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Associations between urinary biomarkers of polycyclic aromatic hydrocarbon exposure and reproductive function during menstrual cycles in women

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Abstract

Essentially all women are exposed to polycyclic aromatic hydrocarbons (PAHs), formed during incomplete combustion of organic materials, including fossil fuels, wood, foods, and tobacco. PAHs are ovarian toxicants in rodents, and cigarette smoking is associated with reproductive abnormalities in women. Biomonitoring of hydroxylated PAH (OH-PAH) metabolites in urine provides an integrated measure of exposure to PAHs via multiple routes and has been used to characterize exposure to PAHs in humans. We hypothesized that concentrations of OH-PAHs in urine are associated with reproductive function in women. We recruited women 18–44 years old, living in Orange County, California to conduct daily measurement of urinary luteinizing hormone (LH) and estrone 3-glucuronide ($E_1$3G) using a microelectronic fertility monitor for multiple menstrual cycles; these data were used to calculate endocrine endpoints. Participants also collected urine samples on cycle day 10 for measurement of nine OH-PAHs. Models were constructed for

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[3]The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health (NIOSH). Mention of any company or product does not constitute endorsement by NIOSH.

eight endpoints using a Bayesian mixed modeling approach with subject-specific random effects allowing each participant to act as a baseline for her set of measurements. We observed associations between individual OH-PAH concentrations and follicular phase length, follicular phase LH and $E_1$3G concentrations, preovulatory LH surge concentrations, and periovulatory $E_1$3G slope and concentration. We have demonstrated the feasibility of using urinary reproductive hormone data obtained via fertility monitors to calculate endocrine endpoints for epidemiological studies of ovarian function during multiple menstrual cycles. The results show that environmental exposure to PAHs is associated with changes in endocrine markers of ovarian function in women in a PAH-specific manner.

Keywords

polycyclic aromatic hydrocarbon; ovarian function; reproduction; luteinizing hormone; estradiol; menstrual cycle

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants formed during incomplete combustion of organic materials such as wood, tobacco, fossil fuels, and food (ATSDR 1995). Data from the National Health and Nutrition Examination Survey (NHANES) on concentrations of hydroxylated PAHs (OH-PAHs) in the urine of representative samples of Americans show that essentially all Americans are exposed to PAHs (Li et al. 2008; NHANES 2009). For non-smokers who do not consume grilled or roasted foods, air pollution is the largest source of exposure. Residents of urban areas have higher inhalation exposure to PAHs than do residents of rural areas (Menzie et al. 1992).

Exposure to tobacco smoke can be the principal source of PAH exposure for smokers. Sidestream smoke has 10-fold higher concentrations of PAHs than mainstream tobacco smoke, and second hand smoke is another major source of exposure (Lodovici et al. 2004; Lu et al. 2008; Shopland et al. 2001). PAHs have been measured in human serum and ovarian follicular fluid (Neal et al. 2008). The latter study showed that follicular fluid of women smokers had significantly elevated levels of the PAH benzo[a]pyrene (BaP) compared to follicular fluid of non-smoking women.

Many PAHs are mutagenic and carcinogenic (ATSDR 1995; IARC 1983; 2010) and are potent ovarian toxicants and ovarian tumorigens in rodents. Both single high doses (Mattison 1979; Mattison and Nightingale 1982; Mattison and Thorgeirsson 1979; Mattison et al. 1980; Takizawa et al. 1984) and multiple lower doses (Borman et al. 2000) of BaP, 9,12-dimethyl-1,2-benzanthracene (DMBA), and 3-methylcholanthrene destroy immature primordial and primary follicles in mice and rats. DMBA concentration-dependently increases reactive oxygen species and induces apoptosis in cultured rat antral follicles (Tsai-Turton et al. 2007). BaP inhibits growth, survival, and estradiol and anti-Müllerian hormone secretion of cultured mouse secondary follicles (Neal et al. 2007; Sadeu and Foster 2011).

Epidemiological studies have linked smoking to decreased fecundability (probability of pregnancy) (Alderete et al. 1995; Baird and Wilcox 1985; Hassan and Killick 2004; Jensen

et al. 1998) and earlier onset of menopause (Harlow and Signorello 2000; Mattison et al. 1989; van Noord et al. 1997). In a prospective study in which urinary estrogen and progesterone metabolites were measured daily during multiple menstrual cycles, exposure to environmental tobacco smoke in nonsmoking women was associated with lower concentrations of estrone conjugates during the 20-day window around ovulation (Chen et al. 2005).

PAHs are readily absorbed through the lungs, gut, and skin (ATSDR 1995). PAHs generally require metabolic activation to exert toxicity. They are first oxidized by cytochrome P450 (CYP) enzymes to epoxides, which undergo hydrolysis by epoxide hydrolase to diols, which can be oxidized by CYPs to diol epoxides or converted to catechols by aldo-keto deductases (Penning 2004; Verma et al. 2012; Xue and Warshawsky 2005). The diol epoxide metabolites are DNA damaging mutagens, and the catechols undergo redox cycling, generating reactive oxygen species that can damage cellular macromolecules (Denissenko et al. 1996; Penning 2004; Verma et al. 2012; Xue and Warshawsky 2005). The epoxides can also spontaneously rearrange to monohydroxylated intermediates, which are excreted as conjugates in the urine or bile (Verma et al. 2012). Biomonitoring of these hydroxylated PAH metabolites in urine provides an integrated measure of PAH exposure via multiple exposure routes (Li et al. 2008).

Although the altered reproductive function observed in women who smoke or who are exposed to environmental tobacco smoke has been postulated to be due to exposure to PAHs in tobacco smoke, no studies have directly examined the associations between PAH exposure biomarkers and measures of hypothalamic-pituitary-ovarian axis function in women. We hypothesized that PAH exposure causes ovarian dysfunction manifested as altered urinary luteinizing hormone (LH) and estrogen metabolite profiles and even anovulatory menstrual cycles.

2. METHODS

2.1 Study Participants

Study participants were recruited for a pilot study to lay the groundwork for a subsequent larger, adjunct study to the planned National Children's Study (NCS). The purpose of the pilot study was 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 PAH metabolites measured once per menstrual cycle and urinary reproductive hormone concentrations measured daily using a microelectronic dipstick monitor for six menstrual cycles.

Initially, women were recruited based on NCS Vanguard Study protocols (Baker et al. 2014; Montaquila et al. 2010). For the Vanguard Study, Orange County, California was divided into 15 geographical strata, which were each then further divided into 11 geographical units (GUs). Each GU was then divided into 10 segments or 'neighborhoods'. In a two-stage sampling process, 15 of those segments, totaling 15,000 households, were sampled by the NCS Coordinating Center for screening. The remaining segments were not further studied under the NCS Vanguard Study. Women for the current study were recruited from four

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 to assure representation of the two largest ethnic groups in Orange County. Initially eligible women were identified and recruited 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, eligible women for the present study were recruited at public events such as health fairs at universities and colleges, work places and events sponsored by community groups. The current study population is thus a convenience sample. Baseline study visits and sample collection occurred between October 2010 and July 2012.

2.2 Inclusion and exclusion criteria

Eligible women were between the ages of 18–44, residing in Orange County California, who were not pregnant, currently not planning to conceive, not using hormonal contraception, did not have a history of surgical sterilization, treatment with antineoplastic drugs or radiation therapy to the pelvis and did not have conditions known to cause infertility by mechanisms other than ovarian failure (pelvic inflammatory disease, endometriosis). Women who had recently been pregnant or breastfeeding were asked to delay starting the study until they had one full menstrual cycle after the birth if not breastfeeding or after they stopped breastfeeding.

