Supplementary Materials

Nonpersistent pesticide exposure and circulating sex hormones in postmenopausal women: evidence from NHANES 2013-2016

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Supplementary Note 1. NHANES laboratory methods for pesticide biomarkers and sex hormones.

Urinary pesticide biomarker measurements

Urinary pesticide biomarker data were obtained from the publicly available NHANES laboratory datasets for the 2013–2014 and 2015–2016 survey cycles. The laboratory measurements were performed as part of NHANES, and no additional laboratory analyses were conducted by the authors of the present study.

Four urinary biomarkers with relatively high detection frequencies were included: 3-phenoxybenzoic acid (3-PBA), a common metabolite of several pyrethroid insecticides; 3,5,6-trichloro-2-pyridinol (TCPY), a metabolite of chlorpyrifos; para-nitrophenol (PNP), a biomarker associated with exposure to selected organophosphate pesticides; and 2,4-dichlorophenoxyacetic acid (2,4-D), a phenoxy herbicide.

Single spot urine specimens were collected at the NHANES Mobile Examination Center. The specimens were processed and stored according to standardized NHANES protocols and were maintained at −20°C or below until laboratory analysis.

Urinary concentrations of 3-PBA, TCPY, PNP, and 2,4-D were determined using online solid-phase extraction coupled with high-performance liquid chromatography–tandem mass spectrometry. Isotope-dilution calibration was used for quantitative measurement. Concentrations were reported in micrograms per liter (μg/L).

The lower limits of detection used in the present analysis were 0.10 μg/L for 3-PBA, 0.10 μg/L for TCPY, 0.10 μg/L for PNP, and 0.15 μg/L for 2,4-D. For measurements below the lower limit of detection, the imputed values provided in the NHANES datasets, calculated as the lower limit of detection divided by the square root of two, were used.

NHANES laboratory quality-assurance and quality-control procedures included standardized specimen collection and handling, instrument calibration, the use of isotope-labeled internal standards, analysis of quality-control samples, and routine monitoring of analytical accuracy and precision. Complete analytical procedures and quality-control protocols are available in the corresponding cycle-specific NHANES laboratory documentation. The principal laboratory parameters are summarized in Table S1.

Serum sex hormone measurements

Serum sex hormone data were obtained from the publicly available NHANES laboratory datasets for the 2013–2014 and 2015–2016 survey cycles. Serum specimens were collected at the Mobile Examination Center, processed according to standardized NHANES procedures, and stored frozen until laboratory analysis.

Total testosterone and estradiol were measured using isotope-dilution liquid chromatography–tandem mass spectrometry. Sex hormone-binding globulin was measured using a chemiluminescent immunoassay.

The reported lower limits of detection were 0.75 ng/mL for total testosterone, 2.994 pg/mL for estradiol, and 0.800 nmol/L for sex hormone-binding globulin. Values below the lower limit of detection were handled using the values supplied in the publicly available NHANES laboratory datasets.

The free androgen index was derived from total testosterone and sex hormone-binding globulin according to the following equation:

FAI = [TT (nmol/L)/SHBG (nmol/L)] × 100.

Total testosterone values were converted to nmol/L, where necessary, before calculation of the free androgen index.

Laboratory calibration, quality-control assessment, specimen handling, and verification of results were performed by NHANES laboratories in accordance with the corresponding cycle-specific protocols. Detailed standard operating procedures, reagent information, and quality-control criteria are available in the official NHANES laboratory documentation. Key analytical parameters are presented in Table S1.

Supplementary Note 2. Construction and coding of covariates.

Potential covariates were selected on the basis of substantive knowledge and prior evidence regarding factors associated with urinary pesticide biomarker concentrations or circulating sex-hormone concentrations in postmenopausal women. The selected variables were organized within a study-specific directed acyclic graph to guide the revised adjustment strategy. Demographic, socioeconomic, lifestyle, reproductive, hormone-use, dietary, and anthropometric variables were obtained from the publicly available National Health and Nutrition Examination Survey demographic, questionnaire, examination, laboratory, dietary, and prescription-drug files.

Demographic and socioeconomic variables

Age was obtained from the demographic questionnaire and was included as a continuous variable in years. Race/ethnicity was based on participants' self-reported race and ethnic affiliation and was categorized as Mexican American, other Hispanic, non-Hispanic White, non-Hispanic Black, or other race, including multiracial participants.

The family poverty-income ratio (PIR) represented the ratio of family income to the applicable poverty threshold and was used as an indicator of household socioeconomic status. For descriptive and regression analyses, PIR was classified as low or high according to the prespecified cutoff used in the analytical dataset.

Educational attainment was obtained from the demographic questionnaire and classified into five categories: less than ninth grade; ninth to eleventh grade, including participants who attended twelfth grade but did not receive a diploma; high-school graduate, General Educational Development certificate, or equivalent; some college education or an associate degree; and college graduate or above.

Urinary creatinine and anthropometric variables

Urinary creatinine (UCRE) was measured in spot urine specimens using the NHANES laboratory method. UCRE was included in the statistical models to account for variation in urine dilution related to factors such as fluid intake and urine concentration. UCRE was treated as a urine-dilution correction variable rather than as a back-door confounder in the directed acyclic graph.

Body mass index (BMI) was calculated from standardized examination measurements as weight in kilograms divided by height in meters squared (kg/m^2). BMI was entered as a continuous variable in the expanded Model 3.

Serum cotinine and physical activity

Tobacco-smoke exposure was assessed using serum cotinine, the principal metabolite of nicotine. Serum cotinine was categorized as below versus at or above the lower limit of detection of 0.015 ng/mL. The latter category may reflect active smoking, passive exposure to tobacco smoke, or other recent nicotine exposure.

