UNIVERSITY OF CALIFORNIA,
IRVINE

The Effect of Polycyclic Aromatic Hydrocarbons on Ovulatory Status in Women

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Environmental Health Sciences

by

Lynn Morrissey Flowers, DO

Thesis Committee:
Ulrike Luderer, MD, PhD, MPH, Chair
Dean Baker, MD, MPH
Jun Wu, PhD

2015

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my committee chair, Dr. Ulrike Luderer for her patience and guidance through this thesis. I sincerely thank her for sharing her time, tremendous knowledge, and support through this process.

I would like to thank my committee members, Dr. Dean Baker and Dr. Jun Wu for their immensely helpful expertise, comments and insights.

ABSTRACT OF THESIS

The Effect of Polycyclic Aromatic Hydrocarbons on Ovulatory Status in Women

By

Lynn Morrissey Flowers, DO

Master of Science in Environmental Health Sciences

University of California, Irvine, 2015

Dr. Ulrike Luderer, Chair

The objective of this study was to investigate the hypothesis that environmental exposure to polycyclic aromatic hydrocarbons (PAH) in reproductive age women would be associated with abnormal ovarian function, with focus on ovulation status. Urinary hydroxy-metabolites of PAHs were measured in 150 menstrual cycles of 51 women who were not on hormonal contraception and not planning on becoming pregnant. This was a convenience sample of women aged 18-44 and residing in Orange County, CA. Participants were given a fertility monitor and asked to perform daily urinary dipstick testing for measurements of urinary $E_1$3G and LH. Ovulatory status was determined from the monitor data and classified as ovulatory, anovulatory or indeterminate. Bivariate analysis including correlation and analysis of variance, and logistic regression were used to investigate relationships between PAH metabolites and ovulatory status with adjustment for age. When analyzed, it was found that there were statistically significant associations of PAH exposure and ovulatory status. Two metabolites had positive associations with anovulatory cycles, and one metabolite had a negative association with anovulatory cycles.

1.0 INTRODUCTION

1.1 PAHs: what are they, how are they formed and human exposure

Polycyclic aromatic hydrocarbons (PAHs) are products of incomplete combustion. Typical sources of exposure include foods cooked in certain processes, diesel exhaust, and tobacco smoke. PAHs are also a known water contaminant from soil, industrial accidents and oil shipping vessels (Gehle, 2009). The soil's main source of PAHs is airborne fallout from nearby industrial sites such as refineries (Gehle, 2009).

Diet is also a potential source of exposure, as one study showed urinary concentrations after consumption of barbecued chicken rivaled that of concentrations in known high occupationally exposed groups (Li et al., 2012). Up to 70% of PAH exposure in non-occupational settings for non-smokers comes from their diet (Skupinska, Misiewicz, & Kasprzycka-Guttman, 2004). Tea, roasted peanuts, coffee, refined vegetable oil, cereals, spinach, and many other foodstuffs contain PAHs. Some crops, such as wheat, rye, and lentils, may synthesize PAHs or absorb them via water, air, or soil (Gehle, 2009).

Dietary exposure of PAHs in humans can rival that of occupational exposure as a study showed that a 100 to 250-fold increase in a dietary benzo[*a*]pyrene (BaP) dose paralleled a four to 12-fold increase in urinary 1-hydroxypyrene elimination (Buckley and Lioy, 1992). In the average American diet, the intake of carcinogenic PAHs is estimated to be between 1 and 5 mcg/day (Menzie et al., 1992). Ingested PAHs are taken up by the gastrointestinal tract in fat-soluble compounds (ATSDR, 1995).

PAHs are also found in topical medications to treat eczema, psoriasis, dandruff, seborrheic dermatitis and other dermatologic conditions. In analyses on creams containing coal-tar, seven PAHs were identified in the cream: naphthalene, biphenyl, acenaphthylene,

fluorene, phenanthrene, fluoranthene and pyrene (Disdier et al., 2000). Percutaneous absorption of PAHs occurs through passive diffusion (ATSDR, 1995).

Another important route of exposure in the United States is inhalation, with PAHs in ambient air reported to be 0.02–1.2 nanograms/m^3 in rural areas and 0.15–19.3 ng/m^3 in urban areas (ATSDR, 1995). Absorption of the PAHs is thought to occur through the mucous lining of bronchi (ATSDR, 1995).

1.2 PAHs: mechanism of action, metabolism, biologic fate, and biomonitoring

The aryl hydrocarbon receptor's (AhR) activity is known to be induced as a result of PAH exposure and activation of the AhR increases their adverse effects. Many PAHs bind and activate the AhR, inducing expression of some cytochromes P450 to activate the promutagenic parent compounds (Machala et al., 2001). The AhR translocates to the nucleus and interacts with genes that contain a common nucleotide sequence (the *Ahr* response element), after ligand binding, resulting in altered expression of AhR regulated genes (Matikainen et al., 2001).

PAHs are metabolized mainly by cytochrome P450 enzymes in the liver and to a lesser extent in the kidneys, adrenal glands, testes, ovaries, thyroid, lungs, skin, sebaceous glands, and small intestines (Gehle, 2009; ATSDR, 1995). In studies, utilizing intraovarian injection of benzo[*a*]pyrene, it was demonstrated that ovaries have the capability to metabolize benzo[*a*]pyrene to ovotoxic products, resulting in primordial oocyte destruction in mice (Shiromizu and Mattison1984).

PAHs are metabolized mainly by cytochrome P450 enzymes in the liver and to a lesser extent in the kidneys, adrenal glands, testes, ovaries, thyroid, lungs, skin, sebaceous

glands, and small intestines (Gehle, 2009; ATSDR, 1995). In studies by Shiromizu and Mattison, intraovarian injection of benzo[*a*]pyrene demonstrated that ovaries have the capability to metabolize benzo[*a*]pyrene to ovotoxic products, resulting in primordial oocyte destruction in mice (1984).

PAHs are initially transformed into epoxides by P450s, and then converted to dihydrodiol derivatives and phenols. Glucuronide and sulfate conjugates of these metabolites are excreted in the bile and urine. Glutathione conjugates are further metabolized to mercapturic acids in the kidney and are excreted in the urine. The hydroxylated metabolites of the PAHs are excreted in human urine both as free hydroxylated metabolites and as hydroxylated metabolites conjugated to glucuronic acid and sulfate (Gehle, 2009). In urine of battery workers, the excretion of 1-hydroxypyrene occurred with a half-life of 6-35 hours (Jongeneelen et al., 1990). In rats, 60% of pyrene is eliminated as metabolites in urine by 24 hours after injection while 20% is excreted in the feces over the same time period (Viau et al., 1999).

PAHs are widespread in the environment with every person having some level detected through monitoring/screening. Monitoring of PAHs can be done through analysis of serum or urine specimens. Monitoring metabolites in the urine can be a useful indicator of PAH exposures from multiple exposure routes. Multiple human worker studies have shown that urinary metabolites reflected exposure satisfactorily (Buratti et al., 2007; Jeng et al., 2011), although exposure misclassification can become an issue when metabolites are also derived from exposure to non-PAH compounds.

1.3 PAH Metabolites

PAHs are present in the atmosphere in the gaseous phase or sorbed onto particulates. PAHs having two to three rings (naphthalene, acenaphthene, acenaphthylene, anthracene, fluorene, and phenanthrene) are typically present in air predominantly in the vapor phase. PAHs that have four rings (fluoranthene, pyrene, chrysene, and benz[*a*]anthracene) exist both in the vapor and particulate phase. PAHs having five or more rings (benzo[*a*]pyrene, and benzo[*g,h,i*]perylene) are found predominantly in the particle phase (ATSDR, 1995).

2-hydroxyfluorene, 3-hydroxyfluorene and 9-hydroxyfluorene are all metabolites of the PAH fluorene and thus are present in the air predominantly in the vapor phase. Fluorene is a chemical intermediate most often used in the manufacture of dyes, plastics, pesticides, explosives, and chemotherapeutic agents (ATSDR, 1995).

1-hydroxyphenanthrene, 2-hydroxyphenanthrene, and 3-hydroxyphenanthrene are all metabolites of the PAH phenanthrene, having three rings and are present in the air predominantly in the vapor phase. Phenanthrene is most commonly used in the manufacture of dyestuffs and explosives and in biological research. It is one of the most prominent PAHs found in diesel exhaust, and the most abundant and frequently detected PAH in samples of fly ash and bottom ash collected from municipal refuse incinerators in the United States (ATSDR, 1995).

1-hydroxypyrene is a metabolite of the PAH pyrene, and has four rings. It exists in both in the vapor and particulate phase. Pyrene is prominent in diesel exhaust, coke ovens, cigarette smoke, and coal tar (ATSDR, 1995).

