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Supplementary Information for

Mono (2-ethyl-5-hydroxyhexyl) phthalate promotes uterine leiomyoma cell survival through tryptophan-kynurenine-AHR pathway activation

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[Methods]

Weighted Quantile Sum modeling to define the bad actor (BAD) effect-based phthalate mixture

We conducted WQS modeling following the four-step method described by Bornehag *et al* (1). WQS is a statistical model for highly correlated data such as those encountered in environmental exposures. It is a mixture effect strategy that functions as dimensionality reduction to assess the association of the mixture with an outcome and determine the contributions of each component in the mixture to the overall effect. We focused on WQS modeling Step 1 "identification of bad-acting chemicals," and Step 2 "construction of a typical mixture" for our experimental studies. In Step 1, the WQS model is built to test associations between urinary phthalate levels and serum estradiol concentrations based on results from the MWHS cohort (2). Briefly, for each study participant (all women), urinary phthalates were measured by HPLC-MS/MS from four separate spot urine samples that were pooled. The measured levels were specific gravity-adjusted and presented in ng/mL. Serum samples were collected at four visits over four consecutive weeks and used for steroid hormone measurements. Serum estrogen levels were averaged across the menstrual cycle to obtain an average hormone concentration for each woman. For statistical modeling, phthalate levels were divided into quartiles and estradiol levels were log-transformed. Both positive and negative associations were tested. Individual chemicals were also tested using linear models. Co-variates used were menopausal status, race, body mass index (BMI), and smoking. Statistical analyses were done using the gWQS package in R. To compare WQS findings with individual chemical analyses, linear models were used. The WQS model was built on the subset of women from the MWHS cohort where data on outcomes and covariates were available (n=654; Table S7). The BAD mixture was based on the geometrical mean values from the full cohort (N=765) (Table S8).

RNA-seq data analysis

To explore the gene expression of SLC7A5 and SLC7A8, we analyzed previouly published three RNA-seq datasets containing LM and matched MM tissue samples: GSE120854 (3), GSE128242 (4), and GSE142329 dataset (5). The matched MM tissue sample was used as the control to normalize the gene expression.