Physical activity was assessed using questionnaire information on participation in moderate- and vigorous-intensity activities. Participants were classified into three mutually exclusive categories: no physical activity, moderate activity, or vigorous activity. The no-activity category was used as the reference group in regression analyses.

Alcohol intake

Alcohol intake was assessed using questionnaire information on alcohol consumption during the previous 12 months. Participants were classified into three categories: none, rare, or occasional consumption; greater than 0 to 1 drink per day; and more than 1 drink per day. The none, rare, or occasional category was used as the reference group.

Menopause classification

Menopause type was derived from self-reported menstrual history and hysterectomy and oophorectomy information. Women who reported menstruation during the previous 12 months were not classified as postmenopausal and were excluded from the analytical sample, as shown in **Figure S1**.

Among the included postmenopausal women, menopause type was categorized as natural menopause, bilateral-oophorectomy-related surgical menopause, hysterectomy without bilateral oophorectomy, or other or uncertain menopause. Natural menopause included women whose cessation of menstruation was not attributed to hysterectomy or bilateral oophorectomy. Bilateral-oophorectomy-related surgical menopause included women reporting removal of both ovaries. Women reporting hysterectomy without evidence of bilateral oophorectomy were classified separately because the absence of menstrual periods alone does not establish loss of ovarian function. Participants with insufficient, inconsistent, or otherwise unclassifiable menstrual or surgical information were assigned to the other or uncertain category.

Previous and current hormone use

Ever use of female hormones was derived from the reproductive-health questionnaire and was coded as yes or no according to participants' self-reported previous use of female hormones.

Current hormone use was identified separately using prescription-drug information for medications taken during the previous 30 days. Participants with a recorded hormone-containing prescription medication were classified as current hormone users. Previous hormone use and current prescription hormone use were retained as separate variables because they represented different exposure periods and were obtained from different NHANES components.

Dietary variables

Dietary variables were included in the expanded models because dietary intake may influence both urinary pesticide biomarker concentrations and circulating sex-hormone concentrations. Day 1 total fruit and total vegetable intakes were obtained from the Food Patterns Equivalents Database and were expressed as cup equivalents per day. Total energy intake was obtained from the first 24-hour dietary recall and was expressed in units of 1,000 kcal/day for analysis. Fruit intake, vegetable intake, and total energy intake were entered as continuous variables. These Day 1 variables represent short-term dietary intake and should not be interpreted as measurements of habitual long-term diet.

Missing-data handling and model inclusion

Participants with missing data for the survey-design variables or any covariate required for the complete-case analysis were excluded. No imputation of covariate values was performed. After application of the final eligibility and complete-case criteria, the analytical sample comprised 593 postmenopausal women.

Model 1 was the primary directed acyclic graph-informed model and included age, race/ethnicity, educational attainment, PIR, and UCRE. Model 2 additionally included serum cotinine category, physical activity, alcohol intake category, menopause type, ever use of female hormones, and current hormone use. Model 3 additionally included Day 1 total fruit intake, Day 1 total vegetable intake, total energy intake, and BMI. The expanded models were used to examine the sensitivity of the primary estimates to additional lifestyle, reproductive, hormone-use, dietary, and anthropometric adjustment.

Supplementary Table 1. Laboratory information for urinary pesticide biomarkers and serum sex hormones in NHANES 2013–2016

Analyte	Specimen	Analytical method	Unit	LLOD	Treatment of values below LLOD
3-PBA	Urine	Online SPE-HPLC-MS/MS with isotope-dilution calibration	μg/L	0.10	NHANES-provided value, LLOD/sqrt[2]
TCPY	Urine	Online SPE-HPLC-MS/MS with isotope-dilution calibration	μg/L	0.10	NHANES-provided value, LLOD/sqrt[2]
PNP	Urine	Online SPE-HPLC-MS/MS with isotope-dilution calibration	μg/L	0.10	NHANES-provided value, LLOD/sqrt[2]
2,4-D	Urine	Online SPE-HPLC-MS/MS with isotope-dilution calibration	μg/L	0.15	NHANES-provided value, LLOD/sqrt[2]
Total testosterone	Serum	Isotope-dilution LC-MS/MS	ng/dL	0.75	NHANES-provided value
Estradiol	Serum	Isotope-dilution LC-MS/MS	pg/mL	2.994	NHANES-provided value
SHBG	Serum	Chemiluminescent immunoassay	nmol/L	0.800	NHANES-provided value

Supplementary Table 2. Survey-weighted pesticide–hormone associations and percentage differences associated with twofold and interquartile-range increases in urinary pesticide biomarkers.

Biomarker	Outcome	Model	β (95%CI)	P value	% difference per twofold increase (95% CI)	% difference per IQR increase (95% CI)
3-PBA	TT	Model 1	−0.0282 (−0.1043, 0.0480)	0.456	−1.9% (−7.0%, 3.4%)	−4.3% (−15.1%, 7.8%)
		Model 2	−0.0145 (−0.0855, 0.0566)	0.680	−1.0% (−5.8%, 4.0%)	−2.2% (−12.5%, 9.2%)
		Model 3	−0.0181 (−0.0893, 0.0530)	0.607	−1.2% (−6.0%, 3.7%)	−2.8% (−13.0%, 8.6%)
3-PBA	FAI	Model 1	−0.0469 (−0.1155, 0.0218)	0.173	−3.2% (−7.7%, 1.5%)	−7.1% (−16.5%, 3.5%)
		Model 2	−0.0380 (−0.0975, 0.0216)	0.203	−2.6% (−6.5%, 1.5%)	−5.8% (−14.1%, 3.4%)
		Model 3	−0.0458 (−0.0953, 0.0037)	0.069	−3.1% (−6.4%, 0.3%)	−6.9% (−13.8%, 0.6%)
3-PBA	E2	Model 1	0.0060 (−0.0839, 0.0959)	0.892	0.4% (−5.6%, 6.9%)	0.9% (−12.3%, 16.2%)
		Model 2	0.0182 (−0.0638, 0.1002)	0.654	1.3% (−4.3%, 7.2%)	2.9% (−9.5%, 16.9%)
		Model	0.0127	0.709	0.9% (−3.8%,	2.0% (−8.4%,