1-hydroxynaphthalene and 2-hydroxynaphthalene are both metabolites of the PAH naphthalene. 1-hydroxynaphthalene is also a main metabolite of the wide-spectrum carbamate insecticide carbaryl, the herbicide napropamide, and the widely used beta-blocker propranolol (Li et al., 2015). PAHs like naphthalene have 2 rings and are present in air predominantly in the vapor phase. Naphthalene is commonly found in mothballs and coal tar (ATSDR, 1995), although use of naphthalene in mothballs and pesticides in CA has been reduced significantly the major source in California is now traffic-related emission (Lu et al., 2005).

2.0 FEMALE OVARIAN FUNCTION, MENSTRUAL CYCLE AND HORMONES

2.1 Hypothalamic-Anterior Pituitary-Gonadal Axis

The hypothalamic anterior pituitary gonadal axis (HPG) regulates reproductive function. The hypothalamus produces and releases the peptide hormone, gonadotropin releasing hormone (GnRH) in a pulsatile manner. GnRH has a positive effect on the anterior pituitary gonadotrope cells, which produce the gonadotropins, lutenizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are glycopeptides that regulate folliculogenesis, ovulation and sex steroid production. Progesterone and estrogen produced in the ovaries are involved in feedback loops to the hypothalamus and the anterior pituitary. Estrogen directly inhibits GnRH in negative feedback loop, while inhibin indirectly inhibits FSH production at the pituitary level. There is a positive feedback loop from estrogen to GnRH, initiating the mid-cycle LH and FSH surges. Progesterone is involved in a negative feedback loop to the hypothalamus and anterior pituitary after ovulation, as it inhibits LH. If conception occurs, progesterone production is taken over by

the corpus luteum. If conception does not occur, progesterone decreases and LH and FSH prepare for the next ovulatory follicle.

2.2 Oocyte growth maturation and development

The oocyte is arrested in prophase I of meiosis during the second trimester of fetal development until the first meiosis is completed just prior to ovulation. Second meiosis does not occur until fertilization.

Oogenesis in the ovary is a continuous process starting with the small primordial follicle which consists of an oocyte that is surrounded by a single layer of flat granulosa cells. Next the mitotic primary oocyte, slightly larger in diameter is surrounded by a layer of cuboidal granulosa cells. The secondary follicle, approximately twice the size in diameter than the primary oocyte, is marked by the appearance of theca cells and many layers of cuboidal granulosa cells. Follicle growth after the primary stage is influenced by FSH. Antral follicles are ones with an antrum- a fluid filled cavity next to the oocyte- and they are gonadotropin-dependent. Finally the preovulatory follicule ends with a mid-cycle LH surge that completes the first meiotic division and triggers ovulation within twenty-four hours. Once the egg is released a corpus luteum forms. The corpus luteum produces progesterone and persists to support a pregnancy if fertilization occurs, or atrophies if fertilization does not occur. Granulosa cells produce both progesterone and estrogen (estradiol) and theca cells produce progesterone and androgens.

Early follicular development is paracrine regulated and anti-Müllerian hormone (AMH) from growing follicles inhibits recruitment of primordial follicles (Durlinger et al., 1999). Phosphoinositol-3-kinase (PI3K) signaling controls dormancy, activation, and loss

of primordial follicles (Zheng et al., 2012). It is apparent that there are many steps in the oocyte maturation process that allows for susceptibility to toxic insult.

2.2 Menstrual cycle

The start of the menstrual cycle is marked by the first day of blood flow, which lasts approximately five days. Next the follicular phase involves increasing levels of estrogen, produced by the developing the follicle to just before ovulation. Estradiol exerts negative feedback until a threshold concentration is exceeded, at which point it exerts positive feedback and triggers the LH and FSH surges with resultant ovulation within 24 hours of the surge. The luteal phase occurs after ovulation and last for approximately 14 days as the ovum transits the tube in anticipation of fertilization. During the luteal phase, the corpus luteum produces progesterone and estrogen, which act to maintain a pregnancy, or the corpus luteum degenerates in preparation for the next menstrual cycle. Estrogen actually refers to a group of hormones in women that include estrone (E1), estradiol (E2), and estriol (E3). Estrone-3-glucuronide ($E_1$3G) is a glucuronide conjugate metabolite of estrogen and is measured in the urine, typically used in home monitoring kits.

2.5 Fecundity and Fertility

Fertility refers to the rate at which women have babies. Fecundity is the ability to produce offspring, which can be affected by infertility, but also contraception. Infertility affects approximately 10.9% of women ages 15-44, and increases with age, with 30-40% resultant of ovulatory failure (CDC, 2015).

3.0 PAHS AND OVARIAN FUNCTION EFFECT IN HUMAN STUDIES

Environmental tobacco smoke exposure, a source of PAHs, was associated with significantly lower urinary reproductive hormone (estrone conjugate, E1C) levels among non-conception cycles (Chen, 2005). Additionally using data on smoking history and number of noncontracepting cycles until conception from 678 pregnant women it was found that thirty-eight percent of nonsmokers conceived in their first cycle compared with 28% of smokers. Smokers were 3.4 times more likely to have taken greater than a year to conceive compared with nonsmokers (Baird & Wilcox, 1985).

The PAH benzo[*a*]pyrene has been measured in the follicular fluid and is found in a much higher concentration in women who smoke and especially in those women who were unsuccessful at achieving conception with in-vitro fertilization (IVF) (Neal et al., 2008). In a meta-analysis by Augood et al. (1998), the overall value of the odds ratio (OR) for risk of infertility in women smokers versus non-smokers was 1.60 [95% confidence interval (CI) 1.34-1.91]. Additionally, studies of subfertile women undergoing in-vitro fertilization (IVF) treatment also show a reduction in fecundity among women smokers. A meta-analysis of nine studies found an OR of 0.66 (95% CI 0.49-0.88) for pregnancies per number of IVF-treated cycles in smokers versus non-smokers (Augood et al., 1998).

4.0 PAHS AND OVARIAN FUNCTION EFFECT IN ANIMAL STUDIES

It was shown that low-dose repeated exposures to PAHs are substantially more toxic in terms of cumulative dose to the ovary than a single high-dose exposure in murine models (Borman et al., 2000). Small oocyte destruction after intraovarian injection of benzo[*a*]pyrene was observed in murine models (Takizawa et al., 1984) and primordial

oocyte destruction was shown in rats by single high-doses of PAHs (Mattison, 1979). Mattison also demonstrated treatment with benzo[*a*]pyrene at doses of 10, 50, 100, and 500 mg/kg destroyed 20%, 58%, 88%, and 100%, respectively, of the primordial oocytes in DBA/2N mouse ovaries (1979). In a study by Jurisicova et al. (2007), ovaries of offspring born to mice exposed to PAHs before gestation contained only a third of the ovarian follicle pool compared with offspring of unexposed female mice, demonstrating that maternal exposure to PAHs prior to pregnancy and/or during lactation compromises ovarian reserve of female offspring and, raising the concern about the multigenerational impact of maternal smoking on ovarian function in the human. Neal et al. (2008) quantified benzo[*a*]pyrene levels quantified in the serum and follicular fluid of women undergoing IVF exposed to mainstream smoke (n = 19) and not exposed to smoke (n = 10) by gas chromatography mass spectrometry. These concentrations were used to model human exposure in isolated rat follicles cultured with increasing concentrations of benzo[*a*]pyrene (1.5–300 ng ml^{-1}), and follicle diameter was measured daily. The treatment significantly reduced rat follicle growth in a concentration dependent manner. An in vitro exposure study on benzo[*a*]pyrene effects on folliculogenesis showed negative effects on follicle development and survival (Sadeu, 2011). In a murine model it was demonstrated that PAHs induce apoptosis in developing female germ cells, with the *bax* gene being a likely target for the PAH-activated AHR in fetal oocytes showing positive correlation between increased Bax expression and fetal germ cell death (Matikainen et al., 2002). These animal studies demonstrate the effect of PAHs on follicle/oocyte development. It humans, this effect is likely happening and is the result of interactions of PAHs at the genetic level as well as hormone regulation level.

5.0 STUDY OBJECTIVE

The objective of this study was to assess ovulatory status in women (anovulatory/ovulatory) in relation to PAH exposure using urinary OH-PAH metabolite measurements as the indicators of exposure and LH and $E_1$3G measurements to determine ovulatory status by menstrual cycle. It was hypothesized that greater concentrations of urinary PAH metabolites have a negative association with ovulation (anovulatory cycles) compared to those with lower PAH exposure levels.

6.0 METHODS

6.1 Study Design

This is a prospective cohort study that was designed to investigate if overall PAH exposure, measured by urinary PAH metabolite concentrations, is associated with decreased fertility. This study aimed to analyze urinary reproductive hormone profiles and urinary PAH metabolites in women who did not plan to become pregnant. Fifty-one women who were not on hormonal contraception were in the urinary hormone monitoring group. The women were selected as a convenience sample using public events, such as health fairs, that targeted two ethnic groups (non-Hispanic white and Hispanic) in three cities of Orange County, CA. The recruited women made an appointment to be seen at the study center 5-9 days prior to their next menstrual cycle. Urinary hormone home monitoring was begun on the first day of subsequent menstrual cycles after that appointment, and continued for 6 months.