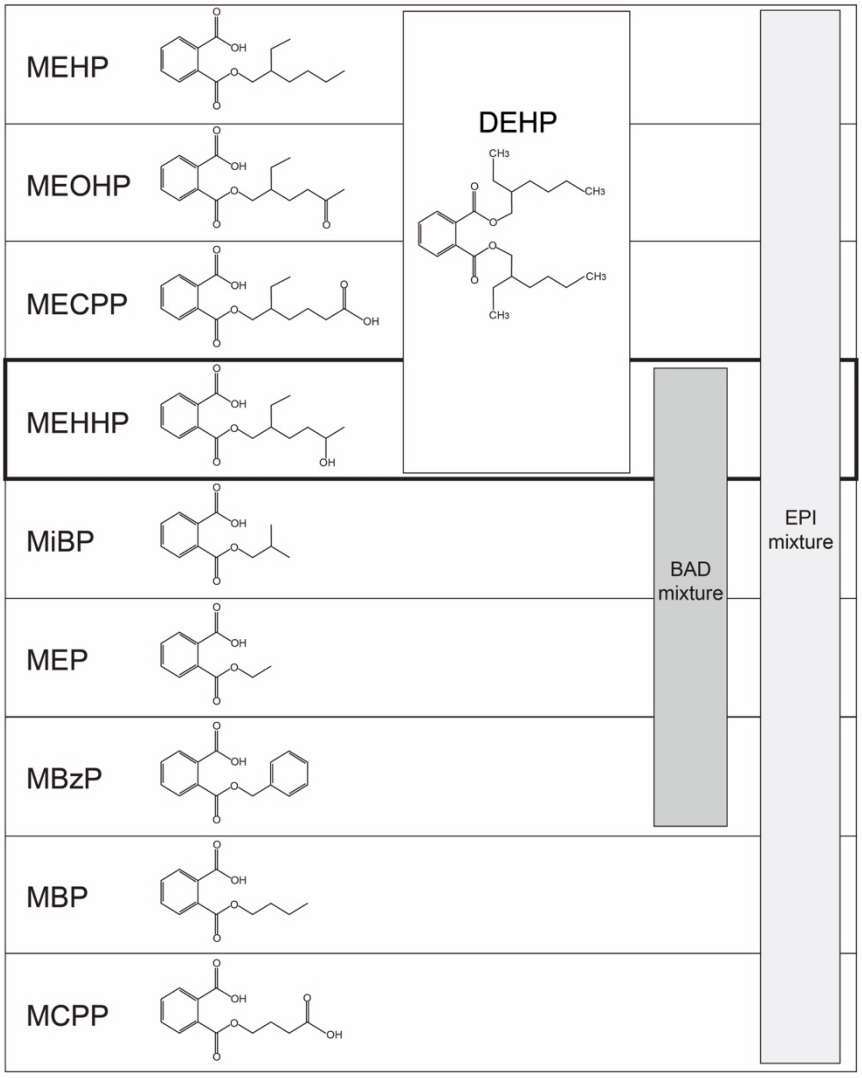

Figure S1. The composition of DEHP metabolites in the BAD (effect-based) mixture and EPI (exposure-based) mixture.
The EPI mixture (contains all nine phthalate metabolites shown), BAD mixture (contains MEHHP, MiBP, MEP, and MBzP), MEHHP alone, and DEHP alone (the parent compound of MEHP, MEOHP, MECPP, and MEHHP) were used in this study. BAD, bad actor (effect-based) mixture; EPI, epidemiological (exposure-based) mixture; DEHP, bis(2-ethylhexyl)phthalate; MBP, mono-butyl phthalate; MBzP, mono-benzyl phthalate; MCPP, mono-(3-carboxypropyl) phthalate; MECPP, mono-(2-ethyl-5-carboxypentyl) phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate; MiBP, mono-isobutyl phthalate.

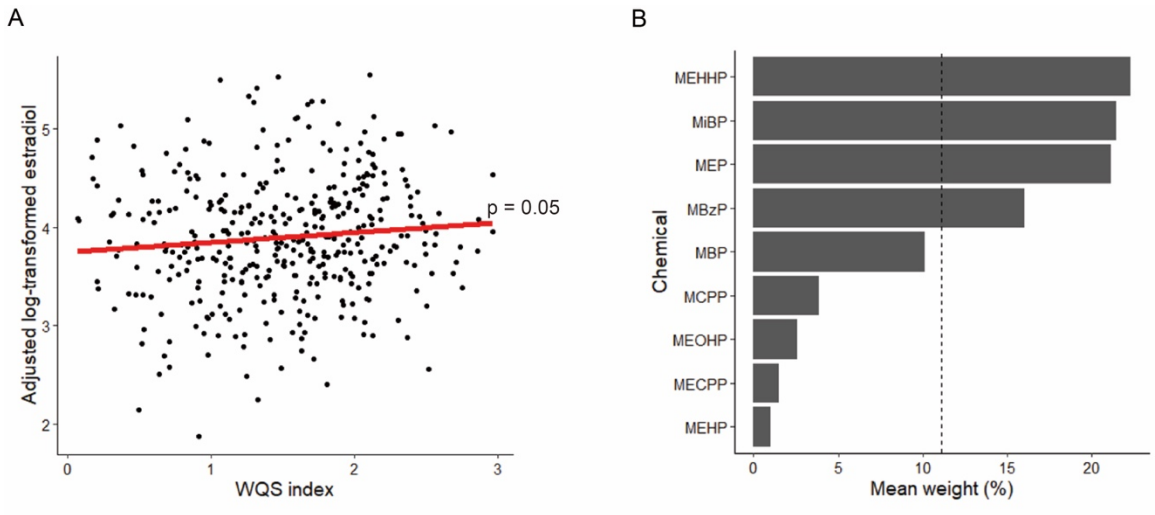

Figure S2. Association of serum estradiol with mixtures and weights of phthalate metabolites in urine.

A: Weighted Quantile Sum (WQS) model demonstrating the association between urinary levels of nine phthalate metabolites (MEHHP, MiBP, MEP, MBzP, MBP, MCPP, MEOHP, MECPP, and MEHP) and serum estradiol concentrations. The model shows a 0.1 increase in log-transformed estradiol concentration for every quartile increase in the WQS index. B: Bar graph showing the weights for each metabolite; the four metabolites ranked at the top of the list comprise the BAD (effect-based) mixture.