		3	(−0.0559, 0.0813)		5.8%)	13.5%)
3-PBA	SHBG	Model 1	0.0187 (−0.0426, 0.0800)	0.538	1.3% (−2.9%, 5.7%)	3.0% (−6.4%, 13.3%)
		Model 2	0.0235 (−0.0351, 0.0821)	0.419	1.6% (−2.4%, 5.9%)	3.7% (−5.3%, 13.7%)
		Model 3	0.0277 (−0.0251, 0.0805)	0.292	1.9% (−1.7%, 5.7%)	4.4% (−3.8%, 13.4%)
TCPY	TT	Model 1	−0.1192 (−0.2081, −0.0304)	0.010	−7.9% (−13.4%, −2.1%)	−14.0% (−23.1%, −3.8%)
		Model 2	−0.1306 (−0.2150, −0.0461)	0.004	-8.7% (-13.8%, -3.1%)	-15.2% (-23.8%, -5.7%)
		Model 3	−0.1387 (−0.2275, −0.0500)	0.003	-9.2% (-14.6%, -3.4%)	-16.1% (-25.0%, -6.1%)
TCPY	FAI	Model 1	−0.1343 (−0.2192, −0.0495)	0.003	-8.9% (-14.1%, -3.4%)	-15.6% (-24.2%, -6.1%)
		Model 2	−0.1482 (−0.2284, −0.0680)	0.001	-9.8% (-14.6%, -4.6%)	-17.1% (-25.0%, -8.2%)
		Model 3	−0.1729 (−0.2557, −0.0901)	<0.001	-11.3% (-16.2%, -6.1%)	−19.6% (−27.6%, −10.7%)
TCPY	E2	Model 1	−0.0153 (−0.1417, 0.1112)	0.807	-1.1% (-9.4%, 8.0%)	-1.9% (-16.4%, 15.1%)

		Model 2	−0.0375 (−0.1405, 0.0656)	0.463	-2.6% (-9.3%, 4.6%)	-4.6% (-16.3%, 8.6%)
		Model 3	−0.0619 (−0.1680, 0.0441)	0.242	-4.2% (-11.0%, 3.1%)	-7.5% (-19.1%, 5.7%)
TCPY	SHBG	Model 1	0.0151 (−0.0586, 0.0888)	0.679	1.1% (-4.0%, 6.3%)	1.9% (-7.1%, 11.9%)
		Model 2	0.0176 (−0.0546, 0.0898)	0.622	1.2% (-3.7%, 6.4%)	2.2% (−6.7%, 12.0%)
		Model 3	0.0342 (−0.0258, 0.0941)	0.254	2.4% (-1.8%, 6.7%)	4.4% (-3.2%, 12.6%)
PNP	TT	Model 1	−0.0896 (−0.1632, −0.0160)	0.019	-6.0% (-10.7%, -1.1%)	-11.5% (-20.0%, -2.2%)
		Model 2	−0.1176 (−0.1920, −0.0432)	0.003	-7.8% (-12.5%, -2.9%)	-14.9% (-23.1%, -5.7%)
		Model 3	−0.1165 (−0.1873, −0.0457)	0.002	-7.8% (-12.2%, -3.1%)	-14.7% (-22.6%, -6.1%)
PNP	FAI	Model 1	−0.1241 (−0.2032, −0.0450)	0.003	-8.2% (-13.1%, -3.1%)	-15.6% (-24.3%, -6.0%)
		Model 2	−0.1467 (−0.2329, −0.0606)	0.002	-9.7% (-14.9%, -4.1%)	-18.2% (-27.3%, -8.0%)
		Model 3	−0.1254 (−0.2008,	0.002	-8.3% (-13.0%,	-15.8% (-24.0%, -6.6%)