Participants were asked to contact the study office if they planned to actively seek pregnancy, or at least monthly. Participants were given the options to contact the study office to answer questions about pregnancy intention and current contraceptive use via secure website, telephone, or by returning a hard copy questionnaire in stamped, self-addressed envelope. If a participant did not contact the office for more than two months, the study staff contacted her by telephone and email.

Pregnancy intention was assessed using the following two questions: "How would you feel about a possible pregnancy? How would you describe your conversations with your partner about a possible pregnancy? (Morin et al., 2003). Women were considered to be intending pregnancy if they answered the first question "I want a baby" and the second question, "I tell him I want to get pregnant and we discuss what we could do." By ascertaining intention rather than actual planning, the study attempted to enroll women into a time-to-pregnancy sub-study before they stopped using contraception to capture the most fecund women who would become pregnant the most quickly after stopping contraception. When a woman indicated that she intended to become pregnant, she was enrolled in the time-to pregnancy study. These women continued urinary hormone monitoring and urine collection for PAH metabolite measurement until they had a positive pregnancy test, stopped trying to become pregnant, or the study ended.

6.2 Study Participants

The study population was recruited as part of a pilot study designed to test the feasibility of recruiting women who were not intending to become pregnant and not using hormonal contraception for a study of the association between urinary reproductive

hormone concentrations measured daily using a microelectronic dipstick monitor and urinary PAH metabolites measured once per menstrual cycle during six menstrual cycles.

The intent of the pilot study was to lay the groundwork for a subsequent larger, adjunct study to the planned National Children's Study (NCS). Initially, women were recruited based on NCS Vanguard Study protocols. For the Vanguard Study, Orange County was divided into 15 geographical strata, which were then further divided into 11 geographical units (GUs), which were then divided into 10 segments or "neighborhoods" based on socio-economic, environmental, and neighborhood vulnerability characteristics. The measures of size (MOS) of the strata, GUs, and segments respectively were based on the number of births during the prior five years in the census blocks within the geographical boundaries according to mother's place of residence at the time of delivery. The MOS at each sampling stage were equal, so theoretically every potential future birth during a recruitment period would have an equal probability of being sampled. Fifteen of these segments, one from each GU, totaling 15,000 households, were sampled by the NCS Coordinating Center for screening. The remaining 135 segments from the 15 GUs were characterized, but were not further studied in the NCS Vanguard Study. Women for the current study were recruited from segments that were not selected for the NCS Vanguard Study. Two segments from predominantly non-Hispanic white GUs (in Irvine, CA) and two segments from predominantly Hispanic GUs (in Santa Ana and Costa Mesa, CA) were selected at random to assure representation of the two largest ethnic groups in Orange County.

The initial recruitment strategy was to identify eligible women by door-to-door contact in the home with follow-up telephone and email contact by study staff.

Subsequently, when the NCS shifted to other recruitment strategies, it was decided to recruit via public events such as health fairs at universities and colleges, work places and those sponsored by community groups. The study population is, therefore, a convenience sample of women living in Orange County, CA.

Women between the ages of 18-44, residing in Orange County California, who were not pregnant, not using hormonal contraception, and currently not planning to conceive were eligible for the study. Women were excluded if they were pregnant or actively trying to become pregnant, had a history of surgical sterilization, were undergoing treatment for infertility, had a history of treatment with antineoplastic drugs or radiation therapy to the pelvis, or had conditions known to cause infertility by mechanisms other than ovarian failure (PID, endometriosis, etc.).

After completing informed consent, participants were asked to come to one of the two Orange County locations of the UC Irvine Institute for Clinical and Translational Science (ICTS) for the baseline visit 5-9 days before their expected next menstrual period onset, when they were to begin daily urinary hormone monitoring. ICTS Nursing staff measured height, weight, and blood pressure and drew baseline blood samples. Study staff administered the questionnaires and taught subjects how to use the Clearblue® Easy monitors, home pregnancy tests, and menstrual diaries at the ITCS during the baseline visit. Participants were given the option of keeping the Clearblue Easy monitors upon completion of the study or of receiving a payment of $100 with return of the monitor.

6.3 Baseline Study Visit

At the initial appointment, study staff administered the standardized preconception

questionnaire developed for the National Children's Study (NCS) (NCS, 2007), which included demographic variables (age, birthplace, ethnicity, household income, education), medical history (cancer, chronic illnesses, medication use), reproductive history (menstrual history, pregnancy and contraceptive history, fertility and reproductive problems), environmental tobacco smoke exposure, exercise history, occupational history (job title, industry, exposure to specific chemicals and hazards), residential history (location and proximity to traffic, farms and industrial facilities), housing characteristics (age of house, gas cooking stove, gas heating, space heater, etc.), use of cleaning agents and pesticides in the home and yard, and pets. Questions relating to tobacco, alcohol, and illicit drug use were taken from the NCS First Trimester Maternal In-Person Questionnaire (NCS, 2007). Height and weight were also measured. At the same visit, a blood sample was collected and women were given urinary hormone monitors, urine collection kits, and home pregnancy tests.

6.4 Participant Diary

Participants were instructed to fill out a daily diary during the study period. The diary was given to them during their baseline visit. Questions included information regarding menstrual bleeding, heaviness of menstrual flow (assessed with the help of a menstrual pictogram, as described by Wyatt et al. (2001), use of contraception, number of cigarettes smoked, illness, use of medications, use of alcohol, and whether they had sexual intercourse.

6.5 Home Urine collection for PAH metabolites

Participants were given urine collection kits, which they were instructed to use to collect one urine sample per menstrual cycle (on cycle day 10) into a plastic beaker, pour 10 ml from the beaker into each of 4 tubes, and to store them in the box provided with the kit in their home freezers for pickup by study staff every month. The urine samples were used for the measurement of hydroxylated PAH metabolites as biomarkers of PAH exposure (Li et al., 2008).

6.6 Urinary hormone data measurements

Participants were given a Clearblue® Easy Fertility Monitor (Swiss Precision Diagnostics, Bedford, UK) and were asked to perform daily urinary dipstick testing for measurements of urinary $E_1$3G and LH beginning on the first day of their next menstrual cycle. The Clearblue® Easy Fertility Monitor permits monitoring of daily urinary hormone concentrations without having to collect or store samples of urine. Women held a disposable test stick in the first morning void urine stream for 3 seconds. The dual-assay test stick was then inserted into the monitor, which takes 5 minutes to read the LH and $E_1$3G concentrations. For each menstrual cycle the monitor was started on the first day of blood flow counted as day one. The monitor requested test strips from cycle day 6 until it detected a peak LH surge or exceeded a certain number of test strips. The monitors assayed LH and $E_1$3G concentrations, and would report fertility as low, high, or peak on the monitor. The monitors utilize a sandwich immunoassay to measure LH and a competitive assay to measure $E_1$3G (Robinson et al., 2007). The monitors are capable of storing up to two months of data, including date and time of sample, LH level, and $E_1$3G level. Data were

collected from the monitor via download onto a data card that was inserted into the monitor. Data from the card were then uploaded to a computer file, which was sent to the manufacturer for conversion into values representing relative LH and $E_1$3G concentrations (Rockett et al., 2004).

Data cards were exchanged every one or at most two months by study staff during visits made to pick up stored urine so as not to lose large amounts of data in the case of a monitor malfunction or loss. Women were also asked to perform human chorionic gonadotropin Clearblue® Easy pregnancy tests (Swiss Precision Diagnostics) if their menses onset did not occur within 10 days of the expected date. Clearblue® Easy pregnancy tests measure urinary human chorionic gonadotropin. They consist of disposable dipsticks that are held for 5 seconds into the urinary stream, incubated for 1 minute, and then read as positive (blue line in the square result window) or negative (no blue line).

Daily urinary hormone monitoring and once per cycle hCG and urine collection continued for 6 menstrual cycles.

6.7 PAH urine metabolite testing

Urinary OH-PAH metabolites were measured for each subject for three (49 participants) or two (2 participants) cycles during the testing period. Urine specimens were collected from subjects in standard urine collection cups. A minimum of 5 milliliters of urine was poured into each of four sterile polypropylene vials with screw-cap tops by the participants and stored in the home freezer. After pick up by study staff, the specimens were transferred to the ICTS, where they were stored at -80 ºC, until shipping on dry ice to

the California Department of Public Health Environmental Health Laboratory (EHL) in Richmond, CA. At the EHL, the urine samples were enzymatically deconjugated and subjected to solid phase extraction, followed by gas chromatography/isotope dilution high resolution mass spectrometry for nine PAH metabolites (Romanoff et al., 2006; Li et al., 2008). Ion transitions specific to each analyte and carbon-13 labeled internal standards were monitored, and the abundances of each ion was measured. These procedures were developed by the Centers for Disease Control and Prevention NHANES study (CDC, 2013).