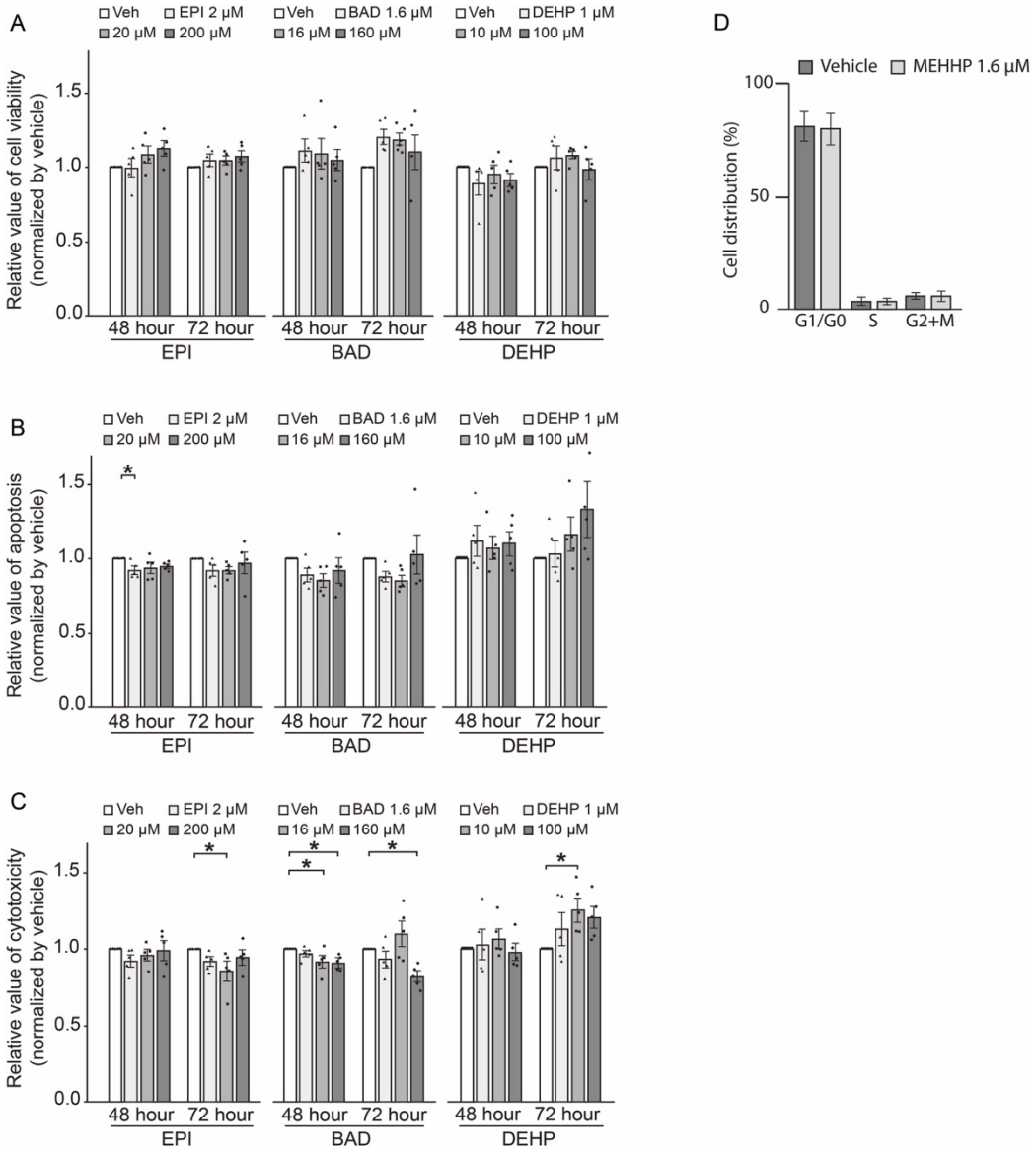

Figure S3. Phthalate metabolites increase viability and decrease apoptosis in leiomyoma cells

A to D: LM cells were treated with various concentrations of the EPI mixture (2, 20, 200 µM), BAD mixture (1.6, 16, 160 µM), and DEHP alone (1, 10, 100 µM) for 48 or 72 h. Cell viability (A), apoptosis (B), and cytotoxicity (C) were assessed with the ApoTox-Glo Triplex Assay kit (n = 5). D: LM cells were treated with vehicle or MEHHP (1.6 µM) for 72 h, and BrdU was added for the last 24 h. The cells were then analyzed by flow cytometry. Values are presented as mean ± SEM (n = 8). Statistical analysis was performed using student's t-test or Dennett's multiple comparison test. * $p < 0.05$. BAD, bad actor (effect-based) mixture; EPI, epidemiological (exposure-based) mixture.