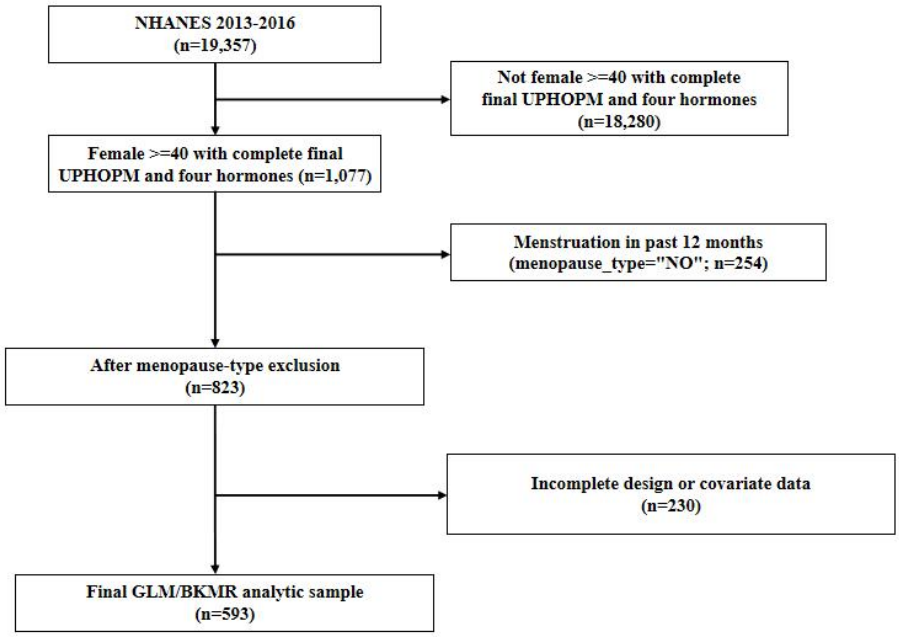
			−0.0500)		-3.4%)	
PNP	E2	Model 1	−0.1062 (−0.2198, 0.0074)	0.066	-7.1% (-14.1%, 0.5%)	-13.5% (-26.0%, 1.0%)
		Model 2	−0.1250 (−0.2366, −0.0134)	0.029	-8.3% (-15.1%, -0.9%)	-15.7% (-27.7%, -1.8%)
		Model 3	−0.1010 (−0.1907, −0.0113)	0.029	-6.8% (-12.4%, -0.8%)	-12.9% (-23.0%, -1.5%)
PNP	SHBG	Model 1	0.0345 (−0.0164, 0.0854)	0.177	2.4% (-1.1%, 6.1%)	4.8% (-2.2%, 12.4%)
		Model 2	0.0292 (−0.0163, 0.0746)	0.200	2.0% (-1.1%, 5.3%)	4.1% (-2.2%, 10.7%)
		Model 3	0.0089 (−0.0444, 0.0622)	0.735	0.6% (-3.0%, 4.4%)	1.2% (-5.9%, 8.9%)
2,4-D	TT	Model 1	0.0611 (−0.0768, 0.1991)	0.373	4.3% (−5.2%, 14.8%)	9.6% (−10.8%, 34.6%)
		Model 2	0.0412 (−0.0699, 0.1523)	0.455	2.9% (−4.7%, 11.1%)	6.3% (−9.9%, 25.5%)
		Model 3	0.0275 (−0.0755, 0.1305)	0.590	1.9% (−5.1%, 9.5%)	4.2% (−10.7%, 21.5%)
2,4-D	FAI	Model 1	0.0660 (−0.0777, 0.2097)	0.356	4.7% (-5.2%, 15.6%)	10.4% (-11.0%, 36.8%)

		Model 2	0.0537 (−0.0695, 0.1768)	0.381	3.8% (-4.7%, 13.0%)	8.3% (-9.9%, 30.2%)
		Model 3	0.0074 (−0.1040, 0.1187)	0.894	0.5% (-7.0%, 8.6%)	1.1% (-14.4%, 19.4%)
2,4-D	E2	Model 1	0.0247 (−0.1465, 0.1959)	0.770	1.7% (−9.7%, 14.5%)	3.8% (−19.7%, 34.0%)
		Model 2	−0.0115 (−0.1550, 0.1320)	0.871	−0.8% (−10.2%, 9.6%)	−1.7% (−20.7%, 21.8%)
		Model 3	−0.0643 (−0.2058, 0.0772)	0.361	−4.4% (−13.3%, 5.5%)	−9.2% (−26.5%, 12.2%)
2,4-D	SHBG	Model 1	−0.0049 (−0.0719, 0.0621)	0.882	−0.3% (−4.9%, 4.4%)	−0.7% (−10.2%, 9.7%)
		Model 2	−0.0125 (−0.0770, 0.0521)	0.696	-0.9% (-5.2%, 3.7%)	-1.8% (-10.9%, 8.1%)
		Model 3	0.0201 (−0.0388, 0.0791)	0.491	1.4% (-2.7%, 5.6%)	3.1% (-5.6%, 12.5%)

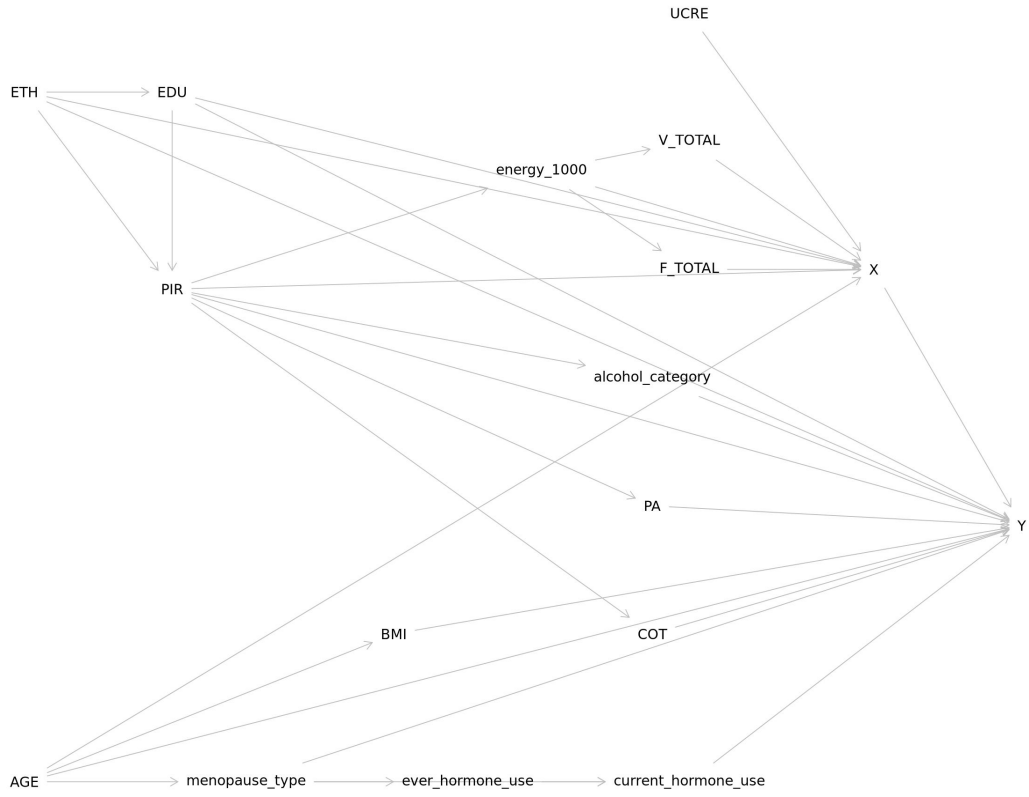
Supplementary Table 3. List of abbreviations