6.8 Conversion of Clearblue monitor output to concentrations of LH and $E_1$3G

Standard curves were generated for LH using WHO 3500 U/L standard (WHO 2nd International Standard for Pituitary LH for Immunoassay, coded 80/552), which was diluted into water to to the following concentrtions: 0.386, 1.17, 1.68, 3.73, 11.93, 44.74, 175.9, and 700.8 U/L. These eight dilutions and the 3500 U/L standard were tested in triplicate using dipsticks on three Clear blue Easy Fertility monitors. Triplicate LH readings from the three monitors testing the ten samples were averaged and the standard curve is shown in Figure 1. Standard curves were generated for $E_1$3G using ~0.6 ml in a 2 ml cryovials of standard concentrations of 0, 1.562, 3.125, 12.5, 50, and 200 ng/ml $E_1$3G. These six concentrations were tested using dipsticks on three Clear Blue Easy Fertility monitors used by participants. $E_1$3G readings from the three monitors testing the six concentrations were averaged, and the standard curve is shown in Figure 2.

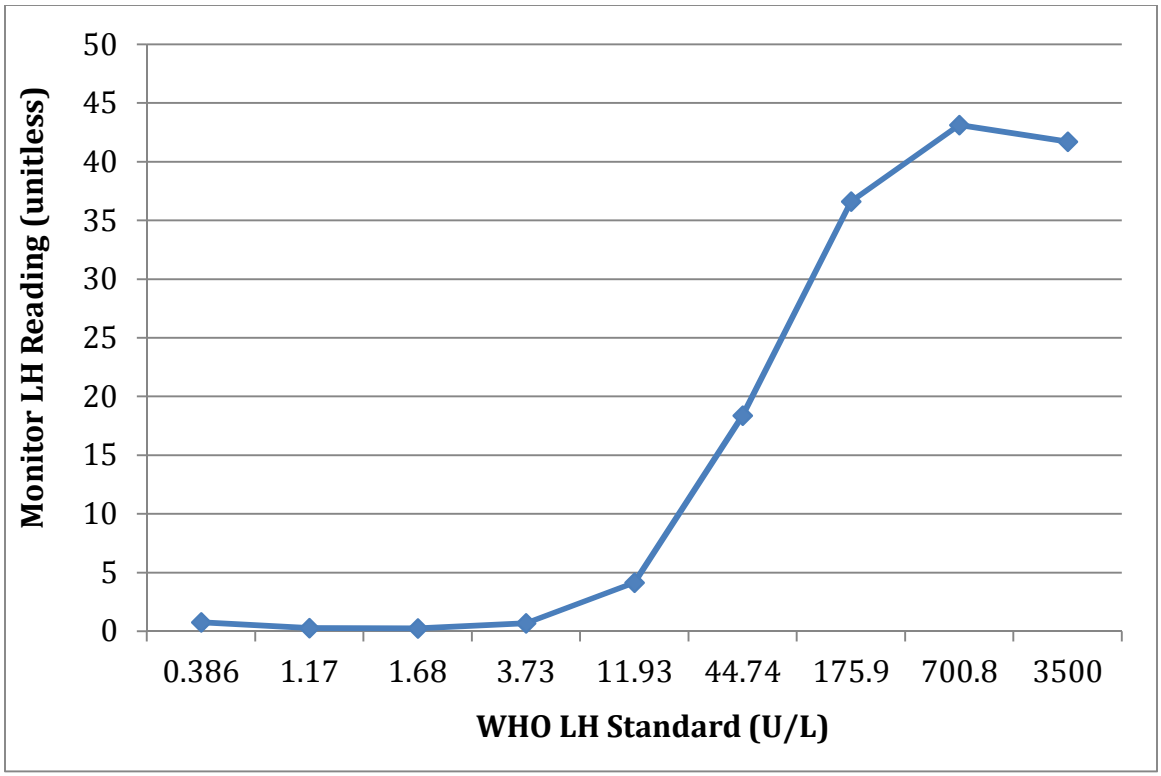

Figure 1: Clearblue LH standard curve

In a prior study, results for detection of the LH surge and for detection of peak fertility days based on rise of $E_1$3G using the Clear Blue Easy monitors compared very favorably with laboratory-based radioimmunoassay of urinary and serum LH and serum estradiol and fluoroimmunoassay of urinary $E_1$3G (Tanabe et al., 2001). These authors demonstrated that 97% of LH surge peaks detected by the monitor occurred within 0 to 2 days after the peak LH level was detected in serum (Tanabe et al., 2001).

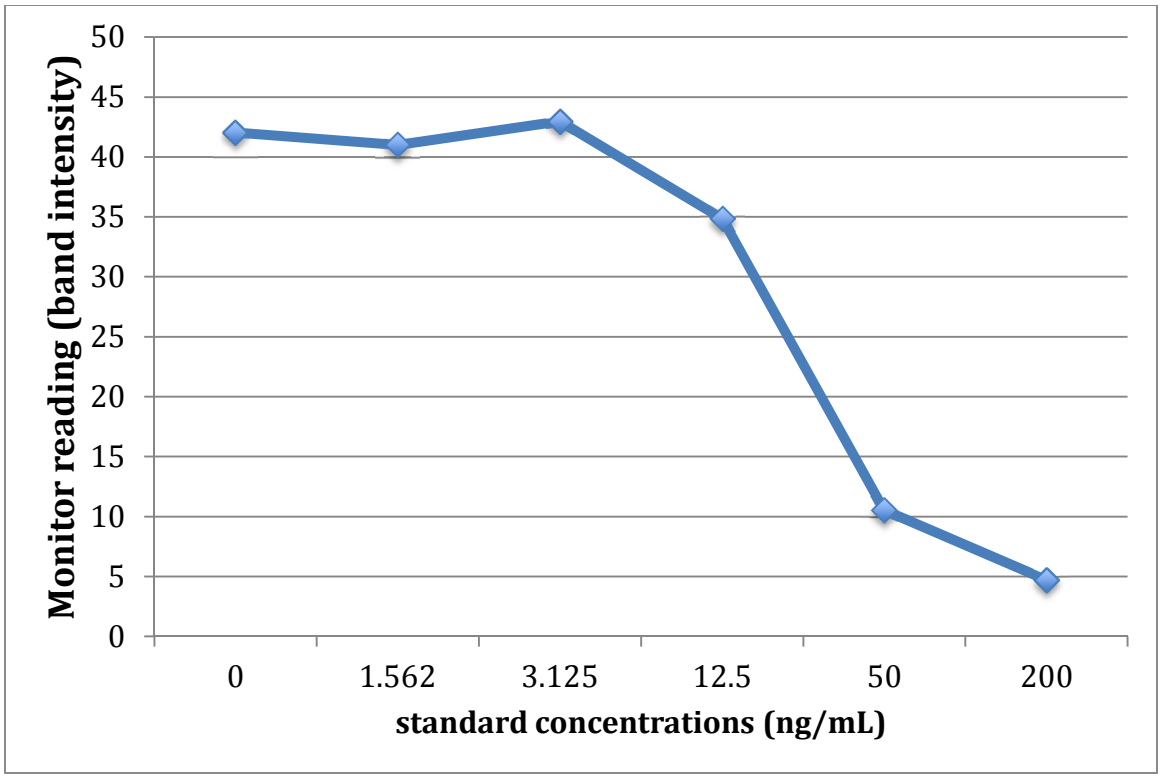

Figure 2: Clearblue E_13G standard curve

6.9 Ovulation status determination algorithm

Ovulatory status was determined from the monitor data and classified as ovulatory, anovulatory or indeterminate. An ovulatory cycle has a defined LH surge onset. The LH surge onset was defined as the first LH rise ≥ 2.5-fold above the mean of the previous 3-7 day baseline. An anovulatory cycle was determined to be one with no apparent LH surge onset that had no missing LH values from the eleventh day of the cycle through the ninth day before the next menses onset. Indeterminate cycles were cycles that did not fit the criteria for either ovulatory or anovulatory. Cycles also must have had a start and end menses date to be valid for analysis.

6.10 Statistical methods

OH-PAH urine metabolites were analyzed for frequency distributions, showing left skewed data. All metabolite concentrations were natural log transformed for normal distribution. Log transformed metabolite concentrations were used for all further analyses.

One-way-ANOVA was used to test for bivariate associations between smoking status and PAH metabolites. Correlation using Pearson coefficient was performed across the three cycles using log transformed creatinine corrected metabolites to test for associations between metabolites and between metabolites and continuous covariates of BMI and age. Bivariate analysis using one-way-ANOVA was used to test for associations between categorical variables of race/ethnicity, alcohol consumption, and education level to metabolites.

