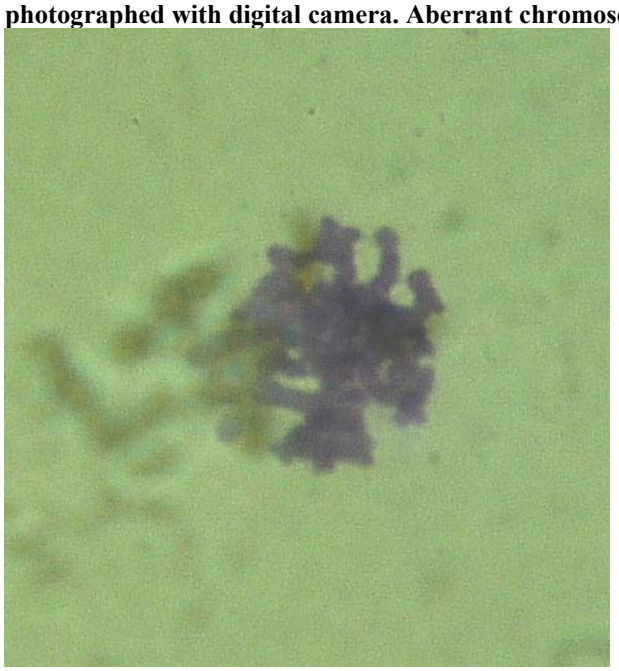


Figure 11. Treated group, dose 1 (cells treated with 1µM B(α)P). Chromosomes prepared from B(α)P-treated human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. Aberrant chromosomes are hypoploidic and appear condensed and contracted. The arrow indicates a ringed chromosome.

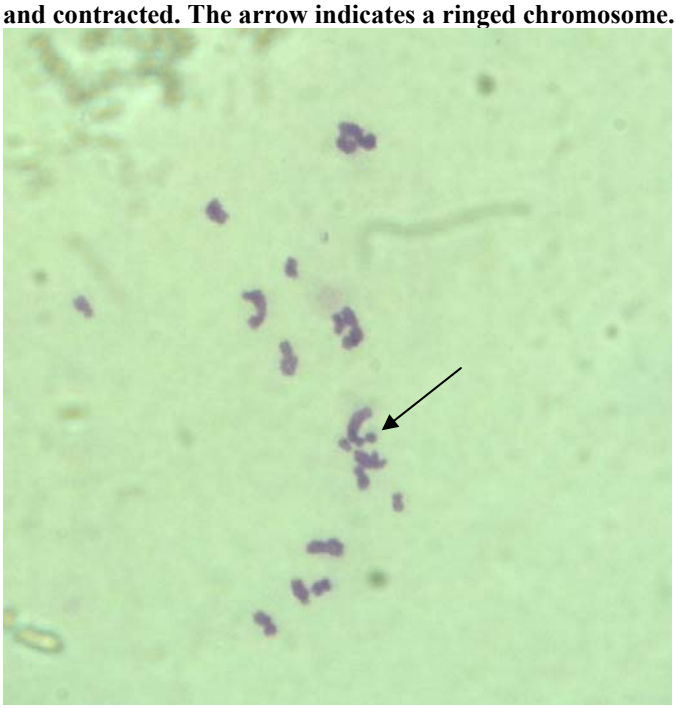


Figure 12. Treated group, dose 2 (cells treated with 5µM B(α)P). Chromosomes prepared from B(α)P-treated human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. Aberrant chromosomes are polyploidic.

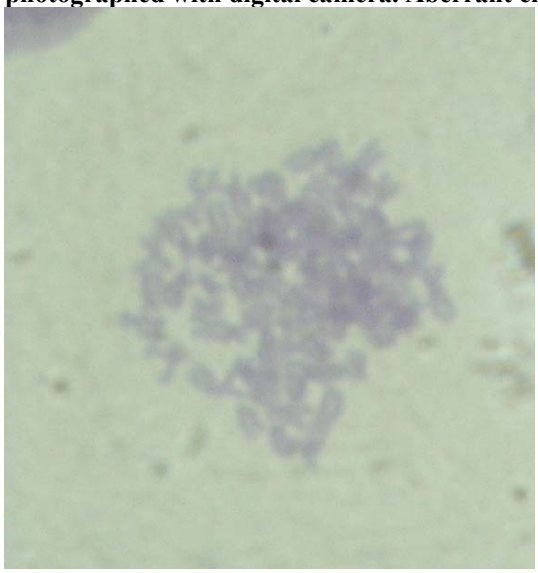
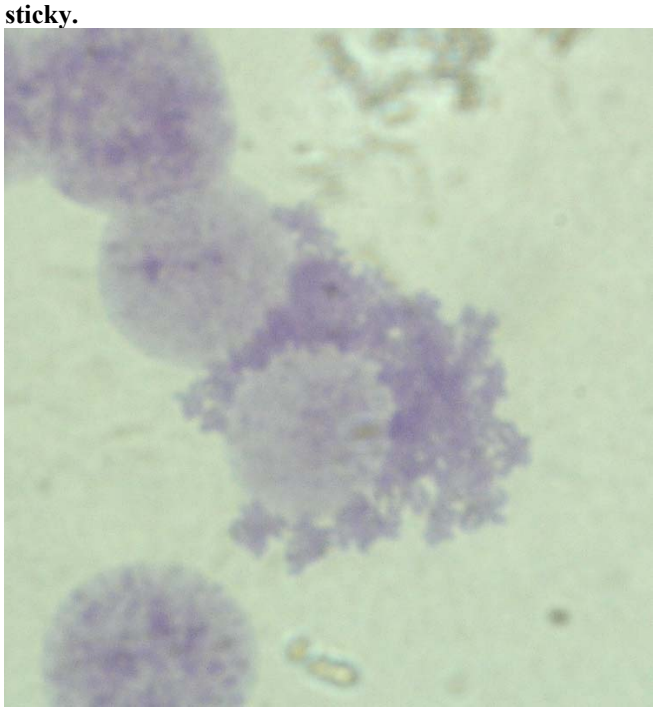


Figure 13. Treated group, dose 2 (cells treated with 5µM B(α)P). Chromosomes prepared from B(α)P-treated human placental cell line; Giemsa stain. Viewed under light microscopy (1000x) and photographed with digital camera. Aberrant chromosomes are polyploidic and appear clumped and sticky.



The mitotic index analysis showed that mitosis was significantly reduced in the sample treated with BαP (figure 14). A student t-test gave a *P* value of 0.0447, 95% confidence interval (0.024-1.236).