2.3 Participation and compensation

Recruits were told that they would be responsible for testing their first morning void urine daily for reproductive hormones using a dipstick fertility monitor, collecting one urine sample during each of the study menstrual cycles, completing a daily diary and a monthly questionnaire, and potentially taking one or more home pregnancy tests. Participants were given the option of keeping the fertility monitors upon completion of the study or of receiving a payment of $100 upon returning the monitor. Written, informed consent was obtained from all participants in person by a member of the study staff. All participants were fluent in English or Spanish. The study was approved by the University of California Irvine Institutional Review Board (Study Number 2009–7034).

2.4 Baseline Study Visit

After completing informed consent, participants were instructed to go to one of the two Orange County locations of the UC Irvine Institute for Clinical and Translational Science (ICTS) for their baseline visit 5–9 days prior to their next menses onset. Study staff administered the standardized NCS preconception questionnaire (NCS 2007) to obtain information about the participant’s demographics, medical history, reproductive history, tobacco smoke exposure, exercise history, occupational history, residential history, housing characteristics, use of chemicals (e.g. cleaning agents, pesticides) in and around 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). We refer to these variables hereafter as baseline covariates.

Participants were instructed to fill out a daily diary during the study period to provide information about days and amount of menstrual bleeding (guided by a menstrual pictogram) (Wyatt et al. 2001), contraception use, number of cigarettes smoked, illness, use of medications, number of alcoholic drinks, and sexual intercourse.

ICTS nursing staff measured height, weight, and blood pressure and collected a blood sample. Study staff gave participants urinary hormone monitors, urine collection kits, and home pregnancy tests and provided instruction in how to use them.

2.5 Urinary Reproductive Endocrine Testing

Participants were given a Clearblue Easy Fertility Monitor (Swiss Precision Diagnostics, Bedford, UK) and instructed to perform daily urinary dipstick tests to measure estrone 3-glucuronide (E13G) and LH beginning on the first day of their next menstrual cycle. The Clearblue Easy Fertility Monitor measures daily urinary E13G and LH without any need for collection or storage of urine samples. For each menstrual cycle, the participant pushes the monitor “M button” if she had onset of menstrual bleeding during the preceding 24 h (cycle day one). The time of day that she pushes the “M” button becomes the center of the six hour testing window for subsequent days of that cycle. If the monitor is turned on outside the testing window or is not turned on at all on a given day, no test is requested and the result is recorded as a “fail”. The monitors request daily test sticks starting on cycle day 6 and continuing until an LH surge has been identified or until 20 days have passed, whichever is first. In subsequent cycles, the monitor requests tests based on the timing of the LH surges in prior cycles. To conduct a test, the participant holds a disposable test stick in the urine stream of the first morning void for 3 seconds. The test stick is then inserted into the monitor to measure LH using a two-site noncompetitive immunoassay and E13G by a 1-site competitive immunoassay (Robinson et al. 2007). The monitor displays a fertility status (low, high, or peak), derived by proprietary algorithmic interpretations of the LH and E13G concentrations. The monitor readings do not include any adjustments for urine concentration.

The monitors are capable of storing up to two months of data, including date and time the monitor was turned on, days a test stick was requested and completed, and LH and $E_1$3G readings. Study staff downloaded data from the monitors to a data card and then to a computer file, which was sent to the manufacturer for conversion to unitless LH and $E_1$3G values.

Study staff visited participants at 1–2 month intervals to collect each participant’s monthly urine samples and download data from the monitor to data cards. Participants were also instructed to perform a human chorionic gonadotropin Clearblue Easy Pregnancy Test (Swiss Precision Diagnostics) if their menses onset did not begin within 10 days of their expected date. If the pregnancy test was positive, the participants were removed from the current study

2.6 Calibration of Monitor LH and $E_1$3G Values

Standards were created to calibrate the Clearblue Easy Fertility Monitor LH and $E_1$3G unitless values sent by the manufacturer. A stock solution of WHO 2nd International

Standard for Pituitary LH for Immunoassay (80/552) with a concentration of 3,500 mIU/mL in water was serially diluted in a urine pool containing 1 mIU LH/mL to generate standards with calculated concentrations of 0.4, 1.2, 1.7, 3.7, 11.9, 44.7, 175.9, and 700.8 mIU LH/mL. $E_1$3G purified by recrystallization (Kesner et al. 1992) was used to generate $E_1$3G standards: 0, 1.6, 3.1, 12.5, 50, 200 ng $E_1$3G/mL. These LH and $E_1$3G standards were submitted to the Clearblue Easy Fertility Monitors and dipsticks in triplicate (LH) and duplicate ($E_1$3G) using three and two separate Clearblue Easy monitors, respectively. The means of the monitor readings were used to construct LH and $E_1$3G standard curves (Supplemental Figure S1).

The monitor LH and $E_1$3G values were calibrated using the standard curves. In consultation with the manufacturer on the bases of monitor readings, rules were established to deal with LH and $E_1$3G readings that were off the low or high ends of the standard curves or “0” readings. If the LH and $E_1$3G readings for a single sample were both “0,” the test was considered flawed and set to missing. If LH reading was “0” or off the low end of the standard curve, the reading was accepted as low and set to half the lowest LH standard level. If an $E_1$3G reading was off the low end of the standard curve the reading was accepted as low and set to half the lower limit of the lowest $E_1$3G standard level. E13G readings of “0” or greater than 100 ng/mL were considered flawed and set to missing. If an LH peak reading coincided with an $E_1$3G level greater than 100 ng/mL, the $E_1$3G value was set to missing and LH was evaluated by a panel of four experts on a case-by-case basis to determine whether the LH peak was a valid peak based on the details of that cycle and the participant’s other cycles.

2.7 Calculation of summary endocrine endpoints

Algorithms for menstrual cycle function endpoints were modified from those previously described (Luderer et al. 2013) due to the limited sampling window in this study. Endpoints describing the cycle and phase lengths and LH and $E_1$3G secretion patterns are defined in Table 1. LH Surge Onset and Day of $E_1$3G Peak were used only to calculate other endpoints and were not further analyzed. Menses onset for each cycle was determined from the monitor output and/or the daily diary. Analysis indicated that almost all quantitative endocrine endpoints had approximately normal distributions, with the sole exception of menstrual cycle length, which was heavily right-skewed.

2.8 Measurement of urinary hydroxylated PAH metabolites

Participants were instructed to collect one urine sample on the 10^{th} day after menses onset into a polypropylene beaker and to pour 10 mL of that sample from the beaker into each of 4 cryogenic polypropylene vials to be stored in their home freezer until picked up by study staff every month.

Hydroxylated PAH (OH-PAH) metabolites were measured in the urine samples as biomarkers of PAH exposure. We chose cycle day 10 because it is approximately in the middle of the follicular phase days when the monitor requests test sticks.