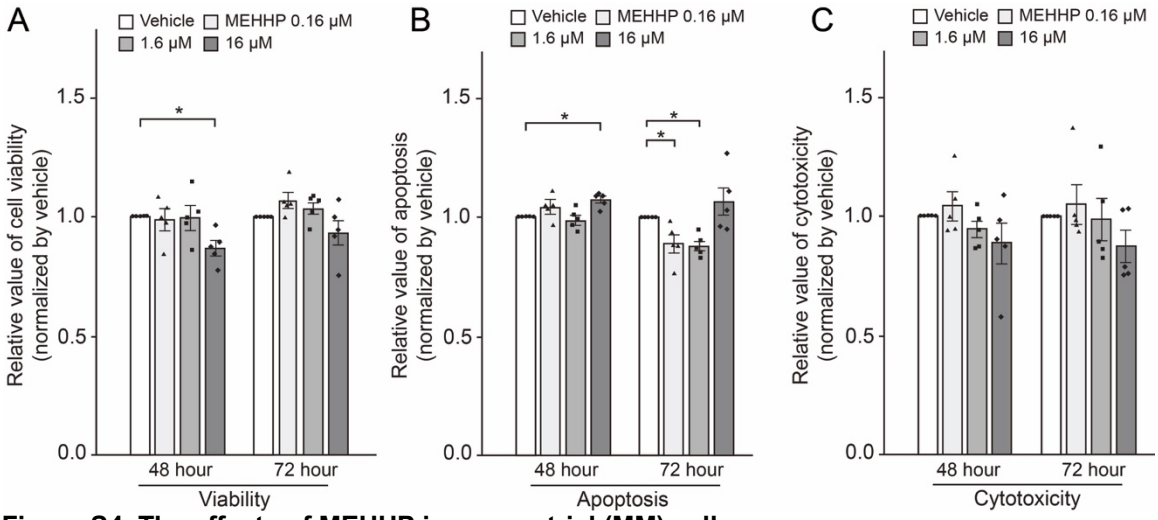

Figure S4. The effects of MEHHP in myometrial (MM) cells

A to C: MM cells were treated with various concentrations of the MEHHP (0.16, 1.6, 16 μM) for 48 or 72 h. Cell viability (A), apoptosis (B), and cytotoxicity (C) were assessed using the ApoTox-Glo Triplex Assay kit. Values were presented as mean ± SEM (n = 5, in duplicate). Statistical analysis was performed using Dennett's multiple comparison test. * $p < 0.05$.

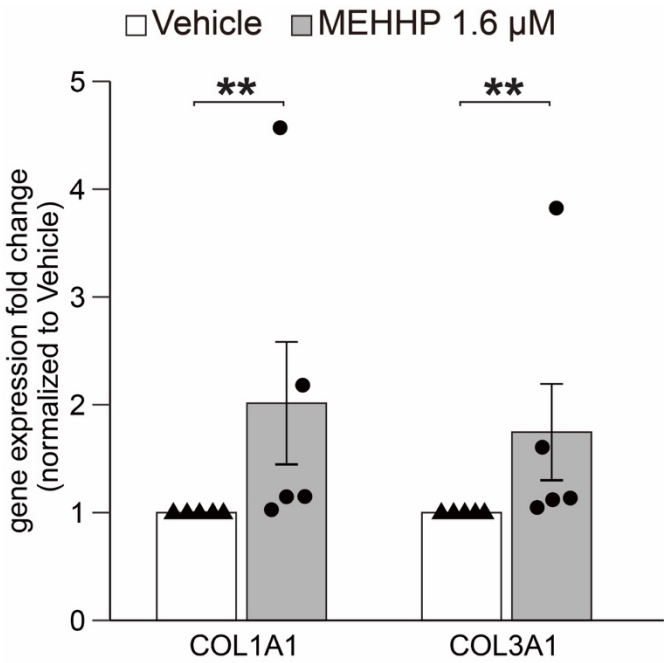

Figure S5. Bar graph showing RT-qPCR quantification of the expression of extracellular matrix genes COL1A1 and COL3A1 in LM cells treated with MEHHP (1.6 µM) or vehicle for 8 h. Statistical analysis was performed using Wilcoxon signed rank test (n=5). ** $p < 0.01$.