Figure 14. Mitotic indices for control group versus dose 1 of the BαP-treated group. Mitotic index (MI) is the fraction of cells in a microscope field which contain condensed chromosomes. MI is a method of quantifying cellular proliferation. For mitotic index analysis in this study, slides containing prepared chromosomes from a human placental cell line were viewed under light microscopy (Carl Zeiss, Inc., Thornwood, NY). Ten random fields were viewed and the number of metaphasic cells per 100 cells were counted for each field. An unpaired student t-test showed a *p*-value of 0.0447, 95% confidence interval (0.024-1.236).

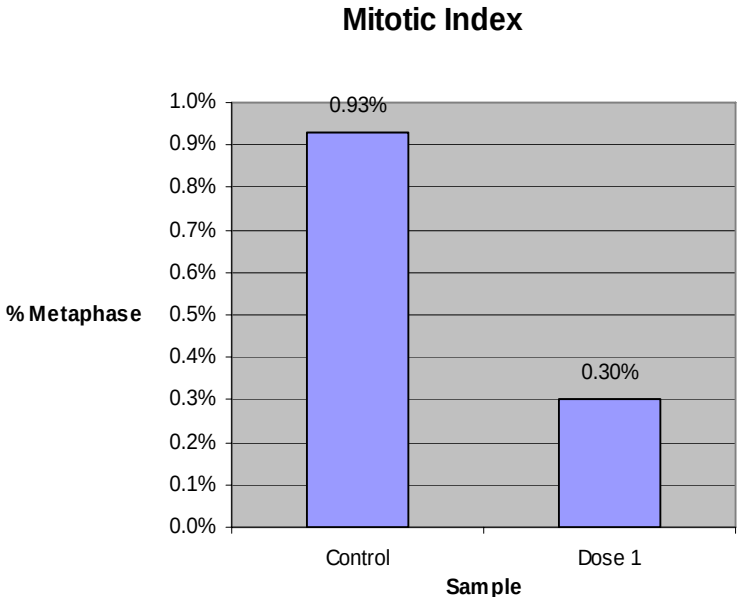
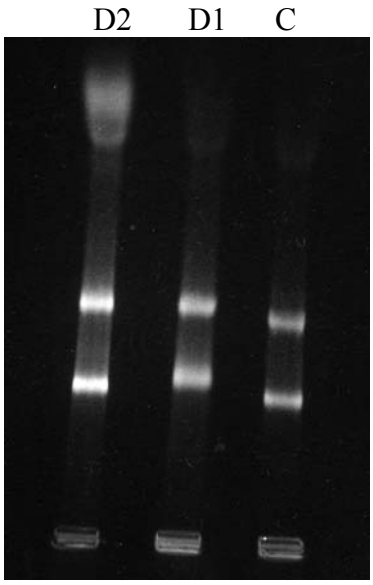


Figure 15 shows the gel electrophoresis of RNA extracted from HTR human placental cell line. Figure 16 shows the gel electrophoresis of DNA extracted from Trizol. Traditional PCR of DNA shown in figure 17 displays the amplification of IGF-I exons 1-4. The lane labeled *L* is the ladder (PCR 100bp Low Ladder, Sigma P1473). The IGF-I exons are between 200 and 400 base pairs. The sequence of each exon is given in Table 3. It is important to note that the IGF-I gene consists of 6 exons, however, this study was performed in a laboratory that only had access to exons 1-4.

Figure 15. Gel electrophoresis of RNA extracted from human placental cell line.



D2	Dose 2: 5µM BαP
D1	Dose 1: 1µM BαP
C	Control: untreated

Figure 16. Gel electrophoresis of DNA extracted from human placental cell line.

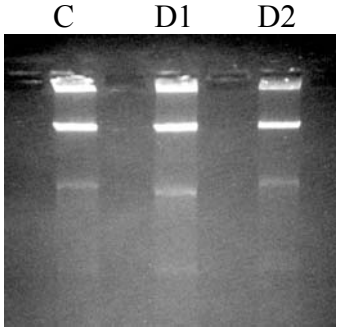
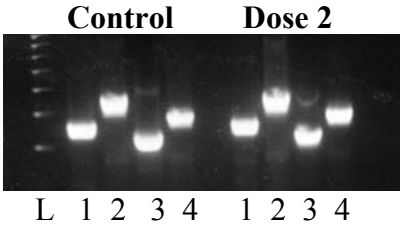


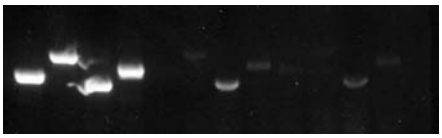
Figure 17. Gel electrophoresis of DNA polymerized chain reaction (PCR) products of insulin-like growth factor-I (IGF-I) exons 1-4.



L = Ladder (100 bp)
1 = IGF-I exon 1
2 = IGF-I exon 2
3 = IGF-I exon 3
4 = IGF-I exon 4

Traditional PCR of cDNA synthesized from RNA (as shown in Figure 15) is shown in Figure 18. Samples from all groups (control, dose 1, and dose 2) were run and IGF-I primers 1-4 were amplified from all groups. Samples were run containing the primer of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal normalizer. For optimal viewing of the IGF-I exon PCR bands, the samples were run again without the GAPDH, as shown in Figure 18. The amplification bands were analyzed using the NIH *Image*, Scion Image for Windows software and the results are shown in Figure 19. The area under each curve represents the relative brightness of each band as compared to the internal normalizer, GAPDH. The brightness of the bands from the treated groups is diminished compared to the control group. Of note, this traditional method of PCR is only semi-quantative and real-time PCR is needed for complete quantitative analysis.

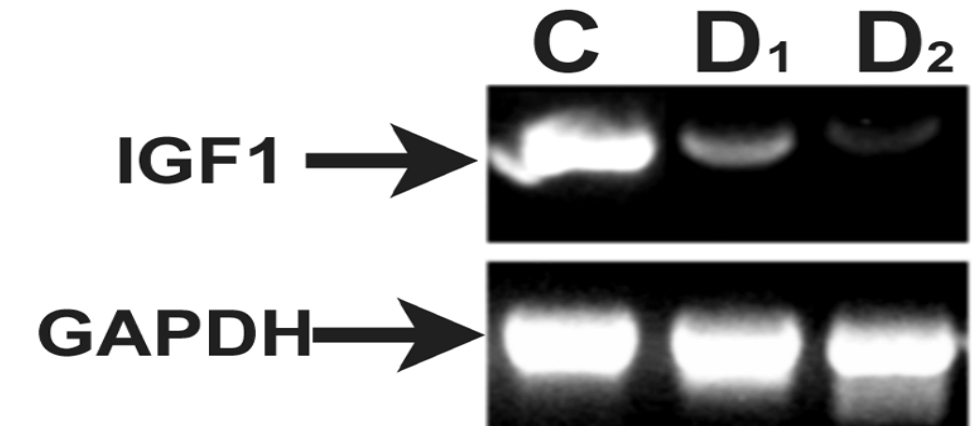
Figure 18. Gel electrophoresis of cDNA polymerized chain reaction (PCR) products of insulin-like growth factor-I (IGF-I) exons 1-4. The cDNA was synthesized from RNA extracted from human placental cells. Each lane number represents the exon number. The first four lanes are from the control group (untreated); the second four lanes are from the dose 1 group (treated with 1µM BαP); and the third four lanes are from the dose 2 group (treated with 5µM BαP).