Urinary OH-PAH metabolites were measured for each subject for two cycles (3 participants) or three cycles (48 participants) during the testing period by the California Department of

Public Health Environmental Health Laboratory using procedures developed by the Centers for Disease Control and Prevention NHANES study (Li et al. 2008; Romanoff et al. 2006). If a participant only had collected two or three cycles of urinary hormone data, then OH-PAHs were measured in all cycles. If they had collected urinary hormone data for more than three cycles, then the first three cycles with the fewest missing days of urinary hormone data were selected for OH-PAH measurements using isotope dilution gas chromatography/ high resolution mass spectrometry (GC-HR-MS). Urine samples were first enzymatically deconjugated and subjected to liquid phase extraction and silylation by N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), analyzed by GC-HR-MS for nine PAH metabolites, 1- and 2-hydroxy naphthalene (1-NAP, 2-NAP), 1-hydroxy pyrene (1-PYR), 1-, 2-, and 3-hydroxy phenanthrene (1-PHEN, 2-PHEN, 3-PHEN), and 2-, 3-, and 9-hydroxy fluorene (2-FLUO, 3-FLUO, 9-FLUO) (Li et al. 2008; Romanoff et al. 2006). Additional details regarding the OH-PAH measurement methods can be found in Supplemental Materials.

A small percentage of analytes were below the limits of detection (LOD) of the assay. These were set to the LOD/√2 (Ogden 2010).

2.9 Statistical analyses

Descriptive statistics (arithmetic means and standard deviations and geometric means for continuous variables and percentages in each group for categorical variables) were calculated for demographic variables (Table 2), endocrine endpoints (Table 3) and OH-PAH concentrations (Table 4). Intra-class correlation coefficients (ICCs) were calculated using mean squares from one-way random effects ANOVA models with participant as the independent variable (Bartko 1966) to assess the correlation of individual OH-PAH metabolite concentrations among repeated samples within participants. To assess the correlations among the OH-PAH metabolite concentrations within the same urine sample, pairwise Pearson correlations were calculated (Table 5).

Observed metabolite concentrations were right-skewed, but were approximately normal after a log-transformation was applied. These log transformed values were then standardized by subtracting the corresponding sample mean and dividing by the corresponding standard deviation so that each new transformed variable has sample mean zero and sample standard deviation one. Then, in part because metabolites of phenanthrene (1-PHEN, 2-PHEN, 3-PHEN) and of fluorene (2-FLUO, 3-FLUO, 9-FLUO) can be collinear, additional transformations were considered for each. For each woman and time at which these measurements were taken, an overall average concentration was computed for PHEN, and another for FLUO. These variables were multiplied by a constant for technical reasons and were included in models as PHENs and FLUOs. Additional details are provided in Supplemental Methods.

For phenanthrene and fluorene, metabolite-specific variables were calculated as differences between the specified (standardized log) metabolite and the corresponding overall average concentrations (PHENs and FLUOs), for each woman-time combination. Each of these differences was multiplied by a suitable constant for technical reasons. These transformed variables were termed the same as the original variables for simplicity. So the variable we

analyzed subsequently termed 1-PHEN involved the difference between the originally standardized log of 1-PHEN minus the average of the corresponding 1-, 2-, and 3-PHENs, for each of the woman-time combinations, and multiplied by a constant. This was all done to make clearer the difference between an overall effect for a compound like phenanthrene and the effect for having unusually high (or low) concentration of one metabolite relative to the average concentration of phenanthrene metabolites observed in the individual.

The two naphthalene metabolites were not combined. 1-NAP, in addition to being a metabolite of naphthalene, is generated by metabolism of the pesticides carbaryl and napropamide and the beta-blocking medication propranolol (Hill et al. 1995; Shealy et al. 1997; Walle and Gaffney 1972). 2-NAP, in addition to being a metabolite of naphthalene, is also a metabolite of naphthoanilide and (2-naphthyloxy)acetic acid (Hill et al. 1995).

To investigate the role of OH-PAH concentrations in predicting the aforementioned endocrine endpoints, we performed a two-stage procedure. Models for all endpoints were constructed in the same way—through a Bayesian mixed modeling approach with subject-specific random effects allowing each participant to act as a baseline for her set of measurements. In the first stage we built a model involving only non-PAH baseline covariates, using a backwards stepwise algorithm to select a parsimonious model that fits the data. The starting baseline model included the covariates age, race, educational attainment, stress, body-mass index (BMI), alcohol use, caffeine intake, and measures of how many minutes the participant walked and engaged in vigorous physical activity each week. Estimates of regression coefficients are their posterior means. These were numerically approximated using Markov Chain Monte Carlo (MCMC) simulation (Christensen et al. 2011), Chapter 6). This allowed us to directly examine the posterior probability that each covariate in the model was associated with a non-zero regression coefficient by determining the proportion of simulated coefficient values that were above zero.

The second stage model then began from the selected baseline covariate model, adding all of the OH-PAH variables and an indicator variable denoting whether the participant was a smoker to that model. Because OH-PAH information was only available for 2–3 cycles per participant, we used a shared-parameter modeling strategy to take full advantage of our available endpoint data: mean-centering the OH-PAH covariates, fitting models for the cycles without PAH information using only baseline covariates, fitting models for the cycles with PAH information using both baseline and OH-PAH covariates, and constraining the baseline model coefficients to be equal between models for the same endpoint. As in the first stage, we used a backwards selection procedure to again pick a parsimonious model that fit the data well with any additional terms that had been selected. Our primary research interest was to assess the effect of PAHs on endocrine endpoints, and so our goal in these analyses was to determine whether OH-PAHs could provide any incremental benefit in modeling those endpoints beyond the fit we obtained with the baseline model.

Finally, we compared the deviance information criterion (DIC, a common Bayesian measure of model fit) for the baseline and final models. DIC for these models was calculated manually from the MCMC iterates to ensure that only covariates of interest were considered in assessing model complexity. Generally, a decrease of 3 or more in DIC is suggestive that a

lower-scoring model is preferred to a higher-scoring model (Spiegelhalter et al. 2002). In this way, we addressed a primary goal of this paper by assessing the added benefit of OH-PAH terms over and above the model involving only baseline characteristics. Analyses were performed using R and WinBUGS, two publicly available statistical analysis programs. Additional details about the statistical methods are provided in Supplemental Materials.

3. RESULTS

3.1 Demographics and participant characteristics

Three participants were eliminated from the analyses because they did not collect any urine for PAH measurements (one woman) or they had performed so few urine hormone test sticks that the no endocrine endpoints could be calculated. Demographic and other characteristics of the 51 remaining participants are shown in Table 2. The mean age of the participants was 29.9 years. Non-Hispanic white women made up the largest racial/ethnic group among the participants, followed by Asian and Hispanic, in that order. More than 92% of the participants had some education beyond high school graduation, with 21.6% having a graduate degree. The mean BMI was 24.9. More than a third of the participants had engaged in more than two hours of vigorous exercise, while nearly 24% had not engaged in any vigorous exercise, during the week prior to the baseline interview. Forty-one percent of the participants reported walking more than 4 h during the week prior to the baseline interview. Fewer than 12% of the participants smoked, and 23.5% reported drinking alcoholic beverages on 2 or more days per week.

3.2 Menstrual cycle endpoints

We had monitor data for 305 menstrual cycles from the 51 participants. Of these, 150 cycles had OH-PAH measurements. Representative menstrual cycle LH and $E_1$3G concentrations for a participant with regular cycles with clearly defined LH peaks are shown in Figure 1A, while representative data for a participant with some anovulatory cycles are shown in Figure 1B. Both of these participants missed very few days of sampling. In contrast, some participants had many missing days. Of 4,726 potential test days recorded by the monitors, tests were not performed on 1,168 days. Most of these were because the participant did not turn the monitor on at all that day or turned it on outside of the test window; 10 participants had one to several cycles when they took a hiatus for travel or other reasons and resumed thereafter.