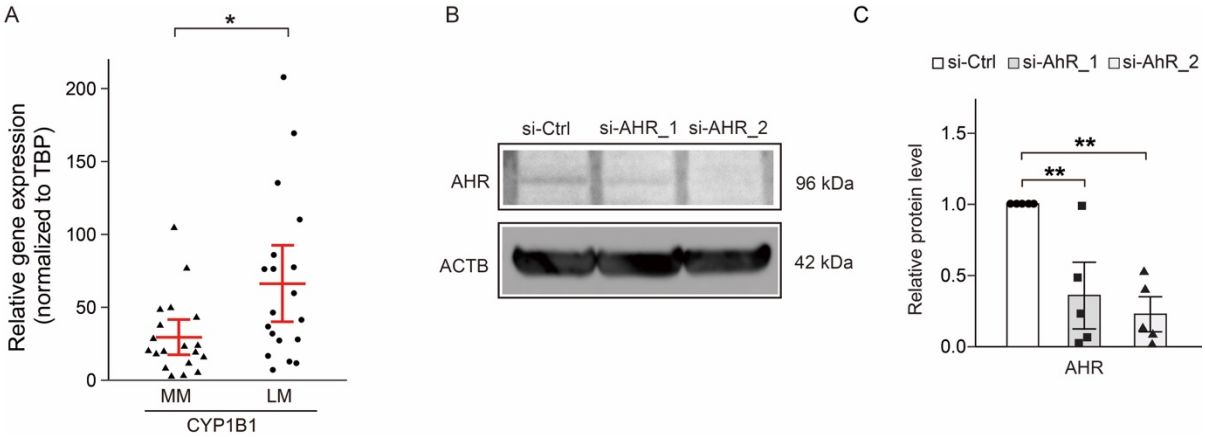

Figure S6.

A. Dot plot shows the distribution of CYP1B1 gene expression in LM and matched MM tissue. Data are expressed as means ± 95 % confidence interval (n=19 patient samples).
B: Representative Immunoblot image showing AHR protein expression in LM cells which were transfected with control siRNA (si-Ctrl) or two different AHR siRNAs (si-AHR_1 and si-AHR_2). C: Image J quantification of AHR protein levels in B. Values are presented as mean ± SEM (n = 5). Statistical analysis was performed using paired student's t-test or Dennett's multiple comparison test compared with control. * $p < 0.05$, ** $p < 0.01$.