Generalized estimating equation models with binary logistic regression link functions and with participant as the subject variable were used to assess associations between ovulation status and PAH metabolite concentrations with adjustment for potentially important covariates in repeated cycles per woman. Initial analyses were done separately for the three separate cycles to explore the associations. Ovulation status was determined as ovulatory or anovulatory (indeterminate cycles were excluded). Covariates were selected using a cutoff value of p-value <0.20 for associations of potential confounders with the exposure variables. Preliminary cycle-specific logistic regression was done to assess associations between ovulatory status and urinary PAH metabolites with potential confounders as covariates. All statistical analysis was performed using SPSS v22.

7.0 RESULTS

Forty three percent of the participants were non-Hispanic white and 54% had a bachelor's degree or higher. Most participants were within the normal BMI category (62.5%) and consumed alcohol on a seldom to occasional basis (56.2%). Selected population characteristics are shown in Table 1.

Table 1: Population characteristics for study subjects

	N	**(%)**
Ethnicity		
Non-Hispanic White	22	43.1%
Hispanic	13	25.5%
Asian	10	19.6%
Black/Other	6	11.8%
Age		
18-25	13	25.5%
26-29	13	25.9%
30-34	13	25.2%
35-44	12	23.4%
BMI		
18.5-24.9	30	62.5%
25-29.9	12	25.0%
>30-35	6	12.5%
Alcohol use		
1 to >5 times per week	19	38.8%
Less than once monthly to 1-4 times per month	19	38.8%
Never	11	22.4%
Smoking		
Yes	5	9.8%
No	46	90.2%
Education		
HS/GED or less	3	6.0%
Some college- Associates	20	40.0%
Bachelors	16	32.0%
Masters/Doctoral	11	22.0%

7.1 PAH metabolite concentrations

Metabolites from the study as compared to NHANES geometric means are shown in

Table 2. Across all three cycles and metabolites, the study geometric means were less than the NHANES geometric means.

Table 2: Geometric means of OH-PAH Metabolites from NHANES and study data

	NHANES (95% CI)	**Cycle 1**	**Cycle 2**	**Cycle 3**
			(arithmetic mean)	
Metabolites of Fluorine				
2-hydroxyfluorene	284 (255-316)	172 (203)	192 (226)	171 (200)
3-hydroxyfluorene	108 (94.7-123)	87.0 (105)	91.7 (113)	81.8 (106)
9-hydroxyfluorene	297 (269-329)	221 (264)	228 (346)	218 (288)
Metabolites of Phenanthrene				
1-hydroxyphenanthrene	163 (148-180)	104 (118)	102 (127)	95.2 (113)
2-hydroxyphenanthrene	72.5 (66.9-78.6)	29.4 (33.8)	32.1 (38.4)	29.0 (32.1)
3-hydroxyphenanthrene	73.2 (67.0-80.0)	51.6 (59.1)	54.6 (64.3)	49.0 (55.1)
Metabolites of Pyrene				
1-hydroxypyrene	141 (130-154)	82.3 (97.4)	101 (125)	90.2 (107)
Metabolites of Naphthalene				
1-hydroxynaphthalene	2040 (1680-2470)	1071 (1525)	1382 (2280)	925 (1572)
2-hydroxynaphthalene	5530 (5010-6110)	3644 (4980)	5509 (7550)	4705 (6156)

Geometric means of OH-PAH metabolites in ng/g creatinine corrected. NHANES OH-PAH 2011-2012 female geometric mean.

Upon log transformation the frequencies of metabolite concentrations were more normalized with a few notable outliers in the higher concentrations.

Correlation using Pearson coefficient was performed across the three cycles using log transformed creatinine corrected metabolites (Tables 3-5). Metabolites tended to be

highly correlated with each other and within parent compound groups. One exception is 2-hydroxynaphthalene, which is not significantly correlated with the other metabolites including the 1-hydroxynaphthalene that comes from the same parent compound.

Table 3: Correlation among PAH metabolites in cycle 1

	2-FLUO	3-FLUO	9-FLUO	1-PHEN	2-PHEN	3-PHEN	1-PYR	1-NAP	2-NAP
2-FLUO	1								
3-FLUO	**.877****	1							
9-FLUO	**.406****	**.354***	1						
1-PHEN	**.691****	**.601****	**.613****	1					
2-PHEN	**.643****	**.659****	**.643****	**.732****	1				
3-PHEN	**.587****	**.642****	**.435****	**.716****	**.776****	1			
1-PYR	**.333***	**.419****	.268	**.544****	**.629****	**.583****	1		
1-NAP	.148	.184	**.329***	.243	.009	-.026	-.198	1	
2-NAP	.059	.111	-.218	-.078	.092	.049	.187	.057	1

Table 4: Correlation among PAH metabolites in cycle 2

	2-FLUO	3-FLUO	9-FLUO	1-PHEN	2-PHEN	3-PHEN	1-PYR	1-NAP	2-NAP
2-FLUO	1								
3-FLUO	**.894****	1							
9-FLUO	**.480****	**.471****	1						
1-PHEN	**.620****	**.518****	**.738****	1					
2-PHEN	**.525****	**.447****	**.604****	**.726****	1				
3-PHEN	**.667****	**.625****	**.740****	**.804****	**.687****	1			
1-PYR	**.414****	**.433****	**.489****	**.637****	**.584****	**.716****	1		
1-NAP	**.423****	**.523****	**.362****	**.412****	.134	**.494****	.281	1	
2-NAP	.081	.065	-.065	-.044	.015	-.083	.072	-.025	1

Table 5: Correlation among PAH metabolites in cycle 3

	2-FLUO	3-FLUO	9-FLUO	1-PHEN	2-PHEN	3-PHEN	1-PYR	1-NAP	2-NAP
2-FLUO	1								
3-FLUO	**.899****	1							
9-FLUO	**.566****	**.555****	1						
1-PHEN	**.631****	**.605****	**.609****	1					
2-PHEN	**.547****	**.493****	**.669****	**.732****	1				
3-PHEN	**.590****	**.709****	**.692****	**.671****	**.691****	1			
1-PYR	**.444****	**.510****	**.518****	**.701****	**.606****	**.721****	1		
1-NAP	**.443****	**.553****	**.530****	**.536****	**.537****	**.499****	**.547****	1	
2-NAP	-.001	.075	-.183	-.011	.121	.020	.097	.267	1

Tables 4-6: 2-hydroxyfluorene=2-OHFLUO, 3-hydroxyfluorene= 3-FLUO, 9-hydroxyfluorene= 9-FLUO, 1-hydroxyphenanthrene= 1-PHEN, 2-hydroxyphenanthrene= 2-PHEN,
3-hydroxyphenanthrene= 3-PHEN, 1-hydroxypyrene= 1-PYR, 1-hydroxynaphthalene= 1-NAP,
2-hydroxynaphthalene= 2-NAP
Pearson correlation:
**. Correlation significant at 0.01 level (2-tailed)
*. Correlation significant at 0.05 level (2-tailed)

7.2 Bivariate analysis

Pearson correlation was performed on age at time of questionnaire and the PAH metabolites. There was medium to high negative correlation between age and 2-hydroxynaphthalene in cycle one and two (-0.356, p=0.039; -0.505, p=0.002). There were medium positive correlations of age and 1-hydroxyphenanthrene, 3-hydroxyphenanthrene and 9-hydroxyfluorene in cycle three (0.367, p=0.042; 0.384, p= 0.033; 0.367, p=0.046).

There were high positive correlations of BMI and 2-hydroxyfluorene and 3-hydroxyfluorene (0.543, p=0.003; 0.411, p=0.030) in cycle 1. In cycle 3, there was moderate positive correlation of BMI and 2-hydroxyfluorene (0.427, p=0.024). Pearson correlation of metabolite by cycle and continuous variables of age and BMI are shown in table 6. Bivariate analysis of metabolite by cycle and categorical variables are shown in Table 7. Education and alcohol use had the most significant associations with metabolites, whereas smoking and race/ethnicity had relatively few.