We applied the algorithms summarized in Table 1 to the urinary LH and $E_1$3G concentrations and menses onset data to generate endocrine endpoints for each cycle. These endocrine endpoints are summarized in Table 3. This table also shows for how many cycles each endpoint could be calculated. Cycle length was calculable for the largest number of cycles, 297 of 305 cycles. The mean follicular phase LH and $E_1$3G concentrations were calculable for the next largest numbers of cycles because these variables do not depend on having identified a mid-cycle LH surge. Because ovulation status was indeterminate for 29% of cycles due to missing urinary LH data during mid-cycle, we had low statistical power to detect effects of OH-PAHs on ovulation and therefore do not present models for this endpoint.

3.3 PAH exposures

OH-PAHs were measured around menstrual cycle day 10 for three cycles (48 participants) or two cycles (3 participants) per participant. Table 4 shows the arithmetic and geometric mean concentrations and LODs of urinary OH-PAH metabolites. All participants had detectable concentrations of each of the measured OH-PAHs in at least one urine sample, which is consistent with ubiquitous exposure to PAHs. Overall, very few urine samples had concentrations of the measured PAH metabolites below the LOD. 1- and 2-NAP concentrations were above the LOD in all samples assayed. 2- FLUO, 9-FLUO, 1-PHEN, and 3-PHEN were below the LOD in one urine sample each (0.7% of the samples). 1-PYR was below the LOD in 6 samples (4%); 2-PHEN in 12 samples (7.9%) and 3-FLUO in 5 samples (3.3%).

ICCs ranged from 0.23 for 3-PHEN to 0.67 for 2-FLUO (creatinine adjusted), indicating that concentrations of the urinary OH-PAH metabolites were minimally to moderately correlated among repeated samples within participants. This provides justification for analyzing multiple cycles within participants for associations between OH-PAHs and endocrine endpoints.

Pairwise correlations among the different OH-PAHs within urine samples are shown in Table 5. All of the measured metabolites tended to have moderate correlation with each other, with higher correlations for metabolites of the same parent compound. 2-NAP was the exception; it had relatively low correlation with the other metabolites including 1-NAP. The moderately high collinearity among metabolites of fluorene and among metabolites of phenanthrene demonstrated in Table 5 provides some justification for the combination and difference-score forms we used for these metabolites in the endocrine endpoint analyses.

3.4 Associations between OH-PAH metabolites and endocrine endpoints

Table 6 presents the final OH-PAH models for each endocrine endpoint, PAH coefficient estimates, the proportion of positive coefficients from the MCMC, a list of baseline covariates identified by the stepwise modeling procedure for which the models have been adjusted, and DIC scores for comparing the baseline and final models. Creatinine-adjusted OH-PAH measurements were used for all models displayed. The models for all endpoints were refit with unadjusted OH-PAH measurements, and the results did not differ in any appreciable respect, and thus are not presented here.

For all eight endpoints considered, addition of OH-PAHs or smoking status improved the model fit (decreased DIC by more than 3) beyond that provided by the covariates in the baseline models. Smoking status played a role in modeling the three $E_1$3G endpoints, but directly measured OH-PAHs were selected into all eight models even when smoking status was accounted for.

Because understanding what these models are saying can be difficult, we provide graphical interpretations using the follicular phase length (Figure 2) and highest LH (Supplemental Figure S3) endpoints. Let us consider a hypothetical participant with urinary 1-PYR concentration of 85 ng/g Cr for her first menstrual cycle, and 280 ng/g Cr for her second cycle. A 1-PYR concentration of 85 ng/g Cr is approximately the median concentration in

our dataset, and a concentration of 280 ng/g Cr reflects a value two standard deviations higher on the log scale. Then the expected increase in follicular phase length is $2\beta_{1PYR}$ = 1.156 days longer in the second cycle than in the first (Figure 2). In addition, the graphs in Figure 2 show that a shift in 2-FLUO or 9-FLUO from their median to their 75th percentile concentrations is associated with approximately a half day decrease in follicular phase length, while a similar shift in 3-FLUO is associated with about a one day increase. For a sense of how large these effects are, based on our data we find that given identical covariate values, about half the time a woman's follicular phase length will vary by 3.3 days or less. Similarly, for about half of the participants the woman-to-woman difference in average follicular phase length is about 3.7 days or less. A predicted difference of 1–2 days based on OH-PAH concentration levels is less than the range of expected cycle-to-cycle variability, but it is still a noteworthy effect.

Highest LH and Peak LH were calculated from the same data when a surge was identified with no missing data. Peak LH was not calculated when the peak of the LH surge could not be confirmed due to missing data. Results for both endpoints are similar, as we would hope, and show roles for all naphthalene and fluorene metabolites studied, as well as some naphthalene and phenanthrene metabolites. Of particular note, the coefficients corresponding to 2-FLUO and 3-FLUO have opposite signs and similar magnitudes. Recall that these covariates represent the difference between their specific metabolite and the overall shared level of the fluorene compound across all metabolites. What we appear to see here, then, is that highest LH increases when the 2-FLUO metabolite accounts for a greater proportion of the total fluorene in a subject's urine than the 3-FLUO metabolite. We also see that increasing levels of fluorene overall are associated with an increase in the highest LH level. Supplemental Figure S3 provides a graphical representation of the effects of changes in 1-NAP, 2-FLUO, and 3-FLUO on highest LH. We see from the graphs that highest LH decreases with increasing 1-NAP. A shift in 2-FLUO or 9-FLUO from their medians to their 75th percentile concentrations results in approximately a 5 and 1 mIU/mL increase in highest LH, respectively, while a similar shift in 3-FLUO is associated with about a 3 mIU/mL decrease in highest LH.

For the three follicular phase E13G endpoints, we observed positive associations with smoking. On average, the slope of the estradiol rise is steeper in smokers than the slope in non-smokers. In practical terms, for a white woman of average age (in the study) who doesn't walk or exercise much, the slope for nonsmokers shows an increase of about 2 ng/mL per day, and the slope for shows a daily increase of about 4 ng/mL. E13G slope also increases with 1- and 2-NAP and decreases when 1-PHEN is higher than the other phenanthrene metabolites. On average, the periovulatory E13G concentrations are higher for women who smoke by nearly 9 ng/mL. A white woman of average age who doesn't exercise much, doesn't have a college degree, doesn't drink caffeinated beverages, and doesn't drink alcohol will on average have a periovulatory E13G of about 20 ng/mL if she doesn't smoke and 28.5 ng/mL if she does smoke. Periovulatory E13G also increases with fluorene metabolites and decreases with 2-NAP and 1-PYR. On average, follicular phase estradiol is higher for smokers, with our model predicting a 5-unit increase in E13G for smokers compared to nonsmokers. A white woman of average age who doesn't walk much would have an average follicular phase E13G of 15 ng/mL if she doesn't smoke, and 20 ng/mL if

she does. Follicular E13G also increases with 1-NAP or 2-FLUO that is higher than the other fluorene metabolites and decreases with 2-NAP or 3-FLUO higher than the other fluorene metabolites.

The final model for menstrual cycle length did not include any OH-PAHs when all cycles were included in the models. When only cycles with length within the normative range (21–35 days) were analyzed, cycle length increased with 1-PYR and 2-PHEN concentrations and decreased with 9-FLUO concentration.