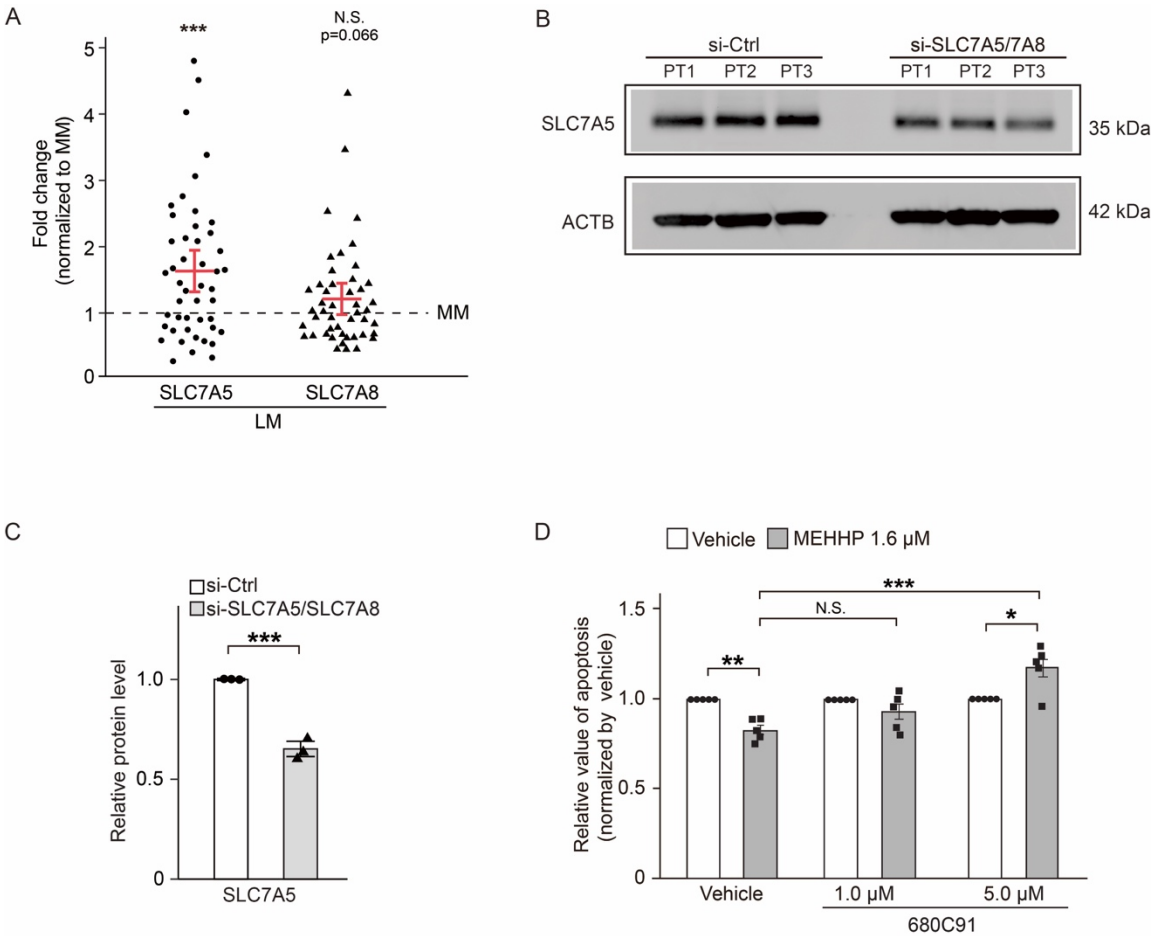

Figure S7.

A: Dot plot shows the fold change for SLC7A5 and SLC7A8 mRNA levels in LM tissue relative to matched MM tissue. Data are expressed as means ± 95 % confidence interval (n=47).
B: Representative Immunoblot image showing SLC7A5 protein expression in LM cells which were transfected with control siRNA (si-Ctrl) or SLC7A5 and SLC7A8 siRNAs. C: Image J quantification of AHR protein levels in B. Values are presented as mean ± SEM (n = 3).
D: LM cells were treated with the TDO2-specific inhibitor 680C91 at 1 μM and 5 μM for 72 h. Apoptosis was assessed by evaluation of Caspase 3/7 activity. Values were normalized to vehicle control for each group and presented as mean ± SEM (n = 5). Statistical analysis was performed using paired student's t-test or Dennett's multiple comparison test compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table S1. Composition of phthalate mixtures used in the study

EPI mixture (exposure-based mixture)				Stock solution		Working concentration (Low dose)	
Parent	**Metabolites**	**%**	**MW**	**mM**	**mg/ml**	**µM**	**ng/ml**
DEHP	MEHP	1.3	278	3.47	0.97	0.026	7.235
	MEOHP	3.1	292	8.28	2.42	0.06	18.13
	MECPP	6.4	308	17.09	5.26	0.12	39.48
	MEHHP	8.1	294	21.63	6.36	0.162	47.69
DiBP	MiBP	5.3	222	14.15	3.14	0.10	23.56
DEP	MEP	65.6	194	175.15	33.98	1.31	254.84
BBzP	MBzP	2.6	256	6.94	1.78	0.05	13.32
DBP	MBP	6.7	222	17.89	3.97	0.13	29.78
DOP	MCPP	0.8	252	2.14	0.54	0.016	4.04
	SUM	99.9		267	58.42	2.00	438.1

BAD mixture (effect-based mixture)				Stock solution		Working concentration (Low dose)	
Parent	**Metabolites**	**%**	**MW**	**mM**	**mg/ml**	**µM**	**ng/ml**
DEHP	MEHHP	9.9	294	21.63	6.36	0.16	47.69
DiBP	MiBP	6.5	222	14.15	3.14	0.10	23.56
DEP	MEP	80.3	194	175.15	33.98	1.31	254.84
BBzP	MBzP	3.2	256	6.94	1.78	0.05	13.32
	SUM	99.9		217.87	45.26	1.63	33.94

MEHHP				Stock solution		Working concentration (Low dose)	
Parent	**Metabolites**	**%**	**MW**	**mM**	**mg/ml**	**µM**	**ng/ml**
DEHP	MEHHP	100.0	294	21.63	6.36	0.162	47.69

DEHP			Stock solution		Working concentration (Low dose)	
	%	**MW**	**mM**	**mg/ml**	**µM**	**ng/ml**
	100.0	294	133.17	52.07	0.100	390.53

DBP, di-butylphthalate; BBzP, butyl benzyl phthalat; DEP, di-ethyl phthalate; DiBP, di-isobutyl phthalate; DPB, di-butyl phthalate;

Table S2. Median (25th, 75th percentiles) concentrations of individual urinary phthalate metabolites and phthalate molar sums of the MWHS.