Abbreviation	Full term
NHANES	National Health and Nutrition Examination Survey
BKMR	Bayesian kernel machine regression
OCPs	organochlorine pesticides
PNP	para-nitrophenol
TCPY	3,5,6-trichloro-2-pyridinol
3-PBA	3-phenoxybenzoic acid
2,4-D	2,4-dichlorophenoxyacetic acid
TT	total testosterone
FAI	free androgen index
E2	estradiol
SHBG	sex hormone-binding globulin
NCHS	National Center for Health Statistics
CDC	Centers for Disease Control and Prevention
MEC	Mobile Examination Center
IRB	Institutional Review Board
LLOD	lower limit of detection
LC-MS/MS	liquid chromatography-tandem mass spectrometry
PIR	family poverty-income ratio
GED	General Educational Development
BMI	body mass index
SD	standard deviation
CI	confidence interval
MCMC	Markov chain Monte Carlo
PIP	posterior inclusion probability
UCRE	urinary creatinine
DAG	directed acyclic graph
IQR	interquartile range
StAR	steroidogenic acute regulatory protein

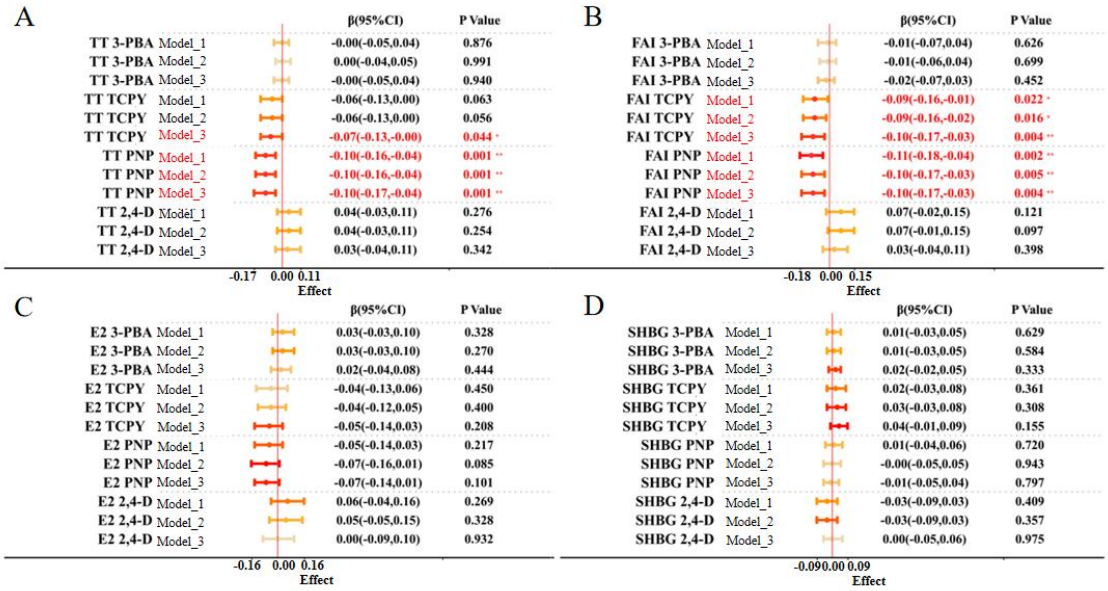
CYP17A1	cytochrome P450 family 17 subfamily A member 1
LH	luteinizing hormone
FSH	follicle-stimulating hormone