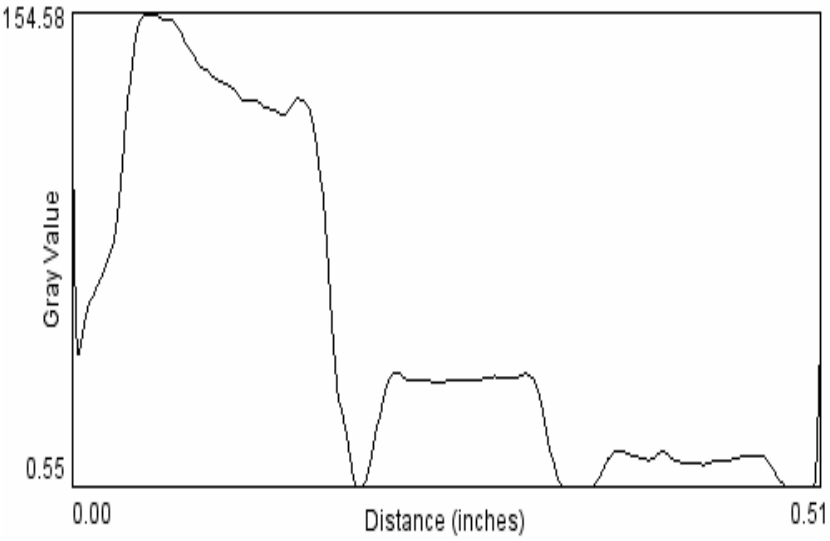


1 2 3 4 1 2 3 4 1 2 3 4
Control Dose 1 Dose 2

1 = IGF-1 exon 1
2 = IGF-1 exon 2
3 = IGF-1 exon 3
4 = IGF-1 exon 4

Figure 19. Analysis of PCR amplification bands using NIH *Image*, Scion Image for Windows software. GAPDH is the internal normalizer. The brightness of the IGF-I exon amplification bands was compared against the brightness of the GAPDH bands. The control group is untreated. Dose 1 and dose 2 were treated with 1µM BαP and 5µM BαP, respectively.





CONTROL DOSE 1 DOSE 2

Discussion

IGF-I has been implicated in IUGR. One common cause of IUGR is cigarette smoking during pregnancy. BαP is a commonly studied mutagen found in cigarette smoke. This study looked at the effects of BαP on the IGF-I gene through methods of cytogenetic analysis, mitotic index determination, and PCR of cDNA. The cells studied were from a human placental cell line.

Cytogenetics is the study of chromosome number, structure, function, and behavior in relation to gene inheritance, organization and expression. Cytogenetic analysis is used to study and diagnose many different diseases, from malignancies, such as head and neck cancer (95) and leukemia, to Trisomy 21 to pesticide exposure (96). In cytogenetic analysis, there are two types of aberrations: structural and numerical. A numerical aberration is a change in the number of chromosomes from the normal number characteristic of the cells utilized. A structural aberration is a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges, and interchanges (97). There are two types of structural aberrations: chromatid and chromosome. Table 5 gives examples of the different types of chromosomal aberrations.

Table 5. Major categories of chromosomal aberrations (98-100).

STRUCTURAL CHROMOSOMAL ABERRATIONS	NUMERICAL CHROMOSOMAL ABERRATIONS
Chromosomal breaks	Aneuploidy
Isochromatid gaps	Hyperploid (polyploidy)
Dicentrics and rings	Hypoploid
Chromatid breaks	
Chromatid gaps	
Fusion	
Deletions	
Translocations	
Fragmentation	
Chromosomal stickiness	
Chromosomal clumping	
Chromosomal contraction	

The chromosomal analysis revealed aberrations amongst the BαP-treated groups. Dose 1 (treated with 1μM BαP) showed hypoploidy, clumping, contraction, stickiness, and ringed chromosomes. Dose 2 (treated with 5μM BαP) showed polyploidy, clumping, and stickiness. Schmidt *et al* performed cytogenetic analysis on benzpyrene-induced osteosarcomas in the rat. They found mainly aneuploidy (abnormal chromosome number) and translocation (interchange of parts between nonhomologous chromosomes) (101). Benzpyrene is another name for BαP (102).

Chromosomal stickiness was first described by Gaulder (103) in 1987. Gaulden proposed stickiness as a possible cause of mutagen-induced structural chromosome aberration without DNA interaction (104). Rayburn and Wetzel describe sticky chromosomes as a consequence of genetic mutations or environmental effects on mitosis and meiosis (105). Chromosomal stickiness has been described in numerous studies (106-108).

Chromosomes from the control group (untreated) are shown in figure 5, revealing normal chromosomes, with the centromeres intact and the correct diploid number of 46. Figures 6 and 7 also show normal chromosomes and reveal similar findings to Figure 5.

Chromosomes from the dose 1 group are shown in Figure 8. It is obvious that the chromosomes appear aberrant. They are hypoploidic and contracted. Like Figure 8, the chromosomes in Figure 9 are hypoploidic and contracted. They also appear to be clumped. Khokhar *et al* exposed plant root tips to radiation and found chromosomal clumping (109). In figure 10 the chromosomes are so clumped as to be almost indistinguishable. The chromosomes in figure 11 reveal a ringed chromosome. Ringed chromosomes result when one broken end of a chromosome becomes sticky and fuses with the other end. Ringed chromosomes are found in malignancies, such as nonrhabdomyosarcoma soft tissue sarcomas (110) and acute myelofibrosis (111).

Two examples of chromosomes treated with dose 2 are shown in figures 12 and 13. Both show polyploidy. In addition to polyploidy, Figure 13 reveals clumping and stickiness. It is interesting to note that the chromosomal aberrations differ amongst the two doses of treatment groups. Dose 1 resulted in mostly hypoploidy, while dose 2 resulted in hyperploidy. Many different types of chromosomal anomalies from BαP exposure have been described here. We speculate that the genetic material of the developing fetus is prone to the same adverse effects of BαP as the chromosomes from this study.