Name	Abbreviation	MWHS (2006-2015) n=728
	Phthalate metabolite	**Median (25th, 75th percentiles) in ng/mL**
Monoethyl phthalate	MEP	97.3 (48.2, 192.0) [#]
Mono-n-butyl phthalate	MBP	19.8 (13.0, 32.8)
Mono-isobutyl phthalate	MiBP	16.4 (10.0, 26.1)
Mono(3-carboxypropyl) phthalate	MCPP	2.5 (1.3, 5.4)
Monobenzyl phthalate	MBzP	9.4 (5.4, 16.1)
Mono(2-ethylhexyl) phthalate	MEHP	4.5 (2.7, 9.3)
Mono(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP	33.5 (20.5, 58.7)
Mono(2-ethyl-5-oxohexyl) phthalate	MEOHP	12.0 (7.3, 22.4)
Mono(2-ethyl-5-carboxypentyl) phthalate	MECPP	25.9 (15.9, 48.0)
	Phthalate molar-converted sum	**Median (25th, 75th percentiles) in nmol/mL**
Di(2-ethylhexyl) phthalate	DEHP	0.3 (0.2, 0.5)

[#] Two samples (0.3%) were <LOD for MEP.

Table S3. Geometric mean of urinary phthalate metabolite concentrations in women with and without LMs.

		MEHP	MEHHP	MEOHP	MECPP	MCPP	MBzP	MEP	MBP	MiBP
LMs	**n**	**GM (SE)**	**GM (SE)**	**GM (SE)**	**GM (SE)**	**GM (SE)**	**GM (SE)**	**GM (SE)**	**GM (SE)**	**GM (SE)**
yes	207	5.88 (0.36)	39.81 (2.04)	15.51 (0.9)	32.55 (1.85)	2.95 (0.22)	9.44 (0.45)	131.11 (11.87)	22.73 (1.08)	19.66 (0.96)
no	547	5.53 (0.21)	34.90 (1.08)	13.65 (0.47)	29.29 (0.98)	3.03 (0.14)	10.41 (0.37)	100.18 (4.71)	21.6 (0.57)	16.83 (0.48)
P-value		0.329	0.011*	0.025*	0.072	0.812	0.200	0.0007***	0.388	0.005**

Values in the table indicate concentrations in urinary phthalate metabolite (ng/ml) of women in MWHS. Statistical analysis was performed using Kruskal Wallis test to compare geometric mean of phthalate metabolite concentrations in women with and without LMs. * $p < 0.05$, ** < 0.01, *** 0.001. GE, geometric mean; SE, standard error.

Table S4. Chemicals and inhibitors used in the study

Chemical	Chemical Name	CAS #	Catalog	Company
DMSO	Dimethyl sulfoxide	67-68-5	D2650	Sigma
MEHHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate	40321-99-1	M542510	TRC
MEHP	Mono-(ethylhexyl) phthalate	4376-20-9	M545290	TRC
MEOHP	Mono-(2-ethyl-5-oxohexyl) phthalate	40321-98-0	M542520	TRC
MECPP	Mono-(5-carboxyl-2-ethylpentyl) phthalate	40809-41-4	M525550	TRC
MiBP	Monoisobutyl phthalate	30833-52-5	M525635	TRC
MBzP	Monobenzyl phthalate	2528-16-7	M524900	TRC
MCPP	Mono-(3-carboxypropyl) phthalate	66851-46-5	M547700	TRC
MEP	Monoethyl phthalate	2306-33-4	M542580	TRC
MBP	Monobutyl phthalate	131-70-4	M525100	TRC
DEHP	Dioctyl phthalate	117-81-7	D201154	Sigma
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin	1746-01-6	ED-901-B	CIL

Inhibitors	Description	Catalog	Company
CH-223191	Antagonist of aryl hydrocarbon receptor	T2448	TargetMol
680C91	TDO2 inhibitor	SML0287	Sigma-Aldrich

TRC, Toronto Research Chemicals; CIL, Cambridge Isotope Laboratories.