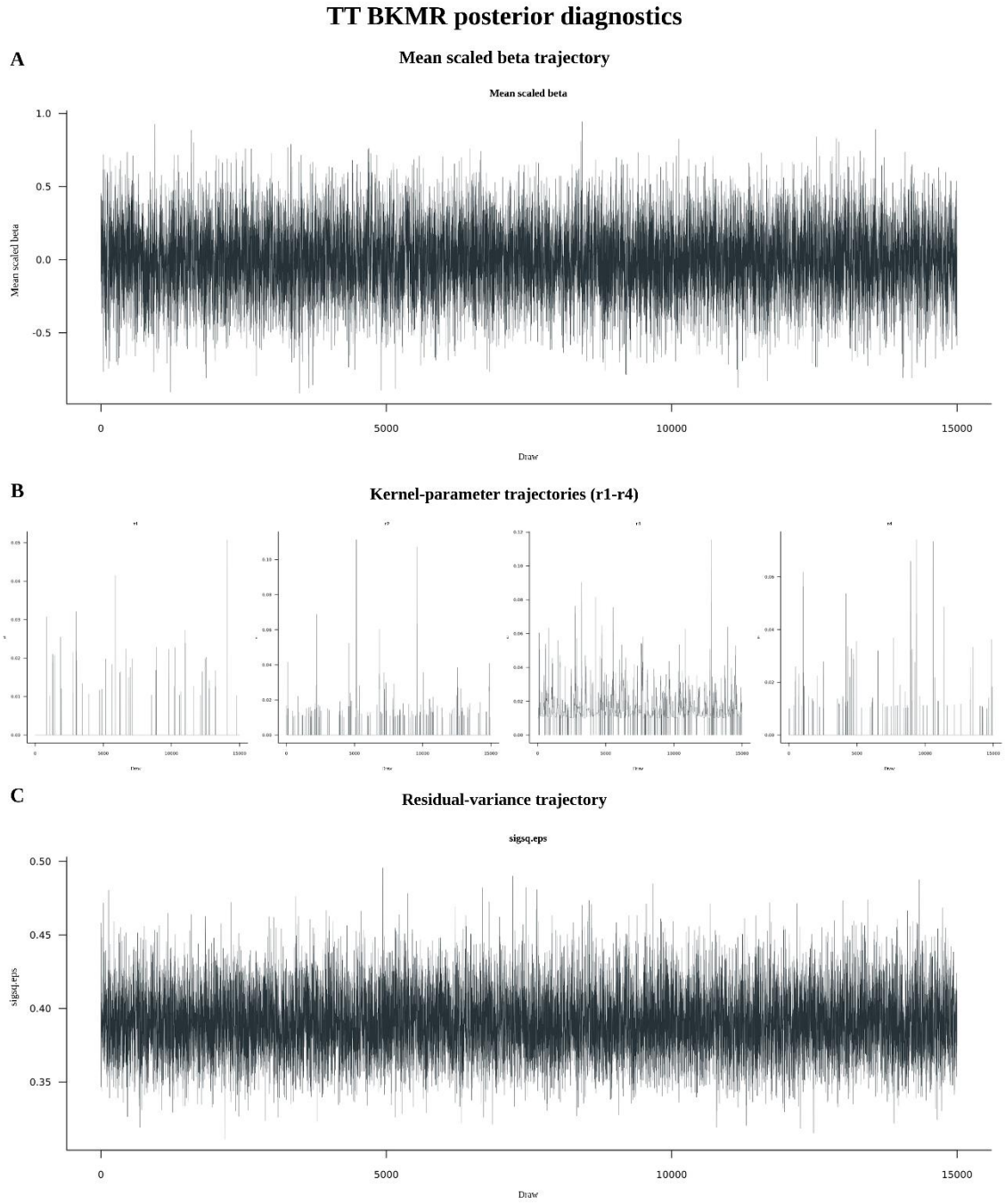
Supplementary Figure 1. Flowchart of population selection in NHANES 2013-2016.



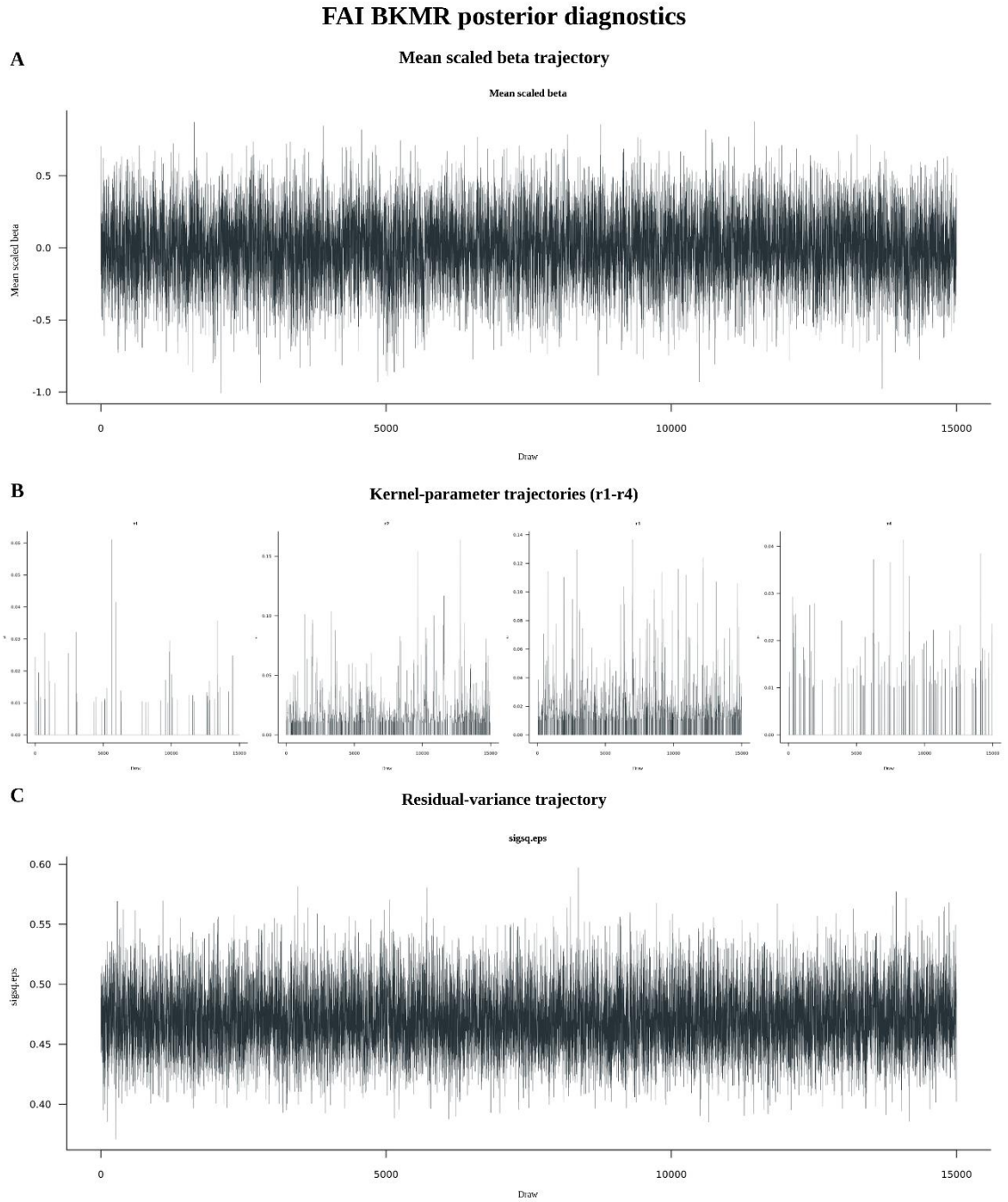
Supplementary Figure 2. Prespecified directed acyclic graph for the adjusted cross-sectional association between urinary pesticide biomarkers and sex-hormone outcomes. The graph was used to identify the minimal adjustment set and to organize lifestyle, reproductive, hormone-use, dietary, and anthropometric covariates included in the expanded models. UCRE was treated as a urine-dilution measurement-correction variable rather than as a back-door confounder. AGE, age; alcohol_category, alcohol intake category; BMI, body mass index; COT, serum cotinine category; current_hormone_use, current hormone use identified from prescription-drug records for the previous 30 days; EDU, educational attainment; energy_1000, total energy intake expressed per 1,000 kcal/day; ETH, race/ethnicity; ever_hormone_use, ever use of female hormones; F_TOTAL, total fruit intake; menopause_type, menopause classification; PA, physical activity; PIR, family poverty-income ratio; UCRE, urinary creatinine; V_TOTAL, total vegetable intake; X, urinary pesticide biomarker exposure; Y, sex-hormone outcome.