BαP affects the cell cycle, as indicated by the mitotic indices of the control and treated cells (see Figure 14). In the mitotic index analysis, cellular proliferation was significantly reduced in the treated group versus the control group (p=0.0447). Mitotic

index (MI) is the fraction of cells in a microscope field which contain condensed chromosomes. MI is a method of quantifying cellular proliferation. In this study, the mitotic index was done in order to look at the effects of BαP on cell proliferation in human placental cells. This type of analysis was done by Bresgan *et al* where they looked at the effects of a ß-carotene breakdown products on rat hepatocytes (112). Another study looked at the effects of BαP on mouse skin and tumor production and found a dose-dependent increase in the mitotic index (113).

This study has shown through traditional PCR analysis that BαP affects DNA at the genomic level. This study focused on the IGF-I gene due to its various implications in fetal growth restriction. A review of the literature on gene mutations and polymorphisms of IGF-I and the IGF family that resulted in developmental impairment is shown in Table 6 and Table 7, respectively.

Table 6. Phenotypic effects of described Insulin-like Growth Factor-I (IGF-I) mutations. Numerous studies have described the phenotypic effects of mutations in the IGF-I gene. The IGF-I gene is found on chromosome 12 (12q22-qter) and contains 6 exons. The mRNA is alternatively spliced to produce IGF-IA (153 amino acids) and IGF-IB (195 amino acids). *Cytosine-adenosine. **diabetes mellitus type II, myocardial infarction. ***wild-type allele 192/192

Genotype	Location	Phenotype	Physiologic Effect	Report
Deletion of exons 4 & 5	IGF-I	Severe growth restriction		Laron 2001 (59)
Polymorphism *Hind*III, *Pvu*II	IGF-I exon 5			Rotwein *et al* 1986 (114)
Homozygous deletion of exons 4 & 5	IGF-I	Growth failure, deafness, mental retardation		Woods *et al* 1996 (70)
CA* repeats	1 kb upstream from transcription start site, IGF-I	Increased risk DMII and MI**		tHart, *et al* 2004 (115)
Polymorphism allele 191***	IGF-I	Persistent short stature		Arends *et al* 2002 (116)
T→A transversion	Polyadenylation signal in 3' untranslated region of exon 6 of IGF-I	Severe growth restriction		Bonapace *et al* 2003 (71)
Missense mutation, $Val^{44}Met$ IGF-I (G247A)	Position 44, methionine instead of valanine	Severe growth restriction and mental retardation, deafness	90-fold ↓ affinity for IGF-IR ; ↓ activation of downstream signaling pathways	Denley *et al* 2005 (117)

Table 7. Phenotypic effects of described Insulin-like Growth Factor (IGF) family mutations. The IGF family consists of IGF-I, IGF-II, the IGF receptors (IGF-IR and IGF-IIR), and the IGF binding proteins (IGFBP) 1-6.

Genotype	Location	Phenotype	Report
IGF2/ApaI	IGF-II	High BMI in young adults	Gomes, *et al* 2005 (118)
Single base pair substitution in codon for amino acids 108 & 115	IGF-IR exon 2	Fetal & postnatal growth failure	Abuzzahab *et al* 2003 (60)
Point mutation CGA to TGA, heterozygous	IGF-IR exon 2	Fetal & postnatal growth failure	Abuzzahab *et al* 2003 (60), Kiess *et al* 2005 (119)
Gly1619arg	IGF-IIR	Small stature, age 3-7 years	Petry *et al* 2005 (120)

The brightness of the PCR amplification bands from each group (control, dose 1, and dose 2) was compared relative to the internal normalizer, GAPDH. There was a difference in the brightness, with the control having the most bright band, dose 1 the second-most bright band, and dose 2 having the least bright band. Since real-time PCR was not done, we can only speculate that there is a dose-dependent response between dose 1 and dose 2. In the future, real-time PCR would need to be done in order to thoroughly analyze the qualitative effect of BαP on the IGF-I gene. As shown in Figure 18, the brightness of the bands for the gene products from treated cells are diminished compared to the control group. We speculate that BαP has deleterious effects on the IGF-I gene. It is impossible to say from this study the mechanism by which BαP disrupts the IGF-I gene. Because of the possible dose-dependent disruption of BαP on the IGF-I gene, women who smoke during pregnancy, and find it difficult to quit, may reduce the risk of harm to the fetus by simply cutting down on the number of cigarettes smoked each day.

Complete smoking cessation would be the best way of decreasing the risk of fetal abnormalities, however.

Intrauterine growth restriction is a serious problem. The immediate effects are harmful and costly. The potential long-term effect of cardiovascular disease is striking. Depending on the cause, there may be no cure for IUGR. Therefore, it is imperative that IUGR be prevented. One of the most preventable causes of IUGR is cigarette smoking during pregnancy. This study has shown that toxins in cigarettes are harmful to the fetus on many levels—at the genomic level, chromosomal level, and at the level of the placenta.