4. DISCUSSION

Animal studies have clearly demonstrated that several higher molecular weight PAHs are potent ovotoxicants that destroy ovarian follicles, causing ovarian failure (Borman et al. 2000; Mattison 1979; Mattison and Nightingale 1982; Mattison and Thorgeirsson 1979; Mattison et al. 1980; Takizawa et al. 1984). Cigarette smoking is associated with decreased fecundity and earlier menopause in women, which may be due to the dozens of PAHs found in tobacco smoke (Alderete et al. 1995; Baird and Wilcox 1985; Harlow and Signorello 2000; Hassan and Killick 2004; Jensen et al. 1998; Mattison et al. 1989; van Noord et al. 1997). However, this is the first study that has directly examined the associations of specific biomarkers of PAH exposure with measures of ovarian function in women. We found overall positive associations of fluorene metabolites and negative associations of naphthalene metabolites with two measures of LH surge amplitude. We found positive associations of 2-NAP and 1-PYR and negative association of phenanthrene metabolites with average follicular LH. Follicular phase length was positively associated with 3-FLUO and 1-PYR. Smoking was positively associated with follicular and periovulatory E13G concentrations and E13G slope, and PAH metabolites were retained in these models.

A few prior studies have examined the effects of cigarette smoking on similar endpoints. Smoking has been associated with shorter cycle and follicular phase lengths (Windham et al. 1999), increased early follicular phase urinary $E_1$3G, non-significantly decreased luteal phase urinary pregnanediol-3-glucuronide, and higher urinary FSH concentrations during the luteal to follicular phase transition (Windham et al. 2005). Similarly, increased early follicular phase serum estradiol and follicular phase progesterone concentrations have been reported in smokers compared to non-smokers (Zumoff et al. 1990). The same study reported decreased follicular phase serum LH concentrations and no differences in peak preovulatory LH concentrations. In contrast, another study reported that exposure to environmental tobacco smoke was associated with decreased mid to late follicular phase urinary $E_1$3G concentrations during non-conception cycles only (Chen et al. 2005).

It is difficult to compare the associations between OH-PAH metabolites and endocrine endpoints in the present study with the reported associations between exposure to tobacco smoke and similar endocrine endpoints in prior studies. Active smoking is associated with significantly increased urinary excretion of all nine OH-PAHs measured in the present study, while exposure to environmental tobacco smoke is associated with lesser or no increase in excretion of these metabolites (Aquilina et al. 2010; Suwan-Ampai et al. 2009). Tobacco smoke contains many high molecular weight PAHs, which were not directly biomonitored in

the present study because their metabolites are primarily excreted in the bile and are below the limit of detection in urine (Li et al. 2008). Tobacco smoke also contains thousands of other compounds (Shopland et al. 2001), and therefore observed associations with smoking may or may not be due to the PAH content of tobacco smoke. Smoking was retained in the second stage of modeling along with specific PAH metabolites only for the three $E_1$3G endpoints in the present study. This indicates that the OH-PAH metabolites contribute additional information to the models above and beyond that contributed by smoking. We observe increased follicular $E_1$3G concentrations with smoking, which is consistent with increased early follicular phase $E_1$3G in smokers reported by Windham and coworkers (Windham et al. 2005). We also observed associations of follicular $E_1$3G with fluorene, naphthalene, and phenanthrene metabolite concentrations. In contrast, our finding that periovulatory $E_1$3G increases with smoking is opposite the findings of Chen et al regarding the association between environmental tobacco smoke exposure and mid to late follicular phase $E_1$3G in non-conception cycles (Chen et al. 2005). We also observed that periovulatory $E_1$3G decreases with 2-NAP and 1-PYR and increases with fluorene. While we find that peak and highest LH decreased with naphthalene and phenanthrene and increased with fluorene, we see no effect for smoking on these endpoints, concordant with prior findings of no effect of smoking on LH surge amplitude (Zumoff et al. 1990). Higher preovulatory LH concentrations have been associated with conception cycles compared to nonconception cycles (Baird et al. 1999). While we find that both pyrene and the fluorene metabolite profile associate with follicular phase length, we see no effect for smoking, which contrasts with prior findings associating shorter follicular phase length with smoking (Windham et al. 1999).

For women who do not smoke, food and exposure to particulate matter air pollution are the major sources of PAH exposures. Consumption of grilled meat is associated with greater increases in urinary 1- and 2-NAP than the other PAH metabolites in the NHANES data (Suwan-Ampai et al. 2009). More detailed studies in which OH-PAHs were monitored repeatedly before and after consumption of grilled chicken (Li et al. 2012) or smoked salmon (Motorykin et al. 2015) showed that urinary levels of all nine OH-PAHs increase rapidly reaching peak concentrations within 8.5 hours and decrease rapidly with median half-lives ranging from 1.7 to 7.0 hours. The OH-PAHs monitored in the present study have also been measured in relation to exposure to wood smoke. Urinary excretion of all nine increased after exposure, but 2-NAP showed the strongest association with personal air monitoring of wood smoke components, and 1-PYR showed no association (Li et al. 2015). Inhalation exposure to lower molecular weight PAHs, including the parent compounds of the metabolites measured in the present study, is more strongly associated with indoor sources, while higher molecular weight PAH exposures are associated with outdoor air pollution sources (Nethery et al. 2012). Compared to other studies that examined the effects of smoking on reproductive endpoints in women, the measurement of biomarkers that integrate PAH exposures from all routes is a strength of our study, and our models demonstrate that individual OH-PAH metabolites may have divergent associations with the same endpoint.

Our study is the first to convert output from urinary hormone monitors to concentrations and to utilize these data to calculate endocrine endpoints independently of the monitor algorithms. Other studies have demonstrated that use of the Clearblue Easy Fertility

Monitors can increase pregnancy rates by identification of the potentially fertile window (Robinson et al. 2007; Tanabe et al. 2001). The monitors, along with daily diaries, have also been used to establish menses dates in a cohort study of the effects of environmental exposures on time to pregnancy (Buck Louis et al. 2011; Buck Louis et al. 2014a; Buck Louis et al. 2012; Buck Louis et al. 2014b). We generated standard curves to convert monitor readings to LH and $E_1$3G concentrations and used these to calculate summary endocrine variables for each menstrual cycle. The dynamic range of the monitor LH assay spanned more than two orders of magnitude, while that of the monitor $E_1$3G assay spanned about one order of magnitude (Supplemental Figure S1). As a result, there were many more $E_1$3G than LH monitor readings above or below the limits of the respective standard curves (282 versus 99). Because the commercially available monitors used for this study are designed to minimize testing days, we only had data for a maximum of 20 days of each cycle, starting no earlier than cycle day 6 and ending soon after the LH surge ended. Therefore, we were not able to calculate any early follicular phase or luteal phase endpoints. In addition, because the monitors do not measure progesterone metabolite concentrations we were not able to confirm that ovulation occurred by identifying a significant luteal phase rise in progesterone or fall in the ratio of $E_1$3G to progesterone metabolites (Baird et al. 1995; Baird et al. 1991; Kassam et al. 1996). Finally, the monitor output did not include outputs (i.e., creatinine, osmolality, specific gravity) to adjust for urine dilution when converting the monitor readings to endocrine concentrations.