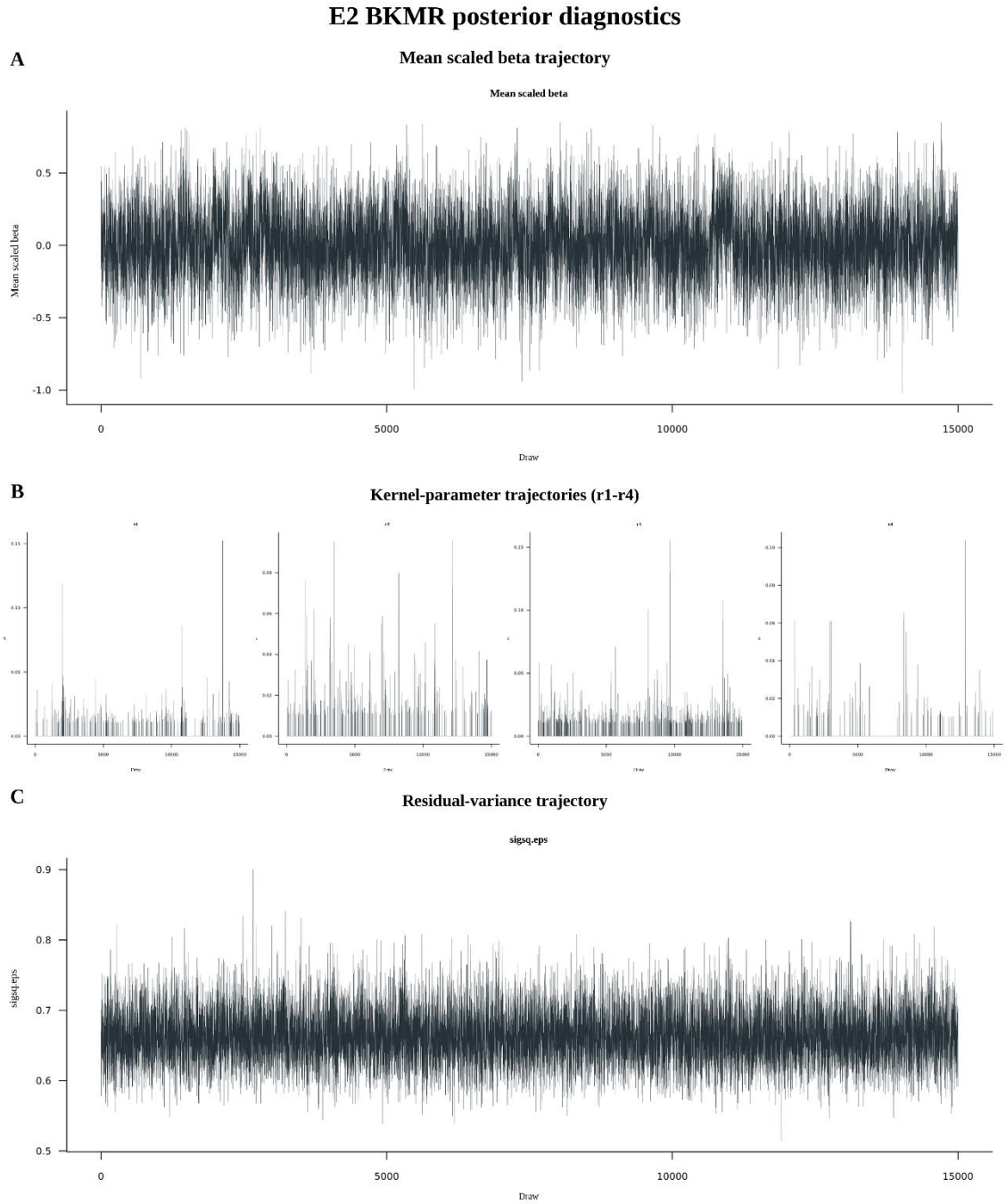
Supplementary Figure 3. Unweighted Independent Associations Between Non-Persistent Pesticide Exposure and Sex Hormone Levels in Postmenopausal Women. (A) Testosterone; (B) Free Androgen Index; (C) Estradiol; (D) Sex Hormone-Binding Globulin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. TT: Testosterone; FAI: Free Androgen Index; E2: Estradiol; SHBG: Sex Hormone-Binding Globulin; TCPY: 3,5,6-trichloro-2-pyridinol; PNP: para-nitrophenol; 2,4-D: 2,4-dichlorophenoxyacetic acid; 3-PBA: 3-phenoxybenzoic acid; PIR: family poverty-income ratio; BMI: body mass index.