References

1. Tan, T.Y., and Yeo, G.S. 2005. Intrauterine growth restriction. *Current Opinion in Obstetrics & Gynecology* 17:135-142.
2. Silver, L.E., Decamps, P.J., Korst, L.M., Platt, L.D., and Castro, L.C. 2003. Intrauterine growth restriction is accompanied by decreased renal volume in the human fetus.[erratum appears in Am J Obstet Gynecol. 2004 Mar;27(3):1062]. *American Journal of Obstetrics & Gynecology* 188:1320-1325.
3. PAC/LAC. 1998. http://www.paclac.org/manuals_guidelines/intrauterine_growth_restriction_final_4.30.98.pdf. Sherman Oaks, CA: Perinatal Advisory Council. Leadership, Advocacy, and Consultation.
4. Barker, D.J., Gluckman, P.D., Godfrey, K.M., Harding, J.E., Owens, J.A., and Robinson, J.S. 1993. Fetal nutrition and cardiovascular disease in adult life.[see comment]. *Lancet* 341:938-941.
5. Aerts, L., and Van Assche, F.A. 2002. Taurine and taurine-deficiency in the perinatal period. *Journal of Perinatal Medicine* 30:281-286.
6. Alexander, B.T. 2006. Fetal programming of hypertension. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* 290:R1-R10.
7. Mi, J., Cheng, H., Zhao, X.Y., Zhang, Z.K., Ding, X.Y., Hou, D.Q., and Zhang, K.L. 2004. [Ponderal index at birth predicts metabolic syndrome in mid-aged Chinese]. *Chung-Hua Yu Fang i Hsueh Tsa Chih [Chinese Journal of Preventive Medicine]* 38:221-225.
8. Noyan-Ashraf, M.H., Wu, L., Wang, R., and Juurlink, B.H. 2006. Dietary approaches to positively influence fetal determinants of adult health. *FASEB Journal* 20:371-373.
9. Pladys, P., Sennlaub, F., Brault, S., Checchin, D., Lahaie, I., Le, N.L., Bibeau, K., Cambonie, G., Abran, D., Brochu, M., et al. 2005. Microvascular rarefaction and decreased angiogenesis in rats with fetal programming of hypertension associated with exposure to a low-protein diet in utero. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* 289:R1580-1588.
10. Rao, S. 2001. Nutritional status of the Indian population. *Journal of Biosciences* 26:481-489.
11. Sayer, A.A., and Cooper, C. 2005. Fetal programming of body composition and musculoskeletal development. *Early Human Development* 81:735-744.
12. Venu, L., Harishankar, N., Prasanna Krishna, T., and Raghunath, M. 2004. Maternal dietary vitamin restriction increases body fat content but not insulin resistance in WNIN rat offspring up to 6 months of age. *Diabetologia* 47:1493-1501.
13. Wideroe, M., Vik, T., Jacobsen, G., and Bakketeig, L.S. 2003. Does maternal smoking during pregnancy cause childhood overweight?[erratum appears in Paediatr Perinat Epidemiol. 2003 Oct;17(4):422]. *Paediatric and Perinatal Epidemiology* 17:171-179.
14. Agostoni, C., Galli, C., Riva, E., Colombo, C., Giovannini, M., and Marangoni, F. 2005. Reduced docosahexaenoic acid synthesis may contribute to growth restriction in infants born to mothers who smoke. *Journal of Pediatrics* 147:854-856.
15. Bada, H.S., Das, A., Bauer, C.R., Shankaran, S., Lester, B.M., Gard, C.C., Wright, L.L., Lagasse, L., and Higgins, R. 2005. Low birth weight and preterm births: etiologic fraction attributable to prenatal drug exposure. *Journal of Perinatology* 25:631-637.
16. Bouhours-Nouet, N., May-Panloup, P., Coutant, R., de Casson, F.B., Descamps, P., Douay, O., Reynier, P., Ritz, P., Malthiery, Y., and Simard, G. 2005. Maternal smoking is associated with mitochondrial DNA depletion and respiratory chain complex III deficiency in placenta. *American Journal of Physiology - Endocrinology & Metabolism* 288:E171-177.
17. Chiolero, A., Bovet, P., and Paccaud, F. 2005. Association between maternal smoking and low birth weight in Switzerland: the EDEN study. *Swiss Medical Weekly* 135:525-530.
18. Collet, M., and Beillard, C. 2005. [Consequences of smoking on fetal development and risk of intra-uterine growth retardation or in utero fetal death]. *Journal de Gynecologie, Obstetrique et Biologie de la Reproduction* 34 Spec No 1:3S135-145.
19. Hammoud, A.O., Bujold, E., Sorokin, Y., Schild, C., Krapp, M., and Baumann, P. 2005. Smoking in pregnancy revisited: findings from a large population-based study. *American Journal of Obstetrics & Gynecology* 192:1856-1862; discussion 1862-1853.

20. Kleijer, M.E., Dekker, G.A., and Heard, A.R. 2005. Risk factors for intrauterine growth restriction in a socio-economically disadvantaged region. *Journal of Maternal-Fetal & Neonatal Medicine* 18:23-30.
21. Okah, F.A., Cai, J., and Hoff, G.L. 2005. Term-gestation low birth weight and health-compromising behaviors during pregnancy. *Obstetrics & Gynecology* 105:543-550.
22. Polanska, K., and Hanke, W. 2004. [Effect of smoking during pregnancy on maternal condition and birth outcome--overview of epidemiologic studies]. *Przeglad Epidemiologiczny* 58:683-691.
23. Salihu, H.M., Aliyu, M.H., and Kirby, R.S. 2005. In utero nicotine exposure and fetal growth inhibition among twins. *American Journal of Perinatology* 22:421-427.
24. Gulgun, S.G., Guven, M., Onaran, I., Ulutin, T., and Hacihanefioglu, S. 2006. Individual sensitivity to cytogenetic effects of benzo[a]pyrene in cultured human lymphocytes: influence of glutathione S-transferase M1 genotype. *Genetic and Molecular Biology* 29:142-147.
25. Vandenbosche, R.C., and Kirchner, J.T. 1993. Intrauterine growth retardation. *American Family Physician* 58:1384-1390.
26. Vandenbosche, R.C., and Kirchner, J.T. 1393. Intrauterine growth retardation. *American Family Physician* 58:1384-1390.
27. Shapiro, S., McCormick, M.C., Starfield, B.H., Krischer, J.P., and Bross, D. 1980. Relevance of correlates of infant deaths for significant morbidity at 1 year of age. *American Journal of Obstetrics & Gynecology* 136:363-373.
28. Cosmi, E., Ambrosini, G., D'Antona, D., Saccardi, C., and Mari, G. 2005. Doppler, cardiotocography, and biophysical profile changes in growth-restricted fetuses. *Obstetrics & Gynecology* 106:1240-1245.
29. Lott, D.G., Zimmerman, M.B., Labbe, R.F., Kling, P.J., and Widness, J.A. 2005. Erythrocyte zinc protoporphyrin is elevated with prematurity and fetal hypoxemia. *Pediatrics* 116:414-422.
30. Gale, C.R., and Martyn, C.N. 2004. Birth weight and later risk of depression in a national birth cohort. *British Journal of Psychiatry* 184:28-33.
31. Chan, S.Y., McCabe, C.J., Boelaert, K., Visser, T.J., Friesema, E.C., et al. 2004. The expression of monocarboxylate transporter 8, as a specific thyroid hormone transporter in human fetal brain and placenta: the effects of intrauterine growth restriction (IUGR). *Endocrine Abstracts* 7:9.
32. Pickard, M.R., Sinha, A.K., Ogilvie, L.M., Leonard, A.J., Edwards, P.R., and Ekins, R.P. 1999. Maternal hypothyroxinemia influences glucose transporter expression in fetal brain and placenta. *Journal of Endocrinology* 163:385-394.
33. Mittendorfer-Rutz, E., Rasmussen, F., and Wasserman, D. 2004. Restricted fetal growth and adverse maternal psychosocial and socioeconomic conditions as risk factors for suicidal behaviour of offspring: a cohort study.[see comment]. *Lancet* 364:1135-1140.
34. CDC. 1994. From Data to Action: CDC's public health surveillance sor women, infants, and children. Centers for Disease Control and Prevention. Atlanta, GA.
35. Huang, S.T., Vo, K.C., Lyell, D.J., Faessen, G.H., Tulac, S., Tibshirani, R., Giaccia, A.J., and Giudice, L.C. 2004. Developmental response to hypoxia. *FASEB Journal* 18:1348-1365.
36. Manikkam, M., Crespi, E.J., Doop, D.D., Herkimer, C., Lee, J.S., Yu, S., Brown, M.B., Foster, D.L., and Padmanabhan, V. 2004. Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. *Endocrinology* 145:790-798.
37. Gray, R.H., and Wu, L.Y. 2000. Subfertility and risk of spontaneous abortion. *American Journal of Public Health* 90:1452-1454.
38. Habib, P. 2005. What are the consequences of smoking on pregnancy and delivery. *Journal de Gynecologie, Obstetrique et Biologie de la Reproduction* 34Spec:3S353-369.
39. Murray, H., Baakdah, H., Bardell, T., and Tulandi, T. 2005. Diagnosis and treatment of ectopic pregnancy. *CMAJ Canadian Medical Association Journal* 173:905-912.
40. CDC. 1999. Prenatal Smoking Data Book. G. Centers for Disease Control and Prevention. Atlanta, editor.
41. CDC. 2004. Summary Health Statistics for U.S. Adults: National Health Interview Survey. G. Centers for Disease Control and Prevention. Atlanta, editor.
42. Peeters, L.L. 1994. The effect of early maternal maladaptation on fetal growth. *Journal of Perinatal Medicine* 22Suppl:9-17.