Table 6: Bivariate correlation between BMI and PAH metabolite, and age and PAH metabolite by cycle

	BMI	Age
2-FLUO		
Cycle 1	**0.195***	-0.102
Cycle 2	0.071	-0.156
Cycle 3	**0.210***	0.030
3-FLUO		
Cycle 1	0.176	-0.005

Cycle 2	0.047	-0.179
Cycle 3	0.190	**0.238***
9-FLUO		
Cycle 1	0.132	-0.096
Cycle 2	-0.071	0.014
Cycle 3	0.089	0.185
1-PHEN		
Cycle 1	0.158	-0.021
Cycle 2	-0.113	-0.068
Cycle 3	0.032	**0.311***
2-PHEN		
Cycle 1	0.177	0.007
Cycle 2	-0.004	0.083
Cycle 3	0.115	**0.275***
3-PHEN		
Cycle 1	**0.199***	0.024
Cycle 2	-0.116	-0.151
Cycle 3	0.130	**0.332***
1-PYR		
Cycle 1	0.028	-0.080
Cycle 2	-0.126	-0.262
Cycle 3	0.013	**0.207***
1-NAP		
Cycle 1	-0.144	-0.047
Cycle 2	-0.126	-0.029
Cycle 3	-0.057	**0.218***
2-NAP		
Cycle 1	-0.150	**-0.222***
Cycle 2	0.019	**-0.339***
Cycle 3	-0.180	-0.109

Pearson correlation:
* correlation significant at 0.2 level (2-tailed)
2-hydroxyfluorene=2-OHFLUO, 3-hydroxyfluorene= 3-FLUO, 9-hydroxyfluorene= 9-FLUO, 1-hydroxyphenanthrene= 1-PHEN, 2-hydroxyphenanthrene= 2-PHEN, 3-hydroxyphenanthrene= 3-PHEN, 1-hydroxypyrene= 1-PYR, 1-hydroxynaphthalene= 1-NAP, 2-hydroxynaphthalene= 2-NAP

Table 7: P-values for bivariate analyses of the association between race/ethnicity, education, alcohol use, smoking and OH-PAH metabolites in cycles 1-3

	Race/ Ethnicity	**Education**	**Alcohol use**	**Smoking**
2-FLUO	P value	P value	P value	P value
Cycle 1	0.330	0.384	0.859	0.290
Cycle 2	0.914	**0.157***	0.423	0.241
Cycle3	0.709	**0.122***	0.256	**0.078***
3-FLUO				

Cycle 1	**0.179***	0.316	0.821	0.213
Cycle 2	0.976	**0.108***	0.405	**0.127***
Cycle3	0.813	**0.073***	0.698	**0.002***
9-FLUO				
Cycle 1	0.930	**0.004***	0.206	0.965
Cycle 2	0.753	**0.009***	**0.066***	0.859
Cycle3	0.780	**0.187***	**0.134***	0.878
1-PHEN				
Cycle 1	0.379	0.864	0.674	0.990
Cycle 2	0.691	**0.185***	0.228	0.502
Cycle3	0.439	0.386	**0.162***	0.702
2-PHEN				
Cycle 1	0.607	0.721	0.596	0.358
Cycle 2	0.618	**0.068***	**0.067***	0.857
Cycle3	0.667	**0.107***	**0.165***	0.711
3-PHEN				
Cycle 1	0.323	0.519	0.917	0.990
Cycle 2	0.476	**0.072***	**0.130***	0.789
Cycle3	0.902	0.478	**0.153***	**0.171***
1-PYR				
Cycle 1	0.258	0.925	0.776	0.631
Cycle 2	0.831	0.576	**0.079***	0.666
Cycle3	0.917	0.772	**0.081***	0.602
1-NAP				
Cycle 1	0.845	0.782	0.549	0.240
Cycle 2	**0.015***	**0.021***	0.585	0.985
Cycle3	1.00	**0.072***	**0.136***	0.225
2-NAP				
Cycle 1	0.660	0.327	**0.097***	0.923
Cycle 2	0.998	0.240	**0.085***	0.861
Cycle3	0.907	0.824	0.260	0.409

* Designates p values ≤ 0.20. One-way ANOVA used for multi-group comparisons.

7.3 Ovulatory status by cycle

Cycle 1 had 2 anovulatory cycles, 17 indeterminate cycles, and 32 ovulatory cycles for analysis. Cycle 2 had 4 anovulatory cycles, 14 indeterminate cycles and 33 ovulatory cycles for analysis. Cycle 3 had 3 anovulatory cycles, 17 indeterminate cycles, and 28

ovulatory cycles for analysis. The outcome variable of ovulatory status was ovulatory versus anovulatory with ovulatory being the reference category.

7.4 Cycle-specific Logistic regression

Alcohol use and education were selected for use in the cycle-specific logistic regression models as they had a majority of p-values ≤ 0.20 (used as cutoff for inclusion in final models) for associations with metabolites throughout cycles. Due to low case numbers and sample population, categorical covariates with more than two categories were converted into two-category variables for use in the logistic regression models. Additionally each categorical variable covariate was run separately from the other with each PAH metabolite, so a solution could be found without maxing iterations.

Table 8 is the logistic regression by alcohol use. The categories of use were collapsed to two categories: yes and no (reference category: yes). There were no statistically significant associations, but some associations were nearly significant in cycle 3, including for 2-hydroxyfluorene (p=0.108) and 2-hydroxyphenanthrene (p=0.055). These two metabolites both had positive B coefficients representing positive association with alcohol consumption, the respective metabolites and ovulatory cycles. However, it should be noted that the regression coefficients and odds ratios has extremely wide 95% confidence intervals, so these estimates are very instable despite being nearly significant.

Table 8: Cycle-specific logistic regression of ovulatory status by alcohol use (yes/no), and metabolites, cycles 1-3

	B	Exp(B)	P value
2-FLUO			
Cycle 1	NS	NS	NS
Cycle 2	0.444	1.559	0.887

Cycle3	8.382	4365.851	**0.108***
3-FLUO			
Cycle 1	NS	NS	NS
Cycle 2	-1.341	0.262	0.578
Cycle3	NS	NS	NS
9-FLUO			
Cycle 1	NS	NS	NS
Cycle 2	0.284	1.329	0.893
Cycle3	2.698	14.851	0.339
1-PHEN			
Cycle 1	NS	NS	NS
Cycle 2	2.309	10.066	0.443
Cycle3	3.752	42.594	0.345
2-PHEN			
Cycle 1	NS	NS	NS
Cycle 2	2.281	9.791	0.417
Cycle3	10.656	42453.202	**0.055***
3-PHEN			
Cycle 1	NS	NS	NS
Cycle 2	0.434	1.544	0.879
Cycle3	0.058	1.059	**0.**204
1-PYR			
Cycle 1	NS	NS	NS
Cycle 2	6.341	576.369	0.219
Cycle3	4.076	58.932	0.232
1-NAP			
Cycle 1	NS	NS	NS
Cycle 2	0.548	1.730	0.676
Cycle3	1.394	4.031	0.382
2-NAP			
Cycle 1	NS	NS	NS
Cycle 2	1.653	5.225	0.467
Cycle3	-2.103	0.122	0.250

* Designates p values ≤ 0.20. Binary logistic regression
NS= No final solution, maximum iterations reached

Table 9 is the logistic regression by education. Categories of education were collapsed to two categories: bachelor's degree and higher or less than bachelor's degree (Bachelor's degree or higher as reference). There were no statistically significant associations in these regressions, but some coefficients were nearly significant for this

regression, including 2-hydroxyfluorene (p=0.120), 2-hydroxyphenanthrene (p=0.060), and 3-hydroxyphenanthrene (p=0.160). For these three metabolites, they all had positive B coefficients representing positive association with education, the respective metabolites and ovulatory cycles. However, similar to the findings using alcohol as a covariate, these regression coefficients and odds ratios has extremely wide 95% confidence intervals, so these estimates are very unstable despite being nearly significant.

Table 9: Logistic regression of ovulatory status by education (two category), and metabolites, cycles 1-3.

	B	**Exp(B)**	**P value**
2-FLUO			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS
Cycle3	7.899	2695.070	**0.120***
3-FLUO			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS
Cycle3	2.199	9.013	0.472
9-FLUO			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS
Cycle3	3.039	20.892	0.288
1-PHEN			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS
Cycle3	3.912	50.023	0.304
2-PHEN			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS
Cycle3	9.260	10511.552	**0.060***
3-PHEN			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS
Cycle3	0.063	1.065	**0.160***
1-PYR			
Cycle 1	NS	NS	NS
Cycle 2	NS	NS	NS

Cycle3	3.938	51.319	0.226
1-NAP			
Cycle 1	NS	NS	NS
Cycle 2	0.824	2.281	0.594
Cycle3	1.023	2.781	0.435
2-NAP			
Cycle 1	NS	NS	NS
Cycle 2	3.729	41.631	0.225
Cycle3	-2.023	0.132	0.262

* Designates p values ≤ 0.20. Binary logistic regression
NS= No final solution, max iterations reached

The preliminary logistic regression model is shown in table 10, using the previously nearly significant metabolites in cycle 3 with the covariates of education and alcohol use. This final model did not reveal any statistically significant effects of 2-FLUO, 2-PHEN, or 3-PHEN urinary concentrations on ovulatory status.

Table 10: Logistic regression by two-category alcohol, education and 2-hydroxyfluorene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene metabolites and ovulatory status.