Our observations of associations of some OH-PAHs with follicular and periovulatory $E_1$3G concentrations are consistent with experimental studies that have reported effects of the OH-PAHs measured in the present study or their parent compounds on ovarian steroid synthesis in several species. Dietary phenanthrene exposure dose-dependently decreased plasma estradiol concentrations in female flounder and decreased androstenedione, testosterone, and estradiol synthesis in cultured flounder ovaries via inhibition of the enzymatic activities of key steroidogenic enzymes (Monteiro et al. 2000a; b). 9-PHEN, 1-NAP, 1-PYR all inhibited steroidogenic cytochrome P450 enzyme activities in carp ovary and testis subcellular fractions (Fernandes and Porte 2013). Phenanthrene exposure also inhibited ovarian development and down-regulated ovarian aromatase, estrogen and androgen receptor expression in another fish species, the marine medaka (Sun et al. 2015). In cultured rat ovarian luteal cells, phenanthrenequinone, a photooxidation product of phenanthrene, inhibited LH-stimulated progesterone secretion and increased reactive oxygen species (Nykamp et al. 2001).

While the different associations we observed between various OH-PAH metabolites and several endocrine endpoints are interesting, we must be cautious in interpreting these associations because most of the OH-PAHs were highly correlated with one another. Only 2-NAP was not highly correlated with the other OH-PAHs. Interestingly, 2-NAP was a significant predictor for five of the eight endocrine endpoints. For the other OH-PAHs retained in seven of the nine models, their combined effects may be considered to be representative of the combined effects of all of the highly correlated OH-PAHs. In addition, because we examined a large number of endpoints and PAH metabolites, some of the associations may be spurious and should be confirmed in larger studies. It is also possible that associations were missed because we were only able to measure biomarkers of PAH

exposure once per cycle during the follicular phase, reflecting exposures during the preceding day to a few days. In the future, it would be ideal to conduct a similar study in which PAH metabolites are measured more frequently during each cycle.

Another limitation of the present study is that 29% of the cycles were indeterminate for ovulatory status due to missed testing days. As a result, we had reduced power to detect effects of OH-PAHs on ovulatory status. Missed test days also decreased the number of cycles for which some of the other endpoints could be calculated. A larger study will be required to confirm the associations between OH-PAHs and endocrine endpoints we observed.

In summary, we have demonstrated the feasibility of using urinary reproductive hormone data obtained via microelectronic fertility monitors to calculate endocrine endpoints for epidemiological studies of ovarian function during multiple menstrual cycles. We observed associations between biomarkers of environmental PAH exposure and follicular phase length, follicular phase LH and $E_1$3G concentrations, preovulatory LH surge amplitude, and periovulatory $E_1$3G concentration and slope. The results show that environmental exposure to PAHs is associated with endocrine markers of ovarian function in women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Urinary reproductive hormones were measured using fertility monitors in cycling women.
2. Urinary metabolites of polycyclic aromatic hydrocarbons were measured once per cycle.
3. Environmental exposure to PAHs is associated with follicular phase length, follicular LH and E13G concentrations, preovulatory LH levels, and periovulatory E13G.

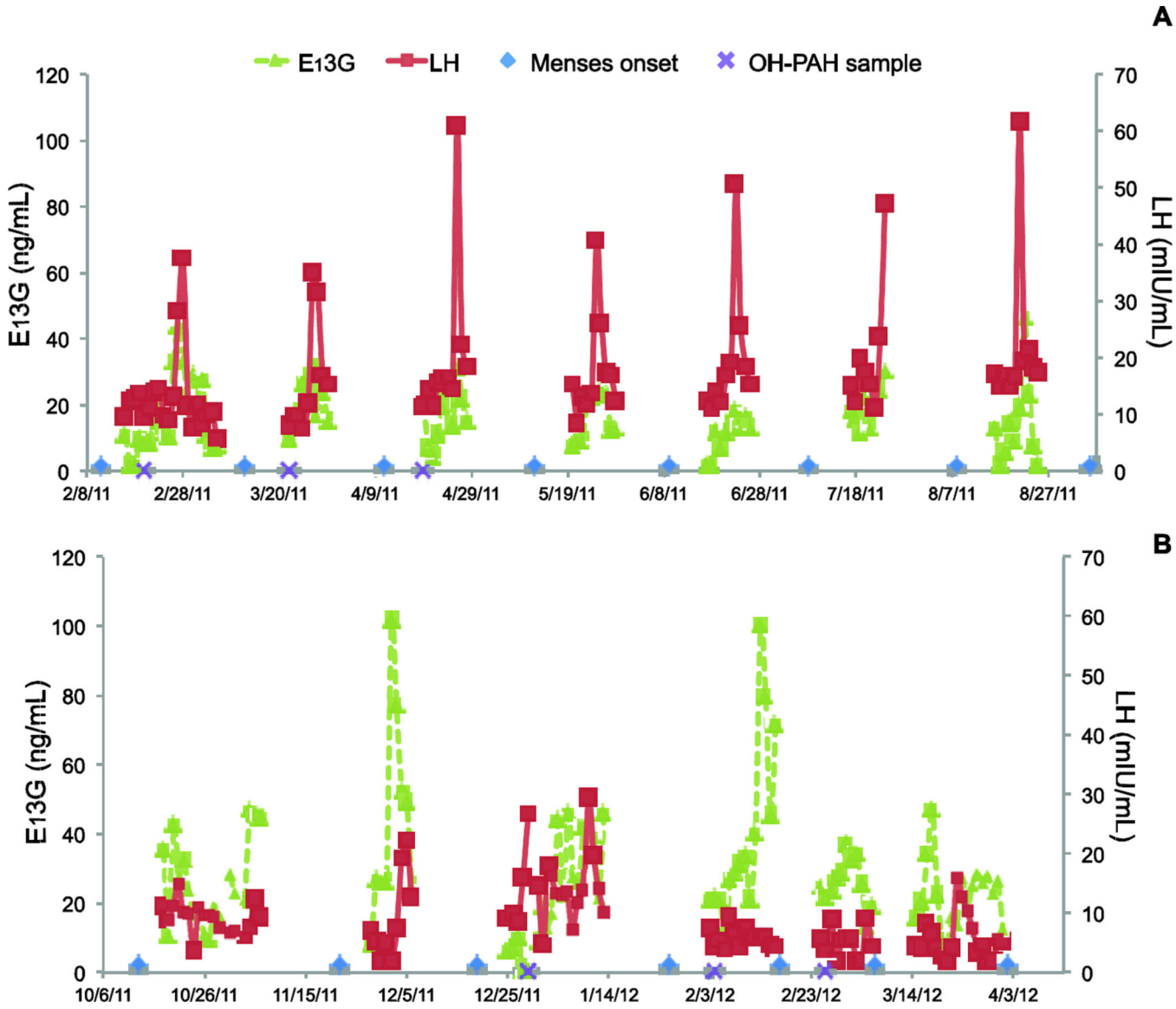

Figure 1. Representative urinary LH and $E_1$3G concentrations for two participants across multiple menstrual cycles

A) A participant with regular cycles with clearly defined LH peaks. B) A participant with several ostensibly anovulatory cycles without clear LH peaks. Blue diamonds indicate the onset of menses. Days for which urinary OH-PAHs were measured are indicated by a purple X.