43. Wang, X., Zuckerman, B., Pearson, C., Kaufman, G., Chen, C., Wang, G., Niu, T., Wise, P.H., Bauchner, H., and Xu, X. 2002. Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight.[see comment]. *JAMA* 287:195-202.
44. CDC. 2001. Women and Smoking: A report of the Surgeon General. G. Centers for Disease Control and Prevention. Atlanta, editor.
45. Boffetta, P., Jourenkova, N., and Gustavsson, P. 1997. Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. *Cancer Causes & Control* 8:444-472.
46. Hecht, S.S. 1999. Tobacco smoke carcinogens and lung cancer. *Journal of the National Cancer Institute* 91:1194-1210.
47. Salama, S.A., Sierra-Torres, C.H., Oh, H.Y., Hamada, F.A., and Au, W.W. 2001. Variant metabolizing gene alleles determine the genotoxicity of benzo[a]pyrene. *Environmental & Molecular Mutagenesis* 37:17-26.
48. Lin, P., Chang, J.T., Ko, J.L., Liao, S.H., and Lo, W.S. 2004. Reduction of androgen receptor expression by benzo[alpha]pyrene and 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[alpha]pyrene in human lung cells. *Biochemical Pharmacology* 67:1523-1530.
49. Rubin, H. 2001. Synergistic mechanisms in carcinogenesis by polycyclic aromatic hydrocarbons and by tobacco smoke: a bio-historical perspective with updates. *Carcinogenesis* 22:1903-1930.
50. CASA. 2006. Polycyclic Aromatic Hydrocarbons (PAHs). CASA, Clean Air Strategic Alliance. Http:www/casadata.org/pollutants/poly_hydrocarbons.asp.
51. Okona-Mensah, K.B., Battershill, J., Boobis, A., and Fielder, R. 2005. An approach to investigating the importance of high potency polycyclic aromatic hydrocarbons (PAHs) in the induction of lung cancer by air pollution. *Food & Chemical Toxicology* 43:1103-1116.
52. Charles, G.D., Bartels, M.J., Zacharewski, T.R., Gollapudi, B.B., Freshour, N.L., and Carney, E.W. 2000. Activity of benzo[a]pyrene and its hydroxylated metabolites in an estrogen receptor-alpha reporter gene assay. *Toxicological Sciences* 55:320-326.
53. Kim, J.H., Stansbury, K.H., Walker, N.J., Trush, M.A., Strickland, P.T., and Sutter, T.R. 1998. Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1.[erratum appears in Carcinogenesis 1999 Mar;20(3):515]. *Carcinogenesis* 19:1847-1853.
54. Le Roith, D., and Butler, A.A. 1999. Insulin-like growth factors in pediatric health and disease. *Journal of Clinical Endocrinology & Metabolism* 84:4355-4361.
55. Giudice, L.C., de Zegher, F., Gargosky, S.E., Dsupin, B.A., de las Fuentes, L., Crystal, R.A., Hintz, R.L., and Rosenfeld, R.G. 1995. Insulin-like growth factors and their binding proteins in the term and preterm human fetus and neonate with normal and extremes of intrauterine growth. *Journal of Clinical Endocrinology & Metabolism* 80:1548-1555.
56. Kajimura, S., Aida, K., and Duan, C. 2005. Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. *Proceedings of the National Academy of Sciences of the United States of America* 102:1240-1245.
57. Heinz, D.W., Baase, W.A., Dahlquist, F.W., and Matthews, B.W. 1993. How amino-acid insertions are allowed in an alpha-helix of T4 lysozyme. *Nature* 361:561-564.
58. Cooke, R.M., Harvey, T.S., and Campbell, I.D. 1991. Solution structure of human insulin-like growth factor 1: a nuclear magnetic resonance and restrained molecular dynamics study. *Biochemistry* 30:5484-5491.
59. Laron, Z. 2001. Insulin-like growth factor 1 (IGF-1): a growth hormone. *Molecular Pathology* 54:311-316.
60. Abuzzahab, M.J., Schneider, A., Goddard, A., Grigorescu, F., Lautier, C., Keller, E., Kiess, W., Klammt, J., Kratzsch, J., Osgood, D., et al. 2003. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation.[see comment]. *New England Journal of Medicine* 349:2211-2222.
61. Yakar, S., Liu, J.L., Stannard, B., Butler, A., Accili, D., Sauer, B., and LeRoith, D. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proceedings of the National Academy of Sciences of the United States of America* 96:7324-7329.
62. Rivadeneira, F., Houwing-Duistermaat, J.J., Vaessen, N., Vergeer-Drop, J.M., Hofman, A., Pols, H.A., Van Duijn, C.M., and Uitterlinden, A.G. 2003. Association between an insulin-like growth factor I gene promoter polymorphism and bone mineral density in the elderly: the Rotterdam Study. *Journal of Clinical Endocrinology & Metabolism* 88:3878-3884.