	B	**Exp(B)**	**P value**
2-FLUO			
Cycle3	9.764	17401.367	0.396
2-PHEN			
Cycle 3	7.469	1753.029	0.278
3-PHEN			
Cycle 3	-0.017	.983	0.860

* Designates p values ≤ 0.05. Binary logistic regression

7.5 Generalized estimating equations

The final model used a logistic regression generalized estimating equations due to the

repeated measures of OH-PAH metabolites and ovulatory status across menstrual cycles in this study. Age, BMI, race/ethnicity, alcohol use, and education were tested in a preliminary model to see if there was significant association with outcome. Only age was significant at the p-value cutoff of <0.20, so age was the only covariate included in the regression models. All metabolites were initially included in the model with age to test for associations with the PAH metabolites. In a stepwise manner, the regression models were repeated after removing the least significant metabolites. The resultant model with significant metabolites < 0.10 were 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 1-hydroxypyrene, 3-hydroxyfluorene, and 3-hydroxyphenanthrene, shown in table 11.

Table 11: Logistic regression using generalized estimating equations for ovulation status and PAH metabolites, adjusted for age.

	B	**CI**	**p-value**
2-FLUO	-4.201	0.001 to 0.298	**0.006***
3-FLUO	2.716	0.600 to 381.212	**0.099***
1-PYR	-1.750	0.030 to 0.990	**0.049***
3-PHEN	1.891	1.004 to 47.746	**0.049***
1-NAP	-0.575	0.302 to 1.049	**0.070***
2-NAP	1.165	1.588 to 6.471	**0.001***

*p<= 0.10

Although the regression model shown in Table 11 converged and all of the PAH metabolites remaining in the model were statistically significant, it may not be the optimal model due to residual collinearity, among the PAH metabolites. This possibility is suggested by the strongly different coefficients and odds ratios for PAH metabolites in the same sub-groups. For example, the coefficient for 2-hydroxyfluorene is negative, while the

coefficient for 3-hydroxyfluorene is positive. Similarly, the coefficient for 1-hydroxynaphthalene is negative, but the coefficient for 2-hydroxynaphthalene is positive. When looking at the coefficients for this model there is a paired effect. This paired effect is where the coefficient is very large for one metabolite and very small for another metabolite in the same parent compound group. This happened for each of the metabolites that had more than one metabolite per parent compound group. Therefore, a final logistic regression model was run using only the most statistically significant urinary PAH metabolite from each of the metabolite sub-groups. The result of this model is shown in Table 12. The narrower 95% confidence intervals indicate that the estimates of this model are more stable. In this model, 2-hydroxynaphthalene has a statistically significant positive association with anovulatory cycles, while 1-hydroxypyrene had a nearly significant negative association and 3-hydroxyphenanthrene had a nearly significant positive association with anovulatory cycles.

Table 12: Final model logistic regression using generalized estimating equations for ovulation status and PAH metabolites, adjusted for age.

	B	**CI**	**p-value**
2-FLUO	-1.529	-3.906-0.849	0.208
1-PYR	-1.492	-3.163-0.179	0.088
3-PHEN	1.672	-0.185-3.530	0.078
2-NAP	1.085	0.493-1.676	**0.001***

*p<= 0.05

8.0 DISCUSSION

In this study our hypothesis was that overall PAH exposure was associated with

decreased fertility. In relation to the hypothesis, the findings suggest that some PAHs (metabolites) have an opposite association, while some have the expected association.

The logistic regression analysis was limited by case numbers and sample sizes. For example cycle one only had 2 anovulatory and 32 ovulatory cycles, for a total of 34 cases. This was an issue with lack of convergence of the logistic regressions. As the final result, only cycle three had suitable outputs from the cycle-specific crude regression to be used in the final model only looking at ovulatory status of cycle 3. Even for the models that did converge, many of the coefficients were not plausible. Not surprisingly, with the small case numbers, the outcome was not significant even with forward conditional regression (not shown).

Due to the nature of repeated measures in this study, the final model for analysis used generalized estimating equations with binary logistic regression link function. Binary logistic regression was done by cycle as an exploratory exercise.

The result of the final adjusted logistic regression GEE model (Table 12) shows that 2-hydroxynaphthalene has a statistically significant positive association with anovulatory cycles and 3-hydroxyphenanthrene had a nearly significant positive association with anovulatory cycles. With respect to these metabolites, compared to ovulatory, anovulatory cycles had higher concentrations. This metabolite is associated with the greater likelihood to have anovulatory cycles (supports our hypothesis). Alternatively, 1-hydroxypyrene had a nearly significant negative association, demonstrating that anovulatory cycles had lower concentrations. These findings are contrary to our hypothesis and can be due to a variety of reasons. First, we are relying on LH surge to classify cycles as ovulatory as we do not have progesterone measurement to further support the

classification. So it is a possibility that ovulatory cycles were classified as such when they were not. Another potential explanation is that these PAHs (metabolites) provide some unknown protective effect, where as the known larger PAHs that we cannot measure in urine do have a deleterious effect. Also measurements of the PAH metabolites done at different parts of the cycle may contribute to misclassification of status. Finally there may be some residual unexplained confounding with these results and residual multi-collinearity, among the PAH metabolites that affects the regression models.

Our cohort overall had lower means than the NHANES data (Table 2). This is could be explained by few factors. First, our population was Southern California women who may have a reduced exposure to PAHs due to measures such as reducing naphthalene in mothballs or pesticides (Lu et al., 2005). Secondly we had very few smokers in our cohort, which likely reflects a lower percentage than was found in the NHANES sample.

We would suspect that the smokers, with higher exposure to PAHs via cigarette smoke, would have higher levels of OH-PAH metabolites in their urine. When looking for a correlation between smoking status and OH-PAH metabolites, very few significant correlations were found, with exception to the fluorene metabolites and a phenanthrene metabolite (Table 7). In a study measuring the levels of 14 PAHs in mainstream smoke, naphthalene was highest in concentration followed by fluorene and phenanthrene at 100–200 ng/cigarette (Ding et al., 2005). Otherwise all the other metabolites did not show a significant association with smoking status, and this can be explained by our low number of smokers (n=5) and the low numbers of cigarettes per day they reportedly smoked.

Generally, correlation between the various PAH metabolites ranged from moderate to high with notable exception of 2-hydroxynaphthalene. The latter could be explained by a

unique parent compound or route of exposure such as dermal, which is different from the largely inhalational exposure of all the other compounds. Overall parent groups of metabolites dictated stronger correlation between metabolites. One exception is 1-hydroxynaphtalene and 2-hydroxynaphthalene and this is likely due to 1-hydroxynaphthalene having other potential parent compounds of insecticide, herbicides and medications, in addition to naphthalene, which is the only parent compound for 2-hydroxynaphthalene's one parent (ATSDR, 1995). Interestingly, 2-hydroxynaphthlene had weak correlations with other metabolites, yet it was the only significant positive correlation with anovulatory cycles in the final model. This may be due to a solitary parent compound effect on cycles versus multiple parent compounds with multiple highly correlated metabolites.

Correlations between age and PAH metabolites, and BMI and PAH metabolites were few in numbers or not significant. This could be due to the distributions of BMI which fell largely in the normal category and age of participants that did not have a very wide range. Likewise the same could be said for no significant correlation with race/ethnicity. This could also be due to there being no association with these variables, and low power of the study due to low sample size.

Education was used as a proxy for socioeconomic status (SES) and higher education was associated with some of the PAH metabolites. One would expect lower SES to have higher association with PAHs as they have a greater smoking prevalence (Hiscock et al., 2012). This could be due to greater commute times by higher educated women as they commute from their homes in suburban areas to workplaces in urban areas.

Alcohol use was also associated with some of the PAH metabolites. The effect of

alcohol consumption on the metabolism of xenobiotics in general has been investigated, and it is thought that acute alcohol consumption has an inhibitory effect whereas induction of metabolism occurs when alcohol is used chronically. These effects are based on competition for, and stimulation of, the microsomal cytochrome P-450 system, respectively. In animal studies, a decrease in PAH metabolite concentrations were found in animals addicted to alcohol (Van Rooij et al., 1994).

Lastly commercial home fertility monitors were used by the participants. These monitors use an algorithm that attempts to minimize the number of days per cycle that a test stick is requested. A research version of the monitor, which requests test sticks everyday, would provide more information, especially about the luteal phase of the cycle, which could be used for more accurate determination of ovulatory status. Unfortunately, research monitors were not available when we performed our study.

9.0 CONCLUSION

In conclusion we analyzed the effects of exposure to PAHs on the ovulatory status of a cohort of women. It was hypothesized that higher PAH exposure would result in more anovulatory cycles than ovulatory cycles. The results of the study did support the hypothesis with respect to two PAH metabolites, as there was significant association of PAH metabolite concentrations with ovulatory status. One of the metabolites was associated with more ovulatory cycles contrary to the hypothesis. The small n of 51, and many menstrual cycles being categorized as indeterminate ovulatory status, as well as collinearity of the metabolite concentrations, could add to the dichotomous nature of the results. To further investigate this relationship it would be helpful to have a larger sample

size. Additionally, better methods for collecting menstrual cycle data such a clinical monitors versus commercially available monitor may yield better results in determination of cycle ovulatory status.