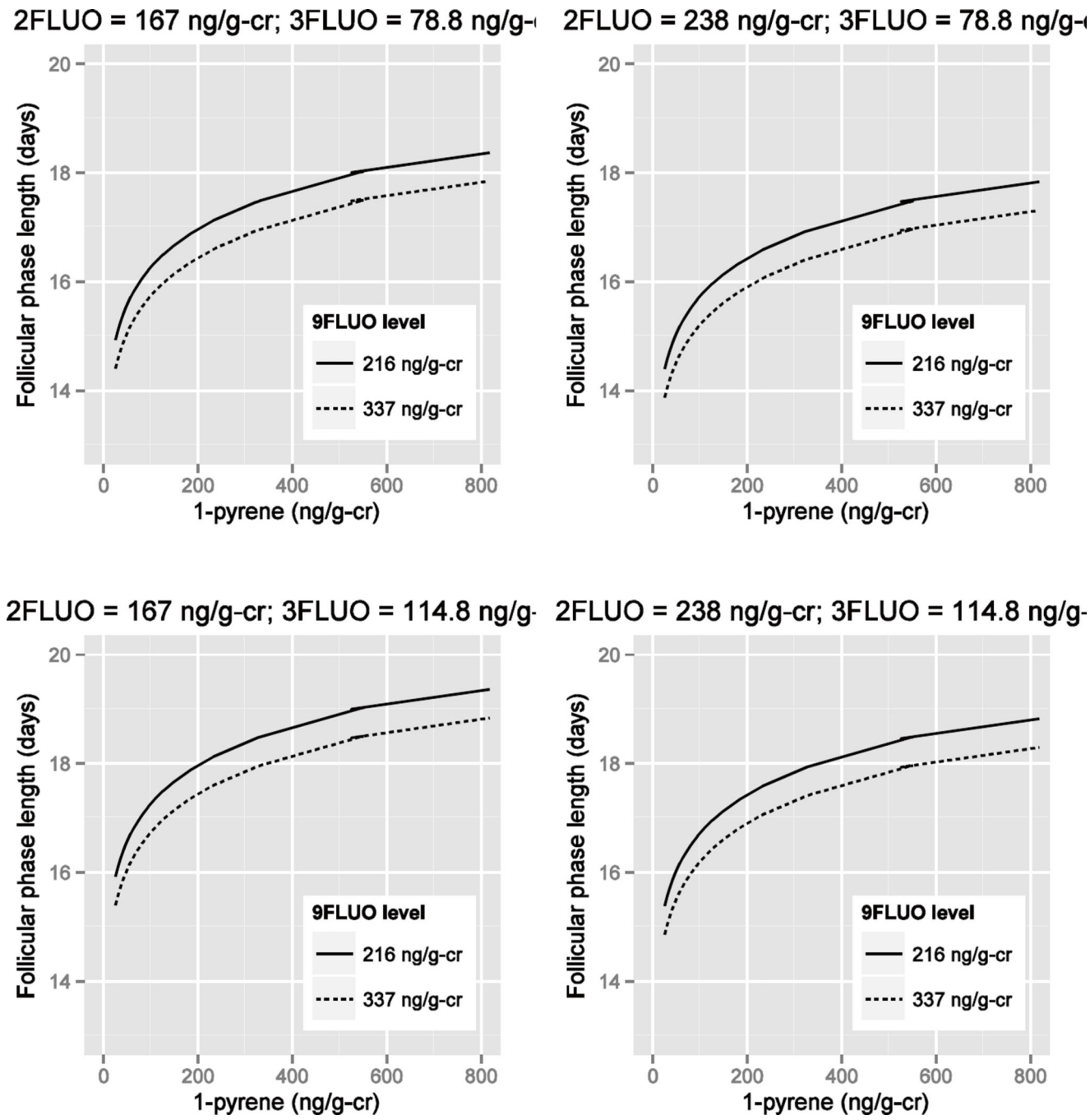

Figure 2. Graphical representation of changes in follicular phase length with changes in 1-PYR and 2-, 3-, and 9-FLUO derived from the final model

All four graphs show the changes in follicular phase length with increasing urinary concentration of 1-PYR at two different concentrations of 9-FLUO, the study median concentration (solid line) and the 75th percentile concentration (dotted line). 2-FLUO was held constant at its study median concentration in the two graphs on the left and at its 75th percentile in the two graphs on the right. 3-FLUO was held constant at its study median concentration in the two upper graphs and at its 75th percentile in the two lower graphs. The graphs show that follicular phase length increases with increasing 1-PYR. A shift in 2-

FLUO or 9-FLUO from the median to the 75th percentile results in approximately a half day decrease in follicular phase length, while a similar shift in 3-FLUO is associated with a one day increase.

Table 1

Endocrine Endpoint Algorithms

Endpoint (abbreviation)	Algorithm
Cycle length	Number of days from first day of menstrual bleeding through the day before next onset of menstrual bleeding.
Follicular phase length	Number of days from first day of menstrual bleeding to the day of the LH surge onset.
Luteal phase length	Cycle length minus follicular phase length.
LH surge onset	Day of the first LH rise ≥2.5-fold above the mean of the previous 3–7 day LH baseline.
Highest LH surge level (Highest LH)	Highest LH value of the cycle that is ≥2.5-fold above the LH surge onset baseline and ≤4 days after the LH surge onset.
Peak LH surge level (Peak LH)	Same as highest LH surge level except it is omitted if there is a missing value on an adjacent day.
Follicular LH level (Follicular LH)	Mean for all days before LH surge onset, or cycle days 6 through 10 for cycles without an LH surge. Omit if <3 values.
Ovulatory status	Ovulatory cycle = Has a defined LH surge onset; Anovulatory cycle = No LH surge onset for cycles with no missing LH values from cycle day 11 through the 9th day before the next menses onset. Must have start and end menses Indeterminate cycle = Cycles that are neither ovulatory nor anovulatory
Mid-follicular $E_1$3G level (Follicular $E_1$3G)	Mean for cycle day 1 through day −2 from LH surge onset, or for $E_1$3G values on days 6 through 10 for cycles with no LH surge onset. Omit if <3 values.
Periovulatory $E_1$3G level (Periovulatory $E_1$3G)	Mean for 7 days centered around the day of the LH surge onset. Omit if <3 values.
Preovulatory $E_1$3G rise ($E_1$3G slope)	Slope for 3 days prior to day of $E_1$3G peak. Omit if the first or third value is missing.
Day of $E_1$3G peak	Day of the highest $E_1$3G value ±3 days from the day of the LH surge onset. For cycles with no surge, select highest cycle $E_1$3G value of the cycle.

Table 2

Characteristics of study participants

Characteristic	Mean ± SD	Range	N
Age at baseline	29.9±6.5	18, 44	51
Height (m)	1.63±0.07	1.50, 1.83	48
Weight (kg)	66.6±15.7	49.2, 124.9	49
BMI	24.9±5.3	18.6, 42.6	48
	Number	**Percent**	
Ethnicity/Race			
Hispanic	11	21.6	
Non-Hispanic white	22	43.1	
Non-Hispanic Asian	14	27.5	
Non-Hispanic black	4	7.8	
Education - highest level attained			
high school diploma or less	3	5.9	
some college or vocational	20	39.2	
Bachelor’s degree	16	31.4	
Graduate degree	11	21.6	
Family income/year			
<$20,000	4	7.8	
≥$20,000<$50,000	12	23.5	
≥$50,000<$75,000	10	19.6	
≥$75,000<$100,000	9	17.6	
≥$100,000	9	17.6	
Current smoking			
Yes	6	11.8	
No	45	88.2	
Alcohol			
≥2 days/wk	12	23.5	
1–4 days/month	16	31.4	

Table 3

Means, standard deviations, and geometric means of menstrual cycle endpoint variables averaged over repeated measures within participant

Endpoint Variable	Mean (SD)	Geometric Mean	N*	n**
Cycle Length (days)	33.2 (10.7)	32.0	51	297
Follicular Phase Length (days)	16.3 (3.3)	16.0	47	199
Luteal Phase Length (days)	14.3 (5.3)	13.6	47	193
Mean Follicular Phase $E_1$3G (ng/mL)	20.2 (7.5)	18.7	50	219
Mean Follicular Phase LH (mIU/mL)	9.1 (2.0)	8.9	50	233
Preovulatory $E_1$3G Rise (slope)	3.2 (3.7)	3.2	49	178
Periovulatory $E_1$3G (ng/mL)	31.1(10.3)	29.4	47	191
Peak LH (mIU/mL)	37.9 (12.1)	36.1	45	132
Highest LH (mIU/mL)	36.6 (10.7)	35.3	47	199
Ovulatory Status of Cycles	**N (%)**			
Ovulatory	199 (67.0)			
Anovulatory	12 (4.0)			
Indeterminate	86 (29.0)			

Generated using all cycles available for each woman, regardless of whether that cycle had OH-PAH measurements. Calculated by taking the mean for each participant for each endpoint, then calculating the means, SDs, and geometric means of those new variables.