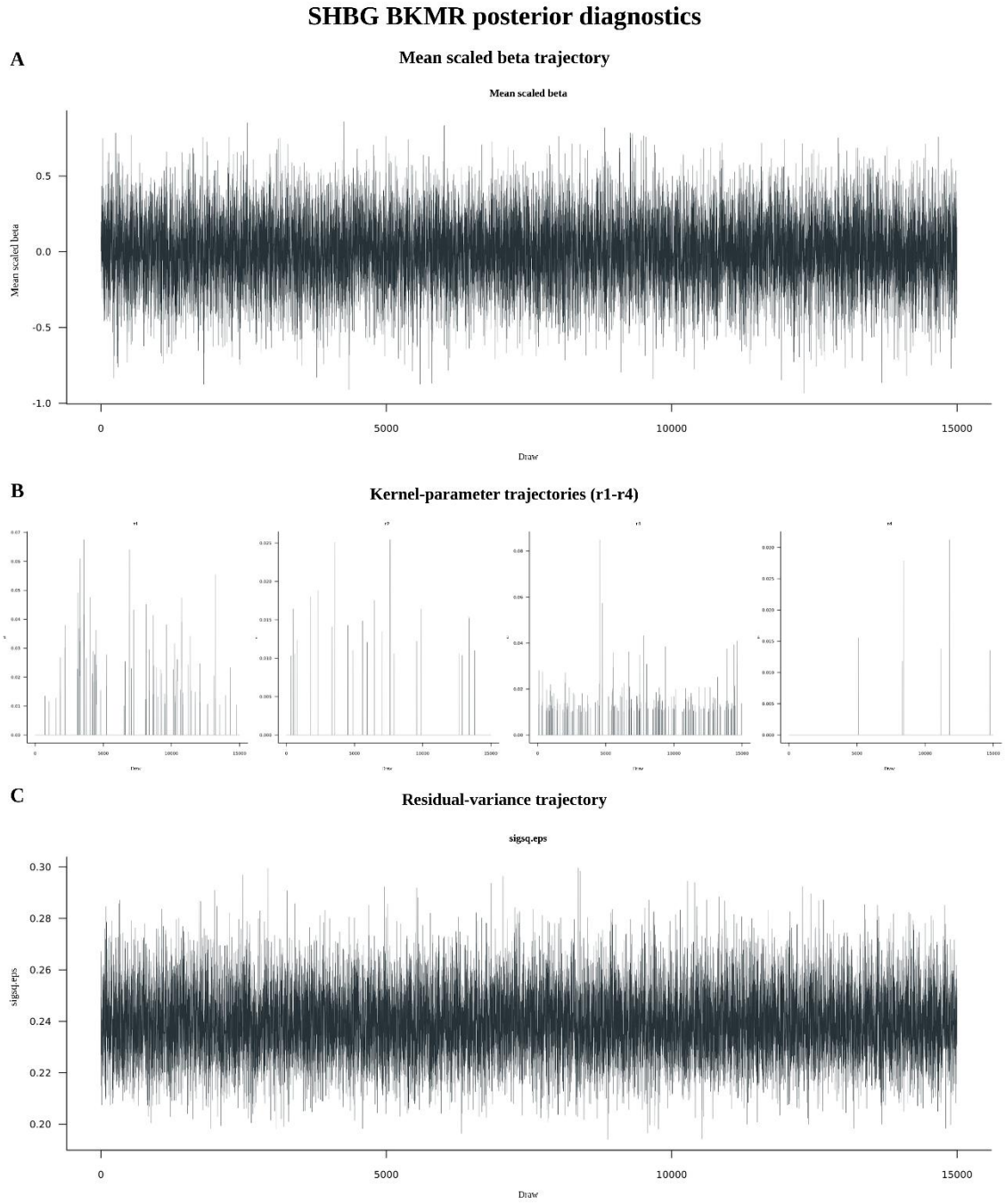
Supplementary Figure 4. BKMR posterior diagnostic plots for TT. (A) Mean scaled covariate-coefficient trajectory; (B) kernel-parameter trajectories r1–r4 corresponding to 3-PBA, TCPY, PNP, and 2,4-D; (C) residual-variance trajectory. The plots show retained posterior draws 5,001–20,000 from the selected chain. TT: Total Testosterone.



Supplementary Figure 5. BKMR posterior diagnostic plots for Free Androgen Index. (A) Mean scaled covariate-coefficient trajectory; (B) kernel-parameter trajectories r1–r4 corresponding to 3-PBA, TCPY, PNP, and 2,4-D; (C) residual-variance trajectory. The plots show retained posterior draws 5,001–20,000 from the selected chain. FAI: Free Androgen Index.



Supplementary Figure 6. BKMR posterior diagnostic plots for Estradiol. (A) Mean scaled covariate-coefficient trajectory; (B) kernel-parameter trajectories r1–r4 corresponding to 3-PBA, TCPY, PNP, and 2,4-D; (C) residual-variance trajectory. The plots show retained posterior draws 5,001–20,000 from the selected chain. E2: Estradiol.



Supplementary Figure 7. BKMR posterior diagnostic plots for sex hormone-binding globulin. (A) Mean scaled covariate-coefficient trajectory; (B) kernel-parameter trajectories r1–r4 corresponding to 3-PBA, TCPY, PNP, and 2,4-D; (C) residual-variance trajectory. The plots show retained posterior draws 5,001–20,000 from the selected chain. SHBG: Sex Hormone-Binding Globulin.