63. Vaessen, N., Heutink, P., Janssen, J.A., Witteman, J.C., Testers, L., Hofman, A., Lamberts, S.W., Oostra, B.A., Pols, H.A., and van Duijn, C.M. 2001. A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes and myocardial infarction. *Diabetes* 50:637-642.
64. Baker, J., Liu, J.P., Robertson, E.J., and Efstratiadis, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75:73-82.
65. D'Ercole, A.J., Dai, Z., Xing, Y., Boney, C., Wilkie, M.B., Lauder, J.M., Han, V.K., and Clemmons, D.R. 1994. Brain growth retardation due to the expression of human insulin like growth factor binding protein-1 in transgenic mice: an in vivo model for the analysis of igf function in the brain. *Brain Research Developmental Brain Research* 82:213-222.
66. Du, J., and Delafontaine, P. 1995. Inhibition of vascular smooth muscle cell growth through antisense transcription of a rat insulin-like growth factor I receptor cDNA. *Circulation Research* 76:963-972.
67. Ashton, I.K., and Vesey, J. 1978. Somatomedin activity in human cord plasma and relationship to birth size, insulin, growth hormone, and prolactin. *Early Human Development* 2:115-122.
68. Bennett, A., Wilson, D.M., Liu, F., Nagashima, R., Rosenfeld, R.G., and Hintz, R.L. 1983. Levels of insulin-like growth factors I and II in human cord blood. *Journal of Clinical Endocrinology & Metabolism* 57:609-612.
69. Lassarre, C., Hardouin, S., Daffos, F., Forestier, F., Frankenne, F., and Binoux, M. 1991. Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. Relationships with growth in normal subjects and in subjects with intrauterine growth retardation. *Pediatric Research* 29:219-225.
70. Woods, K.A., Camacho-Hubner, C., Savage, M.O., and Clark, A.J. 1996. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene.[see comment]. *New England Journal of Medicine* 335:1363-1367.
71. Bonapace, G., Concolino, D., Formicola, S., and Strisciuglio, P. 2003. A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. *Journal of Medical Genetics* 40:913-917.
72. Gembruch, U., and Gortner, L. 1998. Perinatal aspects of preterm intrauterine growth restriction. *Ultrasound in Obstetrics & Gynecology* 11:233-239.
73. Corstius, H.B., Zimanyi, M.A., Maka, N., Herath, T., Thomas, W., van der Laarse, A., Wreford, N.G., and Black, M.J. 2005. Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. *Pediatric Research* 57:796-800.
74. Everson, R.B., Randerath, E., Santella, R.M., Cefalo, R.C., Avitts, T.A., and Randerath, K. 1986. Detection of smoking-related covalent DNA adducts in human placenta. *Science* 231:54-57.
75. U.S. Human Genome Project. Accessed 4/2006. http://www.ornl.gov/sci/techresources/Human_Genome/faq/snps.shtml.
76. Schut, A.F., Janssen, J.A., Deinum, J., Vergeer, J.M., Hofman, A., Lamberts, S.W., Oostra, B.A., Pols, H.A., Witteman, J.C., and van Duijn, C.M. 2003. Polymorphism in the promoter region of the insulin-like growth factor I gene is related to carotid intima-media thickness and aortic pulse wave velocity in subjects with hypertension. *Stroke* 34:1623-1627.
77. Wadsworth, M., Butterworth, S., Marmot, M., Ecob, R., and Hardy, R. 2005. Early growth and type 2 diabetes: evidence from the 1946 British birth cohort. *Diabetologia* 48:2505-2510.
78. Soto, I.N., and Mericq, G.V. 2005. [Fetal growth restriction and insulin resistance. New findings and review of the literature]. *Revista Medica de Chile* 133:97-104.
79. Phillips, D.I. 2004. Fetal programming of the neuroendocrine response to stress: links between low birth weight and the metabolic syndrome. *Endocrine Research* 30:819-826.
80. Fagerberg, B., Bondjers, L., and Nilsson, P. 2004. Low birth weight in combination with catch-up growth predicts the occurrence of the metabolic syndrome in men at late middle age: the Atherosclerosis and Insulin Resistance study. *Journal of Internal Medicine* 256:254-259.
81. Hausberg, M., Barenbrock, M., and Kosch, M. 2004. Elevated sympathetic nerve activity: the link between low birth size and adult-onset metabolic syndrome?[comment]. *Journal of Hypertension* 22:1087-1089.
82. Barker, D.J. 1995. Fetal origins of coronary heart disease. *BMJ* 311:171-174.
83. Stoffers, D.A., Desai, B.M., DeLeon, D.D., and Simmons, R.A. 2003. Neonatal exendin-4 prevents the development of diabetes in the intrauterine growth retarded rat. *Diabetes* 52:734-740.

84. Vickers, M.H., Breier, B.H., McCarthy, D., and Gluckman, P.D. 2003. Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* 285:R271-273.
85. Adair, L.S., and Cole, T.J. 2003. Rapid child growth raises blood pressure in adolescent boys who were thin at birth. *Hypertension* 41:451-456.
86. Gerberding, J.L. 2006. Heart Disease and Stroke: The Nation's Leading Killers. G. Centers for Disease Control and Prevention. Atlanta, editor.
87. Huxley, R.R., Shiell, A.W., and Law, C.M. 2000. The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of the literature. *Journal of Hypertension* 18:815-831.
88. Vickers, M.H., Ikenasio, B.A., and Breier, B.H. 2002. Adult growth hormone treatment reduces hypertension and obesity induced by an adverse prenatal environment. *Journal of Endocrinology* 175:615-623.
89. Rogers, I., Emmett, P., Ness, A., and Golding, J. 2004. Maternal fish intake in late pregnancy and the frequency of low birth weight and intrauterine growth retardation in a cohort of British infants. *Journal of Epidemiology & Community Health* 58:486-492.
90. Kuljukka-Rabb, T., Peltonen, K., Isotalo, S., Mikkonen, S., Rantanen, L., and Savela, K. 2001. Time- and dose-dependent DNA binding of PAHs derived from diesel particle extracts, benzo[a]pyrene and 5-methychrysene in a human mammary carcinoma cell line (MCF-7). *Mutagenesis* 16:353-358.
91. Larsen, J.C. Accessed 4/2006. Benzo(a)Pyrene. Institute of Toxicology, National Food Agency of Denmark. http://www.inchem.org/documents/jecfa/jecmono/v28je18.htm.
92. Melcher, U. 2000. Sister Chromatid Exchanges. *Molecular Genetics.* Pg. 1223. http://opbs.okstate.edu/~melcher/MG/MGW1/MG1223.html.
93. Trier, J.S., and Browning, T.H. 1996. Morphologic response of the mucosa of human small intestine to x-ray exposure. *Journal of Clinical Investigation* 45:194-204.
94. Grant, W.F. 1978. Chromosome aberrations in plants as a monitoring system. *Environmental Health Perspectives* 27:37-43.
95. Bergamo, N.A., da Silva Veiga, L.C., dos Reis, P.P., Nishimoto, I.N., Magrin, J., Kowalski, L.P., Squire, J.A., and Rogatto, S.R. 2005. Classic and molecular cytogenetic analyses reveal chromosomal gains and losses correlated with survival in head and neck cancer patients. *Clinical Cancer Research* 11:621-631.
96. Bhalli, J.A., Khan, Q.M., Haq, M.A., Khalid, A.M., and Nasim, A. 2006. Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry. *Mutagenesis* not yet in print.
97. EPA. 1998. Health Effects Test Guidelines: *In vitro* mammalian chromosome aberration test. U.S. Envirnomental Protection Agency.
98. Carrano, A.V., and Heddle, J.A. 1973. The fate of chromosome aberrations. *Journal of Theoretical Biology* 38:289-304.
99. Smalls, E., and Patterson, R.M. 1982. Reduction of benzo(a)pyrene induced chromosomal aberrations by DL-alpha-tocopherol. *European Journal of Cell Biology* 28:92-97.
100. Weissenborn, U., and Streffer, C. 1988. Analysis of structural and numerical chromosomal anomalies at the first, second, and third mitosis after irradiation of one-cell mouse embryos with X-rays or neutrons. *International Journal of Radiation Biology* 54:381-394.
101. Schmidt, H., and Rath, F.W. 1993. Cytogenetic analysis of a benzpyrene induced osteosarcoma in the rat (Rattus norvegicus). *Cancer Genetics & Cytogenetics* 71:139-143.
102. Wang, Y., Fan, A., Brown, J., Morry, D., and DiBartolomeis, M. 1997. Public Health Goal for Benzo(a)Pyrene in Drinking Water. California Environmental Protection Agency. Pesticide and Environmental Toxicology Section. 9.
103. Gaulden, M.E. 1987. Hypothesis: some mutagens directly alter specific chromosomal proteins (DNA topoisomerase II and peripheral proteins) to produce chromosome stickiness, which causes chromosome aberrations. *Mutagenesis* 2:357-365.
104. Tateno, H., and Kamiguchi, Y. 2002. Abnormal chromosome migration and chromosome aberrations in mouse oocytes during meiosis II in the presence of topoisomerase II inhibitor ICRF-193. *Mutation Research* 502:1-9.