REFERENCES

ATSDR Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs)." *Atlanta, GA* (1995): 1-487.

Augood, C., K. Duckitt, and A. A. Templeton. "Smoking and female infertility: a systematic review and meta-analysis." *Human Reproduction* 13.6 (1998): 1532-1539.

Baird, Donna Day, and Allen J. Wilcox. "Cigarette smoking associated with delayed conception." *Jama* 253.20 (1985): 2979-2983.

Buckley, Timothy J., and Paul J. Lioy. "An examination of the time course from human dietary exposure to polycyclic aromatic hydrocarbons to urinary elimination of 1-hydroxypyrene." *British journal of industrial medicine* 49.2 (1992): 113-124.

Buratti, M., et al. "Urinary hydroxylated metabolites of polycyclic aromatic hydrocarbons as biomarkers of exposure in asphalt workers." *Biomarkers* 12.3 (2007): 221-239.

CDC Laboratory Procedure Manual, Monohydroxy-Polycyclic Aromatic Hydrocarbons (OH-PAHs) (2013).

CDC/National Center for Health Statistics. Reproductive Health. 2015.

Chen, Changzhong, et al. "Effect of environmental tobacco smoke on levels of urinary hormone markers." *Environmental health perspectives* (2005): 412-417.

de Vet, Annemarie, et al. "Antimüllerian hormone serum levels: a putative marker for ovarian aging." *Fertility and sterility* 77.2 (2002): 357-362.

Ding, Yan S., et al. "Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes." *Environmental science & technology* 39.2 (2005): 471-478.

Disdier, B., et al. "Analysis by GC-MS of Polycyclic Aromatic Hydrocarbons in a Cream Containing Coal Tar." *Polycyclic Aromatic Compounds* 20.1-4 (2000): 169-177.

Durlinger, Alexandra LL, et al. "Control of Primordial Follicle Recruitment by Anti-Müllerian Hormone in the Mouse Ovary 1." *Endocrinology* 140.12 (1999): 5789-5796.

Gehle, K. "Agency for Toxic Substances and Disease Registry (ATSDR) Case Studies in Environmental Medicine Toxicity of Polycyclic Aromatic Hydrocarbons (PAHs)." (2009).

Hiscock, Rosemary, et al. "Socioeconomic status and smoking: a review." *Annals of the New York Academy of Sciences* 1248.1 (2012): 107-123.

Jeng, Hueiwang Anna, et al. "Repeated measurements for assessment of urinary 2-naphthol levels in individuals exposed to polycyclic aromatic hydrocarbons." *Journal of Environmental Science and Health, Part A* 46.8 (2011): 865-873.

Jongeneelen, F. J., et al. "Ambient and biological monitoring of cokeoven workers: determinants of the internal dose of polycyclic aromatic hydrocarbons." *British journal of industrial medicine* 47.7 (1990): 454-461.

Jurisicova, Andrea, et al. "Maternal exposure to polycyclic aromatic hydrocarbons diminishes murine ovarian reserve via induction of< i> Harakiri</i>." Journal of Clinical Investigation 117.12 (2007): 3971-3978.

Kevenaar, Marlies E., et al. "Serum anti-mullerian hormone levels reflect the size of the primordial follicle pool in mice." *Endocrinology* 147.7 (2006): 3228-3234.

Li, Zheng, et al. "Urinary polycyclic aromatic hydrocarbon metabolites as biomarkers to woodsmoke exposure—results from a controlled exposure study." *Journal of Exposure Science and Environmental Epidemiology* (2015).

Li, Zheng, et al. "Excretion profiles and half-lives of ten urinary polycyclic aromatic hydrocarbon metabolites after dietary exposure." *Chemical research in toxicology* 25.7 (2012): 1452-1461.

Li, Zheng, et al. "Concentration and profile of 22 urinary polycyclic aromatic hydrocarbon metabolites in the US population." *Environmental research* 107.3 (2008): 320-331.

Lu, Rong, et al. "Naphthalene distributions and human exposure in Southern California." *Atmospheric Environment* 39.3 (2005): 489-507.

Machala, Miroslav, et al. "Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay." *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 497.1 (2001): 49-62.

Matikainen, Tiina M., et al. "Ligand activation of the aromatic hydrocarbon receptor transcription factor drives Bax-dependent apoptosis in developing fetal ovarian germ cells." *Endocrinology* 143.2 (2002): 615-620.

Matikainen, Tiina, et al. "Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals." *Nature genetics* 28.4 (2001): 355-360.

Mattison, D. R. "Difference in sensitivity of rat and mouse primordial oocytes to destruction by polycyclic aromatic hydrocarbons." Chemico-biological interactions 28.1 (1979): 133-137.

Mattison, D. R., N. B. White, and M. R. Nightingale. "The effect of benzo[*a*]pyrene on fertility, primordial oocyte number, and ovarian response to pregnant mare's serum gonadotropin." Pediatric pharmacology (New York, NY) 1.2 (1979): 143-151.

Menzie, Charles A., Bonnie B. Potocki, and Joseph Santodonato. "Exposure to carcinogenic PAHs in the environment." *Environmental science & technology* 26.7 (1992): 1278-1284.

Neal, Michael S., Jiping Zhu, and Warren G. Foster. "Quantification of benzo[*a*]pyrene and other PAHs in the serum and follicular fluid of smokers versus non-smokers." *Reproductive Toxicology* 25.1 (2008): 100-106.

Mukherjee, Sutapa, et al. "1-hydroxypyrene as a biomarker of occupational exposure to polycyclic aromatic hydrocarbons (PAH) in boilermakers." *Journal of occupational and environmental medicine* 44.12 (2002): 1119-1125.

NCS. The National Children's Study Research Plan. (2007).

NHANES Anthropometry Procedures Manual, National Health and Nutrition Examination Survey. (2004): 1-65.

NHANES Fourth National Report on Human Exposure to Environmental Chemicals. (2015): 463-499.

Niedbala, R. Sam, et al. "Automated homogeneous immunoassay analysis of cotinine in urine." *Journal of analytical toxicology* 26.3 (2002): 166-170.

Robinson, Janet E., Melanie Wakelin, and Jayne E. Ellis. "Increased pregnancy rate with use of the Clearblue Easy Fertility Monitor." *Fertility and sterility* 87.2 (2007): 329-334.

Rockett, John C., et al. "The value of home-based collection of biospecimens in reproductive epidemiology." *Environmental health perspectives* 112.1 (2004): 94.

Romanoff, Lovisa C., et al. "Automated solid-phase extraction method for measuring urinary polycyclic aromatic hydrocarbon metabolites in human biomonitoring using isotope-dilution gas chromatography high-resolution mass spectrometry." *Journal of Chromatography B* 835.1 (2006): 47-54.

Sadeu, J. C., and W. G. Foster. "Effect of in vitro exposure to benzo[*a*]pyrene, a component of cigarette smoke, on folliculogenesis, steroidogenesis and oocyte nuclear maturation." *Reproductive Toxicology* 31.4 (2011): 402-408.

Shiromizu, Kenji, and Donald R. Mattison. "The effect of intraovarian injection of benzo[*a*]pyrene on primordial oocyte number and ovarian aryl hydrocarbon [benzo[*a*]pyrene] hydroxylase activity." *Toxicology and applied pharmacology* 76.1 (1984): 18-25.

Skupinska, Katarzyna, Irena Misiewicz, and Teresa Kasprzycka-Guttman. "Polycyclic aromatic hydrocarbons: physicochemical properties, environmental appearance and impact on living organisms." *Acta Pol Pharm* 61.3 (2004): 233-240.

Takizawa, Ken, et al. "Murine strain differences in ovotoxicity following intraovarian injection with benzo[*a*]pyrene, (+)-(7r, 8s)-oxide, (-)-(7r, 8r)-dihydrodiol, or (+)-(7r, 8s)-diol-(9s, 10r)-epoxide-2." Cancer research 44.6 (1984): 2571-2576.

Tanabe, K., et al. "Prediction of the potentially fertile period by urinary hormone measurements using a new home-use monitor: comparison with laboratory hormone analyses." *Human Reproduction* 16.8 (2001): 1619-1624.

Van Rooij, Joost GM, et al. "Smoking and dietary intake of polycyclic aromatic hydrocarbons as sources of interindividual variability in the baseline excretion of 1-hydroxypyrene in urine." *International archives of occupational and environmental health* 66.1 (1994): 55-65.

Viau, C., et al. "The toxicokinetics of pyrene and its metabolites in rats." *Toxicology letters* 108.2 (1999): 201-207.

Wyatt, Katrina M., et al. "Determination of total menstrual blood loss." *Fertility and sterility* 76.1 (2001): 125-131.

Zheng, Wenjing, et al. "Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary." *Molecular and cellular endocrinology* 356.1 (2012): 24-30.