*N refers to number of women for whom there was at least one cycle of data for that variable.

**n refers to the total number of cycles for which each endpoint could be calculated, out of the 305 cycles observed.

Table 4

Means, standard deviations, and geometric means of OH-PAH concentrations

OH-PAH	Unadjusted concentration (pg/mL)			Creatinine adjusted concentration (ng/g creat)	
	mean (SD)	geometric mean	LOD	mean (SD)	geometric mean
1-NAP	2252 (2005)	1632	<25	1785 (1245)	1387
2-NAP	8738 (10085)	5797	<20	6192 (5037)	4965
1-PYR	142 (124)	110	<20	107 (64)	95
1-PHEN	150 (89)	125	<10	119 (60)	107
2-PHEN	41 (22)	35	<10	33 (14)	31
3-PHEN	75 (42)	64	<10	59 (23)	55
2-FLUO	272 (204)	213	<20	209 (121)	184
3-FLUO	140 (139)	105	<20	106 (64)	91
9-FLUO	361 (316)	278	<37	296 (259)	240

(Calculated by taking the mean concentration for each participant for each OH-PAH, then calculating the means, SDs, and geometric means of those new variables.)

Table 5

Correlations among concurrent measurements of OH-PAHs*

	1-NAP	2-NAP	1-PYR	1-PHEN	2-PHEN	3-PHEN	2-FLUO	3-FLUO	9-FLUO
1-NAP	1	0.105	0.234	0.429	0.285	0.352	0.374	0.446	0.424
2-NAP		1	0.163	−0.038	0.030	−0.011	0.072	0.067	−0.113
1-PYR			1	0.597	0.574	0.654	0.385	0.465	0.379
1-PHEN				1	0.738	0.738	0.635	0.565	0.655
2-PHEN					1	0.764	0.573	0.536	0.615
3-PHEN						1	0.638	0.683	0.631
2-FLUO							1	0.887	0.470
3-FLUO								1	0.459
9-FLUO									1

*Pearson correlations for all 151 urine samples for which OH-PAHs were measured.

Table 6

Baseline and PAH models for endocrine endpoints

Endpoint	DIC (w/o PAHs)	DIC (w/ PAHs)	
Cycle Length	1211.5	1203.3	
Baseline:	*Race + Age + Walking + Vigorous Activity*		
PAHs:	*9-FLUO*	β = −0.507	Pr(β < 0)[3] = 0.960
	2-PHEN	β = 0.323	Pr(β > 0) = 0.889
	1-PYR	β = 0.728	Pr(β > 0) = 0.994
Follicular Phase Length	977.9	961.6	
Baseline:	*Race + Age + Stress + Vigorous Activity*		
PAHs:	*3- FLUO*	β = 1.040	Pr(β > 0) = 0.999
	1-PYR	β = 0.578	Pr(β > 0) = 0.982
Follicular LH	1072.6	1068.3	
Baseline:	*Race + Age + Caffeine* [1] *+ BMI + Alcohol + Vigorous Activity*		
PAHs:	*2-NAP*	β = 0.446	Pr(β > 0) = 0.962
	PHENs	β = −0.963	Pr(β < 0) = 0.996
	1-PYR	β = 0.548	Pr(β > 0) = 0.945
Highest LH	1563.2	1557.3	
Baseline:	*Race + Age + Caffeine* [1,2] *+ BMI* [2] *+ Alcohol* [1] *+ Walking* [1] *+ Education*[1]		
PAHs:	*2-FLUO*	β = 2.057	Pr(β > 0) = 0.932
	3-FLUO	β = −2.000	Pr(β < 0) = 0.941
	FLUOs	β = 5.308	Pr(β > 0) = 0.983
	1-NAP	β = −1.977	Pr(β < 0) = 0.924
	2-NAP	β = −3.143	Pr(β < 0) = 0.936
Peak LH	1055.7	1035.8	
Baseline:	*Race + Age + Caffeine* [1,2] *+ BMI* [1,2] *+ Alcohol* [1] *+ Walking* [1] *+ Education*[2]		
PAHs:	*2-FLUO*	β = 2.755	Pr(β > 0) = 0.949
	3-FLUO	β = −2.988	Pr(β < 0) = 0.981
	FLUOs	β = 7.262	Pr(β > 0) = 0.992
	1-NAP	β = −3.735	Pr(β < 0) = 0.981
	2-NAP	β = −1.823	Pr(β < 0) = 0.893
	2-PHEN	β = −2.371	Pr(β < 0) = 0.950
	PHENs	β = −4.280	Pr(β < 0) = 0.967
Follicular E13G	1428.7	1420.0	

Endpoint	DIC (w/o PAHs)	DIC (w/ PAHs)	
Baseline:	*Race + Age + Caffeine* [1] *+ Walking*		
PAHs:	*2-FLUO*	β = 1.325	Pr(β > 0) = 0.979
	3-FLUO	β = −1.854	Pr(β < 0) = 0.999
	1-NAP	β = 0.830	Pr(β > 0) = 0.924
	2-NAP	β = −0.680	Pr(β < 0) = 0.878
	2-PHEN	β = −0.613	Pr(β < 0) = 0.870
	Smoking	β = 4.975	Pr(β < 0) = 0.940
Periovulatory E13G	1322.9	1318.8	
Baseline:	*Race + Age + Caffeine + Alcohol + Vigorous Activity + Education*		
PAHs:	*FLUOs*	β = 1.595	Pr(β > 0) = 0.920
	2-NAP	β = −1.381	Pr(β < 0) = 0.954
	1-PYR	β = −0.985	Pr(β < 0) = 0.853
	Smoking	β = 8.585	Pr(β > 0) = 0.969
E13G Slope	1097.7	1094.4	
Baseline:	*Race + Age + Stress* [1] *+ Alcohol* [1] *+ Walking + Vigorous Activity*		
PAHs:	*1-NAP*	β = 0.967	Pr(β > 0) = 0.954
	2-NAP	β = 0.815	Pr(β > 0) = 0.944
	1-PHEN	β = −0.952	Pr(β < 0) = 0.939
	Smoking	β = 1.809	Pr(β > 0) = 0.878

[1]These variables were included in the baseline model but dropped in the PAH model under our selection criteria.

[2]Because Highest LH and Peak LH use the same data whenever a peak was detected (see Table 1), we chose to force BMI and caffeine into the Peak LH baseline model to make it agree with the Highest LH baseline model.

[3]Posterior probability that covariate is associated with a non-zero regression coefficient (proportion of simulated coefficient values that were above zero).