105. Rayburn, A.L., and Wetzel, J.B. 2002. Flow cytometric analyses of intraplant nuclear DNA content variation induced by sticky chromosomes. *Cytometry* 49:36-41.
106. Pagliarini, M.S. 2000. Meiotic behavior of economically important plant species: the relationship between fertility and male sterility. *Genetics and Molecular Biology* 23.
107. Rao, P.N., and Rao, R.N. 1977. Gamma-ray induced meiotic chromosome stickiness in tomato. *Theoretic Application in Genetics* 50:247-252.
108. Rayburn, A.L., Wetzel, J.B., and Baligar, V.C. 2002. Mitotic analysis of sticky chromosomes in aluminum tolerant and susceptible wheat lines grown in soils of differing aluminum saturation. *Euphytica* 127:193-199.
109. Khokhar, M.T., and Oliver, R. 1975. An investigation of chromosome damage in Vicia faba root tips after exposure to 1-5 MHz ultrasonic radiation. *International Journal of Radiation Biology & Related Studies in Physics, Chemistry & Medicine* 28:373-383.
110. Crouch, G.D. 2003. Nonrhabdomyosarcoma soft tissue sarcomas. E-medicine, WebMD. http://www.emedicine.com/ped/topic2764.htm.
111. Mohren, M., Essbach, U., Franke, A., Klink, A., Maas, C., Markmann, I., Pelz, A.F., and Jentsch-Ullrich, K. 2003. Acute myelofibrosis in a patient with diffuse large cell non Hodgkin's lymphoma and renal cancer. *Leukemia & Lymphoma* 44:1603-1607.
112. Alija, A.J., Bresgen, N., Sommerburg, O., Siems, W., and Eckl, P.M. 2004. Cytotoxic and genotoxic effects of beta-carotene breakdown products on primary rat hepatocytes. *Carcinogenesis* 25:827-831.
113. Albert, R.E., Miller, M.L., Cody, T., Andringa, A., Shukla, R., and Baxter, C.S. 1991. Benzo[a]pyrene-induced skin damage and tumor promotion in the mouse. *Carcinogenesis* 12:1273-1280.
114. Rotwein, P., Pollock, K.M., Didier, D.K., and Krivi, G.G. 1986. Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. *Journal of Biological Chemistry* 261:4828-4832.
115. t Hart, L.M., Fritsche, A., Rietveld, I., Dekker, J.M., Nijpels, G., Machicao, F., Stumvoll, M., van Duijn, C.M., Haring, H.U., Heine, R.J., et al. 2004. Genetic factors and insulin secretion: gene variants in the IGF genes. *Diabetes* 53 Suppl 1:S26-30.
116. Arends, N., Johnston, L., Hokken-Koelega, A., van Duijn, C., de Ridder, M., Savage, M., and Clark, A. 2002. Polymorphism in the IGF-I gene: clinical relevance for short children born small for gestational age (SGA). *Journal of Clinical Endocrinology & Metabolism* 87:2720.
117. Denley, A., Wang, C.C., McNeil, K.A., Walenkamp, M.J., van Duyvenvoorde, H., Wit, J.M., Wallace, J.C., Norton, R.S., Karperien, M., and Forbes, B.E. 2005. Structural and functional characteristics of the Val44Met insulin-like growth factor I missense mutation: correlation with effects on growth and development. *Molecular Endocrinology* 19:711-721.
118. Gomes, M.V., Soares, M.R., Pasqualim-Neto, A., Marcondes, C.R., Lobo, R.B., and Ramos, E.S. 2005. Association between birth weight, body mass index and IGF2/ApaI polymorphism. *Growth Hormone & Igf Research* 15:360-362.
119. Kiess, W., Kratzsch, J., Keller, E., Schneider, A., Raile, K., Klammt, J., Seidel, B., Garten, A., Schmidt, H., and Pfaffle, R. 2005. Clinical examples of disturbed IGF signaling: intrauterine and postnatal growth retardation due to mutations of the insulin-like growth factor I receptor (IGF-IR) gene. *Reviews in Endocrine & Metabolic Disorders* 6:183-187.
120. Petry, C.J., Ong, K.K., Wingate, D.L., Brown, J., Scott, C.D., Jones, E.Y., Pembrey, M.E., Dunger, D.B., and Alspac Study, T. 2005. Genetic variation in the type 2 insulin-like growth factor receptor gene and disparity in childhood height. *Growth Hormone & Igf Research* 15:363-368.