Table S5. Primers and siRNAs used in the study

Primers for PrimeTime Std® qPCR Assay (Purchased from Integrated DNA Technologies)	
AHR	Hs.PT.56a.38998805
CYP1A1	Hs.PT.58.219047
CYP1B1	Hs.PT.58.25328727.g
HPRT1	Hs.PT.58v.45621572
SLC7A8	Hs.PT.58.40151777
SLC7A5	Hs.PT.58.25972702
TDO2	Hs.PT.58.3092178
COL1A1	Hs.PT.58.15517795
COL3A1	Hs.PT.58.4249241
On-target plus siRNA (purchased from Horizon Discovery)	
Human AHR	J-004990-05-0005 J-004990-06-0005
Human SLC7A5	J-004953-12-0005
Human SLC7A8	J-007618-09-0005
Human TDO2	J-008506-09-0005 J-008506-10-0005
Non-targeting Pool	D-001810-10-05

Table S6. Antibodies used in the study

Antibodies (Dilution)	Catalog No.	Company
AHR [RPT1] (1/1,000)	GTX22770	GeneTex
SLC7A5 (1/1,000)	28670-1-AP	Proteintech
Beta Actin (1/15,000)	HRP-60008	Proteintech
Anti-mouse IgG, HRP-linked Antibody (1/5,000)	7076S	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked Antibody (1/5,000)	7074S	Cell Signaling Technology

Table S7. Weighted quantile sum (WQS) cohort characteristics

Characteristic	N=654
Estradiol, median (min-max) (ng/ml)	56.2 (5.6-355.1)
BMI, median (min-max)	26.5 (15.6-67.7)
Menopausal status, n (%)	
Premenopausal	384 (59)
Perimenopausal	270 (41)
Race, n (%)	
White	447 (68)
Black	207 (32)
Smoking status, n (%)	
Never smoker	358 (55)
Former smoker	229 (35)
Current smoker	67 (10)

BMI, body mass index

Parent	Metabolite	Arithmetic mean	Geometric mean	Min.	1st Qu.	Median	3rd Qu.	Max.
DEHP	MEHHP	53.83	44.40	5.92	29.81	45.18	67.08	244.17
	MEHP	8.99	6.66	0.40	4.10	6.36	10.28	51.97
	MEOHP	21.75	17.00	2.81	10.80	15.45	25.80	128.35
	MECPP	46.24	36.36	7.60	22.71	33.73	56.37	267.13
DiBP	MiBP	27.11	22.40	1.40	14.86	21.79	33.20	132.63
BBzP	MBzP	16.60	12.80	1.55	7.87	12.64	20.08	95.56
DOP	MCPP	5.73	3.66	0.24	1.92	3.44	6.91	41.52
DEP	MEP	376.05	245.04	0.43	133.60	340.09	552.75	2342.40
DBP	MBP	33.03	27.80	3.00	19.09	27.32	39.59	154.80

Values in the table indicate concentrations in urinary phthalate metabolite concentration (ng/ml) of women in MWHS. Qu, quartile.

Table S8. Summary statistics for the full cohort, N=742-765

Parent	Metabolite	n	Arithmetic mean	Geometric mean	Min.	1st Qu.	Median	3rd Qu.	Max.
DEHP	MEHHP	750	56.80	45.98	5.92	29.93	46.27	69.03	306.00
	MEHP	742	9.42	6.90	0.40	4.14	6.47	10.60	53.87
	MEOHP	744	22.86	17.53	2.81	10.94	15.79	26.83	128.35
	MECPP	745	48.74	37.69	7.60	23.35	35.28	58.80	289.60
DiBP	MiBP	755	27.44	22.67	1.40	15.09	22.29	33.54	132.63
BBzP	MBzP	750	16.67	12.90	1.55	7.95	12.72	20.08	95.56
DOP	MCPP	749	5.90	3.74	0.24	1.97	3.55	7.29	41.52
DEP	MEP	765	373.69	244.55	0.29	136.70	330.67	548.57	2342.40
DBP	MBP	759	34.12	28.65	3.00	19.37	27.69	41.63	154.80

Values in the table indicate concentrations in urinary phthalate metabolite concentration (ng/ml) of women in MWHS. Qu, quartile.

References

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